Exercise, electrical stimulation and ionic effects on Na^+, K^+-ATPase isoform gene and protein expression in mammalian skeletal muscle

SUBMITTED BY

KATE TAMARIND MURPHY

B. APPL. SCI. (HONS.)

A THESIS SUBMITTED TO VICTORIA UNIVERSITY OF TECHNOLOGY

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

SCHOOL OF HUMAN MOVEMENT, RECREATION AND PERFORMANCE

AUGUST, 2005

ABSTRACT

The Na^+,K^+-ATPase enzyme has major regulatory functions, including maintenance of intra- to extracellular $[Na^+]$ and $[K^+]$ gradients, membrane excitability and muscle contractility. The Na^+,K^+-ATPase comprises a catalytic α subunit and a glycosylated β subunit, and belongs to a multigene family, with different genes encoding four α (α_1, α_2, α_3, α_4) and three β isoforms (β_1, β_2, β_3). This thesis examines the effects of acute and chronic exercise, electrical stimulation and explores factors regulating Na^+,K^+-ATPase isoform transcription and translation in skeletal muscle.

Study 1. Characterisation of expression of, and consequently also the acute exercise effects on Na^+,K^+-ATPase isoforms in human skeletal muscle remains incomplete and were therefore investigated. Fifteen healthy subjects (eight males, seven females) performed fatiguing, knee extensor exercise at ~40% of their maximal work output per contraction. A vastus lateralis muscle biopsy was taken at rest, fatigue, 3 and 24 h post-exercise, and analysed for Na^+,K^+-ATPase α_1, α_2, α_3, β_1, β_2 and β_3 mRNA and crude homogenate protein expression, using Real-Time RT-PCR and immunoblotting, respectively. Each individual expressed gene transcripts and protein bands for each Na^+,K^+-ATPase isoform. Each isoform was also expressed in a primary human skeletal muscle cell culture. Intense exercise lasting 352 ± 69 s (mean ± SEM) immediately increased α_3 and β_2 mRNA by 3.4- and 2.7-fold, respectively ($P < 0.05$), whilst α_1 and α_2 mRNA were increased by 3.5- and 4.5-fold at 24 h and 3 h post-exercise, respectively ($P < 0.05$). No significant change occurred for β_1 and β_3 mRNA, reflecting variable time-dependent responses. When the average post-exercise value was contrasted to rest, mRNA increased for all α_1-α_3, β_1-β_3 isoforms, by 2.4-, 3.2-, 2.4-, 2.1-, 2.0- and 2.0-fold, respectively ($P < 0.05$). However, exercise did not alter the protein abundance of the α_1-α_3

and β_1-β_3 isoforms. Thus, human skeletal muscle expresses each of the Na^+,K^+-ATPase α_1, α_2, α_3, β_1, β_2 and β_3 isoforms, evidenced at both transcription and protein levels. Whilst brief intense exercise increased Na^+,K^+-ATPase isoform mRNA expression, there was no effect on isoform protein expression, suggesting that the exercise challenge was insufficient for muscle Na^+,K^+-ATPase up-regulation.

Study 2. This study investigated the effects of prolonged submaximal exercise on Na^+,K^+-ATPase isoform mRNA and protein expression, maximal activity and content in human skeletal muscle. We also investigated the effects on mRNA expression of the transcription initiator gene, RNAP II, and key genes involved in protein translation, eIF-4E and 4E-BP1. Eleven healthy subjects (six males, five females) cycled at 75.5 ± 1.5% peak O_2 uptake, continued until fatigue. A vastus lateralis muscle biopsy was taken at rest, fatigue, 3 and 24 h post-exercise. Muscle was analysed for Na^+,K^+-ATPase α_1, α_2, α_3, β_1, β_2 and β_3 mRNA and protein expression, homogenate maximal activity (3-*O*-MFPase activity) and content (^{3}[H]-ouabain binding site content), as well as for RNAP II, eIF-4E and 4E-BP1 mRNA expression. Cycling to fatigue, lasting 54.5 ± 6.1 min, immediately increased Na^+,K^+-ATPase α_3 ($P < 0.05$) and β_2 mRNA ($P < 0.05$) by 2.2- and 1.9-fold, respectively, whilst α_1 mRNA was elevated by 2.0-fold at 24 h post-exercise ($P < 0.04$). Exercise increased protein abundance of the α_3 isoform ($P < 0.05$), and transiently depressed maximal Na^+,K^+-ATPase activity, but did not alter Na^+,K^+-ATPase content, including in the 24 h period following exercise. Finally, exercise immediately increased RNAP II mRNA expression by 2.5-fold ($P < 0.02$), but had no effect on eIF-4E and 4E-BP1 mRNA expression. Thus, a single bout of prolonged exercise induced isoform-specific Na^+,K^+-ATPase transcriptional and translational regulation. Exercise

also increased transcriptional regulation of the gene initiating transcription, but not of key genes initiating protein translation.

Study 3. The effects of high-intensity, intermittent exercise and training (HIT) on Na^+,K^+-ATPase mRNA and protein expression in already well-trained endurance athletes are unknown and were consequently investigated. Twelve endurance trained male cyclists underwent baseline testing (Baseline) and 4 wks later, commenced HIT comprising seven sessions over 3 wks, of high-intensity, intermittent exercise (8 x 5 min at 80% Peak Power Output, PPO). A vastus lateralis muscle biopsy was taken at rest (Baseline) and both rest and immediately post-exercise during the first (Pre-HIT) and seventh (Post-HIT) HIT session, and analysed for Na^+,K^+-ATPase α_1, α_2, α_3, β_1, β_2 and β_3 mRNA and protein expression. High-intensity, intermittent exercise increased α_1, α_2 and α_3 mRNA expression by 2.0-, 2.4-, and 4.0-fold, respectively Pre-HIT, and also β_2 mRNA by 2.7-fold Post-HIT ($P < 0.05$). There was no effect of acute exercise on protein abundance of any Na^+,K^+-ATPase isoforms. HIT increased PPO by 2.7 ± 0.1% ($P < 0.05$), and increased resting α_3 and β_3 mRNA expression by 4.6- and 2.5-fold, respectively ($P < 0.03$), but had no effect on resting α_1, α_2, β_1 and β_2 mRNA expression or on the protein abundance of any Na^+,K^+-ATPase isoforms. HIT did not alter these acute responses to exercise. In conclusion, the acute exercise Na^+,K^+-ATPase response was isoform-specific and persisted in already well-trained athletes after HIT.

Study 4. This study investigated the effects of chronic endurance training status and gender on skeletal muscle Na^+,K^+-ATPase mRNA expression, content and activity. Forty-five chronic endurance trained males (ETM), eleven recreationally active males (RAM), and nine recreationally active females (RAF) had a vastus lateralis muscle biopsy and analysed for Na^+,K^+-ATPase α_1, α_2, α_3, β_1, β_2 and β_3 mRNA expression, maximal Na^+,K^+-ATPase activity

and Na^+,K^+-ATPase content. ETM demonstrated lower α_1, α_3, β_2 and β_3 mRNA expression by 0.7-, 0.6-, 0.7- and 0.8-fold, respectively, than RAM ($P < 0.03$). In contrast, [^{3}H]-ouabain binding and 3-*O*-MFPase activity were higher in ETM than in RAM, by 16.1% and 15.7%, respectively ($P < 0.03$). RAM demonstrated a 2.3- and 3.6-fold higher α_3 and β_3 mRNA expression ($P < 0.05$), respectively than RAF, but no gender difference was found for α_1, α_2, β_1 or β_2 mRNA, [^{3}H]-ouabain binding or 3-*O*-MFPase activity. No significant correlation was found between years of endurance training and either [^{3}H]-ouabain binding or 3-*O*-MFPase activity, however a weak correlation was found between incremental exercise $\dot{V}O_{2peak}$ and both [^{3}H]-ouabain binding (r = 0.31, $P < 0.01$) and 3-*O*-MFPase activity (r = 0.25, $P < 0.02$). There was also a significant negative correlation between $\dot{V}O_{2peak}$ and mRNA expression for each of the α_1 (r = -0.46, $P < 0.01$), α_2 (r = -0.42, $P < 0.03$), α_3 (r = -0.39, $P < 0.04$) and β_2 (r = -0.38, $P < 0.05$) isoforms. In conclusion, isoform-specific differences in Na^+,K^+-ATPase transcriptional regulation are found with both chronic training status and gender, but only chronic training status influences Na^+,K^+-ATPase content and maximal activity in human skeletal muscle.

Study 5. This study investigated the effects of muscle fibre-type and electrical stimulation on Na^+,K^+-ATPase isoform mRNA expression in rat skeletal muscle. This study also explored factors regulating Na^+,K^+-ATPase transcription in intact rat EDL muscle by utilising interventions designed to induce each of increased intracellular [Na^+] ($[Na^+]_i$), cytosolic free [Ca^{2+}] ($[Ca^{2+}]_{cyto}$), membrane depolarisation and scavenging of intracellular reactive oxygen species (ROS). Soleus and EDL muscles were mounted on force transducers and stimulated electrically (3 x 10 s every 10 min, 60 Hz) to evoke short tetani at regular intervals. Ouabain (10^{-3} M, 120 min), veratridine (10^{-4} M, 30 min) and monensin (10^{-4} M, 30 min) were used to

increase $[Na^+]_i$, with muscle Na^+ content measured by flame photometry. Increased $[Ca^{2+}]_{cyto}$ and membrane depolarisation were induced using caffeine (5 mM, 30 min) and high extracellular $[K^+]$ ($[K^+]_e$) (13 mM, 60 min), respectively. *N*-acetylcysteine (NAC, 10 mM, 30 min) was used to attenuate ROS during electrical stimulation (90 s, 60 Hz). Muscles were then analysed for Na^+,K^+-ATPase α_1, α_2, α_3, β_1, β_2 and β_3 mRNA expression. The EDL contained a 1.5- and 1.7-fold greater abundance of α_2 and β_3 mRNA, respectively, but a 0.3-fold lower abundance of β_1 mRNA, than the soleus ($P < 0.04$). Electrical stimulation had no immediate effect on Na^+,K^+-ATPase mRNA in either the EDL or the soleus, but at 3 h post-stimulation increased α_1, α_2 and α_3 mRNA by 3.2-, 7.2- and 9.9-fold, respectively, in the EDL ($P < 0.02$). Ouabain increased muscle Na^+ content by 90% ($P < 0.01$), but did not increase Na^+,K^+-ATPase isoform mRNA, whilst neither muscle Na^+ content nor isoform mRNA were increased with veratridine or monensin. Caffeine elevated α_1, α_2 and β_1 mRNA by 2.6-, 2.8- and 2.7-fold, respectively ($P < 0.03$), while high $[K^+]_e$ did not affect Na^+,K^+-ATPase isoform mRNA. NAC increased α_2 and β_1 mRNA expression in resting muscles by 4.6- and 4.1-fold, respectively ($P < 0.01$). In muscles incubated without NAC, electrical stimulation increased α_1, α_2 and α_3 mRNA expression, by 3.7-, 5.3- and 24.0-fold, respectively ($P < 0.01$). However, in muscles incubated with NAC, electrical stimulation decreased α_2, β_1 and β_2 mRNA expression, by 3.3-, 4.1- and 2.2-fold, respectively ($P < 0.01$). In conclusion, the relative expression of the rat α_2, β_1 and β_3 gene transcripts differed between EDL and soleus muscles, while the effect of three bouts of high-frequency electrical stimulation on Na^+,K^+-ATPase isoform mRNA expression was both isoform- and muscle-specific. Furthermore, Na^+,K^+-ATPase transcription appears to be regulated by different stimuli in rat skeletal

muscle, including the cellular changes associated with caffeine (such as elevated $[Ca^{2+}]_{cyto}$) and NAC.

In summary, this thesis demonstrated that human skeletal muscle expresses six Na^+,K^+-ATPase isoforms. Studies with brief intense and prolonged submaximal exercise showed that the acute exercise effects on Na^+,K^+-ATPase isoform mRNA and protein expression depended on the exercise stimulus. In striking contrast, chronically endurance trained athletes demonstrated higher Na^+,K^+-ATPase content and maximal activity, but lower isoform mRNA expression than recreationally active subjects. Genders differed in isoform-specific Na^+,K^+-ATPase mRNA expression, but not in Na^+,K^+-ATPase content or maximal activity. In rats, expression of the Na^+,K^+-ATPase isoform gene transcripts varied between EDL and soleus muscles, while the effects of electrical stimulation on Na^+,K^+-ATPase isoform mRNA expression were both isoform- and muscle specific. Finally, $[Ca^{2+}]_{cyto}$ and ROS appear to be involved in the regulation of Na^+,K^+-ATPase transcription in rat skeletal muscle.

DECLARATION

I, Kate Murphy, declare that the PhD thesis entitled, *Exercise, electrical stimulation and ionic effects on Na^+,K^+-ATPase isoform gene and protein expression in mammalian skeletal muscle*, is no more than 100,000 words in length, exclusive of tables, figures, appendices, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work.

Signature 

Date 5/9/05

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my supervisor, Associate Professor Mike McKenna, for taking a chance on me and I am forever grateful for that opportunity. Most importantly, his knowledge, vision, enthusiasm, belief and guidance ensured not only the completion of this thesis, but also a passion for my work.

Thank you to my co-supervisor, Dr Rod Snow, and also to Dr David Cameron-Smith at Deakin University for their expert knowledge, their assistance on experimental trial days, and imperatively, for their positive comments that were so forthcoming at pivotal times.

I would also like to thank Professor Mark Hargreaves at Deakin University for allowing me into his laboratory to perform several of the muscle assays. Thank you to Dr Andrew Garnham from Deakin University, who performed the majority of muscle biopsies in this thesis. I am particularly thankful for the generosity of his time, and for his most reassuring bedside manner.

The human experimental trials required the assistance of a large number of people. For that, I would also like to thank my colleagues, Aaron Petersen, Rob Aughey, Dr. Craig Goodman, Jim Leppik, Xiaofei Gong, Ivan Medved, Anula Costa, Jason Bennie, Collene Steward and also Bente Weidmann. In particular, I would like to thank Aaron who collaborated on Study 1, as well as Rob and Professor John Hawley, Dr Sally Clark and Jemma Christie from RMIT University for their collaboration on Study 3. In particular, thanks to Rob and Aaron for performing some of the [^{3}H]-ouabain binding and 3-*O*-MFPase assays included in Study 4. Special thanks to my "old" colleagues, Rob, Ivan and Aaron for the many forms of support each of you provided. Your knowledge, advice, patience and empathy were invaluable.

Thank you also to Simon Sostaric and Cadeyrn Gaskin for their support and especially for their ears, which I managed to chew off on all too many occasion.

I would also like to thank Dr Craig Goodman, Dr Jong-Sam Lee and Janelle Mollica for teaching me the majority of muscle assays performed in this thesis. Special thanks also to Janelle for preparation of the cell cultures utilised in Study 1.

To Dr Robyn Murphy, thank you for performing the deglycosylation experiments and also for your skilled assistance, willingness to share your knowledge and ideas, for your words of wisdom and for your friendship.

Thank you also Professor Torben Clausen and Associated Professor Ole Bækgaard Nielsen at the University of Aarhus, for allowing me into their laboratory and for their commitment to study 5. I would also like to thank Dr William Macdonald at the University of Aarhus for his expertise and tireless work in continuing this collaboration. Also at the University of Aarhus, I thank Marianne Stürup Johansen, Tove Lindahl Andersen, Vibeke Uhre and Ann-Charlotte Andersen for their skilled technical assistance.

I am especially thankful to all of the subjects who participated in the studies in this thesis. Without you, of course this thesis would not have been possible, and I cannot thank you enough for your generosity and for your patience.

On a personal note to Will, you have helped changed everything. Thank you.

Finally, to my parents, for your support, your sacrifices, your acceptance and most importantly, your unconditional love. We have been through some tough times, particularly the past 12 months, but we have grown closer and for that alone, it has been worthwhile. I can never thank you enough for all that you have done for me.

LIST OF PUBLICATIONS

The following publications and presentations are presented in support of this thesis:

Refereed papers

1. **Murphy KT,** Snow RJ, Petersen AC, Murphy RM, Mollica J, Lee JS, Garnham AP, Aughey RJ, Leppik JA, Medved I, Cameron-Smith D and McKenna MJ. Intense exercise up-regulates Na^+,K^+-ATPase isoform mRNA, but not protein expression in human skeletal muscle. *J Physiol* 556: 507-519, 2004.

2. **Murphy, KT**, Petersen, AC, Goodman, C, Gong, X, Leppik, JA, Garnham, AP, Cameron-Smith, D, Snow, RJ and McKenna, MJ. Acute endurance exercise induces transcriptional and translational responses of the Na^+,K^+-ATPase isoforms in human skeletal muscle. *FASEB J* 19: A115-A115, 2005.

3. Petersen AC, **Murphy KT**, Snow RJ, Leppik JA, Aughey RJ, Garnham AP, Cameron-Smith D and McKenna MJ. Depressed Na^+,K^+-ATPase activity in skeletal muscle at fatigue is correlated with increased Na^+,K^+-ATPase mRNA expression following intense exercise. *Am J Physiol* 289: R266-274, 2005.

4. Clark SA, Chen Z-P, **Murphy KT**, Aughey RJ, McKenna MJ, Kemp BE and Hawley JA. Intensified exercise training does not alter AMPK signaling in human skeletal muscle. *Am J Physiol* 286: E737-743, 2004.

Papers in review

1. **Murphy KT,** Macdonald WA, McKenna MJ and Clausen T. Ionic mechanisms of excitation-induced regulation of Na^+,K^+-ATPase mRNA expression in isolated rat EDL muscle. *J Physiol,* in review.

2. **Murphy KT,** Petersen AC, Goodman C, Gong X, Leppik JA, Garnham AP, Cameron-Smith D, Snow RJ and McKenna MJ. Prolonged submaximal exercise induces isoform-specific Na^+,K^+-ATPase transcriptional and translational responses in human skeletal muscle. *Am J Physiol Regul Physiol*, in review.

3. **Murphy KT,** Aughey RJ, Petersen AC, Clark SA, Goodman C, Hawley JA, Cameron-Smith D, Snow RJ and McKenna MJ. Chronic endurance training but not gender modulate Na^+,K^+-ATPase mRNA expression, content and maximal activity in human skeletal muscle. *J Appl Physiol*, in review.

4. Aughey RJ, **Murphy KT,** Clark SA, Garnham AP, Snow RJ, Cameron-Smith D, Hawley JA and McKenna MJ. Muscle Na^+,K^+ATPase isoform, content and activity responses to interval exercise and training in well-trained athletes. *J Physiol*, in review.

Conference presentations

1. **Murphy, KT**, Macdonald, WA, McKenna, MJ and Clausen, T. Electrical and ionic regulation of Na^+,K^+-ATPase isoform mRNA expression in isolated fast-twitch muscle in the rat. *Joint International Meeting of the Physiological Society and the Federation of European Physiological Societies, July 2005.* Bristol; UK.

2. **Murphy, KT**, Macdonald, WA, McKenna, MJ and Clausen, T. Electrical and ionic regulation of Na^+,K^+-ATPase isoform mRNA expression in isolated fast-twitch muscle in the rat. *The Na,K,Cl-homeostasis and Na,K-pumps of muscle and heart in exercise and disease, June 2005.* Sandbjerg; Denmark.

3. **Murphy KT,** Petersen AC, Goodman C, Gong X, Leppik JA, Garnham AP, Cameron-Smith D, Snow RJ & McKenna MJ. Prolonged submaximal exercise induces isoform-specific Na^+,K^+-ATPase transcriptional and translational responses in human skeletal muscle [poster]. *XXXV Congress of the International Union of Physiological Sciences (IUPS). San Diego; USA. April 2005.*

4. **Murphy KT,** Macdonald WA, McKenna, MJ and Clausen, T. Electrical and ionic regulation of Na^+,K^+-ATPase mRNA expression in fast-twitch and slow-twitch skeletal muscle [poster]. *The Na,K,Cl-homeostasis and Na,K-pumps in muscle and heart in exercise and disease. Sandbjerg; Denmark. June 2005.*

5. **Murphy, KT**, Aughey, RJ, Petersen, AC, Clark, SA, Goodman, C, Garnham, AP, Christie, JJ, Hawley, JA, Cameron-Smith, D, Snow, R & McKenna, MJ. (2004). Gender and chronic endurance training effects on Na^+,K^+-ATPase mRNA, activity and content in human skeletal muscle. *9th Annual Congress of the European College of Sports Science. Clermont-Ferrand; France. July 2004.*

6. **Murphy, KT**, Snow, RJ, Petersen, AC, Murphy, RM, Mollica, J, Lee, JS, Garnham, AP, Aughey, RJ, Leppik, JA, Medved, I, Cameron-Smith, D & McKenna, MJ. (2003). Intense exercise up-regulates Na^+,K^+-ATPase isoform mRNA, but not protein expression in human skeletal muscle. *Proceedings of the Australian Physiological and Pharmacological Society.* 33: 92P.

7. **Murphy, KT**, Snow, RJ, Petersen, AC, Murphy, RM, Mollica, J, Lee, JS, Garnham, AP, Aughey, RJ, Leppik, JA, Medved, I, Cameron-Smith, D & McKenna, MJ. (2003).

Intense exercise up-regulates Na^+,K^+-ATPase isoform mRNA, but not protein expression in human skeletal muscle. *Curtin Conference, Australian National University. Canberra; Australia. April 2003.*

LIST OF ABBREVIATIONS

ATP	adenosine triphosphate
BLAST	basic local alignment sequence tool
Ca^{2+}	calcium ion
Ca^{2+}-ATPase	calcium adenosine triphosphatase
$[Ca^{2+}]_{cyto}$	cytosolic free calcium concentration
$[Ca^{2+}]_e$	extracellular calcium concentration
$[Ca^{2+}]_i$	intracellular calcium concentration
cDNA	complementary deoxyribonucleic acid
Cl^-	chloride
[ ]	concentration of an ion
CrP	creatine phosphate
CSQ	calsequestrin
d	day
DHPR	dihydropyridine receptor
DNA	deoxyribonucleic acid
E-C coupling	excitation-contraction coupling
EDL	extensor digitorum longus
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycol-bis(β-aminoethylether)-N,N'-tetraacetic acid
eIF-4E	eukaryotic initiation factor-4E
4E-BP1	4E-binding protein 1

FKBP	FK506-binding protein
HCl	hydrogen chloride
K^+	potassium ion
$[K^+]_e$	extracellular potassium concentration
$[K^+]_i$	intracellular potassium concentration
kDa	kilo daltons
Lac^-	lactate ion
Mg^{2+}	magnesium ion
mRNA	messenger ribonucleic acid
Na^+	sodium ion
$[Na^+]_e$	extracellular sodium concentration
$[Na^+]_i$	intracellular sodium concentration
NAC	*N*-acetylcysteine
Na^+,K^+-ATPase	sodium-potassium adenosine 5'triphosphatase
N-terminal	amino terminal
Pi	inorganic phosphate ion
p-NPP	*p*-nitrophenyl phosphate
RER	respiratory exchange ratio
RNAP II	RNA polymerase II
ROS	reactive oxygen species
rpm	revolutions per minute
RT-PCR	reverse transcription-polymerase chain reaction
RyR	ryanodine receptor
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SR	sarcoplasmic reticulum
SRP	signal recognition particle
SRPR	signal recognition particle receptor
3-*O*-MFP	3-*O*-methylfluoroscein phosphate
[^{3}H]-ouabain binding	tritiated ouabain binding
T-tubules	tranverse tubules
T_3	triiodothyronine
$\dot{V}O_{2peak}$	peak oxygen consumption
$\dot{V}CO_2$	carbon dioxide output
W	watts
wks	weeks
wt	weight

N.B. All changes reported in this thesis will be expressed as a % change, except for mRNA expression, which will be expressed as a fold change, as per standard use in journals.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1: INTRODUCTION

Sustained muscle contractions depend on the generation and propagation of action potentials in the surface membrane (sarcolemma) and the tranverse tubules (T-tubules). Action potential generation is dependent on steep trans-membrane concentration gradients for sodium (Na^+) and potassium (K^+) ions. However, repeated muscle contractions are associated with a cellular loss of K^+ and gain of Na^+, thereby altering these ionic gradients necessary for action potential generation. The sodium-potassium adenosine 5'triphosphatase (Na^+,K^+-ATPase) enzyme counters these fluxes by transporting 3 Na^+ ions out of, and 2 K^+ ions back into the cell, via energy released from the hydrolysis of one ATP molecule. Thus, the Na^+,K^+-ATPase is critical for maintaining membrane excitability and hence, the ability to sustain repeated muscle contractions.

The Na^+,K^+-ATPase enzyme is ubiquitously expressed in mammalian tissues. In skeletal muscle, the Na^+,K^+-ATPase has major regulatory functions, including maintenance of trans-membrane [Na^+] and [K^+] gradients, membrane excitability and contractility, as well as secondary transport processes (Blanco & Mercer, 1998; Nielsen & Clausen, 2000; Clausen, 2003). The Na^+,K^+-ATPase comprises a catalytic α subunit and a glycosylated β subunit, and belongs to a multigene family, with different genes encoding four α (α_1, α_2, α_3, α_4) and three β isoforms (β_1, β_2, β_3) (Blanco & Mercer, 1998). Expression of these isoforms is tissue-, developmental- and species-specific, suggesting a diversity of isoform-specific functions (Orlowski & Lingrel, 1988; Blanco & Mercer, 1998; Wang *et al.*, 2001). Characterisation of the Na^+,K^+-ATPase isoforms expressed in human skeletal muscle remains incomplete. Furthermore, the effects of acute and chronic exercise, electrical stimulation, and gender on the mRNA and protein expression of these isoforms in skeletal muscle is largely unknown. Finally, the intracellular signals initiating transcriptional

regulation of the Na^+,K^+-ATPase isoforms in skeletal muscle have yet to be investigated. This thesis therefore primarily sought to investigate each of these issues.

Short-term, longitudinal physical training studies have demonstrated an increase in total Na^+,K^+-ATPase content and maximal activity in skeletal muscle, together with an improvement in muscle performance. However cross-sectional studies have been unable to conclusively demonstrate a similar effect. Moreover, the effects of acute exercise and gender on Na^+,K^+-ATPase content and maximal activity in skeletal muscle remain uncertain. The second aim of this thesis was to resolve the apparent controversies regarding the effects of acute and chronic exercise, as well as gender, on the total content and maximal activity of the Na^+,K^+-ATPase.

This thesis comprises a review of literature (Chapter 2) and five experimental studies;

(i) Chapter 3, Study 1: Effects of brief intense exercise on Na^+,K^+-ATPase isoform mRNA and protein expression in human skeletal muscle.

(ii) Chapter 4, Study 2: Prolonged submaximal exercise induces isoform-specific Na^+,K^+-ATPase transcriptional and translational responses in human skeletal muscle.

(iii) Chapter 5, Study 3: Effects of high-intensity, intermittent exercise and training on skeletal muscle Na^+,K^+-ATPase mRNA and protein expression in already well-trained cyclists.

(iv) Chapter 6, Study 4: Chronic endurance training but not gender modulate Na^+,K^+-ATPase mRNA expression, maximal activity and content in human skeletal muscle.

(v) Chapter 7, Study 5: Muscle fibre-type, electrical stimulation and intracellular effects on Na^+,K^+-ATPase mRNA expression in rat skeletal muscle.

This thesis concludes with a general discussion (Chapter 8), conclusions (Chapter 9) and directions for further research (Chapter 10).

CHAPTER 2: LITERATURE REVIEW, AIMS AND HYPOTHESES

This literature review is divided into four sections. Section I briefly describes the events involved in muscle excitation-contraction coupling and explores the roles of ionic transport and membrane excitability in muscle fatigue. Section II characterises Na^+,K^+-ATPase expression and regulation in skeletal muscle, with emphasis on the Na^+,K^+-ATPase isoforms. Section III reviews the effects of physical activity on Na^+,K^+-ATPase adaptability, as well as outlining the potential mechanisms responsible for these effects. Finally, Section IV details the specific aims and hypotheses of each study within this thesis.

SECTION I: MUSCLE CONTRACTION, IONIC REGULATION AND FATIGUE

2.1 Brief overview of excitation-contraction coupling in skeletal muscle

Muscle contraction involves the processes of excitation-contraction coupling, which includes all processes between the generation of an action potential and the shortening of the muscle fibre via cross-bridge cycling. Excitation is initiated by the generation of an excitatory input from the motor nerves, which is transmitted to the synaptic terminal where the neurotransmitter, acetylcholine is released. In skeletal muscle, acetylcholine binds to the nicotinic receptors on the motor end plate (Wessler, 1996), activating the receptors to enable passive ion fluxes. This induces a small depolarisation which activates the voltage-gated Na^+ channels. The subsequent influx of Na^+ induces a large membrane depolarisation, to generate the upstroke of an action potential (Armstrong, 1981). As the membrane potential nears +30 mV, the Na^+ channels become inactivated, and the voltage-gated K^+ channels are activated (Armstrong, 1981). The ensuing efflux of K^+ causes membrane repolarisation, and once the membrane potential reaches –70 mV, the K^+

channels become inactivated (Hocherman & Bezanilla, 1996). Thus, the consequence of an action potential is a cellular Na^+ gain and K^+ loss (Sreter & Woo, 1963), with these changes being magnified with repeated action potentials (Everts *et al.*, 1993). However, the Na^+,K^+-ATPase transports 3 Na^+ ions out of, and 2 K^+ ions back into the cell, exerting an electrogenic effect, to maintain the membrane excitability necessary for subsequent action potential generation. The action potentials originating at the motor end plate spread along the sarcolemma and into the tranverse-tubular (T-tubular) system. An intramembranous T-tubular protein, known as the dihydropyridine receptor (DHPR), senses the voltage change and undergoes a conformational change (Stephenson *et al.*, 1998). This in turn triggers Ca^{2+} release from adjacent sarcoplasmic reticulum (SR) Ca^{2+}-release channels, known as ryanodine receptors (RyR) (Stephenson *et al.*, 1998). The mechanisms responsible for the interaction between the DHPR and RyR are not clearly understood. Studies using isolated single fibres have suggested that interaction of part of the DHPR with the RyR, which may be mediated by the FK506-binding protein (FKBP) (Lamb & Stephenson, 1996), reduces the affinity of magnesium (Mg^{2+}) for the inhibitory site on the RyR (Ritucci & Corbett, 1995). Mg^{2+} competes with ATP and Ca^{2+} to bind to the inhibitory site, in doing so, imposing a strong inhibition on the RyR (Meissner, 1994). A reduction in the affinity of Mg^{2+} for the inhibitory site would induce Mg^{2+} dissociation from the RyR, and at least partial activation of the RyR (Stephenson *et al.*, 1998). The subsequent rise in cytosolic free [Ca^{2+}] ([Ca^{2+}]$_{cyto}$) would increase Ca^{2+} binding to the RyR, leading to a more complete activation of the RyR (Stephenson *et al.*, 1998). The Ca^{2+} released from the SR into the cytoplasm then binds to the protein troponin C and the contraction cycle begins.

The binding of Ca^{2+} to the troponin complex weakens the bond between troponin and actin, enabling the troponin molecule to change position and pull the tropomyosin molecule away from the active sites on the actin filament (Stephenson *et al.*, 1998). This

permits strong binding of the cocked myosin cross-bridge to the actin filament. ATP is then hydrolysed to produce the energy necessary for the myosin filament to slide across the actin filament, thus shortening the sarcomere and eventually, the muscle fibre (Huxley, 2000). The contraction cycle continues as long as there is sufficient free Ca^{2+} to bind to troponin and enough available ATP to be hydrolysed for energy production. The Ca^{2+}-ATPase enzyme, located in the longitudinal tubules of the SR, re-sequesters the Ca^{2+} ions from the cytoplasm, thus lowering $[Ca^{2+}]_{cyto}$ and enabling tropomyosin to move back to its original position, thereby preventing myosin cross-bridge interaction (Stephenson *et al.*, 1998). Muscle contraction ceases in the absence of a nerve impulse at the synaptic terminal.

The focus of this thesis is the Na^+,K^+-ATPase enzyme, which is intimately involved in the membrane excitation processes of muscle contraction. Specifically, Na^+,K^+-ATPase activity is required to maintain the ionic gradients necessary for Na^+ influx and K^+ efflux to generate an action potential. The remainder of this literature review therefore focuses on the Na^+,K^+-ATPase and these electrolytes.

2.2 Roles of ionic transport and membrane excitability in fatigue during muscle contraction

2.2.1 Muscle K^+ fluxes during muscle contraction

In resting skeletal muscle, extracellular $[K^+]$ ($[K^+]_e$) was measured or calculated at around 4.0-4.5 mM (Sjøgaard, 1983; Sjøgaard *et al.*, 1985; Green *et al.*, 2000; Juel *et al.*, 2000b; Nordsborg *et al.*, 2003b), with intracellular $[K^+]$ ($[K^+]_i$) measured at around 145-168 mM (Sahlin *et al.*, 1978; Juel, 1986; Lindinger & Heigenhauser, 1988; Balog & Fitts, 1996).

It was first observed in the 1930's that repeated muscle contractions induce a net muscle K^+ loss (Fenn, 1936). This net muscle K^+ loss during repeated muscle contractions resulted in a reduction in $[K^+]_i$ to 32 and 48 mM in mouse soleus and EDL muscles, respectively

(Juel, 1986), and to 97 mM in frog semitendinosus muscle (Balog & Fitts, 1996). Early human studies investigated the effects of fatiguing knee extensor exercise on both intracellular and extracellular [K^+] (Sjøgaard, 1983; Sjøgaard *et al.*, 1985). They calculated the $[K^+]_e$ based on femoral arterial-venous [K^+] difference and corrected for water shifts, while $[K^+]_i$ was calculated by the difference between muscle [K^+] and $[K^+]_e$ and also corrected for water shifts. Using these calculations, they found that exercise increased muscle $[K^+]_e$ from 4.5 to 6.5 mM, and reduced muscle $[K^+]_i$ from 168 to 129 mM (Sjøgaard *et al.*, 1985). More recent studies have inserted microdialysis probes into the gastrocnemius or vastus lateralis muscles to provide direct measurements of interstitial [K^+] (Green *et al.*, 2000; Nordsborg *et al.*, 2003b). Findings from these studies suggest that the rise in interstitial [K^+] during intense exercise may be much higher, with interstitial [K^+] reaching values of 11-12 mM (Green *et al.*, 2000; Nordsborg *et al.*, 2003b). This loss of K^+ homeostasis indicates that the muscle Na^+,K^+-ATPase cannot keep pace with the exercise-induced K^+-fluxes.

2.2.1.1 Role of muscle K^+ fluxes in fatigue in animal models

Numerous studies have demonstrated the deleterious effects of elevated $[K^+]_e$ on muscle function in animal models. In isolated rat soleus muscle, increasing the $[K^+]_e$ from 4 mM to 10-14 mM induced membrane depolarisation (Cairns *et al.*, 1995; Cairns *et al.*, 1997; Overgaard & Nielsen, 2001), and reduced both twitch (Clausen *et al.*, 1993) and tetanic force (Clausen *et al.*, 1993; Cairns *et al.*, 1995; Cairns *et al.*, 1997; Nielsen *et al.*, 2001; Overgaard & Nielsen, 2001; Pedersen *et al.*, 2003). Studies using mechanically skinned fibres from rat EDL muscle have demonstrated that a reduction in $[K^+]_i$ from 113-126 mM to below 75 mM, reduced twitch (Nielsen *et al.*, 2004b; Pedersen *et al.*, 2004) and tetanic force (Pedersen *et al.*, 2004). Elevated $[K^+]_e$ has also been shown to reduce twitch and tetanic force in isolated frog sartorius muscle (Bouclin *et al.*, 1995), and reduce peak force

amplitude and action potential velocity in isolated mouse muscle, with both effects being greater in the EDL than the soleus muscle (Juel, 1988).

Interestingly, a series of recent studies in isolated rat soleus and EDL muscle (Nielsen *et al.*, 2001; Pedersen *et al.*, 2003; Pedersen *et al.*, 2005), and in mechanically skinned fibres from rat EDL (Pedersen *et al.*, 2004) demonstrated that the deleterious effects of both elevated $[K^+]_e$ and reduced $[K^+]_i$ on tetanic force and membrane excitability were recoverable by inducing intracellular acidosis (Nielsen *et al.*, 2001; Pedersen *et al.*, 2003; Pedersen *et al.*, 2004; Pedersen *et al.*, 2005). They concluded that this effect was mediated by increased chloride permeability (Pedersen *et al.*, 2004; Pedersen *et al.*, 2005).

2.2.1.1.1 Role of reduced $[K^+]_e$ in fatigue in animal models

Whereas repeated muscle contractions induce an increase in $[K^+]_e$, certain diseased states such as K^+-deficiency and hypokalemic periodic paralysis (HPP) result in lowered $[K^+]_e$ (hypokalemia). Two weeks of K^+-deficient fodder reduced plasma $[K^+]$ to only 2.0 mM in rats (Bundgaard & Kjeldsen, 2002), while patients with HPP show a similarly low plasma $[K^+]$, of ~2.2 mM (Lin *et al.*, 2001).

A direct role for hypokalemia in fatigue is seen in patients with HPP, who demonstrate extreme muscle weakness (Lin *et al.*, 2001). Other studies have further investigated this role by experimentally lowering plasma $[K^+]$ using barium (Ba^{2+}), a blocker of K^+ channels. In *in-vitro* mouse and pig skeletal muscle, Ba^{2+} reduced twitch and tetanic force, and in mouse EDL muscle induced a 35 mV depolarisation and increased the duration of a single action potential by reducing the rates of both the rise and fall of the action potential (Gallant, 1983). In isolated rat soleus muscle, Ba^{2+} was also shown to reduce both tetanic force and M-wave area, as well as to induce membrane depolarisation (Clausen & Overgaard, 2000). Thus, the loss of force was thought to reflect the loss of membrane excitability.

2.2.1.2 Role of muscle K^+ fluxes in fatigue during exercise in humans

A role for K^+ fluxes in the development of muscle fatigue has also been suggested in human skeletal muscle. Verburg *et al.*, (1999) measured the time course of net muscle K^+ loss in humans during ~60 min of fatiguing two-legged knee extensor exercise. Net muscle K^+ loss was largest during the first few min of exercise, reached a plateau by 20 min, whilst after 30 min of exercise, an increase in net muscle K^+ loss reappeared. A linear relationship was found between net muscle K^+ loss and force decline during exercise (Verburg *et al.*, 1999).

2.2.2 Muscle Na^+ fluxes during muscle contraction

In resting skeletal muscle, intracellular $[Na^+]$ ($[Na^+]_i$) was measured or calculated at around 7-16 mM (Sjøgaard, 1983; Sjøgaard *et al.*, 1985; Juel, 1986; Balog & Fitts, 1996; Yeung *et al.*, 2003), and extracellular $[Na^+]$ ($[Na^+]_e$) calculated at around 130-135 mM (Sjøgaard, 1983; Sjøgaard *et al.*, 1985).

Repeated muscle contractions induced a rapid rise in $[Na^+]_i$ to 16-23 mM in rodent skeletal muscle (Juel, 1986; Yeung *et al.*, 2003) and to 49 mM in frog semitendinosus muscle (Balog & Fitts, 1996). In humans, intense exercise increased the calculated $[Na^+]_i$ to ~24 mM, but did not significantly alter the calculated $[Na^+]_e$ (Sjøgaard, 1983; Sjøgaard *et al.*, 1985). The authors suggested that the lack of change in $[Na^+]_e$ with repeated muscle contractions was not surprising given the high concentrations of extracellular and interstitial Na^+ (Sjøgaard *et al.*, 1985).

2.2.2.1 Role of muscle Na^+ fluxes in fatigue during stimulation in animal models

2.2.2.1.1 Reduced $[Na^+]_e$

Numerous studies have shown a reduction in muscle function with reduced $[Na^+]_e$, to 25-30 mM. In isolated rat muscle, a reduction in $[Na^+]_e$ from 147 to 30 mM induced a rapid depression in twitch force (Cairns *et al.*, 2003), whilst a reduction in $[Na^+]_e$ from 147 to 25

mM depressed tetanic force (Overgaard *et al.*, 1997; Cairns *et al.*, 2003). In isolated frog skeletal muscle, a 50% reduction in $[Na^+]_e$ decreased both twitch and tetanic force (Bezanilla *et al.*, 1972; Nakajima *et al.*, 1975), and increased the rate of fatigue development during electrical stimulation (Garcia *et al.*, 1991). In isolated rat soleus muscles, fatigability was also greater with reduced $[Na^+]_e$, with a greater decline in peak tetanic force during stimulation (Cairns *et al.*, 2003).

These findings in isolated muscles cannot be extrapolated to those occurring in intact muscle, since extracellular $[Na^+]_e$ is high in intact muscle, and appears to change little with repeated muscle contractions (Sjøgaard, 1983; Sjøgaard *et al.*, 1985). Furthermore, T-tubular $[Na^+]$ is likely to fall during repeated muscle contraction, whilst a reduction in $[Na^+]_e$ may cause a decrease in $[Na^+]_i$ (Overgaard *et al.*, 1997). This finding would contrast the elevated $[Na^+]_i$ seen with repeated muscle contractions (Sahlin *et al.*, 1978; Sjøgaard, 1983; Sjøgaard *et al.*, 1985; Juel, 1986; Balog & Fitts, 1996; Yeung *et al.*, 2003).

2.2.2.1.2 Elevated $[Na^+]_i$

A recent study used carbacholine, an activator of the nicotinic acetylcholine receptors, to investigate the effects of elevated $[Na^+]_i$ on muscle function in isolated rat soleus muscle (Macdonald *et al.*, 2005a). Carbacholine elevated Na^+ influx by 260%, increased intracellular Na^+ content from 15 to 21 μmol.(g wet wt)$^{-1}$, induced a 9 mV depolarisation and reduced tetanic force by 69%. Furthermore, in mechanically skinned fibres from rat EDL muscle, an elevation in $[Na^+]_i$ from 10-20 mM to 30-50 mM reduced the capacity of the T-tubules to support trains of action potentials (Nielsen *et al.*, 2004b).

Thus, both an increase in $[Na^+]_i$ and a reduction in $[Na^+]_e$ results in muscle fatigue via a loss of Na^+ homeostasis and hence, an impaired ability to generate action potentials.

2.2.3 Synergistic muscle Na^+ and K^+ fluxes in fatigue during exercise

The combined effects of increased $[K^+]_e$ and reduced $[Na^+]_e$ have a greater adverse effect on force development than their individual effects. Neither an increase in $[K^+]_e$ from 4 to 9 mM, nor a reduction in $[Na^+]_e$ from 147 to 85 mM affected tetanic force in isolated rat soleus muscle (Overgaard *et al.*, 1999). However, the combination of 9 mM $[K^+]_e$ and 85 mM $[Na^+]_e$ induced a 50% decrease in tetanic force (Overgaard *et al.*, 1999). In frog sartorius muscle, the combined effects on twitch and tetanic force of an increase in $[K^+]_e$ from 3 to 9 mM, and a reduction in $[Na^+]_e$ from 120 to 60 mM, were also greater than the sum of their individual effects (Bouclin *et al.*, 1995).

2.2.4 Water content during exercise

It has been well documented in both animals and humans that within seconds of muscle stimulation, there is an accompanying increase in total muscle water content (Sahlin *et al.*, 1978; Sjøgaard *et al.*, 1985; Lindinger & Heigenhauser, 1988, 1991). The rise in total muscle water content during repeated muscle contractions is typically the result of increases in both the intracellular and extracellular fluid spaces.

2.2.4.1 Water content during stimulation in animal models

In rats, 5 min of intermittent electrical stimulation of the hindlimb increased total muscle water content by 4.9 to 6.1% in the soleus, and red and white gastrocnemius muscles, but did not significantly increase total muscle water content in the plantaris (Lindinger & Heigenhauser, 1988). In cat hindlimb muscle, only 2 min of electrical stimulation increased total water content by 100% immediately post-stimulation, and by 200% at 6 min post-stimulation (Ward *et al.*, 1996). The rise in total water content in both of these studies was predominantly the result of an increase in extracellular water content (Lindinger & Heigenhauser, 1991; Ward *et al.*, 1996).

2.2.4.2 Water content during exercise in humans

Human studies have demonstrated an increase in total water content following exercise in the vastus lateralis muscle (Sahlin *et al.*, 1978; Sjøgaard & Saltin, 1982; Sjøgaard *et al.*, 1985). Intense exercise increased total muscle water content by 12 to 82% (Sahlin *et al.*, 1978; Sjøgaard & Saltin, 1982; Sjøgaard *et al.*, 1985), while submaximal exercise increased total muscle water content by 8% (Sjøgaard *et al.*, 1985). The rise in total muscle water content during intense exercise was predominantly the result of an increase in intracellular water content (Sahlin *et al.*, 1978; Sjøgaard & Saltin, 1982; Sjøgaard *et al.*, 1985), while the rise in total muscle water content during submaximal exercise was predominantly the result of an increase in extracellular water content (Sjøgaard *et al.*, 1985).

2.2.4.3 Implications and mechanisms of water shifts

The water shifts from the plasma to the muscle during repeated muscle contractions have important implications for intracellular and extracellular electrolyte concentrations and hence, also membrane excitability. A small rise in intracellular fluid volume will attenuate the increase in $[Na^+]_i$, and amplify the decrease in $[K^+]_i$ that occur during repeated muscle contractions. Similarly, an elevation in extracellular fluid volume will attenuate the increase in $[K^+]_e$, and amplify the decrease in $[Na^+]_e$. Thus, the deleterious effects of altered K^+ and Na^+ fluxes for membrane potential would be lessened. However, this effect appears to only be small since repeated muscle contractions are associated with membrane depolarisation (Section 2.2.5).

The mechanisms responsible for increases in intracellular fluid volume remain inconclusive. Maximal exercise reduced plasma volume by 10 to 20%, suggesting filtration of water from the vascular bed to the interstitial space (Sjøgaard *et al.*, 1985). Filtration may occur via increased intramuscular capillary pressure, as well as via an elevation in the

number of perfused capillaries (Sjøgaard *et al.*, 1985). In contrast, electrical stimulation of cat skeletal muscle was associated with a reduction in interstitial fluid volume by 1 ml.100 g^{-1} (Ward *et al.*, 1996). A second mechanism potentially explaining the exercise-induced increase in intracellular fluid space relates to osmotic effects of the intracellular accumulation of lactate (Lac^-) and inorganic phosphate (Pi), and the depletion in creatine phosphate (CrP). These changes would result in an osmotic gradient, causing water to diffuse from the extracellular space into the intracellular space. Of these osmolytes, CrP appears the most likely to be responsible for cell swelling, as the intracellular depletion of CrP begins at the onset of exercise and parallels the rapid increase in intracellular fluid space (Sejersted & Sjøgaard, 2000). As discussed below, water content also appears to be regulated by the Na^+,K^+,$2Cl^-$ co-transporter.

2.2.5 Role of the Na^+,K^+,$2Cl^-$ co-transporter in active Na^+ and K^+ transport and cell volume

While the Na^+,K^+-ATPase represents the primary contributor to active Na^+ and K^+ transport in skeletal muscle, there is accumulating evidence that the Na^+,K^+,$2Cl^-$ co-transporter is also important, albeit to a lesser degree, for the active transport of these ions (Wong *et al.*, 1999). In isolated rat soleus muscle, ~15% of $^{86}Rb^+$ uptake (a tracer for K^+ uptake) was both sensitive to bumetanide, an inhibitor of the Na^+,K^+,$2Cl^-$ co-transporter and insensitive to ouabain, an inhibitor of the Na^+,K^+-ATPase. Similar findings were also seen in *in-situ*, vascularly perfused rat hindlimb muscle, a preparation which mimics *in-vivo* conditions (Lindinger *et al.*, 2002). Furthermore, and characteristic of the Na^+,K^+,$2Cl^-$ co-transporter, incubation in Cl^- and Na^+-free medium abolished ~40 and 20% of the ouabain-insensitive component of $^{86}Rb^+$ uptake, respectively (Wong *et al.*, 1999).

The Na^+,K^+,$2Cl^-$ co-transporter has also been shown to regulate cell volume, with bumetanide increasing water loss from perfused rat hindlimb muscle (Lindinger *et al.*,

2002). This regulation appears to involve osmolality-dependent activation of the Na^+,K^+,2Cl^- co-transporter. In L6 rat skeletal muscle cells, hyperosmolality reduced cell volume by 40% and increased activity of the Na^+,K^+,2Cl^- co-transporter by 200% (Zhao *et al.*, 2004).

2.2.6 Muscle membrane potential during muscle contraction

Resting membrane potential is predominantly influenced by the transmembrane [Na^+] and [K^+] gradients, and by K^+ permeability (Sejersted & Sjøgaard, 2000). A single action potential is associated with an entry of ~3-6 nmol Na^+.(g wet wt)$^{-1}$ (Gissel & Clausen, 2000), and a leak of ~2 nmol K^+.(g wet wt)$^{-1}$ (Hallen *et al.*, 1996). At rest, these passive Na^+ and K^+ fluxes are counteracted by Na^+,K^+-ATPase activity to maintain the membrane potential at approximately -80 mV (Sejersted & Sjøgaard, 2000). However, repeated muscle contractions are associated with a net muscle Na^+ gain and a net muscle K^+ loss, and altered transmembrane [Na^+] and [K^+] gradients, resulting in membrane depolarisation. Electrical stimulation has been shown to induce an 8-14 mV membrane depolarisation in perfused rat hindlimb muscle (Lindinger & Heigenhauser, 1988, 1991). Even larger stimulation-induced membrane depolarisations, of 12-20 mV, have been found in isolated mouse soleus and EDL muscles (Juel, 1986) and also in frog toe muscle (Westerblad & Lannergren, 1986; Balog *et al.*, 1994).

In humans, based on the calculations from $[K^+]_i$, $[Na^+]_i$, $[K^+]_e$ and $[Na^+]_e$, 6 min of intense knee extensor exercise induced a 15 mV membrane depolarisation in the vastus lateralis muscle (Sjøgaard *et al.*, 1985).

2.2.6.1 Role of membrane excitability in fatigue during stimulation in animal models

Three studies have shown a direct link between membrane excitability and muscle function in animal models. During electrical stimulation of isolated rat soleus muscle, a linear correlation was found between M wave area and tetanic force (Overgaard *et al.*, 1999),

while the reduction in tetanic force was associated with decreases in deep M wave amplitude and area (Harrison & Flatman, 1999). In frog semitendinosus muscle, high-frequency stimulation was associated with a reduction in force and a 9 mV depolarisation (Balog *et al.*, 1994).

2.2.6.2 Membrane excitability in fatigue during exercise in humans

In humans, numerous studies have implicated failure of action potential propagation in the development of fatigue, during intense dynamic knee extensor exercise (Sjøgaard, 1983; Sjøgaard *et al.*, 1985), isometric knee extensor exercise (Bellemare & Garzaniti, 1988; Fowles *et al.*, 2002b; Hamada *et al.*, 2003), drop-jumps on a sledge-gliding apparatus (Strojnik & Komi, 1998), and during electrical stimulation (Milner-Brown & Miller, 1986). In contrast, 90 min of cycling was not associated with any measurable change in either M wave area, duration or amplitude (Sandiford *et al.*, 2004).

Some studies have concluded that the alterations in membrane potential and action potential characteristics are not of sufficient magnitude to account for the large reductions in force (Balog *et al.*, 1994; Posterino *et al.*, 2000). Furthermore, no relationship was found between force and intramuscularly recorded M wave amplitude during maximal isometric exercise in humans (Bigland-Ritchie *et al.*, 1982).

In summary, changes in transmembrane $[Na^+]$ and $[K^+]$ gradients have important implications for the development of muscle fatigue during exercise and electrical stimulation. In skeletal muscle, Na^+,K^+-ATPase activity maintains these $[Na^+]$ and $[K^+]$ gradients to protect muscle excitability and contractility. The expression and regulation of the skeletal muscle Na^+,K^+-ATPase will be reviewed in Sections II and III.

SECTION II: Na^+,K^+-ATPase IN SKELETAL MUSCLE

2.3 Introduction

In 1997, Danish researcher Jens C. Skou shared the Nobel Prize in Chemistry for his 1957 discovery of the Na^+,K^+-ATPase enzyme. Prior to his discovery, an active Na^+ and K^+ transport had been hypothesised (Dean, 1941). However, Skou was the first to propose a link between active Na^+ and K^+ transport across the sarcolemma, mediated by an ATP- or energy-requiring pump. Since 1957, our understanding of the Na^+,K^+-ATPase has substantially progressed. In skeletal muscle, the Na^+,K^+-ATPase has major regulatory functions, including maintenance of intracellular to extracellular [Na^+] and [K^+] gradients, membrane excitability and contractility, as well as secondary transport processes (Blanco & Mercer, 1998). This section focuses on the structure, synthesis, activation and long-term regulation of the Na^+,K^+-ATPase, as well as the expression and functional properties of the isoforms of the Na^+,K^+-ATPase in skeletal muscle.

2.4 Structure, location and synthesis of Na^+,K^+-ATPase in skeletal muscle

2.4.1 Na^+,K^+-ATPase structure

The Na^+,K^+-ATPase comprises 2 polypeptide chains; a catalytic α subunit and a glycosylated β subunit. The α and β subunits form a functional $\alpha\beta$ complex, which is the minimal functional Na^+,K^+-ATPase unit (Lingrel *et al.*, 1992; Levenson, 1994). The α subunit (100-112 kDa) carries the functional properties of the Na^+,K^+-ATPase; hydrolysing ATP, binding to and transporting cations, and binding cardiac glycosides, as well as being phosphorylated (Lingrel *et al.*, 1992). The β subunit (40-60 kDa) is necessary for the structural and functional maturation of the α subunit, as well as for localisation to the sarcolemma (Ackermann & Geering, 1990; Noguchi *et al.*, 1990). The Na^+,K^+-ATPase belongs to a multi-gene family, with different genes encoding four α (α_1, α_2, α_3, α_4) and three β (β_1, β_2, β_3) isoforms (Blanco & Mercer, 1998). Expression of these isoforms is

tissue-, developmental- and species-specific, suggesting a diversity of isoform-specific functions (Orlowski & Lingrel, 1988; Blanco & Mercer, 1998; Wang *et al.*, 2001).

The possible transmembrane orientation and structure of the Na^+,K^+-ATPase α and β subunits have been determined by analysis of the amino acid sequences, site-specific labelling and studies on antibody reactivity (Geering, 1991; Blanco & Mercer, 1998). A schematic representation of the structure of the Na^+,K^+-ATPase α and β subunits is shown in Figure 2.1. The α-subunit consists of an NH_2-terminal with 4 segments (M1-M4), as well as a carboxy-terminal region with 6 segments (M5-M10). Both the NH_2- and carboxy-terminals of the α-subunit are exposed to the cytoplasm which has a large domain, consisting of ~1/3 of the polypeptide (Blanco & Mercer, 1998). Originally, it was considered that all 10 segments were transmembrane spans. Recent studies employing site-directed mutagenesis combined with immunochemical analyses in rat brain, however, have identified only 6 transmembrane (M1-M5, M10) and 4 sarcolemmal-embedded domains (M6-M9) (Figure 2.1) (Xie *et al.*, 1996). The α-subunit contains the binding sites for ligands such as Na^+, K^+, Mg^{2+}, ATP and Pi, which are located in the cytoplasmic middle region, as well as for cardiac glycosides such as ouabain and digoxin, located between the transmembrane segments M1 and M2 (Figure 2.1) (Arystarkhova *et al.*, 1992). More recent work suggests that binding of ouabain to the α subunit only occurs in the presence of the transmembrane segments M5 and M6 (Koenderink *et al.*, 2000).

The β-subunit is a type II glycoprotein, characterised by a single transmembrane spanning segment, a short NH_2-terminal exposed to the cytoplasmic side, and a large carboxy-terminal endodomain that comprises several *N*-glycosylation sites and three highly conserved disulfide bridges. Glycosylation is a prominent feature of the β-subunit and as

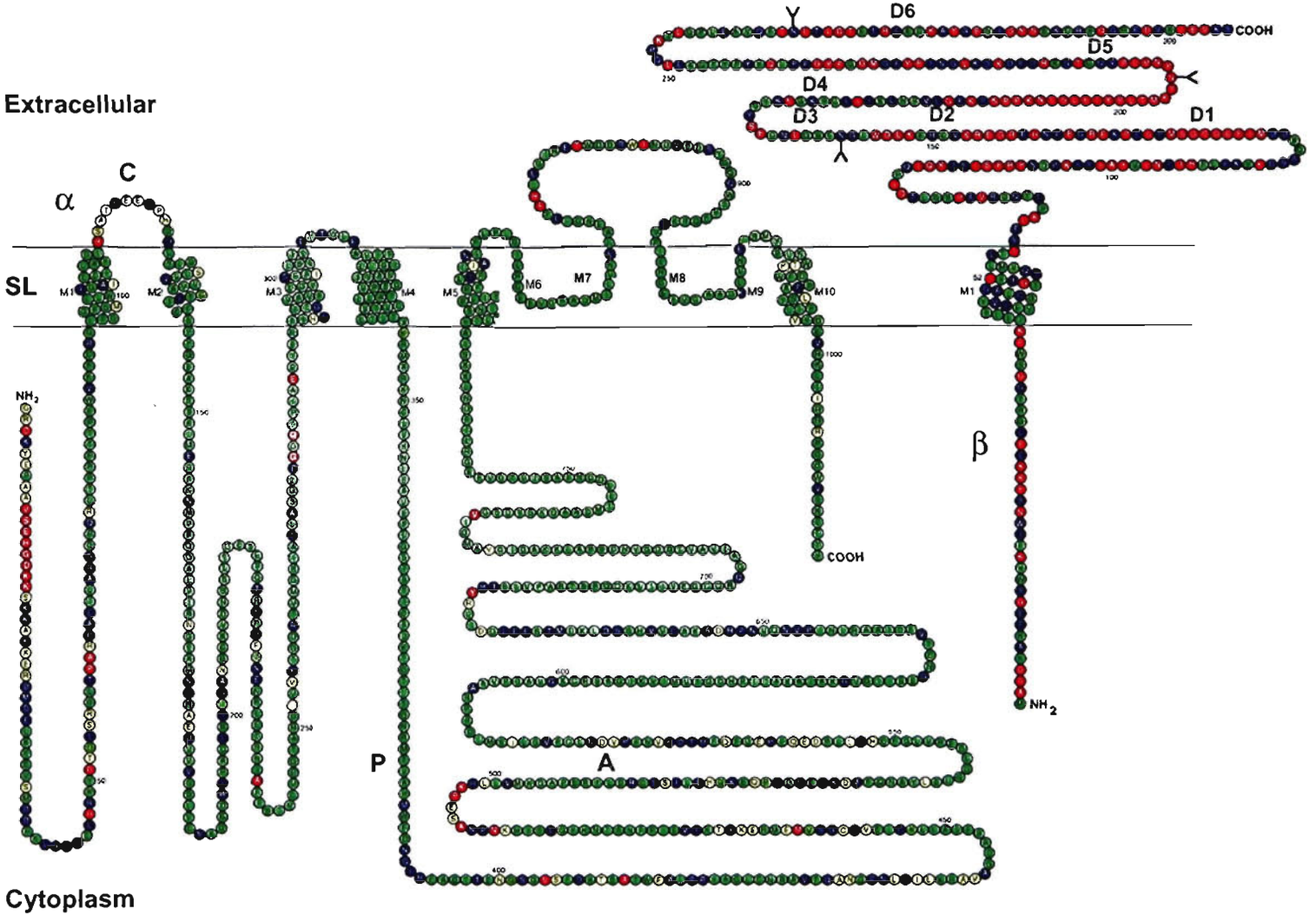


Figure 2.1 Schematic representation of the membrane topology of the Na^+,K^+-ATPase α and β subunits, with amino acid sequence of the rat α_1 and β_1 isoforms (modified from Blanco & Mercer, 1998). SL, sarcolemmal membrane; C, cardiac glycoside binding site; P, phosphorylation site; A, Adenine nucleotide binding site; **Y**, glycosylation sites; D1-6, disulfide bridges (located from *Xenopus* oocytes).

such, the apparent molecular mass on SDS-PAGE of 40-70 kDa is greater than predicted from the core peptide (Chow & Forte, 1995). Each β-subunit isoform is heavily glycosylated; the mammalian β_1 isoform has 3 *N*-linked glycosylation sites, while the number of glycosylation sites for the β_2 isoform is species-dependent (Tamkun & Fambrough, 1986; Kirley, 1989). The human and rat β_2 isoform has eight and seven sites for glycosylation, respectively (Blanco & Mercer, 1998). Although glycosylation does not mediate Na^+,K^+-ATPase transport (Tamkun & Fambrough, 1986; Zamofing *et al.*, 1989; Beggah *et al.*, 1997), it is important for protein folding as mutations of all glycosylation sites on the β subunit attenuated assembly with the α subunit, and increased sensitivity toward proteolysis (Beggah *et al.*, 1997). A second distinctive feature of the Na^+,K^+-ATPase β subunit structure involves the presence of 6 disulfide bridges (Beggah *et al.*, 1997), although the location of these bridges has not been determined for the human β isoform. The disulfide bridges may be required for enzyme function and proper assembly of the αβ complex, since elimination of each one of these bridges structurally altered the β subunit in a way reflected by increased protease sensitivity (Beggah *et al.*, 1997). In particular, the two disulfide bridges closest to the carboxy-terminal may be important in α and β subunit assembly, since mutants deficient in each bridge were unable to at least even partially form stable and functional αβ complexes (Beggah *et al.*, 1997).

A third Na^+,K^+-ATPase protein that was originally considered a contaminant of purification, the γ subunit (~6.5 kDa), has been identified through co-immunoprecipitation with αβ complexes (Forbush *et al.*, 1978; Mercer *et al.*, 1993). Expression of the γ subunit is tissue-specific; being detected in the kidney, pancreas and fetal liver, but not in skeletal muscle (Mercer *et al.*, 1993). The γ subunit is one of seven members of the FXYD protein family, characterised by one transmembrane spanning domain and a signature sequence

that contains the FXYD motif and 3 other conserved amino acids (Sweadner & Rael, 2000). Several members of the FXYD family, including the Na^+,K^+-ATPase γ subunit, play a tissue-specific role in Na^+,K^+-ATPase regulation (Beguin *et al.*, 1997; Pu *et al.*, 2001; Beguin *et al.*, 2002; Crambert *et al.*, 2002). Specifically, the Na^+,K^+-ATPase γ subunit has been shown to modulate the apparent affinity of the Na^+,K^+-ATPase for each of K^+, Na^+ and ATP (Beguin *et al.*, 1997; Arystarkhova *et al.*, 1999; Pu *et al.*, 2001).

The γ subunit consists of a single, hydrophobic transmembrane domain of 19 amino acids, as well as an intracellular and extracellular domain (Mercer *et al.*, 1993). The γ subunit is a type I transmembrane protein, characterised by an extracellular NH_2-terminal and a cytoplasmic carboxy-terminal (Jones *et al.*, 1997). In contrast to the β subunit, the γ subunit contains no sites for *N*-linked glycosylation (Mercer *et al.*, 1993). An interesting feature of the γ subunit structure is that it is detected as two species of similar amino acid composition (Mercer *et al.*, 1993). Contrary to initial belief, the two bands, referred to as γ_a (7.184 kDa) and γ_b (7.354 kDa) are the products of two distinct RNA messages and only differ in their NH_2 termini, with 6 residues for γ_a and 7 residues for γ_b (Kuster *et al.*, 2000). The γ subunit can only interact with assembled αβ complexes, which is required for stable expression of this subunit (Beguin *et al.*, 1997).

2.4.2 Location and quantification in skeletal muscle

2.4.2.1 Quantification of Na^+,K^+-ATPase and location of Na^+,K^+-ATPase enzymes

2.4.2.1.1 Sarcolemmmal Na^+,K^+-ATPase

Na^+,K^+-ATPase quantification is most commonly measured by [^{3}H]-ouabain binding, since ouabain is specific for the Na^+,K^+-ATPase and binds to the extracellular surface of the α subunit with a 1:1 stoichiometry (Hansen, 1984). Based on the early assumption that [^{3}H]-ouabain binding measured Na^+,K^+-ATPase enzymes of sarcolemmal origin, sarcolemmal Na^+,K^+-ATPase density was estimated as being approximately 3,350 molecules/μm^2 in the

soleus muscle of 4 wk old rats (Clausen & Hansen, 1974) and 2,500 molecules/μm^2 in frog sartorius muscle (Venosa & Horowicz, 1981). However, these values are probably vastly overestimated since T-tubule [^{3}H]-ouabain binding site content may be similar to that in the sarcolemma.

2.4.2.1.2 T-tubule Na^+,K^+-ATPase

The first evidence of Na^+,K^+-ATPase localisation in the T-tubules was found when single fibres from frog semitendinosus muscle were used to isolate the T-tubules via removal of the sarcolemma (Costantin & Podolsky, 1967). Using this technique, the T-tubules immediately re-sealed and displayed normal contractile response, indicating normal T-tubule excitability. Furthermore, the contractile response observed in skinned fibres was abolished when ouabain was added before the removal of the sarcolemma to enable access to the T-tubule lumen (Costantin & Podolsky, 1967). These findings provided good evidence for T-tubule Na^+,K^+-ATPase localisation.

Direct evidence for Na^+,K^+-ATPase localisation in the T-tubules was established by [^{3}H]-ouabain binding in isolated T-tubules of rabbit sacrospinalis muscle (Lau *et al.*, 1979). In that study, quantitation of Na^+,K^+-ATPase density in the T-tubules via [^{3}H]-ouabain binding was estimated at 180 sites/μm^2. This was more than an order of magnitude lower than that found in the sarcolemma (Clausen, 2003). In frog sartorius muscle, quantitation of T-tubule Na^+,K^+-ATPase content was attempted by comparing [^{3}H]-ouabain binding sites in normal muscles versus those pre-treated with glycerol, which disrupts the connection between the sarcolemma and T-tubules (Venosa & Horowicz, 1981). In muscles pre-treated with glycerol, [^{3}H]-ouabain binding site content was only 20% of that in untreated muscles. However, since the T-tubules represent a surface area 4-5 times greater than that of the sarcolemma, T-tubule Na^+,K^+-ATPase content was calculated to be only 4 to 5% of that at the sarcolemma. These values were considered too low since

glycerol treatment does not cause complete disruption of the T-tubule and sarcolemmal connections (Clausen, 1986). A subsequent study in frog skeletal muscle proved the hypothesis correct, with similar results obtained for [^{3}H]-ouabain binding in the T-tubules and sarcolemma (Jaimovich *et al.*, 1986). Therefore, at least in frog skeletal muscle, T-tubule Na^+,K^+-ATPase content may be similar to that in the sarcolemma.

2.4.2.1.3 Intracellular Na^+,K^+-ATPase

An intracellular pool of Na^+,K^+-ATPase has also been reported, with immuno-detection of Na^+,K^+-ATPase isoforms in an intracellular membrane fraction, that was obtained via differential centrifugation on a sucrose gradient (Section 2.5). However, the intracellular fraction suffers from a very low protein yield, as well as the presence of T-tubule and SR protein markers, preventing definitive confirmation of an intracellular Na^+,K^+-ATPase pool (see Section 2.5).

2.4.2.1.4 Quantification of Na^+,K^+-ATPase in human muscle

Using [^{3}H]-ouabain binding analysis, human vastus lateralis muscle Na^+,K^+-ATPase content mainly ranges from 260 to 340 pmol.(g wet wt)$^{-1}$ (Clausen, 1990; Nielsen & Clausen, 2000; Clausen, 2003), although individual values range from ~160 pmol.(g wet wt)$^{-1}$ (Schmidt *et al.*, 1994) to the highest reported values of ~560 pmol.(g wet wt)$^{-1}$ found in elite male alpine skiers (Medbø *et al.*, 2001).

2.4.2.2 Quantification of maximal Na^+,K^+-ATPase activity

Skeletal muscle maximal Na^+,K^+-ATPase activity can be quantified via the measurement of K^+-dependent phosphatase activity, which is an enzyme performing a partial reaction of the Na^+,K^+-ATPase. The K^+-dependent phosphatase assay can use either *p*-nitrophenyl phosphate (*p*-NPP) (Hundal *et al.*, 1994) or 3-*O*-methylfluorescein phosphate (3-*O*-MFP) (Fraser & McKenna, 1998) as substrates, and *p*-NPP and 3-*O*-MFP can attain complete membrane and enzyme recovery (Hansen & Clausen, 1996). The 3-*O*-MFPase assay is

currently the preferred method of the two as 50-100 times less tissue is required than that for the *p*-nPP assay (Nørgaard, 1986). The specificity of this assay for the Na^+,K^+-ATPase is also demonstrated by its complete inhibition by ouabain (Nørgaard *et al.*, 1984b; Fraser & McKenna, 1998; Fowles *et al.*, 2004).

Studies employing the 3-*O*-MFPase assay have reported mean results for maximal Na^+,K^+-ATPase activity in rat muscle homogenates ranging from 250 to 765 $nmol.min^{-1}.(g\ wet\ wt)^{-1}$ (Nørgaard *et al.*, 1984b; Fowles *et al.*, 2002a; Fowles *et al.*, 2004), depending on muscle fibre-type composition (Section 2.5.3), age (Section 2.5.4) and possibly training status (Section 2.11.2).

In human vastus lateralis muscle, mean values for maximal 3-*O*-MFPase activity have ranged from 220 to 290 $nmol.min^{-1}.(g\ wet\ wt)^{-1}$ (Fraser & McKenna, 1998; Fraser *et al.*, 2002; Leppik *et al.*, 2004; Aughey *et al.*, 2005).

2.4.3 Na^+,K^+-ATPase synthesis

2.4.3.1 Overview of protein synthesis

This section provides an overview of non-specific protein synthesis. With any protein, synthesis begins with cellular stress. Application of a stimulus, referred to as a transcription factor, increases cellular demand for specific proteins in order to more effectively cope when the particular stimulus is re-applied. Initiation of transcription involves binding of the RNA polymerase II enzyme (RNAP II) to the promoter region of a gene (Orphanides & Reinberg, 2002). Transcription then occurs as RNAP II clears the promoter region and moves 5' to 3' along the gene, promoting hydrogen bonds between the nitrogenous bases of the gene and the complementary nucleotides present in the nucleoplasm. By facilitating the binding together of the nucleotides, RNAP II generates an mRNA transcript. Once RNAP II reaches a stop codon on the gene, the mRNA transcript detaches and transcription is subsequently terminated. The mRNA transcript then

undergoes 3 conformational changes to enable its transportation from the nucleus; including the splicing out of nonsense introns and the subsequent splicing together of the remaining encoded exons, the addition of a cap structure at the 5' terminus to prevent attack from nucleases and to facilitate mRNA translational efficiency (Dever, 2002; Orphanides & Reinberg, 2002), and the generation of a 3' end via the addition of a poly(A) tail (polyadenylation) (Proudfoot *et al.*, 2002). The mRNA transcript is then moved from the nucleus to the cytoplasm via interactions between mRNA- and membrane-bound proteins of the nucleus and nucleare pores, respectively (Reed & Hurt, 2002). Once in the cytoplasm, the mRNA transcript binds a ribosomal complex, comprising a light and heavy subunit. Initiation factors then bind to the transcript and subsequently recognise the cap structure, after which translation begins. Translation involves the attachment of numerous transfer RNA (tRNA), each binding specific amino acids. Enzymes transfer the amino acids from the tRNA to the mRNA transcript, producing a specific amino acid sequence (Ramakrishnan, 2002). Once the stop codon at the end of the mRNA transcript is reached, the ribosomal complex detaches, leaving an intact mRNA transcript to reproduce translation, and a completed polypeptide. Thus, translation of mRNA is tightly regulated to ensure that only the appropriate proteins are synthesised.

2.4.3.2 Na^+,K^+-ATPase synthesis

Synthesis of the Na^+,K^+-ATPase is represented in Figure 2.2. Na^+,K^+-ATPase synthesis begins with the subunit-specific genes, which are encoded with DNA information specific to the respective subunit. The α and β genes are transcribed independently into RNA's, processed into mRNA, and translated into nascent subunit proteins. The next step in synthesis involves translocation of nascent subunit proteins into the SR, which is achieved via interaction with a signal recognition particle (SRP). Nascent subunit proteins contain a signal sequence, located at their N-terminal, which designates the subunit for SR-targeting

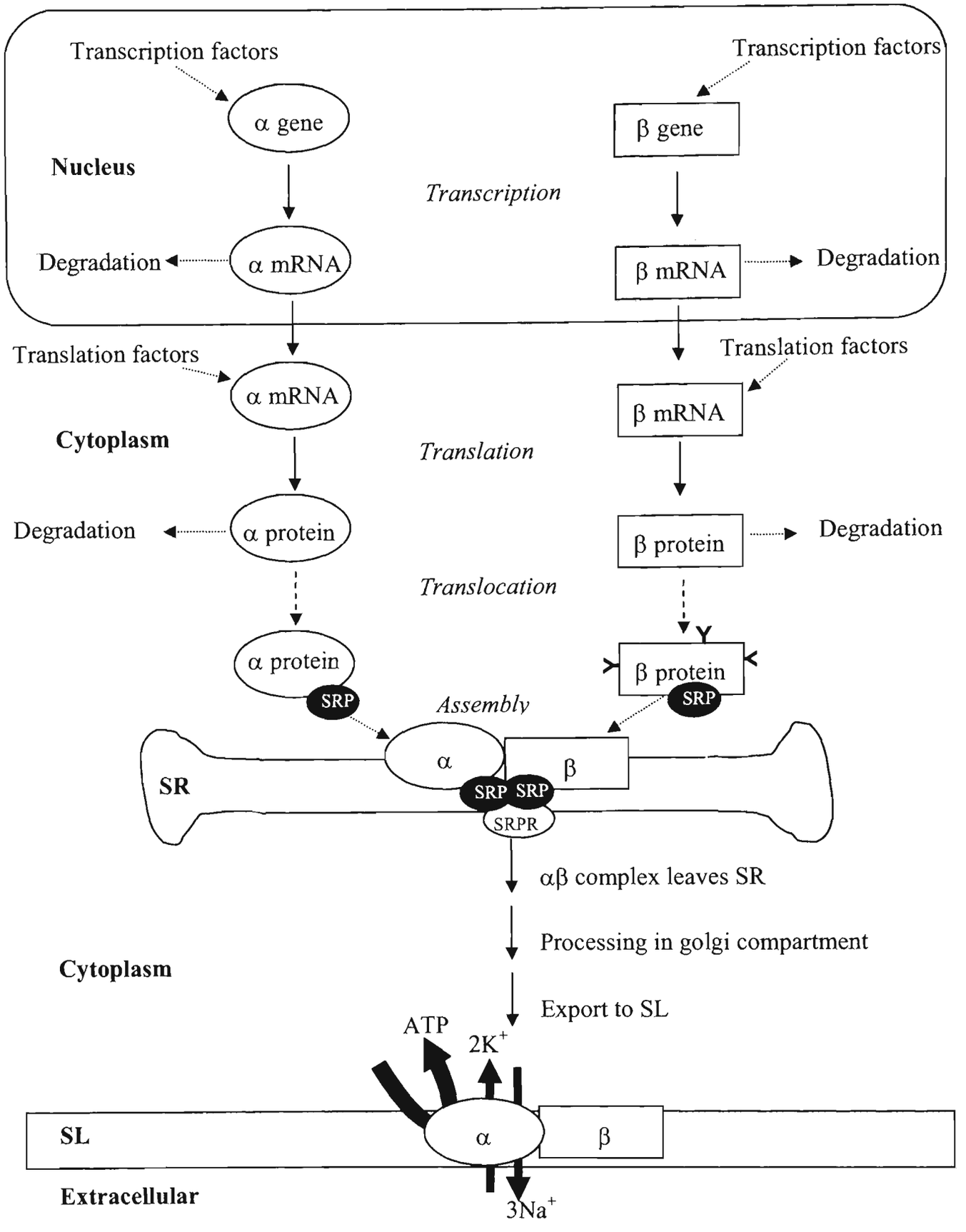


Figure 2.2 Na^+,K^+-ATPase synthesis, membrane insertion and function from the cell nucleus to the sarcolemma. Adapted from McDonough *et al.*, 1992. SR, sarcoplasmic reticulum; SL, sarcolemmal membrane; SRP, signal recognition particle; SRPR, signal recognition particle receptor; **Y**, glycosylation.

(Bovia & Strub, 1996). The SRP interacts directly and transiently with the subunits to discern candidates for SR-targeting. Following recognition of the candidate subunits, SRP binds to the signal sequence, which transiently inhibits translation. Subunits naturally pause at certain sites in mRNA translation and SRP-binding inhibits translation by enhancing the pausing of subunits at these specific sites (Bovia & Strub, 1996). Translocation of the subunit-SRP complex into the SR occurs when SRP interacts with the SRP receptor (SRPR) in the SR membrane. Dissociation of the SRP-SRPR complex from the subunit releases inhibition of translation, allowing protein synthesis to resume at normal speed. Before being allowed to cross the SR membrane, subunits are subjected to a second signal sequence recognition (Jungnickel & Rapoport, 1995). The second recognition event is thought to control efficiency of protein translocation (Belin *et al.*, 1996). Insertion of the Na^+,K^+-ATPase β subunit into the SR membrane is SRP-dependent and has been shown to be co-translational with glycosylation (Kawakami & Nagano, 1988). However, Na^+,K^+-ATPase α subunit membrane insertion remains controversial for a number of reasons. Firstly, Hiatt *et al.*, (1984) proposed that in contrast with the β subunit, the α subunit is initially synthesised on free ribosomes in the cytoplasm and then inserted post-translationally into the SR as a soluble protein. Two yrs later, Caplan *et al.*, (1986) reported that the soluble protein of the α subunit was in fact an artefact of cell homogenisation procedures, suggesting that the α subunit may also be inserted co-translationally. The second controversy surrounding α subunit insertion into the SR involves the SRP-SRPR interaction. Insertion of multi-membrane spanning proteins is complex and often involves functions of the hydrophobic domains alternating as signals and stop-transfer sequences (Wessels & Spiess, 1988). It was initially considered that in multi-membrane spanning proteins, only the first hydrophobic domain needs to be a signal sequence interacting with SRP (Wessels & Spiess, 1988). The following year, it was

suggested that at least two signal sequences are contained within the four transmembrane segments from the NH_2 terminal (Homareda *et al.*, 1989). A more recent study provided an even more fascinating model for α subunit insertion involving alternate initiation and termination of translocation (Beguin *et al.*, 1998). While the first hydrophobic domain, M1, targeted the ribosome to the SR membrane and initiated translocation of the COOH-terminal region, M2, terminated translocation. The second cycle of translocation was initiated by M3 and terminated by M4. The third cycle of translocation predominantly depended on M6 since M5 was an inefficient anchor due to its presence of polar amino acids (Beguin *et al.*, 1998). Packaging, and subsequently translocation of M7 and M8 was facilitated by the β subunit, while the translocation of M9 and M10 depended predominantly on M10 terminating translocation (Beguin *et al.*, 1998). The α subunit only achieved correct membrane insertion when the β subunit was present and acting as a receptor or stabiliser (Noguchi *et al.*, 1990; Beguin *et al.*, 1998). In the absence of β mRNA injection into *Xenopus* oocytes, the α subunit was found to be inserted in an aberrant fashion and not to exhibit any Na^+,K^+-ATPase activity (Noguchi *et al.*, 1990).

The post-translational assembly of the α and β subunits is required for several functions. Firstly, formation of the complex induced a structural change in the α subunit, going from a state that was highly resistant to the digestive enzyme, trypsin pre-complex, to becoming a post-complex trypsin-resistant subunit (Ackermann & Geering, 1990). Secondly, as indicated by a significantly longer enzymatic half-life, integration with the β subunit in the SR prevented the degradation of the α subunit, at least in *Xenopus* oocytes (Ackermann & Geering, 1990). Finally, assembly enabled both subunits to leave the SR. Ackermann and Geering (1990) found that β subunits injected with β cDNA alone remain in their core glycosylated form and thus, confined to the SR for up to 21 h, whereas β subunits injected with both α and β cDNA progress to their fully glycosylated state within 12 h. Once

assembled, the αβ complex can leave the SR, be processed in the golgi compartment and then exported to co-appear at the sarcolemma (Hiatt *et al.*, 1984; McDonough *et al.*, 1990). Passage through the golgi compartment is rapid, requiring only 45-60 min (Tamkun & Fambrough, 1986), while a similar time course is reported for newly synthesised Na^+,K^+-ATPase enzymes to then appear at the sarcolemma (Caplan *et al.*, 1990; Mircheff *et al.*, 1992). Complete recruitment of newly synthesised Na^+,K^+-ATPase enzymes to appear at the sarcolemma occurred within 120 min in canine kidney cells (Mircheff *et al.*, 1992). In chick skeletal muscle cells, there was a 200% increase in the number of Na^+,K^+-ATPase enzymes after 12-18 h treatment with veratridine, an activator of the voltage-gated Na^+-channels (Wolitzky & Fambrough, 1986), whilst in chick sensory neurons, only 55 to 80% Na^+,K^+-ATPase enzymes appear at the sarcolemma after 20 h (Tamkun & Fambrough, 1986).

2.5 Na^+,K^+-ATPase isoform expression in skeletal muscle

A summary of the known expression and properties of the Na^+,K^+-ATPase isoforms in animal and human skeletal muscle, is shown in Tables 2.1 and 2.2.

2.5.1 Difficulties associated with measuring isoform expression

Difficulties and inconsistencies in membrane fractionation and immunoblotting procedures, including the lack of quality monoclonal antibodies, have contributed to the current controversy regarding Na^+,K^+-ATPase isoform expression and localisation in both rat and human skeletal muscle. Isoform localisation techniques combine membrane isolation via differential centrifugation on a sucrose gradient (Hundal *et al.*, 1992; Lavoie *et al.*, 1996; Tsakiridis *et al.*, 1996) or via the formation of giant sarcolemmal vesicles (Juel *et al.*, 2000a; Juel *et al.*, 2001), with immunoblotting using antibodies specific to each Na^+,K^+-ATPase isoform. Although enhanced purification is achieved, these isolation techniques typically yield very low Na^+,K^+-ATPase recoveries, of only 0.2 to 8.9%

Table 2.1 Known expression and properties of Na^+,K^+-ATPase isoforms in rat and mouse skeletal muscle

Isoform	Location and (abundance, %)			Apparent affinities				Specific functions
	SL	IM	TT	Ouabain (K_d, nM)	Na^+ (K_{Na^+}, mM)	K^+ (K_{K^+}, mM)	ATP (K_{ATP}, mM)	
α_1	Yes (91)	Yes (9)		1600	1.2	0.5	0.4	↑ tetanic force, maintain resting E_m, $[Na^+]$, $[K^+]$ gradients (mouse)
α_2	Yes (25)	Yes (75)	Yes (?)	37	1.1	0.4	0.5	↑ muscle relaxation, indicating ↑ Ca^{2+} uptake (mouse)
α_3				1.6	3.1	0.3	0.2	
β_1	Yes (25)	Yes (75)						
β_2	Yes (9)	Yes (91)						
β_3								

Blank, not measured; SL, sarcolemmal membrane fraction; IM, intracellular membrane fraction; TT, T-tubule fraction; ?, unknown; K_d, dissociation constant; E_m, membrane potential. Data taken from He *et al.*, 2001; Hundal *et al.*, 1992; Hundal *et al.*, 1994; Jewell & Lingrel, 1991; Lavoie *et al.*, 1995; O'Brien *et al.*, 1994; Radzyukevich *et al.*, 2004.

Table 2.2 Known expression and properties of Na^+,K^+-ATPase isoforms in human skeletal muscle

Isoform	Location and (abundance, %)			Apparent affinities		
	SL	IM	TT	Ouabain (K_d, nM)	Na^+ ($K_{0.5}$ Na^+, mM)	K^+ ($K_{0.5}$ ATP, mM)
α_1	Yes (96)	Yes (4)		4.9	8.3	0.9
α_2	Yes (57)	Yes (43)	Yes (?)	22.0	12.8	1.3
α_3	Yes (90)	Yes (10)		4.6	24.7	0.9
β_1	Yes (75)	Yes (25)		4.9		0.9
β_2				5.2		1.2
β_3				7.0		1.1

Blank, not measured; SL, sarcolemmal membrane fraction; IM, intracellular fraction; TT, T-tubule fraction; ?, unknown. K_d, dissociation constant. Data taken from Crambert *et al.*, 2000; Hundal *et al.*, 1994; Nielsen *et al.*, 2003.

(Hansen & Clausen, 1988). Such low recoveries raise the possibility of large potential errors in extrapolating these results to the whole muscle Na^+,K^+-ATPase population. Additionally, such small recoveries may be influenced by inclusion of Na^+,K^+-ATPase isoforms that originated from contamination by other tissues such as nervous vascular tissue, adipocytes or fibrocytes (Hansen & Clausen, 1996). Membrane fractionation techniques may also be susceptible to contamination between separated membrane fractions (Table 2.3). For example, the dihydropyridine receptor (DHPR), which is localised in the T-tubules, has been detected in each of the designated sarcolemmal, SR and intracellular membrane fractions obtained using the differential centrifugation technique (Marette *et al.*, 1992; Dombrowski *et al.*, 1996; Nielsen *et al.*, 2003). Furthermore, the SR proteins, Ca^{2+}-ATPase, calsequestrin (CSQ) and the RyR have each been detected in the designated intracellular membrane fraction (Douen *et al.*, 1991; Marette *et al.*, 1992), while the Ca^{2+}-ATPase and RyR were also detected in the T-tubule fraction (Douen *et al.*, 1991; Marette *et al.*, 1992). Therefore, any proteins detected in either of the intracellular or T-tubule membrane fractions, may have originated from the SR.

Fortunately, the concomitant use of immunoelectron microscopy and monoclonal antibodies specific to the Na^+,K^+-ATPase isoforms (Marette *et al.*, 1993; Hundal *et al.*, 1994) provides a more valid method of investigating Na^+,K^+-ATPase isoform expression and localisation.

Table 2.3 Contamination between separated membrane fractions utilising differential centrifugation on sucrose gradients

Protein	Localisation	Detection in membrane fraction			
		SL	TT	SR	IM
DHPR	TT	Yes +	Yes +++	Yes -	Yes -
Ca^{2+}-ATPase	SR	No	Yes +	Yes +++	Yes +
CSQ	SR	No	No	Yes ++	Yes +
RyR	SR	No	Yes ++	Yes +++	Yes +

DHPR; dihydropyridine receptor, CSQ; calsequestrin, RyR; ryanodine receptor, TT; T-tubular membrane, SR; sarcoplasmic reticulum membrane, SL; sarcolemmal membrane, IM; intracellular membrane. + denotes relative abundance in indicated membrane fraction; – denotes expression too low for measurement of relative abundance. Data taken from (Douen *et al.*, 1991; Marette *et al.*, 1992; Dombrowski *et al.*, 1996; Nielsen *et al.*, 2003).

2.5.2 Isoform expression, localisation and abundance in skeletal muscle

2.5.2.1 Isoform gene transcripts

2.5.2.1.1 Rat muscle

Rat skeletal muscle has been reported to express the Na^+,K^+-ATPase α_1, α_2, α_3, β_1 and β_2 gene transcripts (Orlowski & Lingrel, 1988; Hundal *et al.*, 1993; Tsakiridis *et al.*, 1996; Thompson *et al.*, 1999), while the α_4 and β_3 gene transcripts have not been probed.

In 4 wk old rats, the relative α_2 and β_1 mRNA expression was 4.0- and 6.0-fold higher, respectively, than the relative α_1 and α_3 mRNA expression (Orlowski & Lingrel, 1988). Although the relative expression of the rat Na^+,K^+-ATPase gene transcripts appear to vary with age (Section 2.5.4), α_2 and β_1 mRNA expression were clearly greater than α_1 and α_3 mRNA expression from 1 to 15 wks of life (Orlowski & Lingrel, 1988).

2.5.2.1.2 Human muscle

Characterisation of the Na^+,K^+-ATPase gene transcripts expressed in human skeletal muscle also remains incomplete. The α_1, α_2 and β_1 gene transcripts were recently detected in human vastus lateralis muscle (Nordsborg *et al.*, 2003a), whilst the α_4 (Keryanov & Gardner, 2002) and β_3 gene transcripts (Malik *et al.*, 1998) have been detected in human skeletal muscle of unspecified origin. The α_3 and β_2 gene transcripts have not been probed. The relative abundance of the Na^+,K^+-ATPase isoform gene transcripts in human skeletal muscle remains to be investigated.

2.5.2.2 Isoform proteins

2.5.2.2.1 Rat muscle

Protein expression of each of the α_1, α_2, β_1, β_2 and β_3 isoforms has been detected in rat skeletal muscle (Hundal *et al.*, 1993; Tsakiridis *et al.*, 1996; Arystarkova & Sweadner, 1997). Despite the α_3 gene transcript being detected in rat skeletal muscle of undefined origin (Orlowski & Lingrel, 1988), immunoblotting has been unable to detect the α_3

protein in either sarcolemmal or intracellular membrane fractions in rat hindlimb muscle (Hundal *et al.*, 1994), or in homogenates from either rat soleus, red gastrocnemius, EDL, white gastrocnemius or diaphragm muscle (Thompson & McDonough, 1996). These results indicate either pre-translational regulation of the α_3 isoform, or that protein expression of the α_3 isoform is too low to be detected with immunoblotting.

In both red, oxidative and white, glycolytic muscles, the α_1 isoform was located almost exclusively in the sarcolemmal membrane fraction, with only 10% of that in the sarcolemmal fraction being found in the intracellular fraction (Hundal *et al.*, 1992; Lavoie *et al.*, 1995; Tsakiridis *et al.*, 1996). In contrast, the α_2 isoform was predominantly located in the intracellular fraction, with a 300 to 400% greater α_2 abundance in the intracellular fraction than the sarcolemmal fraction (Hundal *et al.*, 1992; Hundal *et al.*, 1994). In mixed hindlimb muscle, 80% of the β_1 isoforms were located in the intracellular fraction, while 20% were located in the sarcolemmal fraction (Hundal *et al.*, 1992). Similarly, when the protein yields were considered, expression of the β_2 isoform was predominantly in the intracellular fraction (Hundal *et al.*, 1992). Expression of the β_3 isoform has only been investigated in crude muscle homogenates (Arystarkova & Sweadner, 1997), preventing analysis of cellular localisation.

Identification of the intracellular site from which the Na^+,K^+-ATPase isoforms detected in the intracellular membrane fraction have originated has been problematic due to the issues of low protein yield and contamination (Section 2.5.1). However, use of immunogold labelling and electron microscopy demonstrated that the α_2 isoform was localised to sub-sarcolemmal vesicular and tubular structures, around mitochondria, at or near triad junctions and in the perinuclear area (Marette *et al.*, 1993). In rabbit hindlimb muscle, membrane fractionation, using the techniques of Dombrowski *et al.*, (1996), and immunoblotting detected the α_2, but not the α_1 isoform, in the T-tubule fraction (Williams

et al., 2001). T-tubule localisation of the α_2 but not the α_1 isoform was confirmed in rat EDL muscle using double immunofluorescence labelling to colocalise DHPR and the α_2, but not the α_1 isoform (Figure 2.3) (Williams *et al.*, 2001). T-tubule α_2 isoform localisation was also supported in mouse diaphragm and hindlimb muscle, where α_2 isoform expression correlated with T-tubule formation (Cougnon *et al.*, 2002). Additionally, Williams *et al.*, (2001) used elegant immunological techniques to colocalise the sarcolemmal Na^+,K^+-ATPase α_1 and α_2 isoforms with β-spectrin and ankyrin 3, which lie over the Z and M lines and are involved in maintaining cell structure and integrity (Figure 2.4). The authors interpreted the colocalisation to indicate a possible role for the Na^+,K^+-ATPase in the maintenance of cell integrity (Williams *et al.*, 2001).

It therefore appears that the Na^+,K^+-ATPase isoforms in rat skeletal muscle are located at the sarcolemma. T-tubules and at intracellular sites. Furthermore, the relative cellular expressions appear to be isoform-specific. The implication of isoform translocation from intracellular sites to the sarcolemma in response to insulin (Section 2.7.3.1) or exercise (see Section 2.12.5) suggests that intracellular Na^+,K^+-ATPase isoform localisation may not be fixed.

2.5.2.2.2 Human muscle

The first study to investigate Na^+,K^+-ATPase isoform protein expression in human muscle obtained soleus muscle from lower limbs amputated from patients, with un-stated pathologies (Hundal *et al.*, 1994). They detected the α_1, α_2, α_3 and β_1 proteins, but not the β_2 protein, and stated that the β_2 protein was also undetected in anterior tibialis muscle (Hundal *et al.*, 1994). Similarly, the β_2 protein was undetected in vastus lateralis muscle obtained from healthy humans (Juel *et al.*, 2000a). The apparent absence of β_2 is surprising

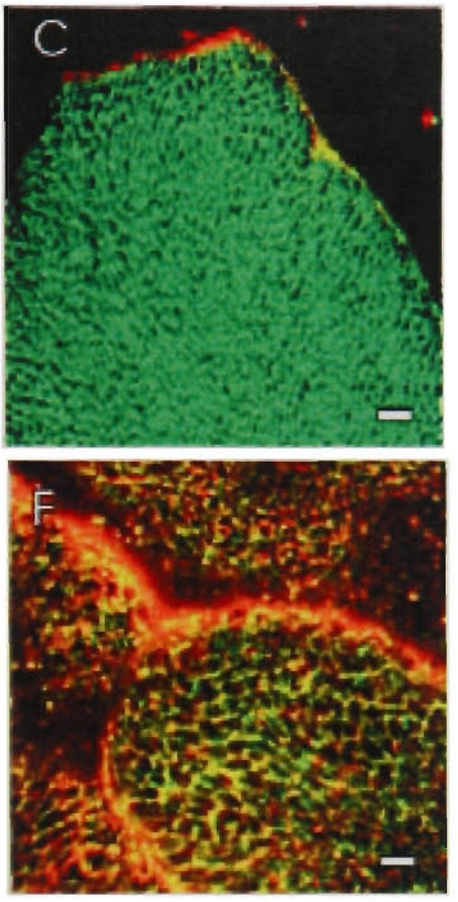


Figure 2.3 Colocalisation of DHPR and the Na^+,K^+-ATPase α_2 (labelled F), but not the α_1 (labelled C) isoform in rat EDL muscle. Areas with both DHPR and the respective Na^+,K^+-ATPase isoform are shown in yellow, areas with only the DHPR are shown in green, areas with only the Na^+,K^+-ATPase isoform are shown in red. Bars, 5 μm. Figure taken from Williams *et al.*, (2001).

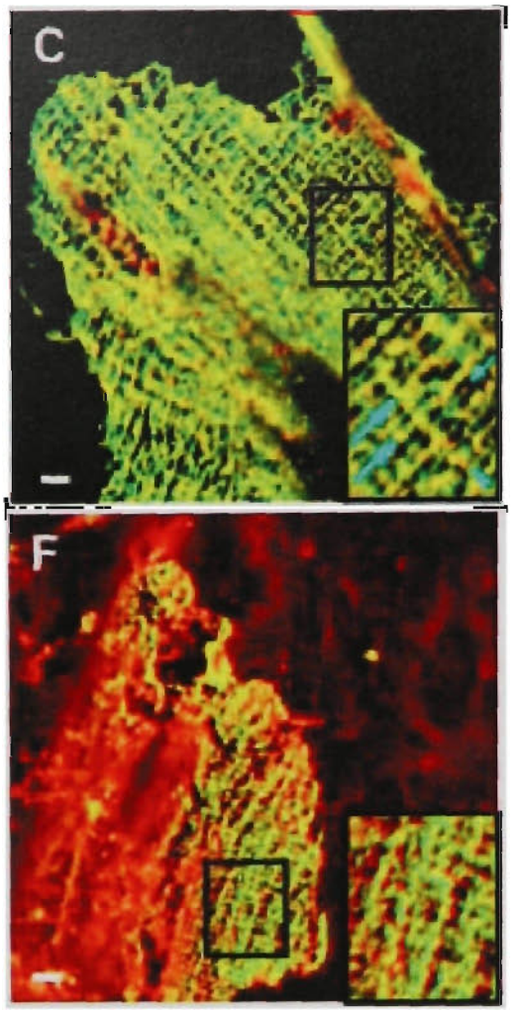


Figure 2.4 Colocalisation of β-spectrin and the Na^+,K^+-ATPase α_1 (labelled C) and α_2 (labelled F) isoforms in rat EDL muscle. Areas with both β-spectrin and the respective Na^+,K^+-ATPase isoform are shown in yellow, areas with only the Na^+,K^+-ATPase isoform are shown in red. Bars, 5 μm. Inset in C; two-fold magnifications of the boxed regions indicate the domains of the costameres: Z line domains (large arrows) and M line domains (small arrow). Figure and legend taken from Williams *et al.*, (2001).

and contrasts β_2 protein expression in rat muscle (Hundal *et al.*, 1993; Tsakiridis *et al.*, 1996). Conflicting data exists on α_3 protein expression in human muscle. Whereas the α_3 protein was detected in soleus muscle from amputated lower limbs (Hundal *et al.*, 1994), it was not found in human skeletal muscle of unstated origin and pathology (Wang *et al.*, 2001). Despite both the α_4 and β_3 gene transcripts being reported in human skeletal muscle (Section 2.5.3.1), α_4 and β_3 protein expression in human muscle has not been investigated.

Due to the problems associated with isolating membrane fractions, few studies have investigated Na^+,K^+-ATPase isoform localisation and abundance in human skeletal muscle (Hundal *et al.*, 1994; Juel *et al.*, 2000a). Hundal *et al.*, (1994) obtained large quantities (20-30 g) of human soleus muscle from amputated lower limbs. Similar to rats, human skeletal muscle demonstrated almost exclusive expression of the α_1 isoform in the sarcolemmal membrane fraction, with α_1 protein abundance in the intracellular fraction being only 4% of that in the sarcolemmal fraction (Hundal *et al.*, 1994). Both the sarcolemmal and intracellular membrane fractions were enriched with α_2 protein, however, in contrast to rats, α_2 protein abundance was 25% greater in the sarcolemmal fraction compared to the intracellular fraction. Immunoblotting detected an abundance of α_3 protein in the sarcolemmal fraction, however lack of a monoclonal α_3 antibody prevented immunocytochemical localisation. The β_1 isoform was detected in both the sarcolemmal and intracellular fractions, with 66% of the β_1 isoforms located in the sarcolemmal fraction. Juel *et al.*, (2000) used giant sarcolemmal vesicles to confirm α_1, α_2 and β_1 proteins expression in the sarcolemma. In contrast, cellular localisation and abundance of the β_2 and β_3 isoforms has yet to be determined.

The origin of the intracellular membrane fraction containing Na^+,K^+-ATPase isoforms in human skeletal muscle remains unclear. However, this intracellular fraction may be

contaminated by T-tubule membrane since the α_2 isoform was detected in a membrane fraction, isolated by differential centrifugation, enriched with the DHPR, a T-tubule protein (Nielsen *et al.*, 2003).

It therefore appears that in contrast with rat skeletal muscle, human skeletal muscle expresses the α_3, but not the β_2 isoform. Moreover, the relative expression of each of the α_1, α_2 and β_1 isoforms appears to vary between rat and human skeletal muscle. However, more research is required to confirm these findings using a muscle preparation that does not suffer from low protein yields or possible contamination. This was therefore investigated in Chapter 3 in this thesis.

2.5.3 Fibre-type specific Na^+,K^+-ATPase expression

2.5.3.1 Isoform mRNA and protein expression

Hundal *et al.*, (1993) used Northern Blotting to investigate possible differences between EDL and soleus muscles for Na^+,K^+-ATPase isoform mRNA expression in rats. The EDL is predominantly comprised of type II fibres (10-20% IIa, 38-40% IId/x, 35-38% IIb), whereas the soleus is predominantly comprised of type I fibres (75-84%) (Delp & Duan, 1996; Suwa *et al.*, 2003). In that study, α_1 mRNA expression was similar between the soleus and EDL, α_2 and β_1 mRNA were 4.0- and 3.0-fold more abundant in the soleus than the EDL, respectively, whilst β_2 mRNA was 8.0-fold more abundant in the EDL than the soleus (Hundal *et al.*, 1993). These differences need to be verified with more sensitive techniques for mRNA analysis, such as Real-Time RT-PCR, and as a result were investigated in Chapter 7 in this thesis.

Possible differences between muscles of different fibre-type composition in Na^+,K^+-ATPase isoform protein abundance was compared using whole muscle homogenates from rats (Thompson & McDonough, 1996). The α_1 and α_2 isoforms were detected in the soleus, red gastrocnemius, EDL, white gastrocnemius and diaphragm muscles. The relative

abundance of the α_1 isoform protein varied considerably between muscles, being 2-4 fold higher in muscles rich in oxidative capacity. However, there was much less variation between muscles in the relative abundance of the α_2 isoform, with relative abundance being the lowest in the white gastrocnemius and being 2-fold higher in the diaphragm. In contrast to the ubiquitous expression of the α_1 and α_2 isoforms, expression of the β_1 and β_2 was muscle-specific. The β_1 isoform was detected in the soleus, red gastrocnemius, EDL and diaphragm, but had very low expression in the white gastrocnemius. Indeed, when the relative abundance of the β_1 isoform was compared between these muscles, soleus, red gastrocnemius and diaphragm expressed a similar abundance of β_1, while abundance in the EDL was less than half of that in the soleus, and was too low to be measured in the white gastrocnemius. Contrasting the higher expression of the β_1 isoform in muscles predominantly containing slow oxidative fibres, expression of the β_2 isoform was highest in muscles predominantly containing fast glycolytic fibres. A similar β_2 relative abundance was found between the red and white gastrocnemius muscles and also the EDL, and was too low to measure in both the soleus and diaphragm. Taken together, these findings suggest that $\alpha_1\beta_1$ and $\alpha_2\beta_1$ are the predominant heterodimers in muscles containing slow oxidative fibres, while $\alpha_2\beta_2$ are the predominant heterodimers in muscles containing fast glycolytic fibres. The same study used co-immunoprecipitation to confirm the existence of both $\alpha_1\beta_1$ and $\alpha_2\beta_1$ heterodimers in the soleus muscle, which predominantly contains slow oxidative fibres (Delp & Duan, 1996). Possible differences between muscles of different fibre-type composition in α_3 and β_3 mRNA and protein expression are unknown.

Whether similar differences between muscles of different fibre-type composition for Na^+,K^+-ATPase isoform mRNA and protein abundance also exist in human skeletal muscle remains to be tested.

2.5.3.2 Na^+,K^+-ATPase content and maximal activity

2.5.3.2.1 Rat muscle

Four studies have contrasted [^{3}H]-ouabain binding site content between the EDL and soleus muscles (Dørup & Clausen, 1997; Bundgaard & Kjeldsen, 2002; Clausen *et al.*, 2004). In three of these studies, [^{3}H]-ouabain binding site content was 23%, 33% and 30% higher in the EDL than the soleus, in rats aged 4, 8 and 10 wks, respectively (Dørup & Clausen, 1997; Bundgaard & Kjeldsen, 2002; Clausen *et al.*, 2004). These findings also suggested that differences in [^{3}H]-ouabain binding site content between muscles of different fibre-type composition do not appear to be dependent on age, since the fibre-type compositions of the EDL and soleus muscles do not vary considerably between rats aged 5 wks and 18-20 wks (Delp & Duan, 1996; Suwa *et al.*, 2003). However, in another study using 10 wk old rats, there was no significant difference between the EDL and soleus for [^{3}H]-ouabain binding site content (Chin & Green, 1993). The reason for such discrepancies between studies is unknown. Furthermore, two recent studies contrasted [^{3}H]-ouabain binding site content (Musch *et al.*, 2002; Fowles *et al.*, 2004) between the white (92% type IIb) and red portions (51% type I) of rat gastrocnemius muscle (Delp & Duan, 1996). A higher [^{3}H]-ouabain binding site content was found in the red portion than the white portion of the gastrocnemius (Musch *et al.*, 2002; Fowles *et al.*, 2004). Thus, possible differences between rat muscles of different fibre-type composition for [^{3}H]-ouabain binding site content remain inconclusive due to inconsistencies between studies.

In accordance with the higher [^{3}H]-ouabain binding site content in muscles rich in type I than type II fibres found in some studies (Musch *et al.*, 2002; Fowles *et al.*, 2004), maximal 3-*O*-MFPase activity was 150-159% higher in the soleus than in the red gastrocnemius and EDL, and 270% higher in the soleus than the white gastrocnemius (Fowles *et al.*, 2004).

2.5.3.2.2 Human muscle

Most studies measuring Na^+,K^+-ATPase expression in humans utilised biopsies taken from the vastus lateralis muscle, which has a mixed fibre-type composition (Nygaard, 1981). Thus, whether possible differences exist between muscles of different fibre-type composition in either Na^+,K^+-ATPase content or maximal activity in human skeletal muscle are unknown. No clear relationship has been found between muscle [^{3}H]-ouabain binding site content and muscle fibre-type composition (Dørup *et al.*, 1988; Madsen *et al.*, 1994; McKenna *et al.*, 2003a).

2.5.4 Aging-specific Na^+,K^+-ATPase expression

2.5.4.1 Isoform mRNA and protein expression

A number of studies have investigated the effects of aging on Na^+,K^+-ATPase isoform mRNA and protein expression in rat skeletal muscle (Orlowski & Lingrel, 1988; Sun *et al.*, 1999; Ng *et al.*, 2003). During the first 5 wks of life, there was an 89-fold increase in α_2 mRNA expression, and a 9-fold increase in non-isoform specific β mRNA expression in skeletal muscle of unspecified origin (Orlowski & Lingrel, 1988).

In the red and white portions of the gastrocnemius, α_1 and β_1 protein abundance increased by 550-620% and 330-1,400%, respectively, from 6 to 29 mo of life (Sun *et al.*, 1999). This was accompanied by a 40% decrease in α_2 protein abundance, and a 60% decrease in β_2 protein abundance (Sun *et al.*, 1999). In the soleus, α_1 protein abundance increased by 200% and α_2 protein abundance decreased by 20% from 6 to 29 mo of life (Sun *et al.*, 1999).

In contrast, 16 mo old rats demonstrated higher α_1 protein abundance, lower β_2 protein abundance and no difference in α_2 or β_1 protein abundance than 29 month old rats (Ng *et al.*, 2003).

Possible effects of aging on Na^+,K^+-ATPase isoform mRNA and protein expression in human skeletal muscle remain to be investigated.

2.5.4.2 Na^+,K^+-ATPase content and maximal activity

2.5.4.2.1 Animal muscle

Mammalian skeletal muscle is at least partially characterised by an aging-specific Na^+,K^+-ATPase expression. In rat soleus muscle, [^{3}H]-ouabain binding site content increased from 2 d to 28 d (4 wks) of life, from 120 to 580 pmol.(g wet wt)$^{-1}$ (Kjeldsen *et al.*, 1982). Muscle [^{3}H]-ouabain binding site content then declined from 4 wks to 21 wks of life, to 150-200 pmol.(g wet wt)$^{-1}$ (Kjeldsen *et al.*, 1982). A similar trend was observed in horse skeletal muscle (Pickar *et al.*, 1993), while muscle [^{3}H]-ouabain binding site content decreased from birth in guinea pigs (Kjeldsen *et al.*, 1984a).

Rat muscle maximal Na^+,K^+-ATPase activity was also shown to be age-dependent in two studies, but with conflicting findings (Nørgaard *et al.*, 1984b; Sun *et al.*, 1999). Maximal 3-*O*-MFPase activity decreased with age in one study, being 70% lower in 11 wk old rats than in 4 wk old rats (Nørgaard *et al.*, 1984b), but increased with age in another, being 60% and 50% higher in 30 mo old rats compared to 6 and 18 mo old rats, respectively (Sun *et al.*, 1999). The reasons for these discrepancies between studies is unknown, but cannot be explained by the use of different muscles since both utilised the mixed gastrocnemius muscle (Nørgaard *et al.*, 1984b; Sun *et al.*, 1999).

2.5.4.2.2 Human muscle

Muscle [^{3}H]-ouabain binding site content was not different in vastus lateralis muscle obtained post-mortem from children aged 0-9 yrs (Kjeldsen & Gron, 1989). There was also no significant difference in vastus lateralis [^{3}H]-ouabain binding site content in a cross-sectional study of humans aged 25-80 yrs (Nørgaard *et al.*, 1984a). There was no significant difference, but there was a suggestion of a trend toward a higher (14%) [^{3}H]-

ouabain binding site content in untrained younger adults (28 ± 0.2 yr) than untrained older adults (68 ± 0.6 yr) (Klitgaard & Clausen, 1989). Whether maximal Na^+,K^+-ATPase activity differs with age in human muscle is unknown. Thus, the effect of aging on at least [^{3}H]-ouabain binding, appears to also be specie-dependent.

2.5.5 Gender-specific Na^+,K^+-ATPase expression

2.5.5.1 Isoform mRNA and protein expression

Possible differences between genders for Na^+,K^+-ATPase isoform mRNA and protein expression have not been investigated in either rat or human skeletal muscle. A possible gender effect on Na^+,K^+-ATPase mRNA expression is therefore investigated in Chapter 6 in this thesis.

2.5.5.2 Na^+,K^+-ATPase content and maximal activity

2.5.5.2.1 Animal muscle

Possible gender effects on muscle Na^+,K^+-ATPase remain unknown, with no study directly investigating the effect of sex hormones on muscle Na^+,K^+-ATPase. In cattle, a recent study found no significant difference between heifers (females) and steers (males) for semitendinosus muscle [^{3}H]-ouabain binding site content (Veeneklaas *et al.*, 2004).

Similar to [^{3}H]-ouabain binding site content, skeletal muscle maximal Na^+,K^+-ATPase activity has not been contrasted between genders. There are studies in rats measuring maximal Na^+,K^+-ATPase activity and using different genders (Nørgaard *et al.*, 1984b; Fowles *et al.*, 2004), but these studies used different assay conditions and consequently, these results cannot be compared.

2.5.5.2.2 Human muscle

In humans, three studies have contrasted the muscle Na^+,K^+-ATPase content between males and females, but with conflicting findings (Nørgaard, 1986; Evertsen *et al.*, 1997; Green *et al.*, 2001). There was no gender difference found in 25 to 80 yr old subjects

(Nørgaard, 1986), or in older (53-61 yr), sedentary subjects (Green *et al.*, 2001). In contrast, in well-trained cross-country skiers, muscle Na^+,K^+-ATPase content was 18% higher in young adult males than females (Evertsen *et al.*, 1997). The reason for the discrepancies between studies is unknown. Another study measured muscle Na^+,K^+-ATPase content in females and males that were either susceptible, non-susceptible or equivocal to malignant hyperthermia (Everts *et al.*, 1992). No attempt was made by the authors to compare muscle Na^+,K^+-ATPase content between genders, however in each of the three groups, males appeared to demonstrate higher muscle Na^+,K^+-ATPase content than females (Everts *et al.*, 1992).

Whether a gender difference exists for maximal Na^+,K^+-ATPase activity in human skeletal muscle is unknown. Further studies are therefore required to clarify possible differences in skeletal muscle Na^+,K^+-ATPase content and maximal activity.

A possible gender effect on [^{3}H]-ouabain binding site content and maximal 3-*O*-MFPase activity is therefore investigated in Chapter 6 in this thesis.

2.6 Isoform-specific functions in mammalian skeletal muscle

The existence of multiple α and β isoforms, as well as the degree of functional diversity displayed by the Na^+,K^+-ATPase suggests that evolutionary adaptations have occurred to render isoform-specific functions. Indeed, comparable analyses of the differential isoform properties suggest the existence of functions specific to each Na^+,K^+-ATPase isoform (see Tables 2.1 and 2.2).

2.6.1 Ouabain affinity

One functional difference observed between the Na^+,K^+-ATPase isoforms in rat skeletal muscle relates to [^{3}H]-ouabain binding affinity (O'Brien *et al.*, 1994). The rat α_1 isoform has a 10,000% and 100,000% lower apparent affinity for ouabain compared to the α_2 and α_3 isoforms, respectively (O'Brien *et al.*, 1994). However, this isoform-specific affinity for

ouabain appears to be species-specific. In human muscle, each of the α_1, α_2 and α_3 isoforms demonstrate similar affinity for ouabain (Crambert *et al.*, 2000; Wang *et al.*, 2001).

2.6.2 Na^+ affinity

In HeLa cells expressed with the rat α isoforms, the α_1 and α_2 isoforms displayed similar affinities for Na^+, whilst the α_3 isoform had a 200 to 300% lower apparent affinity for Na^+ (Jewell & Lingrel, 1991). Similar findings were seen in *Xenopus* oocytes transfected with human Na^+,K^+-ATPase isoforms, with comparable affinities for Na^+ for the α_1 and α_2 isoforms, and a 200 to 300% lower apparent affinity for Na^+ for the α_3 isoform (Crambert *et al.*, 2000).

2.6.3 K^+ affinity

The rat α_1, α_2 and α_3 isoforms did not significantly differ in their relative affinity for K^+ (Jewell & Lingrel, 1991), however the human α_2 isoform demonstrated a 40% higher affinity for K^+ than the α_1 and α_3 isoforms (Crambert *et al.*, 2000). Differences in the relative affinity for K^+ was also seen for the β isoforms, with the β_2 isoform demonstrating a higher K^+ affinity than the β_3 and β_1 isoforms, respectively (Crambert *et al.*, 2000).

2.6.4 ATP affinity

The rat α_3 isoform demonstrated a 130 to 200% greater affinity for ATP than the α_1 and α_2 isoforms (Jewell & Lingrel, 1991). Whether similar differences in the affinity for ATP also exist between the human Na^+,K^+-ATPase isoforms is unknown.

2.6.5 Specific roles of Na^+,K^+-ATPase isoforms in muscle function

In skeletal muscle, the ubiquitous α_1 isoform was previously thought to play a "house-keeping" role, maintaining basal [Na^+] and [K^+] gradients and membrane excitability, as well as lacking adaptability to different stressors (Hundal *et al.*, 1992; McDonough *et al.*, 1992; McDonough, 1994; Thompson *et al.*, 2001). In contrast, the α_2 isoform has been

shown to be adaptable to numerous physiological and environmental stressors, such as exercise (Juel *et al.*, 2000a; Juel *et al.*, 2001; Green *et al.*, 2004) and insulin (Omatsu-Kanbe & Kitasato, 1990; Marette *et al.*, 1993; Aledo & Hundal, 1995). However, a direct role for the α_1 isoform in muscle contractility was recently shown using gene-modified mice (He *et al.*, 2001). Mice lacking one copy of the α_1 gene demonstrated reduced peak tetanic force and a lesser absolute decline in force with fatiguing stimulation. A varying role for muscle contractility was observed for the α_2 isoform, with mice lacking one copy of the α_2 gene showing increased peak twitch and tetanic force, as well as a faster rate of force development (He *et al.*, 2001). However, these contractile changes may have also been due to the 39% increase in α_1 protein abundance that was seen in the α_2 heterozygous mice (He *et al.*, 2001). Nonetheless, the importance of the Na^+,K^+-ATPase α_2 isoform for cell survival is demonstrated in α_2 knockout mice, who do not survive the first day of life (James *et al.*, 1999b). More recent work from the same laboratory has shown that in perinatal mouse diaphragm, the α_1 isoform alone is able to maintain resting membrane potential and Na^+ and K^+ equilibrium gradients, but that the α_2 isoform is important for relaxation following muscle contraction (Radzyukevich *et al.*, 2004). The latter finding suggests that the α_2 isoform may play a role in the extrusion of Ca^{2+} during sustained contractions (Radzyukevich *et al.*, 2004). Therefore, the α_1 isoform appears able to substitute for the α_2 isoform in most cellular functions related to excitability and force, while the α_2 isoform may contribute less at rest than expected from its relative abundance, but can alter contractility during repeated muscle contractions (Radzyukevich *et al.*, 2004). Since none of these studies have probed for the α_3 isoform, specific roles for the α_3 isoform have yet to be determined.

The β subunit has been shown to facilitate correct processing, assembly and membrane insertion of the Na^+,K^+-ATPase (Ackermann & Geering, 1990; Noguchi *et al.*, 1990; Hundal *et al.*, 1994). However, the precise functions of each of the β_1, β_2 and β_3 isoforms remain unknown. In glial cells, the β_2 isoform may have an additional role as an adhesion molecule, where it is tightly bound to α_2 (Ewart & Klip, 1995).

2.7 Acute and chronic regulation of Na^+,K^+-ATPase in skeletal muscle

A combination of ionic, electrical and hormonal factors stimulate increased Na^+,K^+-ATPase activity in skeletal muscle.

2.7.1 Ionic Na^+,K^+-ATPase activation

2.7.1.1 Na^+

An early indication that the Na^+,K^+-ATPase was under ionic regulation was the prediction of a linear relationship between $[Na^+]_i$ and Na^+,K^+-ATPase activity (Sejersted & Hallen, 1987). This finding was supported by a series of experiments in which increased intracellular Na^+ content, induced by pre-incubation in K^+-free buffer, or veratridine, an activator of the voltage-gated Na^+ channels, increased ouabain-suppressible K^+ uptake by 117 to 152% in rat soleus, and by 126 to 194% in rat EDL muscle (Everts & Clausen, 1992). Veratridine was also shown to increase Na^+ efflux by 53% in rat soleus muscle (Everts & Clausen, 1994). Furthermore, a positive relationship was found between intracellular Na^+ content and ouabain-suppressible K^+ uptake in both soleus and EDL muscle in rats (Everts & Clausen, 1994), and Na^+ efflux in rat soleus muscle (Nielsen & Clausen, 1997). A more recent study used carbacholine to activate the nicotinic acetylcholine receptors in isolated rat soleus muscle (Macdonald *et al.*, 2005b). The subsequent 260% and 37% increases in Na^+ influx and intracellular Na^+ content, respectively, were associated with a 36% increase in ouabain-suppressible K^+ uptake (Macdonald *et al.*, 2005b).

2.7.1.2 K^+

A rise in $[K^+]_e$ does not appear to be involved in ionic Na^+,K^+-ATPase activation due to its very low K_m. At an $[K^+]_e$ of 1 mM, the Na^+,K^+-ATPase was already half-saturated (Nielsen & Clausen, 2000). Therefore at the $[K^+]_e$ of ~4 mM in resting skeletal muscle (Section 2.2.1), the Na^+,K^+-ATPase is almost fully saturated (Nielsen & Clausen, 2000). A rise in $[K^+]_e$ from 4 mM to 20 or 50 mM was also shown to increase Na^+,K^+-ATPase activity by only 16% and 28%, respectively (Everts & Clausen, 1994).

2.7.2 Electrical Na^+,K^+-ATPase activation

The Na^+,K^+-ATPase is rapidly activated from low resting levels during repeated muscle contractions to counteract the accelerated Na^+ and K^+ passive fluxes associated with each action potential. This was evidenced by an undershoot in intracellular Na^+ content below resting levels in the recovery period following electrical stimulation, in both rat soleus and EDL muscles (Everts & Clausen, 1992; Nielsen & Clausen, 1997; Buchanan *et al.*, 2002; McKenna *et al.*, 2003b). In rat soleus muscle, only 1 s of stimulation at 60 Hz was sufficient to produce a 10% undershoot in intracellular Na^+ content at 10 min of recovery (Buchanan *et al.*, 2002). Following 10 s of stimulation at 60 Hz, a significant undershoot in intracellular Na^+ content was observed after only 2 min of recovery (Buchanan *et al.*, 2002). Furthermore, after 30 s of stimulation at 60 Hz, intracellular Na^+ content did not return from reduced levels to control levels until 45 min of recovery (Nielsen & Clausen, 1997).

Direct evidence for excitation-induced Na^+,K^+-ATPase activation is the abolishment of the excitation-induced undershoot in intracellular Na^+ content when rat soleus muscles were pre-incubated with ouabain (Buchanan *et al.*, 2002). Furthermore, stimulation at frequencies ranging from 2-60 Hz significantly increased ouabain-suppressible K^+ uptake and Na^+ efflux (Everts & Clausen, 1992, 1994; Buchanan *et al.*, 2002), with the magnitude

of Na^+ efflux at 50 s following stimulation, proportionate to the stimulation frequency (Buchanan *et al.*, 2002). Finally, 1 min of electrical stimulation at 2 and 5 Hz increased the rate of [^{3}H]-ouabain binding by 116% and 124%, respectively (Everts & Clausen, 1994), reflecting increased Na^+,K^+-ATPase activity.

To further define the effect of excitation on Na^+,K^+-ATPase activation, Buchanan *et al.*, (2002) investigated whether action potentials *per se* were sufficient for excitation-induced Na^+,K^+-ATPase activation. Rat soleus muscles were incubated in Na^+-free buffer so as to elicit action potentials with minimal increase in intracellular Na^+ content. They found that 10 s of electrical stimulation at 60 Hz induced an undershoot in intracellular Na^+ content, indicating that stimulation of the muscle *per se* can activate the Na^+,K^+-ATPase in isolated rat muscle.

Based on the rate of Na^+ efflux measured over the 30 s period immediately following isotonic stimulation and the maximum transport capacity of the Na^+,K^+-ATPase, Nielsen & Clausen (1997) calculated a 2,200% increase in Na^+,K^+-ATPase activity with 10 s of stimulation at 120 Hz in rat soleus muscle. This corresponded to the theoretical maximum Na^+,K^+-ATPase transport capacity (Nielsen & Clausen, 1997).

Thus, in isolated rat skeletal muscle, excitation is a rapid and potent activator of the Na^+,K^+-ATPase; increasing Na^+,K^+-ATPase activity after only 1 s of stimulation, and sufficient under certain conditions to increase Na^+,K^+-ATPase activity to its theoretical maximum capacity. Furthermore, excitation-induced Na^+,K^+-ATPase activation may remain activated at high levels even in the face of a reduction in intracellular Na^+ content.

2.7.3 Hormonal Na^+,K^+-ATPase regulation

Hormones induce both acute and chronic Na^+,K^+-ATPase regulation, with the most potent hormonal Na^+,K^+-ATPase stimulators including insulin, epinephrine, norepinephrine and thyroid hormones. Other hormones known to regulate the Na^+,K^+-ATPase in skeletal

muscle include aldosterone (Pfeiffer *et al.*, 1999), insulin-like growth factor (IGF-1) (Matsuda *et al.*, 1993), calcitonin gene-related peptide (CGRP) (Andersen *et al.*, 1993) and amylin (James *et al.*, 1999a).

2.7.3.1 Insulin

Insulin induces both acute and chronic regulation of the Na^+,K^+-ATPase.

2.7.3.1.1 Acute Na^+,K^+-ATPase regulation by insulin

Within 15-30 min of incubation, insulin stimulated a 25% increase in ouabain-sensitive K^+ uptake and a 45% increase in Na^+ efflux in isolated rat soleus muscle (Clausen & Flatman, 1987). The stimulating effect of insulin on Na^+ efflux was unaltered by amiloride, indicating that this effect was not via Na^+/H^+ exchange. The insulin-induced increases in active Na^+ and K^+ transport were reflected in a 22% decrease and 10% increase in the intracellular contents of Na^+ and K^+, respectively, as well as a ouabain-sensitive 4.3 mV hyperpolarisation (Flatman & Clausen, 1979). In isolated rat soleus muscle, insulin increased ouabain-sensitive K^+ uptake in a dose- and time-dependent manner (Weil *et al.*, 1991). That study also found a 74% increase in the rate of [^{3}H]-ouabain binding with insulin (Weil *et al.*, 1991). Insulin has also been shown to induce acute Na^+,K^+-ATPase activation in frog skeletal muscle, as demonstrated by a doubling in the rate of [^{3}H]-ouabain binding after only 60 min of insulin incubation (Omatsu-Kanbe & Kitasato, 1990). In human forearm muscle, insulin induced a 3,100% increase in K^+ uptake (Ferrannini *et al.*, 1988). This effect was abolished with intra-arterial infusion of ouabain, indicating that the stimulatory effect of insulin for K^+ uptake was via Na^+,K^+-ATPase activation (Ferrannini *et al.*, 1988).

The mechanisms responsible for insulin-induced Na^+,K^+-ATPase activation remain unclear, but may involve insulin-induced translocation of Na^+,K^+-ATPase isoforms and is discussed below. The pathways involved in mediating the effects of insulin for acute

Na^+,K^+-ATPase activation may involve protein kinase C (PKC) since inhibition of the protein kinase C (PKC) isoform, PKC-ζ, abolished insulin-induced Na^+,K^+-ATPase activation (Sweeney & Klip, 1998).

Evidence for insulin-induced translocation

The insulin-induced increase in sarcolemmal Na^+,K^+-ATPase content is thought to occur via translocation of pre-existing Na^+,K^+-ATPase isoforms from intracellular sites to the sarcolemma. Evidence for this was demonstrated in rat skeletal muscle when insulin increased α_2 and β_1 protein abundance in the sarcolemmal membrane fraction, and also reduced α_2 protein abundance in the intracellular fraction (Hundal *et al.*, 1992). The lack of decrease in β_1 protein abundance in the intracellular fraction was interpreted to suggest that β_1 translocation may have originated from an organelle not recovered in the intracellular membrane fraction, or that insulin increased the retention of β_1 isoforms already present at the sarcolemma (Hundal *et al.*, 1992). An increase in sarcolemmal α_2 protein abundance with insulin was also found using the more reliable technique of immunoelectron microscopy (Marette *et al.*, 1993).

The insulin-induced increase in sarcolemmal Na^+,K^+-ATPase isoform protein abundance in rat muscle was initially thought to be isoform-specific, with no effect of insulin on the sarcolemmal protein abundance of either the α_1 or β_2 isoforms (Hundal *et al.*, 1992). However, recent evidence suggests that the isoform-specificity of this insulin-induced translocation may reflect the methodologies utilised to isolate sarcolemmal Na^+,K^+-ATPase isoform protein abundance (Al-Khalili *et al.*, 2003). When a sarcolemmal membrane fraction was obtained via differential centrifugation on a sucrose gradient, insulin increased α_2 but not α_1 protein abundance in rat gastrocnemius muscle of mixed fibre-type composition (Al-Khalili *et al.*, 2003). However, when sarcolemmal isoform protein abundance was assessed using sarcolemmal biotinylation techniques, insulin was

found to increase both α_1 and α_2 protein abundance in rat epitrochlearis muscle and also in human skeletal muscle cell cultures (Al-Khalili *et al.*, 2003). This discrepancy between methodologies may reflect the problems associated with the membrane isolation technique, as discussed in Section 2.5.1. Conversely, this discrepancy may also reflect the different muscles used between methodologies. Possible increases in sarcolemmal α_3 or β_3 protein abundance with insulin have yet to be investigated.

Since the insulin-induced translocation of Na^+,K^+-ATPase α_2 from an intracellular site to the sarcolemma was analogous to the recruitment of GLUT4 by insulin (Marette *et al.*, 1992), it was proposed that the Na^+,K^+-ATPase α_2 isoform and GLUT4 may be co-localised in a single intracellular pool, enabling simultaneous translocation to the sarcolemma by insulin. A series of studies employing immunoelectron microscopy to compare the sedimentation and immunological characteristics of the intracellular membrane containing each protein were performed, and reported no evidence for co-localisation (Aledo & Hundal, 1995; Lavoie *et al.*, 1995).

The mechanisms responsible for the purported insulin-induced Na^+,K^+-ATPase isoform translocation are unclear. However, increased intracellular Na^+ content does not appear to be involved since insulin decreases intracellular Na^+ content via activation of the Na^+,K^+-ATPase (Clausen & Hansen, 1977).

Evidence against insulin-induced translocation

Studies measuring functional Na^+,K^+-ATPase enzymes via ouabain-sensitive Na^+,K^+-ATPase activity (Wardzala & Jeanrenaud, 1981) and [^{3}H]-ouabain binding site content (Clausen & Hansen, 1977; McKenna *et al.*, 2003b) found no evidence of insulin-induced Na^+,K^+-ATPase translocation. The findings from the latter two studies are given further credence since they were performed in intact rat skeletal muscle with total protein yield, and therefore do not suffer from the low protein recoveries associated with membrane

isolation protocols (Section 2.5.1) (Hundal *et al.*, 1992; Marette *et al.*, 1992; Marette *et al.*, 1993). Furthermore, the [^{3}H]-ouabain binding site assay used in these studies primarily represents functional α_2 isoforms (O'Brien *et al.*, 1994). Since the α_2 isoform, together with the β_1 isoform, are proposed to be the preferential isoforms translocated with insulin, findings from these studies suggest that any insulin-induced translocation of the α_2 isoform may at least be sub-sarcolemmal, or may not necessarily reflect functional α_2 isoforms.

There are as yet, no reports of insulin-induced Na^+,K^+-ATPase translocation in human skeletal muscle.

2.7.3.1.2 Chronic Na^+,K^+-ATPase regulation by insulin

Insulin induces chronic Na^+,K^+-ATPase regulation by increasing the number of functional Na^+,K^+-ATPase enzymes at the sarcolemma. This was first shown in isolated frog sartorius muscle, when insulin increased [^{3}H]-ouabain binding site content, as well as active Na^+ transport (Grinstein & Erlij, 1974). In humans, a positive relationship was found between muscle [^{3}H]-ouabain binding site content and plasma insulin in both diabetic and non-diabetic subjects (Schmidt *et al.*, 1994).

2.7.3.2 Catecholamines

Within a few min of exercise, the rise in plasma epinephrine and norepinephrine concentrations activate the muscle Na^+,K^+-ATPase. The stimulatory effect of epinephrine on Na^+,K^+-ATPase activity was first shown in rat skeletal muscle, when epinephrine increased the rate of [^{3}H]-ouabain binding (Clausen & Hansen, 1977). In a later study in rat soleus muscle, epinephrine increased ouabain-sensitive K^+ uptake and Na^+ efflux by 130% and 54%, respectively, and induced a 9-22 mV hyperpolarisation (Clausen & Flatman, 1987). Although the stimulatory effect of catecholamines on active K^+ influx and Na^+ efflux occurred in both rat soleus and EDL muscle (James *et al.*, 1999a), the effect was

much more pronounced in muscles containing predominantly type I fibres (Pfliegler *et al.*, 1983; Everts *et al.*, 1988).

Fewer studies have investigated norepinephrine-induced Na^+,K^+-ATPase activation, however the limited studies indicate similar, albeit more modest, effects on active K^+ influx and Na^+ efflux (Lee & Vassalle, 1983).

Although the stimulatory effect of epinephrine and norepinephrine on Na^+,K^+-ATPase activation is not well understood, the mechanism most likely occurs through increased cAMP, and is mediated by activation of the protein kinase A pathway (Ewart & Klip, 1995).

In human studies, β-blockade was found to enhance hyperkalemia, and attenuate K^+ uptake by inactive muscles (Katz *et al.*, 1985). Furthermore, β-blockade induced a 43 to 52% increase in the femoral-venous $[K^+]$ difference during intense knee extensor exercise (Hallen *et al.*, 1996), and accelerated the development of fatigue during incremental cycling by 11% (Hallen *et al.*, 1994).

2.7.3.3 Thyroid hormones

The thyroid hormone triiodothyronine (T_3) is the principle endocrine factor inducing long-term Na^+,K^+-ATPase regulation in skeletal muscle. In the early 1970's, T_3 treatment was shown to increase O_2 consumption in nearly all tissues, while mitochondrial oxidation remained coupled to phosphorylation (Izmail-Beigi & Edelman, 1970; Ismail-Beigi & Edelman, 1971). It was therefore thought that thyroid hormones must be activating another energy-requiring system, one using ATP at a rate sufficient to account for the increase in O_2 consumption with T_3 treatment and one also occurring in nearly all tissues. Active Na^+ transport met both of these requirements, and the pioneering work of Izmail-Beigi & Edelman (1970; 1971) demonstrated that T_3 treatment indeed increased active Na^+ transport. In rat diaphragm muscle and liver slices, the increase in O_2 consumption with T_3

treatment was inhibited with ouabain and also with incubation in each of Na^+-free and K^+-free medium (Izmail-Beigi & Edelman, 1970). Furthermore, T_3 treatment reduced $[Na^+]_i$ and increased $[K^+]_i$ (Izmail-Beigi & Edelman, 1970). In later studies by the same authors, T_3 treatment was found to increase Na^+,K^+-ATPase activity in both thyroidectomised and euthyroid rats (Ismail-Beigi & Edelman, 1971; Edelman, 1974; Asano, 1996).

In rat soleus muscle, T_3 treatment induced a time-dependent increase in ouabain-suppressible K^+ efflux and amiloride-suppressible Na^+ influx, peaking at 3 d of incubation and at values 80% and 40% above controls, respectively (Everts & Clausen, 1988). However, by 8 d of T_3 incubation, the content of [^{3}H]-ouabain binding sites were increased by 103% over control values, suggesting that while hyperthyroidism induces an initial increase in passive Na^+ and K^+ leaks, these leaks stimulate Na^+,K^+-ATPase up-regulation. Harrison & Clausen (1998) attributed the early increase in passive Na^+ and K^+ leaks with hyperthyroidism to an elevated content of Na^+ channels, an event which precedes increased Na^+,K^+-ATPase content by at least 24 h. The effect of thyroid hormones on rat muscle Na^+,K^+-ATPase content is more pronounced in muscles predominantly containing type I fibres, with the EDL, diaphragm, gastrocnemius and soleus muscles of hyperthyroid rats showing a 260%, 350%, 510% and 980% higher [^{3}H]-ouabain binding site content compared to hypothyroid rats, respectively (Kjeldsen *et al.*, 1986a).

The elevation in muscle Na^+,K^+-ATPase content with hyperthyroidism appears to be due to an increase in synthesis of new Na^+,K^+-ATPase enzymes. This is based on the finding that T_3 treatment induced an increase in the mRNA and protein expression of the α_2 and β_2 isoforms in rat hindlimb muscle (Azuma *et al.*, 1993), and also increased α_2 mRNA expression and α_1 and β_2 protein abundance in rat skeletal muscle cells (Sharabani-Yosef *et al.*, 2002).

The long-term regulation of the Na^+,K^+-ATPase by thyroid hormones is also seen in humans, where hyperthyroidism and hypothyroidism induced a 68% increase and a 50% decrease in vastus lateralis [^{3}H]-ouabain binding site content, respectively (Kjeldsen *et al.*, 1984b). Furthermore, when patients became euthyroid with treatment, [^{3}H]-ouabain binding site content returned to normal levels (Kjeldsen *et al.*, 1984b). In a more recent study, [^{3}H]-ouabain binding site content in the vastus lateralis muscle of hyperthyroid patients was 100% higher than that of healthy controls (558 ± 101 vs 278 ± 52 pmol.(g wet wt)$^{-1}$), and 89% higher than that in their euthyroid state (296 ± 34), induced by treatment with methimazole (Riis *et al.*, 2005).

In summary, the Na^+,K^+-ATPase is located at the sarcolemma, T-tubules and at intracellular sites. Expression of the Na^+,K^+-ATPase may be dependent on muscle fibre-type composition, age and gender, while muscle Na^+,K^+-ATPase regulation involves ionic, electrical and hormonal processes. The Na^+,K^+-ATPase is also highly adaptable to altered levels of physical activity, which will be the focus of Section III.

SECTION III: ADAPTABILITY OF Na^+,K^+-ATPase EXPRESSION

This section investigates the effects of altered activity levels on Na^+,K^+-ATPase isoform mRNA and protein expression, and Na^+,K^+-ATPase content, as well as the mechanisms potentially responsible for these effects.

2.8 Effects of acute exercise on Na^+,K^+-ATPase isoform mRNA and protein expression in skeletal muscle

2.8.1 Isoform mRNA expression

2.8.1.1 Rat muscle

Little is known about the effects of acute exercise on Na^+,K^+-ATPase isoform mRNA expression in skeletal muscle, as summarised in Table 2.4. In rats, 1 h of prolonged treadmill running immediately elevated the mRNA expression of the α_1 and β_2 isoforms by 1.5- and 1.6-fold in red, oxidative and white, glycolytic fibres, respectively (Tsakiridis *et al.*, 1996). In contrast, exercise had no effect on α_2 and β_1 mRNA expression in red, oxidative fibres, or on α_1 mRNA expression in white, glycolytic fibres. Whether electrical stimulation also induces an increase in Na^+,K^+-ATPase mRNA expression is unknown and was therefore investigated in Chapter 7.

2.8.1.2 Human muscle

In humans, only α_1 mRNA was significantly increased with high-intensity intermittent one-legged knee extensor exercise, with expression elevated by 3.0-fold at each of 0 h, 1 h and 3 h post-exercise, and returning to resting levels at 5 h post-exercise (Nordsborg *et al.*, 2003a). No effect of exercise on α_2 and β_1 mRNA expression was found. However, their study was limited by low statistical power (Nordsborg *et al.*, 2003a). Furthermore, only the Na^+,K^+-ATPase α_1, α_2 and β_1 gene transcripts were probed (Nordsborg *et al.*, 2003a).

2.8.2 Isoform protein abundance

2.8.2.1 Rat muscle

Very little is also known about the effects of acute exercise on Na^+,K^+-ATPase isoform protein abundance in skeletal muscle (Table 2.4). In rats, 1 h of continuous treadmill running increased sarcolemmal α_1 and α_2 isoform protein abundance in both red, oxidative and white, glycolytic fibres (Tsakiridis *et al.*, 1996). In a separate study, 1 h of intermittent

Table 2.4 Acute exercise effects on Na^+,K^+-ATPase isoform mRNA and protein expression

Specie	Exercise	Muscle	Na^+,K^+-ATPase isoform				Reference No.
			mRNA	Relative increase (fold)	Protein (SL)	Relative increase (%)	
Rat	1 h submaximimal TM running	RO	α_1	1.5	α_1	64	1
			α_2	NS	α_2	63	
			β_1	NS	β_1	NS	
		WG	α_1	NS	α_1	55	
			β_2	1.6	α_2	94	
					β_2	NS	
Rat	1 h submaximimal TM running	RO			α_1	20	2
					α_2	32	
					β_1	27	
					β_2	25	
		WG			α_1	20	
					α_2	25	
					β_2	13	
Human	15 min intense knee extensor	VL	α_1	3.0			3
			α_2	NS			
			β_1	NS			
Human	5 min intense knee extensor	VL			α_1	NS	4
					α_2	70	
					β_1	26	

SL, sarcolemmal fraction/vesicle; RO, red oxidative; WG, white glycolytic; VL, vastus lateralis; TM, treadmill; NS, no significant change. References are; 1, Tsakiridis *et al.*, 1996; 2, Juel *et al.*, 2001; 3, Nordsborg *et al.*, 2003; 4, Juel *et al.*, 2000a.

treadmill running increased sarcolemmal protein abundance of the α_1, α_2, β_1 and β_2 isoforms in muscles predominantly comprising either oxidative or glycolytic fibres (Juel *et al.*, 2001). These elevations were transient, with relative isoform abundance returning to control levels at 3 h post-exercise (Juel *et al.*, 2001). Neither of these studies probed for the Na^+,K^+-ATPase α_3 or β_3 proteins.

2.8.2.2 Human muscle

To date, only a single study has investigated acute exercise effects on Na^+,K^+-ATPase isoform protein abundance in human skeletal muscle, and this only measured the α_1, α_2 and β_1 isoforms (Juel *et al.*, 2000a). They reported that ~5 min of intense one-legged knee extensor exercise elevated the α_2 and β_1 isoform protein abundance in giant sarcolemmal vesicles, by 70% and 26%, respectively (Juel *et al.*, 2000a). Since there was no effect of exercise on the homogenate α_1, α_2 or β_1 isoform protein abundance, the authors interpreted the increase in α_2 and β_1 proteins in the giant sarcolemmal vesicles to reflect translocation (Section 2.12.5) from an intracellular site (Juel *et al.*, 2000a).

Thus, future research is clearly required to investigate the effect of acute exercise on α_3, β_2 and β_3 mRNA and protein expression, as well as to clarify the acute exercise effect on α_1, α_2 and β_1 mRNA and protein expression using a larger sample size. These were therefore investigated in this thesis (Chapters 3-5).

2.9 Effects of chronic physical activity on Na^+,K^+-ATPase isoform mRNA and protein expression in skeletal muscle

2.9.1 Isoform mRNA expression

2.9.1.1 Human muscle

In the only study investigating the effects of physical training on Na^+,K^+-ATPase isoform mRNA expression, previously untrained humans performed 5.5 wks of high-intensity intermittent one-legged knee extensor exercise (Nordsborg *et al.*, 2003a). Training had no

significant effect on resting α_1, α_2 or β_1 mRNA expression (Table 2.5), but abolished the exercise-induced increase in α_1 mRNA that was evident prior to training. Whether long-term physical training also has no effect on Na^+,K^+-ATPase mRNA expression is unknown.

2.9.2 Isoform protein abundance

2.9.2.1 Human muscle

Three recent studies have investigated the effects of physical training on Na^+,K^+-ATPase isoform protein abundance in human muscle homogenates, although each only measured the α_1, α_2 and β_1 isoforms (Table 2.5) (Dela *et al.*, 2003; Green *et al.*, 2004; Nielsen *et al.*, 2004a). Six wks of one-legged strength training elevated α_1, α_2 and β_1 protein abundance by 37%, 21% and 33%, respectively (Dela *et al.*, 2003), while 7 wks of high-intensity, intermittent one-legged knee extensor exercise increased α_1 and α_2 protein abundance by 29% and 15%, respectively (Nielsen *et al.*, 2004a). There was also a tendency toward elevated (16%) β_1 protein abundance with training, however the lack of statistical significance may have reflected the low sample size (n = 6) used in that study (Nielsen *et al.*, 2004a). Green *et al.*, (2004) demonstrated that only 3 and 6 d of prolonged, submaximal cycling training were sufficient to increase α_2 and β_1 protein abundance by 9% and 39%, respectively. Therefore, α_2 protein abundance was elevated with training in each of these three studies, while α_1 and β_1 protein abundance were elevated in two studies.

Thus, the chronic exercise effects on Na^+,K^+-ATPase isoform mRNA and protein expression in human skeletal muscle remains inconclusive due to the limited research performed in this area, and the failure of these studies to probe for the full complement of Na^+,K^+-ATPase isoforms. This thesis therefore investigated the mRNA and protein

Table 2.5 Chronic exercise effects on Na^+,K^+-ATPase isoform mRNA and protein expression

			Na^+,K^+-ATPase isoform				
Specie	Exercise	Muscle	mRNA	Relative increase (fold)	Protein (Hom)	Relative increase (%)	Reference No.
Human	5.5 wk intense knee extensor	VL	α_1	NS			1
			α_2	NS			
			β_1	NS			
Human	6 wk ST	VL			α_1	37	2
					α_2	21	
					β_1	33	
Human	7 wk intense knee extensor	VL			α_1	29	3
					α_2	15	
					β_1	NS	
Human	6 d submaximal cycling	VL			α_1	NS	4
					α_2	9	
					β_1	39	

Hom, whole muscle homogenate; VL, vastus lateralis; ST, strength training; NS, no significant change. References are; 1, Nordsborg *et al.*, 2003a; 2, Dela *et al.*, 2003; 3, Nielsen *et al.*, 2004a; 4, Green *et al.*, 2004.

expression of each of the Na^+,K^+-ATPase isoforms present in human muscle in individuals that had performed short- (Chapter 5) or long-term training.

2.10 Effects of acute exercise on Na^+,K^+-ATPase content and maximal activity in skeletal muscle

2.10.1 Muscle Na^+,K^+-ATPase content

2.10.1.1 Rat muscle

The effects of acute exercise on muscle Na^+,K^+-ATPase content in skeletal muscle are summarised in Table 2.6. In rats, only one study has investigated the acute exercise effects on muscle [^{3}H]-ouabain binding site content (Juel *et al.*, 2001). In that study, [^{3}H]-ouabain binding was performed on purified vesicular membranes from mixed hindlimb muscle. One h of intermittent treadmill running increased muscle [^{3}H]-ouabain binding site content by 30% (Juel *et al.*, 2001). However, a recent study investigated the effects of electrical stimulation of varying frequencies and durations on [^{3}H]-ouabain binding site content in rat soleus muscle (McKenna *et al.*, 2003b). Neither brief high-frequency electrical stimulation, nor prolonged low-frequency electrical stimulation had any significant effect on muscle [^{3}H]-ouabain binding site content (McKenna *et al.*, 2003b). In fact, [^{3}H]-ouabain binding site content expressed per g wet weight muscle was actually decreased following both high-frequency and low-frequency electrical stimulation (McKenna *et al.*, 2003b). However, this was most likely due to fluid shifts since [^{3}H]-ouabain binding site content expressed per g dry weight muscle was not significantly altered with either high- or low-frequency electrical stimulation (McKenna *et al.*, 2003b).

2.10.1.2 Human muscle

In humans, there was no effect of 30 min of maximal isometric knee extensor exercise (Fowles *et al.*, 2002b) or ~72 min of fatiguing cycling (Leppik *et al.*, 2004) on muscle [^{3}H]-ouabain binding site content in the vastus lateralis muscle of recreationally active

Table 2.6 Acute muscle contractions effects on Na^+,K^+-ATPase content and maximal activity

Specie	Intervention	Muscle	[^{3}H]-ouabain binding site content		Maximal 3-*O*-MFPase activity		Ref. No.
			Initial value	Relative change (%)	Initial value	Relative change (%)	
Rat	2 min elec. stim, 120 Hz	Soleus	800	NS			1
	4 h elec. stim, 1 Hz		998 ± 74	NS			
Rat	2 h + 45 min submax running	Soleus, EDL, WVL, RVL			500	-12	2
Rat	1 h submax. running	MG – sarcolemmal fraction		+30			3
Human	50 max. knee extensions	VL			210	-14	4
Human	30 min max. knee extensions	VL	240	NS	238	-35	5
Human	10 h submax.	VL	334 ± 11	+13			6
Human	72 min submax. cycling	VL	333 ± 19	NS	290	-13	7
Human	90 min submax. cycling	VL			250	-21	8
Human	Incremental cycling	VL	307 ± 41	NS	265 ± 27	-13	9

[^{3}H]-ouabain binding site content is expressed in pmol.(g wet wt)$^{-1}$; Maximal 3-*O*-MFPase activity is expressed in nmol.min^{-1}.(g wet wt)$^{-1}$. NS, no significant change; EDL, extensor digitorum longus; WVL, white vastus lateralis; RVL, red vastus lateralis; MG – mixed gastrocnemius; VL, vastus lateralis; elec. stim., electrical stimulation. References are; 1, McKenna *et al.*, 2003b; 2, Fowles *et al.*, 2002a; 3, Juel *et al.*, 2001; 4, Fraser *et al.*, 2002; 5, Fowles *et al.*, 2002b; 6, Overgaard *et al.*, 2002; 7, Leppik *et al.*, 2004; 8, Sandiford *et al.*, 2004; 9, Aughey *et al.*, 2005.

subjects. In well-trained endurance athletes, there was also no significant change in muscle [^{3}H]-ouabain binding site content following fatiguing, incremental cycling (Aughey *et al.*, 2005). Each of these studies sampled muscle biopsies immediately following exercise. Thus, while these findings indicate that muscle Na^+,K^+-ATPase content is unchanged immediately following exercise, little is known about the effects of acute exercise on muscle Na^+,K^+-ATPase content in the 0-24 h period following exercise, when any increase in Na^+,K^+-ATPase content might be expected to occur. One study has shown no change in [^{3}H]-ouabain binding site content in the 4 h period following brief, intense exercise (Fowles *et al.*, 2002), while another found a 13% increase in [^{3}H]-ouabain binding site content at 30 min following ~10 h of running (Overgaard *et al.*, 2002). Thus it appears that only acute exercise of ultra-duration is sufficient to induce an increase in [^{3}H]-ouabain binding site content in human skeletal muscle, and that the recovery period following exercise may be important for this up-regulation.

2.10.2 Muscle maximal Na^+,K^+-ATPase activity

2.10.2.1 Rat muscle

The effects of acute exercise on maximal 3-*O*-MFPase activity were investigated in rat soleus, EDL, white vastus lateralis and red vastus lateralis muscles (Fowles *et al.*, 2002a). Rats performed either only 2 h of submaximal running, or 2 h of submaximal running followed by an additional 45 min of low-intensity running. There was no significant change in maximal 3-*O*-MFPase activity following either exercise protocol for any of the muscles. However, when results were averaged across all muscles, there was a 12% reduction in maximal 3-*O*-MFPase activity in the rats who had performed 2 h of submaximal running followed by an additional 45 min of low-intensity running (Fowles *et al.*, 2002a).

2.10.2.2 Human muscle

Human studies have consistently reported a 13 to 35% depression in maximal 3-*O*-MFPase activity immediately following acute exercise (Fowles *et al.*, 2002b; Fraser *et al.*, 2002; Leppik *et al.*, 2004; Sandiford *et al.*, 2004; Aughey *et al.*, 2005). Leppik *et al.*, (2004) investigated the time course of the exercise-induced depression in maximal 3-*O*-MFPase activity, and found a 7% depression after only 10 min of submaximal cycling, an 11% depression following 45 min of cycling and a 13% depression at fatigue (~72 min) (Leppik *et al.*, 2004). It therefore appears that acute exercise may induce a progressive depression in maximal 3-*O*-MFPase activity. Only one study has investigated the recovery of maximal 3-*O*-MFPase activity following acute exercise, but they had internally inconsistent findings (Fowles *et al.*, 2002b). They found a 35% lower maximal 3-*O*-MFPase activity in the exercised leg than the non-exercised leg immediately following 30 min of maximal, isometric knee extensions, with no difference between legs at 1 h post-exercise. This suggested that exercise only transiently impaired maximal 3-*O*-MFPase activity. However, they also reported no difference in maximal 3-*O*-MFPase activity between pre-exercise in the non-exercised leg, and post-exercise activity in the exercised leg. Furthermore, they also reported no difference in the exercised leg between 0, 1, or 4 h after exercise. Together these suggest either that maximal 3-*O*-MFPase activity was not depressed, or did not recover following exercise. Unfortunately, no comparison was made pre- and post-exercise in the same leg.

Further studies are clearly required to investigate the recovery of skeletal muscle Na^+,K^+-ATPase content and maximal activity following acute exercise, and this was therefore investigated in Chapter 4.

2.11 Effects of chronic physical activity levels on Na^+,K^+-ATPase content and maximal activity in skeletal muscle

2.11.1 Muscle Na^+,K^+-ATPase content

2.11.1.1 Animal muscle

It has been well established that physical training provides a sufficient stimulus to up-regulate Na^+,K^+-ATPase content in animal skeletal muscle, as determined by increased [^{3}H]-ouabain binding site content (see Table 2.7). The first report of a training- induced increase in muscle Na^+,K^+-ATPase content was found in rat muscles (Kjeldsen *et al.*, 1986b). Six wks of swimming training induced a 32%, 46% and 22% increase in [^{3}H]-ouabain binding site content in the soleus, EDL and gastrocnemius muscles, respectively(Kjeldsen *et al.*, 1986b). There was no corresponding increase in [^{3}H]-ouabain binding site content in the diaphragm, suggesting that the effect of training on [^{3}H]-ouabain binding site content was muscle-specific. A later study in foals confirmed this finding, with a significant increase in [^{3}H]-ouabain binding site content in the gluteus medius, but not the masseter muscle, following sprint running training (Suwannachot *et al.*, 1999). Thus, the up-regulatory response of training on Na^+,K^+-ATPase content appears to be confined to the muscles directly trained, and not to simply reflect generalised Na^+,K^+-ATPase up-regulation (Clausen, 2003).

2.11.1.2 Human muscle

In comparison to animal models, physical training appears to induce more moderate increases in Na^+,K^+-ATPase content in human skeletal muscle (see Table 2.8). Training protocols of various modes (swimming, running, cycling, strength training and cross-country skiing), durations (3 d to 5 mo) and intensities (submaximal to maximal) have all elevated [^{3}H]-ouabain binding site content, mainly by 14-18% (range, 9-40%; median,

Table 2.7 Training effects on Na^+,K^+-ATPase content in animal skeletal muscle

Specie	Muscle	Training			Initial [^{3}H]-ouabain binding site content	Relative increase (%)	Reference No.
		Duration	Mode	Frequency			
Rat	Soleus	6 wks	Swimming	5 sessions/wk	372	32	1
	EDL				288 ± 23	46	
	Gastroc.				265 ± 32	22	
	Diaphragm				315 ± 18	2 NS	
Rat	Gastroc.	6 wks	Swimming	5 sessions/wk	~360	25	2
	Soleus				~500	32	2
Horse	GM	5 mo	Sprint running	7 sessions/wk	168 ± 9	30	3
				Mean	324	27	
				SEM	39	5	
				Median	315	30	

EDL, extensor digitorum longus; Gastroc., gastrocnemius; GM, gluteus medius; NS, no significant change. Initial [^{3}H]-ouabain binding site content expressed as pmol.(g wet wt)$^{-1}$. Values are mean ± SEM. References are; 1, Kjeldsen *et al.*, 1986b; 2, Kjeldsen *et al.*, 1988; 3, Suwannachot *et al.*, 1999.

Table 2.8 Training effects on Na^+,K^+-ATPase content in human vastus lateralis muscle

Training			Initial [^{3}H]-ouabain	Relative	Reference
Duration	Mode	Frequency	binding site content	increase (%)	No.
12-17 yrs	Swimming	3 sessions/wk	276 ± 19[a]	30[b]	1
	Running			32[b]	
	Strength training			40[b]	
10 wks	Military training	Not stated	308 ± 13	NS	2
7 wks	Sprint cycling	3 sessions/wk	333 ± 19	16	3
6 d	Prolonged cycling	2 h/d	339 ± 16	14	4
6 wks	Intense endurance	2.7 h/wk	307 ± 43	15	5
5 mo	Cross-country	7 sessions/wk	343 ± 11 (males)	16	6
	skiing		281 ± 14 (females)		
8 wks	Prolonged cycling	3 sessions/wk	326 ± 17	14	7
3 wks	Prolonged cycling	5-6 sessions/wk	~280	22	8
7 wks	Strength training	3 sessions/wk	~280	16	8
3 mo	Strength training	1-3 sessions/wk	356 ± 6	15	9
>2 yr	Endurance	5-6 h/wk	306 ± 14[a]	18[b]	10
	running/cycling				
3 d	Prolonged cycling	2 h/d	~280	9	11
		Mean	304	18	
		SEM	7	2	
		Median	306	16	

[a]Mean [^{3}H]-ouabain binding site content value for untrained controls. [b]% higher [^{3}H]-ouabain binding site content in trained subjects compared to untrained controls. NS, no significant change. Initial [^{3}H]-ouabain binding site content expressed as pmol.(g wet wt)$^{-1}$. Values are mean ± SEM. References are; 1, Klitgaard & Clausen, 1989; 2, Kjeldsen *et al.*, 1990; 3, McKenna *et al.*, 1993; 4, Green *et al.*, 1993; 5, Madsen *et al.*, 1994; 6, Evertsen *et al.*, 1997; 7, Green *et al.*, 1999b; 8, Green *et al.*, 1999a; 9, Medbø *et al.*, 2001; 10, Fraser *et al.*, 2002; 11, Green *et al.*, 2004.

16%), in human vastus lateralis muscle (Table 2.8). Since the magnitude of Na^+,K^+-ATPase up-regulation was similar between these studies, it appears the effect of physical training on muscle Na^+,K^+-ATPase content may be independent of the number of years of training performed. Indeed, studies sampling muscle at various periods throughout their training regime showed that initial increases in Na^+,K^+-ATPase content were not significantly augmented with additional training (Green *et al.*, 1999a; Green *et al.*, 2004). Furthermore, in a cross-sectional study, muscle Na^+,K^+-ATPase content was only 18% higher in young endurance trained adults (age, 26.4 ± 3.1 yr) who had been training continuously for at least 2 yrs, than young untrained adults (26.4 ± 3.9 yr) (Fraser *et al.*, 2002). These findings therefore suggest that endurance training may cause an early increase in muscle Na^+,K^+-ATPase content (~14-18%), that is not further augmented with additional training. However, in another cross-sectional study, muscle Na^+,K^+-ATPase content was 30-32% higher in older endurance trained adults (~69-70 yr) who had been training continuously for the previous 12-17 yrs, than older untrained adults (~68-69 yr) (Klitgaard & Clausen, 1989). Whether the considerably greater Na^+,K^+-ATPase up-regulation observed in that study was due to a further increase in Na^+,K^+-ATPase content that occurs with training extending 12-17 yrs is not clear.

2.11.2 Muscle Na^+,K^+-ATPase activity

2.11.2.1 Rat muscle

Less is known regarding the effects of physical training on skeletal muscle Na^+,K^+-ATPase activity (Ng *et al.*, 2003). Ouabain-sensitive, Na^+- and K^+-stimulated ATPase activity was used for measurement of Na^+,K^+-ATPase activity in the red gastrocnemius, white gastrocnemius and EDL muscles of control rats, and of those who had performed 13-14 wks of prolonged submaximal running training (Ng *et al.*, 2003). Training increased Na^+,K^+-ATPase activity by 29% and 27% in the red and white gastrocnemius muscles,

respectively. However, there was no significant increase for Na^+,K^+-ATPase activity in the EDL muscle. This finding was consistent with the muscle-specific effect of training for Na^+,K^+-ATPase content (see above). The lack of change in Na^+,K^+-ATPase activity in the EDL does not appear to reflect muscle fibre-type composition since Na^+,K^+-ATPase activity was increased with training in the white gastrocnemius muscle, which has a similar fibre-type composition to the EDL (80-90% Type II) (Delp & Duan, 1996).

2.11.2.2 Human muscle

Only two studies have investigated the effects of physical training on maximal Na^+,K^+-ATPase activity in human muscle (Fraser *et al.*, 2002; Green *et al.*, 2004). One longitudinal training study found a 41% increase in maximal 3-*O*-MFPase activity following only 6 d of prolonged, submaximal cycling training (Green *et al.*, 2004). In a cross-sectional study of endurance athletes who had been training continuously for at least 2 yr and untrained subjects, the endurance trained athletes demonstrated a tendency toward a higher (20%, NS) maximal 3-*O*-MFPase activity than the untrained subjects (Fraser *et al.*, 2002). The lack of significance was possibly due to the considerable inter-individual variability of results, as well as the small sample size (Fraser *et al.*, 2002). Therefore, whether physical training induces an increase in muscle maximal Na^+,K^+-ATPase activity remains inconclusive. Furthermore, whether the effects of physical training on muscle Na^+,K^+-ATPase content and maximal activity are dependent on the number of years of training are unclear and this was therefore investigated in Chapter 6 of this thesis.

2.11.3 Down-regulation of Na^+,K^+-ATPase with immoblisation and inactivity

2.11.3.1 Animal muscle

Reduced levels of physical activity via limb immobilisation down-regulate muscle Na^+,K^+-ATPase content. In rat soleus muscle, 1 wk of immobilisation induced a 20-30% reduction in [^{3}H]-ouabain binding site content (Kjeldsen *et al.*, 1986b; Kjeldsen *et al.*, 1988). The

importance of physical activity levels for [^{3}H]-ouabain binding site content in rat muscle were highlighted by the 84% range in [^{3}H]-ouabain binding site content for immobilised versus trained rats (Kjeldsen *et al.*, 1988). A similar finding was also observed in guinea pig gastrocnemius muscle, with a 93% range in [^{3}H]-ouabain binding site content for guinea pigs who were either immobilised or run trained for 3 wks (Leivseth *et al.*, 1992).

In sheep vastus lateralis muscle, 9 wks of immobilisation induced a 39% reduction in [^{3}H]-ouabain binding site content in the immobilised leg, and also a 22% reduction in [^{3}H]-ouabain binding site content in the contralateral leg (Jebens *et al.*, 1995). These findings indicated that in spite of inactivity, the capacity for muscle performance was better maintained in the contralateral leg (Jebens *et al.*, 1995).

2.11.3.2 Human muscle

In humans, the effect of reduced physical activity levels on muscle [^{3}H]-ouabain binding site content was studied in subjects with shoulder impingement syndrome, a condition that hampers the use of the affected shoulder. Subjects had biopsies taken from both their affected and unaffected deltoid muscles (Leivseth & Reikeras, 1994). Muscle [^{3}H]-ouabain binding site content was 27% lower in their affected deltoid than their unaffected deltoid (Leivseth & Reikeras, 1994). These findings indicate that reduced physical activity levels also decrease Na^+,K^+-ATPase content in human muscle.

The effects of reduced physical activity levels on skeletal muscle Na^+,K^+-ATPase activity remain to be investigated.

2.12 Possible mechanisms of Na^+,K^+-ATPase up-regulation

2.12.1 Mechanisms of elevated Na^+,K^+-ATPase mRNA expression

Elevated Na^+,K^+-ATPase mRNA expression can be induced via i) increased mRNA transcription, ii) attenuated mRNA degradation, or ii) a combination of both mechanisms

reflecting improved mRNA stability. This section reviews the likely involvement of transcription and degradation towards elevated Na^+,K^+-ATPase mRNA expression.

2.12.1.1 Increased mRNA transcription and/or attenuated degradation

Only two studies have investigated the mechanisms responsible for elevated Na^+,K^+-ATPase mRNA expression, which was induced by high $[Ca^{2+}]_i$ (1 mM) or ouabain (0.1 mM) (Rayson, 1991; Rayson, 1993). Both studies utilised cultured rat kidney cells and only isoform transcription rates were measured. High $[Ca^{2+}]_i$ was used to induce an elevation in α_1 and β_1 mRNA expression, with peak increases for both isoforms occurring after 1 h of incubation (Rayson, 1991). Elevated α_1 isoform transcription could only account for ~20% of the increase in α_1 mRNA expression, while there was no change in the transcription rate of the β_1 isoform with high $[Ca^{2+}]_i$.

Ouabain, which would inhibit ~60-70% of Na^+,K^+-ATPase (Clausen & Everts, 1991), also induced a 2.0-fold increase in α_1 mRNA expression, which peaked at 1 h of incubation (Rayson, 1993). This was matched both in time-course and magnitude by an elevated α_1 transcription rate (Rayson, 1993). In contrast, a 2.0-fold increase in β_1 mRNA expression induced by ouabain could only partially be explained by an increase in β_1 transcription rate (Rayson, 1993). Since high $[Ca^{2+}]_i$ had previously induced an increase in Na^+,K^+-ATPase mRNA expression (Rayson, 1991), the authors suggested that the ouabain-induced increases in Na^+,K^+-ATPase α_1 and β_1 mRNA expression may have been mediated by an increase in $[Ca^{2+}]_i$ via Na^+/Ca^{2+} exchange (Rayson, 1993).

It therefore appears that elevations in α_1 and β_1 mRNA expression may involve a combination of both enhanced transcription and attenuated degradation, depending on the initial stimuli. Whether such mechanisms are also involved in the mRNA response to muscle contraction are unknown and as such, warrant further investigation.

2.12.2 Mechanisms of transcriptional regulation

Repeated muscle stimulation induces numerous changes in the intracellular environment that could potentially induce an increase in Na^+,K^+-ATPase mRNA expression. Such changes include i) increased $[Na^+]_i$, ii) increased cytosolic free $[Ca^{2+}]$ ($[Ca^{2+}]_{cyto}$), iii) increased $[K^+]_e$, and iv) the production of reactive oxygen species (ROS), and are presented in Figure 2.5. This section reviews the evidence for each of these alterations contributing to Na^+,K^+-ATPase mRNA up-regulation.

2.12.2.1 Increased $[Na^+]_i$

Two interventions have been used to induce elevations in $[Na^+]_i$ for investigation of possible increases in Na^+,K^+-ATPase mRNA expression in cultured rat kidney (Rayson, 1993) or chick skeletal muscle cells (Taormino & Fambrough, 1990). These include ouabain, an inhibitor of the Na^+,K^+-ATPase (Clausen & Hansen, 1974) and veratridine, an activator of the voltage-gated Na^+-channels (Sutro, 1986). Ouabain inhibited Na^+,K^+-ATPase activity (0.01 mM, 61% inhibition) (Clausen & Everts, 1991) and increased intracellular Na^+ content (1 mM, 194%) in isolated rat soleus muscle (Gissel & Clausen, 1999), and also induced an ~2.0-fold (0.1 mM) increase in each of Na^+,K^+-ATPase α_1 and β_1 mRNA expression in rat kidney cells (Rayson, 1993). However, the effect of ouabain on Na^+,K^+-ATPase mRNA expression may be tissue- and/or isoform-specific, since α_3 mRNA expression was reduced, but β_1 mRNA expression was increased in rat cardiac myocytes (Huang *et al.*, 1997; Xie *et al.*, 1999; Kometiani *et al.*, 2000). Furthermore, each of these effects were concentration-dependent (Huang *et al.*, 1997; Xie *et al.*, 1999; Kometiani *et al.*, 2000). Veratridine increased ouabain-suppressible K^+ uptake (0.1 mM, 126%) (Everts & Clausen, 1992) and elevated Na^+ content (0.1 mM, 121%) in isolated rat EDL muscle

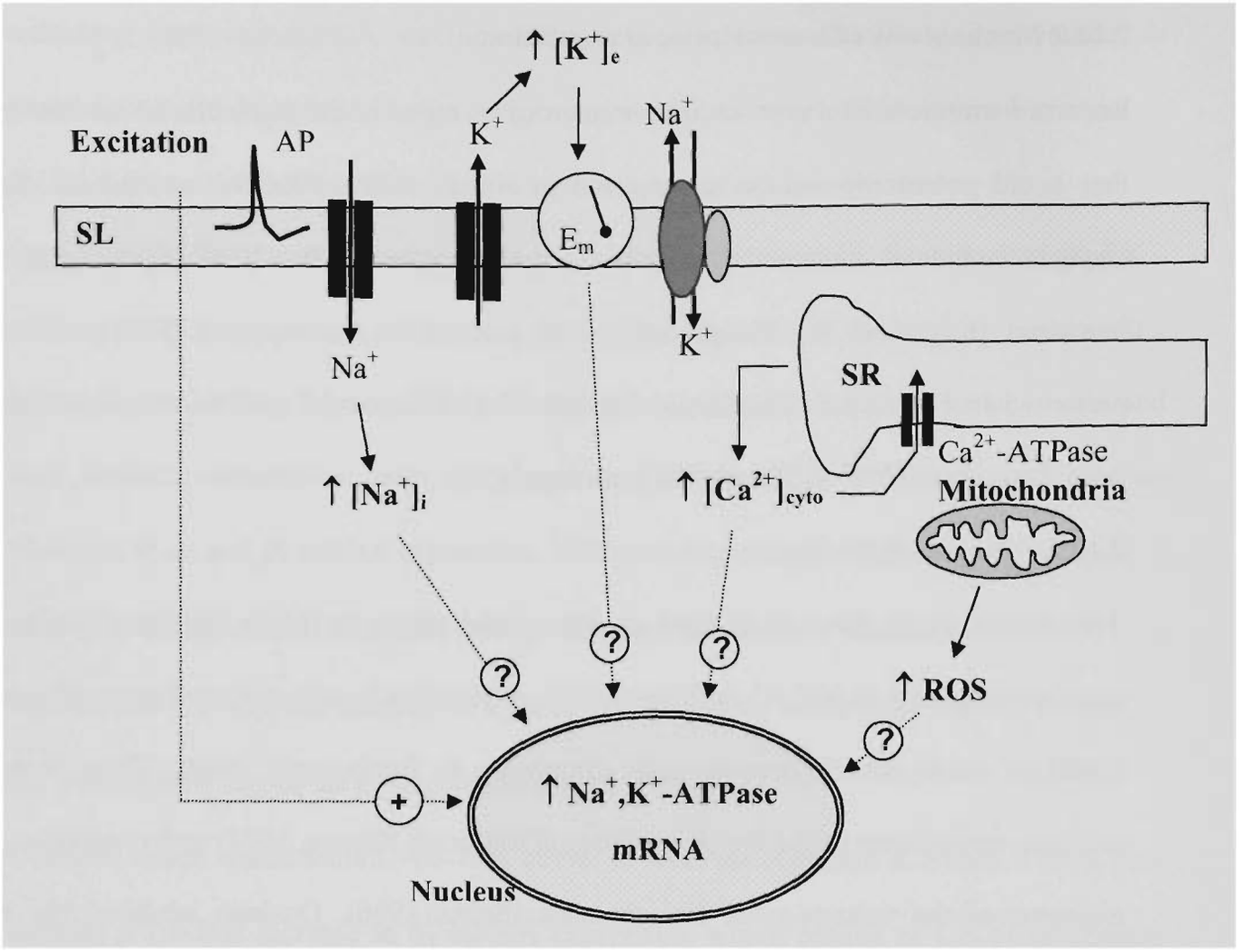


Figure 2.5 Possible intracellular changes involved in up-regulation of skeletal muscle Na^+,K^+-ATPase transcription with muscle contraction. These include an increase in each of; intracellular $[Na^+]$ ($[Na^+]_i$), cytosolic free $[Ca^{2+}]$ ($[Ca^{2+}]_{cyto}$), extracellular $[K^+]$ ($[K^+]_e$) and reactive oxygen species (ROS). SL, sarcolemmal membrane; AP, action potential; SR, sarcoplasmic reticulum. E_m, membrane potential; ↓, depolarisation; ⊕, known stimulatory effect; ⓘ unknown effect.

(Gissel, H. & Clausen, T. personal communication), and also increased (0.01 mM) non-isoform specific α and β mRNA expression, by 1.7-fold and 2.5-fold, respectively, in chick skeletal muscle cells (Taormino & Fambrough, 1990). These results provide strong evidence for a role of increased $[Na^+]_i$ in Na^+,K^+-ATPase transcriptional regulation. However, since these investigations have only taken place in cultured cells or cardiac myocytes, extrapolation of similar effects to *in-vivo* or *in-vitro* mammalian skeletal muscle cannot be assumed and therefore warrants investigation.

2.12.2.2 Increased $[Ca^{2+}]_{cyto}$

Elevated $[Ca^{2+}]_{cyto}$ is known to activate differential signalling pathways, thereby altering the expression of numerous genes (Williams & Neufer, 1996). As $[Ca^{2+}]_{cyto}$ is increased with repeated muscle contractions (Westerblad *et al.*, 1993; Chin & Allen, 1996; Lunde *et al.*, 2001), it is conceivable that elevated $[Ca^{2+}]_{cyto}$ may be involved in Na^+,K^+-ATPase transcriptional regulation. Indeed, in cultured rat kidney cells, high $[Ca^{2+}]_i$ (1.0 μM) induced a 4.0-fold increase in both Na^+,K^+-ATPase α_1 and β_1 mRNA expression in rat kidney cells, with the α_1 and β_1 mRNA degradation rates related to the $[Ca^{2+}]_i$ (Rayson, 1991). Furthermore, the magnitude of the ouabain-induced increases in Na^+,K^+-ATPase α_1 and β_1 mRNA expression in cultured rat kidney cells depended on the $[Ca^{2+}]_i$ (Rayson, 1993). These results therefore provide support for a regulatory role of $[Ca^{2+}]_{cyto}$ for Na^+,K^+-ATPase mRNA expression, although the existence of such regulation in skeletal muscle needs to be determined.

2.12.2.3 Increased $[K^+]_e$

Repeated muscle contractions reduced $[K^+]_i$ and increased $[K^+]_e$, leading to membrane depolarisation (see Section 2.2). No study has elevated $[K^+]_e$ to investigate Na^+,K^+-ATPase isoform mRNA expression. However, several have incubated rat liver (Pressley *et al.*, 1988) and canine kidney cell cultures (Bowen & McDonough, 1987) in low $[K^+]_e$ (0.25-

0.65 mM). Low $[K^+]_e$ induced a 2.0-fold increase in total (non-specific) RNA and a 3.5-fold increase in non-isoform specific Na^+,K^+-ATPase α mRNA expression in one study (Pressley *et al.*, 1988), and a 3.0-fold increase in non-isoform specific Na^+,K^+-ATPase α and β mRNA expression in another study (Bowen & McDonough, 1987). Furthermore, restoration of $[K^+]_e$ to control levels (5.5 mM) for 6 h completely recovered α and β mRNA expression to control values in cultured pig kidney cells (Lescale-Matys *et al.*, 1993a). These results demonstrate that reduced $[K^+]_e$ elevated Na^+,K^+-ATPase mRNA expression in cultured cells, however, whether the increased $[K^+]_e$ that is associated with repeated muscle contractions, also increase Na^+,K^+-ATPase mRNA expression is unknown.

2.12.2.4 Production of ROS

ROS are generated as by-products of oxidative metabolism, and have a deleterious effect on skeletal muscle (Jenkins, 1988). In resting skeletal muscle, ROS effects are minimised due to the presence of endogenous antioxidants (Jackson *et al.*, 1985; van der Poel & Stephenson, 2002). However, during repeated muscle contractions, the greatly enhanced rates of ROS production overwhelm the protective effect of antioxidants, to increase ROS in rat (Jackson *et al.*, 1985; Reid *et al.*, 1992) and in human muscle (Jackson *et al.*, 1985; Bailey *et al.*, 2003).

There is accumulating evidence that increased ROS may alter the expression of numerous genes, by activation of transcription factors (Storz *et al.*, 1990; Schreck *et al.*, 1991; Toledano & Leonard, 1991), and by acting as a second messenger in several signalling pathways involved in transcription (Xie *et al.*, 1999). An increase in ROS in skeletal muscle may be involved in Na^+,K^+-ATPase transcriptional regulation. In rat cardiac myocytes, the non-specific antioxidant compound, *N*-acetylcysteine (NAC), inhibited the

ouabain-induced decrease in α_3 mRNA expression (Xie *et al.*, 1999). Whether ROS are involved in Na^+,K^+-ATPase transcriptional regulation in skeletal muscle is unknown.

The effects of an increase in each of $[Na^+]_i$, $[Ca^{2+}]_{cyto}$, $[K^+]_e$ and ROS on Na^+,K^+-ATPase transcriptional regulation in rat skeletal muscle were therefore investigated in Chapter 7 in this thesis.

2.12.3 Mechanisms of elevated Na^+,K^+-ATPase protein expression

Up-regulation of muscle Na^+,K^+-ATPase protein abundance can be induced via i) increased protein translation, ii) attenuated protein degradation, ii) a combination of both increased translation and attenuated degradation reflecting improved protein stability, and/or iv) translocation of pre-existing Na^+,K^+-ATPase complexes from intracellular compartments. This section reviews the evidence for each of these processes contributing to Na^+,K^+-ATPase isoform protein up-regulation.

2.12.3.1 Increased protein translation and/or attenuated degradation

Only a limited number of studies have investigated the rates of protein synthesis and degradation in Na^+,K^+-ATPase up-regulation. These studies utilised cultured chick skeletal muscle, rat, canine or pig kidney cells, treated with either low $[K^+]_e$ (0.25 – 0.50 mM), ouabain (0.1 mM) or veratridine (0.01 mM) (Wolitzky & Fambrough, 1986; Bowen & McDonough, 1987; Rayson, 1989; Lescale-Matys *et al.*, 1990; Lescale-Matys *et al.*, 1993). Using cultured chick skeletal muscle cells treated with veratridine, Wolitzky & Fambrough (1986) were the first to assess the translation and degradation rates of the Na^+,K^+-ATPase β subunit (specific isoforms not tested). This involved isolating the β subunit by immuno-precipitation and a monoclonal β antibody. Rates of synthesis and degradation were then assessed by pulse-labelling myotubes with [^{35}S]methionine, and measuring the subsequent incorporation of radioactivity into the isolated β subunit (Fambrough *et al.*, 1991). The rate of β subunit translation increased progressively during the first 12-18 h of veratridine

incubation, matching the Na^+,K^+-ATPase up-regulation both in time-course and magnitude. Peak translation rate occurred after 18 h of veratridine incubation, at levels 180% above controls. The rate of translation then slowed and eventually returned to control values by 36 h of veratridine incubation. However, concomitant with the slowing in translation, β subunit degradation rate was attenuated. Thus, the mean half life of Na^+,K^+-ATPase enzymes treated with veratridine was more than double that of controls (64 h vs 31 h). It was therefore concluded that the increase in Na^+,K^+-ATPase content with veratridine was mediated by an initial and transient increase in translation, attaining a peak rate at 18 h of veratridine incubation, followed by an attenuation of protein degradation.

However, the study by Wolitzky & Fambrough (1986) was limited in that it only reflected translation and degradation rates of the Na^+,K^+-ATPase β subunit. Rayson (1989) investigated the specific responses of both the α and β subunits to Na^+,K^+-ATPase up-regulation and produced results consistent with those of Wolitzky & Fambrough (1986). In rat outer medullary kidney tubules treated with ouabain (0.1 mM), translation rates for both α and β subunits were markedly elevated within 12-18 h, before returning to control levels by 24 h ouabain incubation (Rayson, 1989). Despite a transient increase in the rate of degradation at 18 h of ouabain incubation, degradation was significantly attenuated by 24 h incubation, thereby maintaining the Na^+,K^+-ATPase up-regulation (Rayson, 1989).

In another study, Bowen and McDonough (1987) used affinity purified antibodies specific to each of the α and β subunits, and treated canine kidney cultures in low $[K^+]_e$ (0.25 mM K^+). Although the rates of α and β subunit degradation were not measured, the 200% increase in both α and β subunit translation rates at 8 h of incubation accounted for the elevation in sarcolemmal α and β subunit protein abundance. However, since a 300% increase in mRNA expression of each of the α and β subunits preceded these elevations in translation rate, it appears that the increase in α and β subunit protein abundance with low

$[K^+]_e$ was driven by an increase in subunit transcription. In variation to this finding, another study by the same laboratory found that in pig kidney cells, an increase in mRNA expression of the β subunit was sufficient to drive an increase in protein abundance of both of the α and β subunits (Lescale-Matys *et al.*, 1990). This was based on the fact that incubation in low $[K^+]_e$ increased the protein abudance of the α and β subunits and the activity of the Na^+,K^+-ATPase, but that mRNA expression of only the β subunit was increased. The authors therefore concluded that the β subunit is limiting for αβ assembly (Lescale-Matys *et al.*, 1990). This finding was extended three years later when in the same model, the relative abundance of the α and β subunits was determined (Lescale-Matys *et al.*, 1993a). Immediately following incubation in low $[K^+]_e$, β subunits were present in a 3-fold excess over α subunits (Lescale-Matys *et al.*, 1993a). However, over the following 60 min, ~50% of the newly synthesised β subunits were degraded, without any corresponding change in α subunit degradation. (Lescale-Matys *et al.*, 1993a) The stability of α subunit abundance was thought to reflect the assembly of αβ heterodimers, while the excess β subunits were degraded.

The processes involved in mediating Na^+,K^+-ATPase up-regulation in cell cultures therefore appear to involve a combination of increased transcription and translation, together with attenuation of protein degradation. There is also evidence for this up-regulation to be mediated by the β subunit, which is synthesised in excess to ensure the formation of αβ heterodimers. However, whether these processes are similar in *in-vivo* or *in-vitro* skeletal muscle and also, in response to acute and chronic exercise, are unknown. Nonetheless, the time course of up-regulation suggests that an increase in protein abundance within 24 h of exercise is possible. This was therefore investigated in Chapters 3 and 4 in this thesis.

2.12.4 Mechanisms of translational regulation

2.12.4.1 Increased $[Na^+]_i$

Since the three interventions employed to induce increased Na^+,K^+-ATPase content; veratridine, low $[K^+]_e$ and ouabain all induced an elevation in intracellular Na^+ (Bowen & McDonough, 1987; Gissel & Clausen, 1999), increased $[Na^+]_i$ is considered a primary candidate initiating Na^+,K^+-ATPase up-regulation.

In cultured chick ventricular cells, elevated $[Na^+]_i$ induced via reduced $[Na^+]_e$, resulted in a concentration-dependent increase in Na^+,K^+-ATPase content (Kim & Smith, 1986). This effect was blocked by the voltage-gated Na^+-channel inhibitor, tetrodotoxin (Kim & Smith, 1986). Furthermore, in cultured chick skeletal muscle cells, the 60 to 100% increase in Na^+,K^+-ATPase content with veratridine was completely reversed within 6 h exposure to tetrodotoxin (Wolitzky & Fambrough, 1986). In the same study, reducing $[Na^+]_i$ via high $[K^+]_e$ (50 mM) had no effect on Na^+,K^+-ATPase content (Wolitzky & Fambrough, 1986).

2.12.4.2 Increased $[Ca^{2+}]_{cyto}$

Since elevated $[Ca^{2+}]_{cyto}$ may play a role in Na^+,K^+-ATPase transcriptional regulation (see Section 2.12.2.2), it is possible that elevated $[Ca^{2+}]_{cyto}$ is also important for Na^+,K^+-ATPase translational regulation. However, incubation of cultured chick skeletal muscles cells with the specific Ca^{2+}-ionophore A23187, had no effect on sarcolemmal Na^+,K^+-ATPase content (Wolitzky & Fambrough, 1986). Furthermore, a 40% increase in $[Ca^{2+}]_i$ induced via elevated $[Ca^{2+}]_e$ had no effect on either $[Na^+]_i$ or on Na^+,K^+-ATPase content, as measured by $[^3H]$-ouabain binding (see Kim & Smith, 1986).

Elevated $[Na^+]_i$ therefore remain the most plausible intracellular signal to be involved in Na^+,K^+-ATPase translational regulation. It is unlikely that elevated $[Ca^{2+}]_i$, secondary to Na^+/Ca^{2+} exchange may be involved in regulation of Na^+,K^+-ATPase translation. However,

these findings are based on results from cultured cells, which may differ for Na^+,K^+-ATPase regulation in *in-vivo* or *in-vitro* skeletal muscle.

2.12.5 Mechanism of sarcolemmal protein expression: exercise-induced isoform translocation

Exercise-induced Na^+,K^+-ATPase isoform translocation remains controversial in both rat and human skeletal muscle.

2.12.5.1 Exercise effects in rat skeletal muscle

In rats, 1 h of prolonged treadmill running increased the sarcolemmal α_1 and α_2 protein abundance in red, oxidative and white, glycolytic fibres, respectively (Tsakiridis *et al.*, 1996). In that study, differential centrifugation and immunoblotting were used to separate the sarcolemmal and intracellular membrane fractions (Tsakiridis *et al.*, 1996). However, there was no concomitant change in the protein abundance of these isoforms in the intracellular membrane fraction. This finding indicated that exercise increased the retention of α_1 and α_2 isoforms at the sarcolemma and/or induced translocation of α_1 and α_2 from intracellular organelles not detected with the membrane fractionation technique.

There was no effect of exercise on the sarcolemmal or intracellular protein abundance of either the β_1 or β_2 isoforms in red, oxidative and white, glycolytic fibres, respectively (Tsakiridis *et al.*, 1996). The specific increase in sarcolemmal α protein abundance may be explained by the pre-exercise observation of a 600 to 1,300% higher ratio of β to α isoforms at the sarcolemma compared to the intracellular membrane fraction (Tsakiridis *et al.*, 1996). This suggests that a pool of β isoforms may be maintained at the sarcolemma to form functional $\alpha\beta$ complexes with the α isoforms recruited with acute exercise.

However, in a later study using the same exercise protocol, but employing the giant vesicles technique to isolate the sarcolemmal membrane, exercise induced an increase in the sarcolemmal protein abundance of the α_1, α_2, β_1 and β_2 isoforms in red, oxidative

fibres, and the α_1, α_2 and β_2 isoforms in white, glycolytic fibres (Juel *et al.*, 2001). This increase was transient, with protein abundance of each of these isoforms returning to control levels by 3 h post-exercise. The transient increase in Na^+,K^+-ATPase isoforms at the sarcolemma during exercise was interpreted to reflect a translocation to and from the membrane, rather than an overall increase in cellular Na^+,K^+-ATPase expression (Juel *et al.*, 2001). The discrepancy between the afore-mentioned studies regarding the isoforms up-regulated with exercise may reflect the different membrane fractionation techniques utilised and the potential errors associated with each technique (see Section 2.5.1).

Juel *et al.*, (2001) also measured [^{3}H]-ouabain binding site content in sarcolemmal vesicles from mixed rat hindlimb muscle. One h of treadmill running elevated [^{3}H]-ouabain binding site content by 30% in these vesicles, suggesting that at least the increased abundance of α_2 isoforms at the sarcolemma may represent functional isoforms. However, the validity of such a large increase is difficult to determine since physical training induces only a similar magnitude of increase in [^{3}H]-ouabain binding site content (Section 2.11.1).

2.12.5.2 Electrical stimulation effects in rat muscle

The effects of electrical stimulation on Na^+,K^+-ATPase translocation in rat skeletal muscle was investigated in two studies, but with conflicting results (Juel *et al.*, 2001; McKenna *et al.*, 2003b). In mixed hindlimb muscle, 5 min of intermittent electrical stimulation at 30 Hz increased sarcolemmal α_1 and β_1 protein abundance, by 22 and 18%, respectively, but had no effect on sarcolemmal α_2 protein abundance (Juel *et al.*, 2001). Different protocols of electrical stimulation, ranging from durations of 10 s to 4 h at frequencies ranging from 1 to 120 Hz, did not significantly increase the sarcolemmal protein abundance of functional α_2 isoforms, as reflected by [^{3}H]-ouabain binding in isolated rat muscle (McKenna *et al.*, 2003b). In fact, [^{3}H]-ouabain binding site content expressed per g wet weight muscle was actually decreased following electrical stimulation at both 10 and 120 Hz (McKenna *et al.*,

2003b). This was most likely due to water shifts since there was no significant change in [^{3}H]-ouabain binding site content expressed per g dry weight muscle (McKenna *et al.*, 2003b).

2.12.5.3 Exercise effects in human muscle

In humans, the only study investigating exercise-induced Na^+,K^+-ATPase isoform translocation used giant sarcolemmal vesicles (Juel *et al.*, 2000a). In that study, ~5 min of fatiguing one-legged knee extensor exercise increased the sarcolemmal protein abundance of the α_2 and β_1 isoforms. Exercise had no effect on sarcolemmal α_1 protein abundance, which may be explained by the low intracellular expression of α_1 in human skeletal muscle (see Table 2.2) (Hundal *et al.*, 1994).

Therefore, acute exercise appears to induce isoform-specific increases in sarcolemmal Na^+,K^+-ATPase protein abundance. However, whether these increases reflect translocation of pre-existing isoforms from intracellular sites, enhanced retention of sarcolemmal isoforms remains unknown, or methodological problems associated with membrane isolation (Section 2.5.1) remains unknown.

In summary, the Na^+,K^+-ATPase appears to be adaptable to altered levels of physical activity. Both acute and chronic exercise induce alterations in Na^+,K^+-ATPase isoform mRNA and protein expression, Na^+,K^+-ATPase content and maximal activity. However characterisation of these alterations, including the mechanisms responsible for inducing these alterations, remains incomplete due to the failure of these studies to probe for the full complement of Na^+,K^+-ATPase isoforms, as well as the limited research performed in some of these areas.

SECTION IV: AIMS AND HYPOTHESES

The general aim of this thesis was to investigate the effects of acute and chronic exercise, electrical stimulation and possible mechanistic factors regulating Na^+,K^+-ATPase isoform transcription and translation in skeletal muscle. The aims and specific hypotheses of each study are outlined below.

Study 1, Chapter 3

The first aim was to characterise Na^+,K^+-ATPase isoform expression in human skeletal muscle, by measurement of both the gene transcript and protein expression. The second aim was to investigate the effects of brief intense exercise on the Na^+,K^+-ATPase isoform mRNA and protein expression, probing for all isoforms found to be expressed in human skeletal muscle.

Specific hypotheses tested were that:

Hyp 1.1 Each of the Na^+,K^+-ATPase α_1, α_2, α_3, β_1, β_2 and β_3 isoforms would be expressed in the vastus lateralis muscle obtained from healthy individuals.

Hyp. 1.2 Brief intense exercise would transiently increase the mRNA and crude muscle homogenate protein abundance of the Na^+,K^+-ATPase α_1, α_2, α_3, β_1, β_2 and β_3 isoforms within a 24 h post-exercise period.

Study 2, Chapter 4

The first aim was to investigate the effects of prolonged submaximal exercise on Na^+,K^+-ATPase isoform mRNA and protein expression, maximal activity and content in human skeletal muscle. The second aim was to investigate the effects of prolonged submaximal exercise on the mRNA expression of the transcription initiator gene RNAP II, and of the key genes initiating protein translation, eIF-4E and 4E-BP1.

Specific hypotheses tested were that prolonged submaximal exercise would:

Hyp. 2.1 Increase mRNA expression, but not protein abundance of each of the Na^+,K^+-ATPase α_1-α_3 and β_1-β_3 isoforms.

Hyp. 2.2 Transiently depress maximal Na^+,K^+-ATPase activity, with recovery by 3 or 24 h post-exercise, but would not elevate Na^+,K^+-ATPase content in the 24 h period following exercise. The depression in maximal Na^+,K^+-ATPase activity would also be negatively correlated with the increase in Na^+,K^+-ATPase mRNA expression

Hyp. 2.3 Increase the mRNA expression of RNAP II and 4E-BP1, but reduce mRNA expression of eIF-4E.

Study 3, Chapter 5

The first aim of this study was to investigate the effects of high-intensity, intermittent exercise on the mRNA and protein expression of each of the six Na^+,K^+-ATPase isoforms present in human skeletal muscle. The second aim was to investigate the effects of short-term high-intensity, intermittent training on the mRNA and protein expression of each of the Na^+,K^+-ATPase isoforms expressed in skeletal muscle of already well-trained athletes. Specific hypotheses tested were that:

Hyp. 3.1 A single bout of high-intensity, intermittent exercise would increase the mRNA, but not protein abundance of each of the Na^+,K^+-ATPase α_1-α_3 and β_1-β_3 isoforms.

Hyp. 3.2 Short-term high-intensity, intermittent training would increase resting mRNA and protein expression of each of the Na^+,K^+-ATPase α_1-α_3 and β_1-β_3 isoforms, but not significantly alter the response of Na^+,K^+-ATPase isoform mRNA and protein expression to high-intensity, intermittent exercise.

Study 4, Chapter 6

The first aim of this study was to investigate the effects of chronic endurance training on mRNA expression of each of the Na^+,K^+-ATPase isoforms. The second aim was to investigate whether the elevated muscle Na^+,K^+-ATPase content in chronically endurance trained athletes is dependent on years of training. The third aim was to investigate whether chronic endurance training up-regulates the maximal activity of the Na^+,K^+-ATPase in a large cohort of well-trained endurance athletes. The fourth aim was to investigate whether any differences exist between genders for Na^+,K^+-ATPase isoform mRNA expression and maximal activity, and to clarify possible differences in Na^+,K^+-ATPase content.

Specific hypotheses tested were that:

Hyp. 4.1 Chronically endurance trained athletes would demonstrate higher Na^+,K^+-ATPase mRNA expression than recreationally active subjects for each of the six isoforms.

Hyp. 4.2 Muscle Na^+,K^+-ATPase content in chronically endurance trained athletes would not be significantly correlated with years of training.

Hyp. 4.3 Chronically endurance trained athletes would demonstrate higher maximal Na^+,K^+-ATPase activity than recreationally active subjects.

Hyp. 4.4 There would be no difference for either Na^+,K^+-ATPase mRNA expression, maximal activity or content between genders where no gender difference was also found for $\dot{V}O_{2peak}$.

Study 5, Chapter 7

The first aim of this study was to clarify the relative expression of each of the Na^+,K^+-ATPase isoform gene transcripts between muscles of differing fibre-type composition. The second aim was to investigate the effects of three bouts of high-frequency electrical stimulation on the mRNA expression of the Na^+,K^+-ATPase isoforms in rat EDL and soleus muscles. The third aim was to investigate the effects of interventions designed to

increase $[Na^+]_i$, $[Ca^{2+}]_{cyto}$, $[K^+]_e$ and scavenging of ROS, on Na^+,K^+-ATPase transcriptional regulation in rat EDL muscle.

Specific hypotheses tested were that:

Hyp. 5.1 The relative expression of the α_2 and β_1 gene transcripts would be greater in the soleus than the EDL, while in contrast β_2 mRNA expression would be greater in the EDL than the soleus. There would be no difference in α_1, α_3 or β_3 mRNA expression between muscles.

Hyp. 5.2 Three bouts of high-frequency electrical stimulation of isolated rat soleus and EDL muscle would increase the mRNA expression of each of the Na^+,K^+-ATPase α_1, α_2, α_3, β_1, β_2 and β_3 isoforms.

Hyp. 5.3 In rat EDL muscle, mRNA expression would be:

i. elevated for the α_1-α_3 and β_1-β_3 isoforms with ouabain, veratridine and monensin, each designed to increase $[Na^+]_i$.

ii. elevated for the α_1-α_3 and β_1-β_3 isoforms with caffeine, designed to increase $[Ca^{2+}]_{cyto}$.

iii. elevated for the α_1-α_3 and β_1-β_3 isoforms with high $[K^+]_e$, designed to induce membrane depolarisation.

iv. attenuated for the α_1-α_3 and β_1-β_3 isoforms with NAC, designed to scavenge ROS.

CHAPTER 3

STUDY 1: EFFECTS OF BRIEF INTENSE EXERCISE ON Na^+,K^+-ATPase ISOFORM mRNA AND PROTEIN EXPRESSION IN HUMAN SKELETAL MUSCLE

3.1 INTRODUCTION

Characterisation of the Na^+,K^+-ATPase isoforms expressed in human skeletal muscle remains incomplete, at both gene transcription and protein levels. The α_1, α_2, β_1 and β_3 isoforms are the only gene transcripts to be previously investigated and detected in human skeletal muscle (Malik *et al.*, 1998; Nordsborg *et al.*, 2003a). The first study to investigate Na^+,K^+-ATPase isoform protein expression in human muscle obtained soleus muscle from amputated lower limbs from patients with non-specified disease (Hundal *et al.*, 1994). They detected the α_1, α_2, α_3 and β_1 proteins, but not the β_2 protein, and stated that the β_2 protein was also undetected in anterior tibialis muscle (Hundal *et al.*, 1994). Similarly, the β_2 protein was undetected in vastus lateralis muscle obtained from healthy humans (Juel *et al.*, 2000a). The apparent absence of β_2 is surprising and contrasts β_2 protein expression in rat muscle (Hundal *et al.*, 1993; Tsakiridis *et al.*, 1996). Conflicting data exists on α_3 protein expression in human muscle. Whereas the α_3 protein was detected in soleus muscle from amputated lower limb (Hundal *et al.*, 1994), it was not found in human skeletal muscle of unspecified origin and pathology (Wang *et al.*, 2001). Finally, despite very low levels of β_3 mRNA being reported in human skeletal muscle (Malik *et al.*, 1998), β_3 protein expression has not yet been investigated. Thus, research is required to clarify expression of the α_3, β_2 and β_3 isoforms. This knowledge would improve our understanding of the possible $\alpha\beta$ heterodimers expressed in human skeletal muscle. Additionally, if any of the α_3, β_2 and β_3 isoforms are present, this would challenge the isoform-specificity of any

exercise- or insulin-induced translocation of Na^+,K^+-ATPase isoforms since studies investigating this phenomenon have only measured the α_1, α_2 and β_1 isoforms (Tsakiridis *et al.*, 1996; Juel *et al.*, 2000a; Juel *et al.*, 2001)). Previous studies investigating isoform protein expression have isolated sarcolemmal membranes via membrane fractionation on a sucrose gradient (Hundal *et al.*, 1994; Tsakiridis *et al.*, 1996) or via the formation of giant sarcolemmal vesicles (Juel *et al.*, 2000a; Juel *et al.*, 2001), thereby restricting detection of Na^+,K^+-ATPase isoforms to those of sarcolemmal origin. This study therefore firstly aimed to characterise Na^+,K^+-ATPase isoform expression in human skeletal muscle, by measurement of both the gene transcript and protein expression, utilising a crude muscle homogenate to enhance detection of Na^+,K^+-ATPase isoforms in the whole muscle. The hypothesis tested was that each of the Na^+,K^+-ATPase α_1, α_2, α_3, β_1, β_2 and β_3 isoforms would be expressed in the vastus lateralis muscle obtained from healthy individuals.

Little is known about the effects of brief intense exercise on Na^+,K^+-ATPase isoform expression. This in part reflects the inconsistent findings regarding expression of Na^+,K^+-ATPase isoforms in skeletal muscle. In humans, different exercise protocols elevated the mRNA expression in muscle of a variety of genes involved in metabolism (Pilegaard *et al.*, 2000). This raises the possibility that exercise might up-regulate many of the Na^+,K^+-ATPase isoforms. In rats, 1 h of continuous treadmill running elevated α_1 and β_2 mRNA, and also the sarcolemmal membrane α_1 and α_2 isoform protein abundance in both red-oxidative and white-glycolytic fibres (Tsakiridis *et al.*, 1996). In contrast, 1 h of intermittent treadmill running increased the sarcolemmal protein abundance of the α_1, α_2, β_1 and β_2 isoforms (Juel *et al.*, 2001). Interestingly, these elevations were transient with expression of all isoforms returning to control levels at 3 h post-exercise (Juel *et al.*, 2001). The transient increase in Na^+,K^+-ATPase isoforms at the sarcolemma during exercise was interpreted by the authors (Juel et al., 2001) to reflect a translocation to and from the

membrane, rather than an overall increased cellular Na^+,K^+-ATPase expression. In humans, high-intensity intermittent one-legged knee extensor exercise elevated Na^+,K^+-ATPase α_1 mRNA expression by 3.0-fold at 0 h, 1 h and 3 h post-exercise, with expression returning to resting levels at 5 h post-exercise (Nordsborg *et al.*, 2003a). No effect of exercise on α_2 and β_1 mRNA expression was found, however, their study was limited by low statistical power (Nordsborg *et al.*, 2003a). Furthermore, only the Na^+,K^+-ATPase α_1, α_2 and β_1 gene transcripts were probed (Nordsborg *et al.*, 2003a). To date, only a single study has investigated exercise effects on Na^+,K^+-ATPase isoform protein abundance in human skeletal muscle, and this only probed for the α_1, α_2 and β_1 isoforms (Juel *et al.*, 2000a). They reported that intense one-legged knee extensor exercise elevated the sarcolemmal α_2 and β_1 isoform protein abundance by 70% and 26%, respectively (Juel *et al.*, 2000a). The time course of any up-regulation in isoform expression in human skeletal muscle has not been investigated. The second aim of this study was therefore to investigate the effects of brief intense exercise on the Na^+,K^+-ATPase isoform mRNA and protein expression, probing for all isoforms expressed in human skeletal muscle. It was hypothesised that brief intense exercise would transiently increase the mRNA and crude muscle homogenate protein abundance of the Na^+,K^+-ATPase α_1, α_2, α_3, β_1, β_2 and β_3 isoforms within a 24 h post-exercise period.

3.2 METHODS

An overview of the muscle analyses performed and the methods utilised to do so, as well as the sample size used for each of the 5 studies in this thesis is given in Appendix 2.15.

3.2.1 Subjects

Fifteen healthy subjects, comprising 8 males and 7 females (age, 24.7 ± 6.7 yr; height, 174.5 ± 6.8 cm; body mass, 73.2 ± 11.4 kg; mean ± SD) gave written informed consent and

participated in this study. All subjects participated in regular physical activity, but none had any prior experience of isokinetic knee extension exercise. Each subject refrained from vigorous exercise for 48 h, and from caffeine and alcohol consumption for 24 h, prior to each of the exercise tests. All protocols and procedures were approved by the Human Research Ethics Committee at Victoria University of Technology. Cell cultures were grown from a vastus lateralis muscle biopsy sample obtained at rest from one additional healthy female (age, 23 yr; height, 164 cm; body mass, 55 kg). Tissue procurement for cell culture procedures was conducted separately at Deakin University and was approved by the Deakin University Human Research Ethics Committee.

3.2.2 Incremental exercise test

Each subject completed an initial incremental exercise test to determine peak oxygen uptake ($\dot{V}O_{2peak}$) as a marker of aerobic fitness, performing incremental exercise (25 $W.min^{-1}$, 80 rpm) on an electrically braked cycle ergometer (Lode Excalibur, Groningen, Holland), until volitional exhaustion. Subjects breathed through a Hans-Rudolph two-way non-rebreathing valve, with expired air passing through low-resistance plastic tubing into a 4 L mixing chamber and being analysed for expired flow using a flow transducer (KL Engineering K520, California, U.S.A.). Fractions of expired oxygen (F_EO_2) and carbon dioxide (F_ECO_2) were measured continuously by rapidly-responding O_2 and CO_2 analysers (Ametek S-3A/II and Ametek CD-3A, Pittsburgh, USA). Oxygen uptake ($\dot{V}O_2$) was calculated continuously and displayed every 15 s on a personal computer (Turbofit, California, USA). The peak level for $\dot{V}O_2$ was calculated using the mean of the two highest values in the last 60 s bout completed. The ventilometer and gas analysers were calibrated before, and checked for drift after each test, with a standard 3 L syringe and precision reference gases, respectively. Heart rate and rhythm were monitored continuously throughout the exercise test with a 4 - lead electrocardiogram (Mortara, Boston, U.S.A.).

3.2.3 Maximal knee extensor muscle strength and muscle fatigue test

A second test was undertaken to determine maximal muscle strength during isokinetic knee extensor contractions, followed by a third test designed to induce local fatigue of the knee extensor muscles, and designated the muscle fatigue test. Two familiarisation trials were conducted for the muscle strength and fatigue tests. A final third trial for the muscle fatigue test included the muscle biopsy and blood sampling procedures. Intervals of one week between familiarisation trial days and two weeks before the final invasive muscle fatigue trial were utilised, to minimise any local training effects. Subjects consumed standardised meals and fluid intake on the day prior to, during and the subsequent day following the invasive muscle fatigue trial. Specifically, on the day of the invasive muscle fatigue trial, subjects were fasted and meals were only consumed after the biopsy at 3 h post-exercise was taken. On the day following the invasive muscle fatigue trial, subjects were fasted again with meals only consumed after the biopsy at 24 h post-exercise was taken.

The muscle strength and fatigue tests were performed on an isokinetic dynamometer (Cybex Norm 770, Henley HealthCare, Massachusetts, USA), and involved isokinetic knee extensions from knee joint angles of 0° through to 90°, at a speed of $180°.s^{-1}$. All torque data were corrected for gravity, and the dynamometer was calibrated for angle, torque and velocity immediately prior to each test. Subjects were strapped to the dynamometer adjustable chair using belts across the hips and chest to restrict movement of the upper body, and across the right thigh to stabilise the active leg. Identical positions were utilised for each of the muscle strength and fatigue tests for a given individual.

The muscle strength test comprised three maximal isokinetic contractions, with muscle-generated work measured for each contraction. The muscle fatigue test involved contractions repeated every 1.5 s, at a target rate corresponding to ~40% of the maximal work output per contraction, as previously determined during the muscle strength test. The

test was continued until fatigue, defined as the failure to maintain 90% of the target work output for three successive contractions. A visual real-time display of the torque and work for each contraction was provided to the subjects during the maximal strength and muscle fatigue tests. Verbal support was provided to encourage subjects to exert maximal torque during the muscle strength test, as well as to maintain the appropriate work and kicking frequency during the muscle fatigue test.

3.2.4 Muscle biopsy sampling

A muscle biopsy was taken at rest, immediately following the invasive muscle fatigue test, and at 3 h and 24 h post-exercise. A local anaesthetic (1% Xylocaine) was injected into the skin and subcutaneous tissue above the vastus lateralis muscle, a small incision was made through the skin and fascia, and a muscle sample of approximately 120 mg was then excised using a Stille needle. Samples were immediately frozen for later analyses.

3.2.5 Real-time RT-PCR measurement of mRNA

Total RNA was extracted from 5-10 mg muscle using the FastRNA reagents (BIO 101, Vista, CA, USA) using a modification of the phenol/chloroform extraction, isopropanol precipitation protocol using the FastRNA protocol (BIO 101). Following a series of centrifugation and separation steps, the resulting RNA pellet was dissolved in EDTA-treated water and stored at –80 °C. Total RNA concentration was determined spectrophotometrically at 260 nm. The ratio of absorbance at 260 and 280 nm (260/280) was 1.98 ± 0.07.

To stabilise and prepare the extracted RNA for PCR amplification, 1 μg of RNA was transcribed into complimentary DNA (cDNA). RNA was heated at 65 °C for 10 min before first strand cDNA was generated using the Promega AMV Reverse Transcription Kit (kit A3500; Promega, Madison, Wisconsin, USA), with oligo$(dT)_{15}$ primers, in the presence of

1 mM of each dNTP and 20 U of recombinant Rnasin ribonuclease inhibitor (Murphy *et al.*, 2003). The resulting cDNA was stored at –20 °C for subsequent analysis.

Real Time-PCR (GeneAmp 5700 Sequence Detection System) was run for 1 cycle (50 °C for 2 min, 95 °C for 10 min) and 50 cycles (95 °C for 15 s, 60 °C for 60 s). Fluorescence resulted from incorporation of SYBR Green (SYBR Green Master Mix, Applied Biosystems) to double stranded DNA and this fluorescence was measured after each repetitive cycle. Triplicate wells were run for each sample. Measurements included a no-template control, as well as a human muscle sample endogenous control, cyclophilin (CYC). Similar efficiencies of the Na^+,K^+-ATPase genes and CYC were obtained (Appendix 6.2). Primer sequences were designed from published sequences (Table 3.1), where possible spanning exon boundaries to minimise contaminant DNA amplification. Gene expression was quantified from fluorescence emission using a cycle threshold (C_T) method. The relative expression of the genes compared with resting samples was made using the expression, $2^{-\Delta\Delta CT}$, in which the expression of each gene was normalised for input cDNA using the housekeeping gene CYC. Exercise had no significant effect ($P = 0.128$) on the mRNA expression of CYC, when expressed in the linear (2^{-C_T}) form (data not shown). The intra-assay coefficient of variation for each target gene was <15.0% for 2^{-C_T} (Table 3.2). Muscle mRNA are presented for 14 subjects (7 males, 7 females), due to insufficient sample for one subject.

3.2.6 Validity of primers

For each primer used, the validity of using C_T values as a measure of starting cDNA concentrations was established using the analyses reported by Murphy *et al.*, (2003), since a linear relationship was found between the C_T values and the logarithm of the cDNA template concentration (Appendix 2.6). The mean efficiency of the PCR reaction for each gene was also established according to Lekanne Deprez *et al.*, (2002) (Appendix 2.6). A

Table 3.1 Human Na^+,K^+-ATPase α_1-α_3 and β_1-β_3 and CYC gene primer sequences used for mRNA analyses

Gene	GenBank Accession	Identity	Sense Primer (5'-3')	Antisense Primer (5'-3')	Exon boundaries
α_1	NM_000701	ATP1A1	TGTCCAGAATTGCAGGTCTTTG	TGCCCGCTTAAGAATAGGTAGG	4
α_2	NM_000702	ATP1A2	GAATGAGAGGCTCATCAGCAT	CAAAGTAGGTGAAGAAGCCACC	12-13
α_3	NM_152296	ATP1A3	GGTGGCTATGACAGAGCACAA	TGCACACAGTGTGTGTTGTATTT	1-3
β_1	NM_001677	ATP1B1	ACCAATCTTACCATGGACACTG	CGGTCTTTCTCACTGTACCCAAT	3-6
β_2	NM_001678	ATP1B2	CCTGCCAATTCAACCGGA	CTGTAACCATAGTGGGTGGAGT	4
β_3	BC011835	ATP1B3	AGTCTGTCCTGATGGAGCACTT	GCATGCTTGAAGTAATGAAATA	4
CYC	XM_004890	PPIA	CCCACCGTGTTCTTCGACAT	CCAGTGCTCAGAGCACGAAA	

Primer sequences were designed using Primer Express software (Applied Biosystems) from gene sequences obtained from GeneBank. Primer specificity was determined using a BLAST search. CYC, cyclophilin.

Table 3.2 Intra-assay variability of 2^{-C_T} values

Gene	2^{-C_T} CV (%)
α_1	11.0
α_2	14.6
α_3	13.1
β_1	10.6
β_2	11.1
β_3	10.7
Human CYC	13.0

Each sample was run in triplicate wells in the same Real-Time PCR run. n = 52. CV, coefficient of variation; C_T, cycle threshold

100% efficient reaction would yield a slope of –3.3, indicating that twice as many amplicon are being produced per PCR cycle. The mean efficiency for each primer yielded a slope of –2.7 to –6.6, which is within the range of those previously reported for genes involved in cell growth and glycolysis (Lekanne Deprez *et al.*, 2002; Murphy *et al.*, 2003).

3.2.7 Western blotting

3.2.7.1 Protein extraction

Muscle samples (20 – 30 mg) were homogenised for 15 s at a speed rating of 4 (Polytron PT1200; Kinematica, Luzern, Switzerland) on ice in a 1:40 dilution with extraction buffer (25 mM Tris-HCl, pH 6.8, 1% sodium dodecyl sulphate (SDS), 5 mM EGTA, 50 mM NaF, 1 mM sodium vanadate, 10% glycerol, 17.4 μg/ml phenylmethylsulphonyl fluoride (PMSF), 10 μg/ml leupeptin and 1 μg/ml aprotinin). Studies utilising repeated centrifugation of muscle and membrane separation result in very low recovery of Na^+,K^+-ATPase enzymes, thereby yielding a final sample that may be unrepresentative of the whole muscle Na^+,K^+-ATPase population (Hansen & Clausen, 1988). Therefore, muscle sample analyses did not include any membrane isolation steps, to maximize recovery of Na^+,K^+-ATPase enzymes. A portion of each sample was heated for 10 min at 90 °C, and analysed for total protein content, as detailed subsequently, (BCA Assay Kit, Pierce, Rockford, Illinois, USA), with bovine serum albumin (BSA) as the standard. The remaining samples were frozen at –80 °C for immunoblotting.

3.2.7.2 Total protein assay

The total protein content of each sample was determined using the Pierce BCA Total Protein Analysis (Rockford, Illinois, USA), with bovine serum albumin (BSA) used as standards. Muscle samples were diluted 1:25 with distilled water and added to 200 μl solution of Pierce BCA protein assay reagents B and A (1:50 dilution). A series of 7 standards (0, 50, 100, 200, 350, 500, 700 μg/ml) were prepared in a 1:20 dilution with the

BCA protein assay reagents. Samples were then incubated for 30 min at 37 °C, followed by a 15 min cool down period at room temperature. Absorbance was read at 550 nm from a microplate reader (Multiskan RC V1.5-0, Labsystems Genesis V3.03), and a standard curve prepared to determine the sample protein content. To ensure consistency in the protein contents of each sample, a western blot was firstly performed for each isoform to determine the optimal protein content required to produce a clear, un-saturated band, which were as follows; 20 μg of protein for Na^+,K^+-ATPase α_2 and β_1, 80 μg of protein for α_1, α_3, β_2 and β_3.

3.2.7.3 Immunoblotting

SDS-PAGE (10% separating gel, 5% stacking gel) was performed and gels were loaded with their respective protein contents, as detailed above. To test for glycosylation of the β_2 isoform, some protein samples were also treated using an *N*-glycosidase F deglycosylation kit (Roche Molecular Biochemicals USA, Indianapolis, IN) before denaturation. Following electrophoresis (20 min, 100 V and 90 min, 150 V), the protein was transferred (90 min, 100 V) to 0.45 μm nitrocellulose membrane, and blocked for 2 h with blocking buffer (5% non-fat milk in tris-buffered saline Tween (TBST)). Membranes were incubated overnight at 4 °C in primary antibodies diluted in blocking buffer containing 0.1% NaN_3. Membranes were washed in 0.05% TBST, and incubated for 1 h in horseradish peroxidase (HRP) conjugated secondary antibodies (goat anti-mouse immunoglobulins or goat anti-rabbit immunoglobulins) diluted 1:10,000 in TBST buffer. Following three washes in 0.05% TBST, membranes were dried and treated with chemiluminescent substrate (Pierce SuperSignal West Pico, Illinois, USA). The signal was captured and imaged (Kodak Digital Science Image Station 400_{CF}, Eastman Kodak Company, CT, USA). Positive control samples included rat brain and kidney homogenates and these were run on each gel to assess the reactivity and specificity of the antibody (see below). The linearity of the blot

signal versus protein loaded for the experimental conditions was established for each antibody.

3.2.7.4 Antibodies

Blots were probed with antibodies specific to each isoform. These were for α_1: monoclonal α6F (developed by D. Fambrough and obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA, USA); α_2: polyclonal anti-HERED (kindly donated by T. Pressley, Texas Tech University); α_3: monoclonal MA3-915 (Affinity Bioreagents, Golden, CO, USA); β_1: monoclonal MA3-930 (Affinity Bioreagents); β_2: polyclonal 610915 (Transduction Laboratories, Lexington, KY, USA); and β_3: polyclonal 610993 (Transduction Laboratories). For comparative purposes, additional polyclonal antibodies used by other researchers who did not detect α_3 or β_2 in human muscle (Hundal *et al.*, 1994; Juel *et al.*, 2000a) were utilised for the α_3 and β_2 isoforms; these antibodies were for α_3: 06-172 (Upstate Biotechnology, Lake Placid, NY, USA); β_2: 06-171 (Upstate Biotechnology); and β_3: 06-817 (Upstate Biotechnology).

3.2.7.5 Specificity of antibodies

BLAST analysis demonstrated that the antigen sequence of each of the antibodies employed did not cross-react with any other non-Na^+,K^+-ATPase proteins. Isoform specificity of the monoclonal antibodies specific to α_1 (α6F) and α_3 (MA3-915) used in the present study has previously been established in control samples including rat kidney and rat brain homogenates (Arystarkova & Sweadner, 1996). Consequently we ran rat kidney and rat brain homogenates as control samples on each gel. Rat kidney and rat brain have previously been reported to express the α_1 and the α_1-α_3 isoforms, respectively (Hundal *et al.*, 1994). Since the α_2 and α_3 isoforms are both expressed in rat brain, but not rat kidney, to differentiate between α_2 and α_3 I required further evidence of antibody specificity.

BLAST analysis of the antigen sequence for each of the α_2 (anti-HERED) and α_3 (MA3-915) antibodies did not cross-react with the amino acid sequence of the α_3 and α_2 isoforms, respectively. Therefore it is unlikely that the antibodies used for α_1-α_3 isoforms would demonstrate cross-reactivity. BLAST analysis of the antigen sequence for the antibody specific to the β_1 (MA3-930) isoform indicated no cross-reactivity with the β_2 or β_3 isoforms. However, there was a 41% shared sequence identity between the antigen sequence of the antibodies specific to β_2 (Transduction Laboratories 610915) and β_3 (Transduction Laboratories 610993) and the β_3 and β_2 isoforms, respectively. Rat kidney and rat brain samples cannot be used as controls as both samples wouldn't necessarily differentiate between the β_2 and β_3 isoforms (Martin-Vasallo *et al.*, 1989; Hundal *et al.*, 1994; Malik *et al.*, 1996). Thus, whilst unlikely, it is possible that the antibodies specific to β_2 and β_3 may cross-react with the other isoform.

3.2.7.6 Preparation of cell cultures

To verify that Na^+,K^+-ATPase isoform expression in the crude homogenate preparation was unlikely to be a contaminant of vascular or nervous tissues, adipocytes or fibrocytes, I also investigated isoform expression in a primary human skeletal muscle cell culture. The sample (~85 mg) was washed 3 times in serum-free medium to remove blood and then minced finely using a scalpel. A 15 ml solution of 0.05% trypsin and 0.53 mM EDTA was added to the muscle then transferred to a sterile flask, and following shaking at low speed for 20 min at room temperature, the supernatant was collected and placed on ice. This procedure was repeated twice more, the supernatants pooled and fetal bovine serum (FBS) was added at a final concentration of at least 10%. The supernatant was filtered through a 100 μm cell filter to remove any connective tissue, and centrifuged at room temperature. The supernatant was removed and the cell pellet resuspended in 5 ml growth media containing 10% fetal calf serum. Cells were then seeded onto an uncoated 25 cm^2 flask to

remove fibroblasts, and incubated for 20 min at 37°C. The medium containing myoblasts was transferred to an extracellular matrix (ECM)-coated 25 cm^2 flask, with the cells cultured at 37°C and 5% CO_2. The following day, the media was changed once, and thereafter twice per week until 60-70% confluent. Cells were seeded into 6-well plates at a density of 100-150,000 cells per well and incubated in growth medium until they reached ~80% confluency. Cells were then incubated in differentiation medium containing 2% horse serum for 4-5 d and prepared for immunoblotting as described above.

3.2.8 Statistical analysis

All data are presented as mean ± SEM, except population statistics where mean ± SD was reported. Muscle data were analysed using a one-way repeated measures ANOVA, with Newman-Kuels *post-hoc* analyses. To account for individual variability in time responsiveness of mRNA to exercise, the average post-exercise mRNA was calculated as the mean of the fatigue, 3 h and 24 h recovery samples for each individual. This average was contrasted against the resting value and analysed using a paired-samples student *t* test. Correlations were determined by least squares linear regression. Significance was accepted at $P < 0.05$.

3.3 RESULTS

3.3.1 Exercise results

Incremental exercise $\dot{V}O_{2peak}$ was 50.5 ± 2.8 $ml.kg^{-1}.min^{-1}$. Maximal work performed per contraction during the maximal knee extensor muscle strength test was 164 ± 21 J. During the muscle fatigue test, time to fatigue was 352 ± 69 s, work performed per contraction was 66 ± 5 J, and 233 ± 46 contractions were performed.

3.3.2 Muscle Na^+,K^+-ATPase mRNA and protein expression in crude muscle homogenates

Real-Time RT-PCR analysis demonstrated amplification of each of the primer sets specific to each of the Na^+,K^+-ATPase α_1, α_2, α_3, β_1, β_2 and β_3 isoforms (Figure 3.1), no amplification of the no template control samples, and the heat dissociation curve confirmed amplification of only a single gene transcript for each primer set (Figure 3.2). These results indicate the presence of gene transcripts for each of the Na^+,K^+-ATPase α_1, α_2, α_3, β_1, β_2 and β_3 isoforms in human vastus lateralis muscle.

Crude muscle homogenates demonstrated protein bands of apparent molecular weight (α_1-α_3; ~100-105 kDa, β_1-β_3; ~45-52 kDa) specific to each of the α_1, α_2, α_3, β_1, β_2 and β_3 subunit isoforms. Representative immunoblots are shown in Figure 3.3. These results indicate protein expression of each of the α_1-α_3 and β_1-β_3 isoforms in human muscle.

Furthermore, deglycosylation of the antibody specific to the β_2 isoform demonstrated a shift in the apparent molecular weight, from ~50 kDa to ~29 kDa, providing further support for the specificity of this antibody (Figure 3.4)

We also probed for the α_3, β_2 and β_3 proteins using additional polyclonal antibodies previously used by others that were unable to detect their expression in human muscle. Protein bands were detected at each of the apparent subunit-specific molecular weights (α_3; ~100 kDa, β_2, β_3; ~50-52 kDa) for each of these isoforms (Figure 3.5).

3.3.3 Na^+,K^+-ATPase protein expression in human cell cultures

Immunoblots performed on crude homogenates from primary human skeletal muscle cell culture also expressed protein bands of the apparent specific subunit motility (α_1-α_3; ~100-105 kDa, β_1-β_3; ~45-52 kDa) for each of the Na^+,K^+-ATPase α_1, α_2, α_3, β_1, β_2 and β_3 isoforms (Figure 3.6).

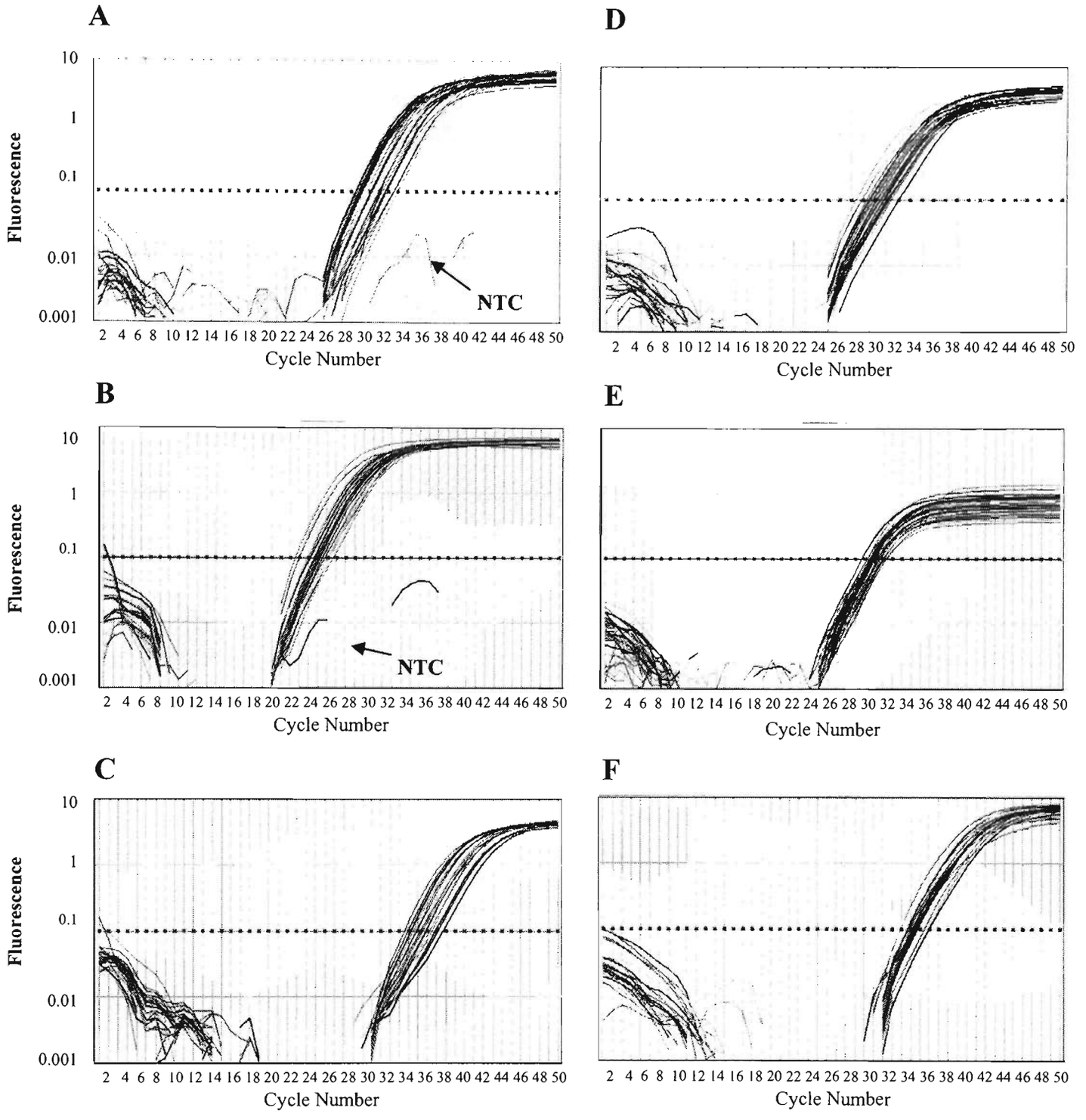


Figure 3.1 Representative amplification plot for Na^+,K^+-ATPase gene transcripts. Na^+,K^+-ATPase (A-F) α_1, α_2, α_3, β_1, β_2, and β_3 isoforms in human skeletal muscle. Samples were subjected to 50 cycles (95 °C for 15 s, 60 °C for 60 s) with fluorescence measured after each cycle. No template control (NTC) samples did not amplify above threshold.

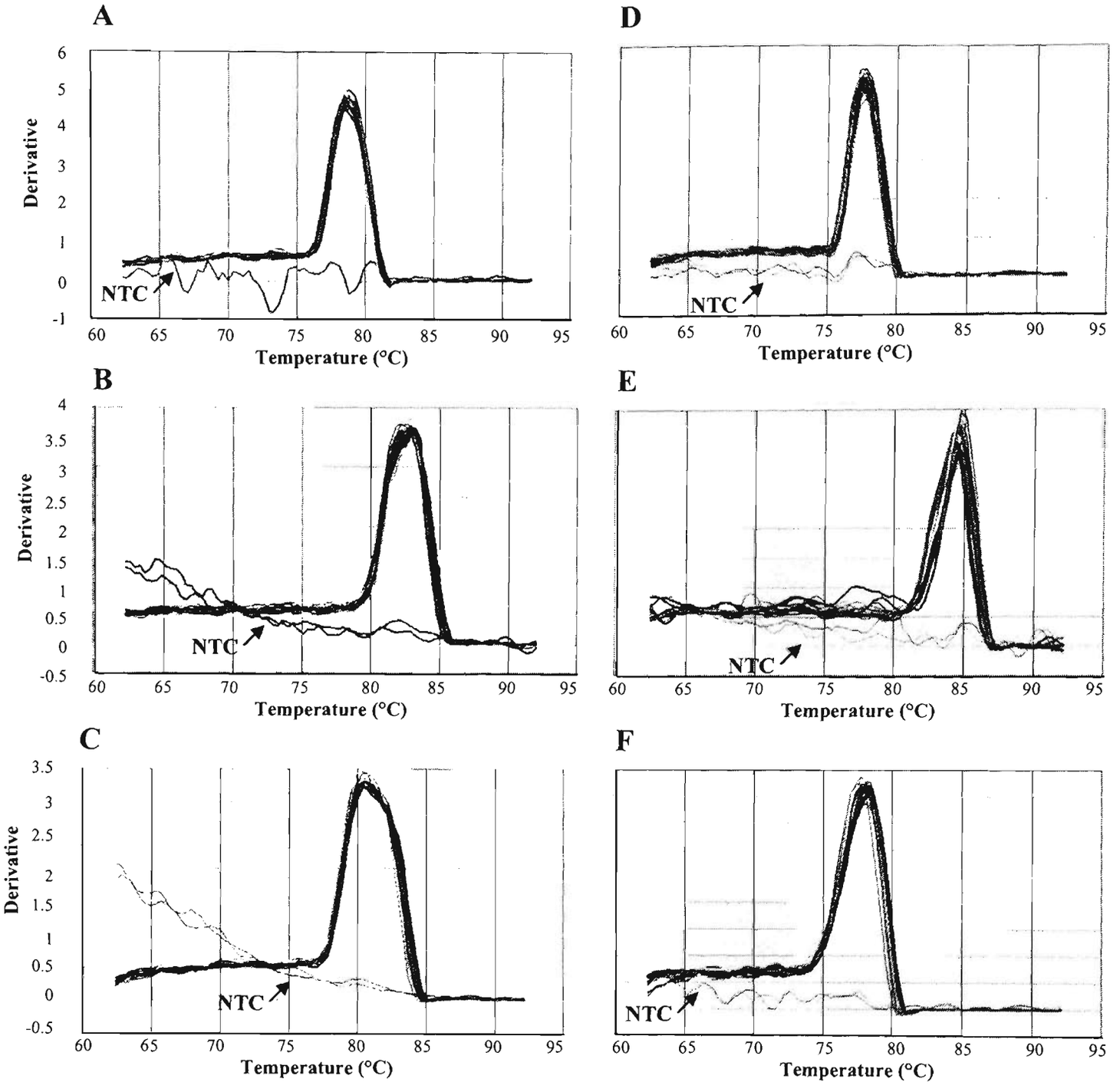


Figure 3.2 Typical heat dissociation curve for Na^+,K^+-ATPase gene transcripts. Na^+,K^+-ATPase (A-F) α_1, α_2, α_3, β_1, β_2, and β_3 isoforms in human skeletal muscle. Following the final PCR cycle, samples and the no template control (NTC) were subjected to a heat dissociation protocol over 60-95 °C, with the derivative being the negative of the rate of change in fluorescence as a function of temperature.

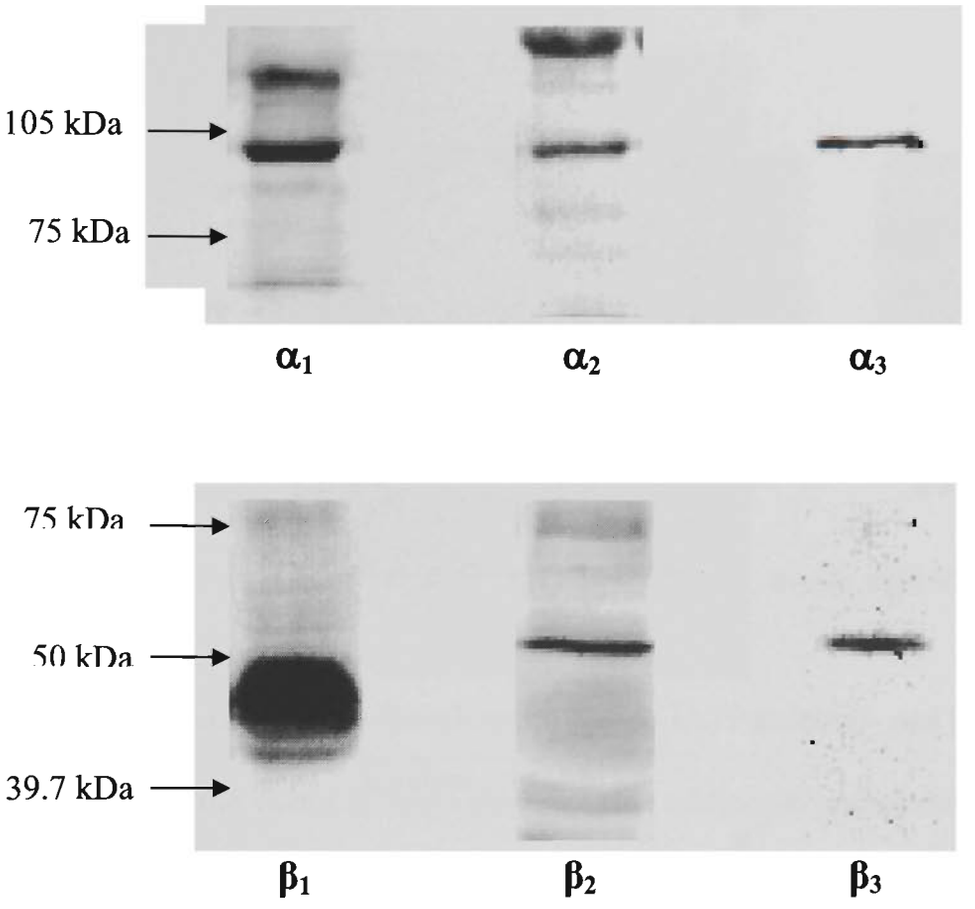


Figure 3.3 Representative immunoblots of Na^+,K^+-ATPase α_1, α_2, α_3, β_1, β_2 and β_3 isoforms in crude muscle homogenates of the human vastus lateralis muscle. Values at left indicate molecular weight of bands.

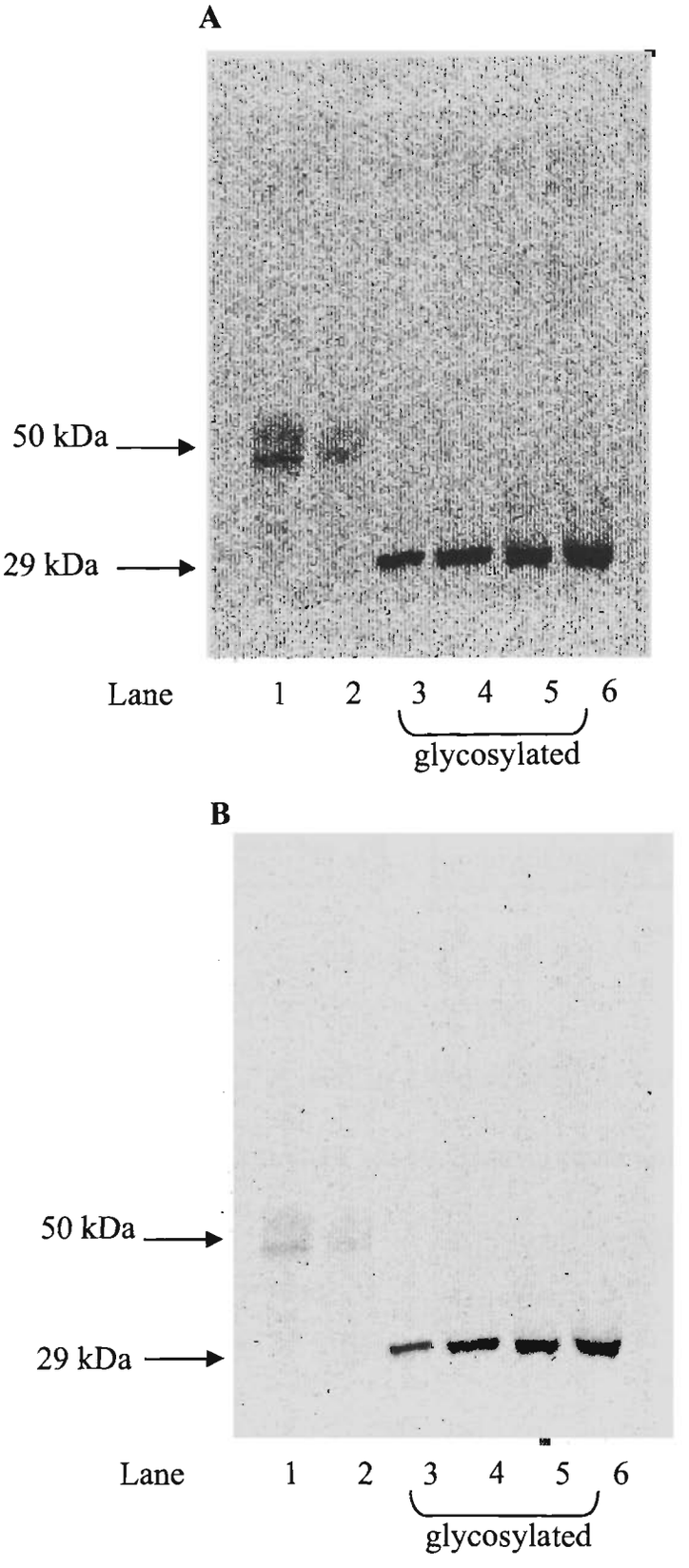


Figure 3.4 Representative immunoblot of the deglycosylated Na^+,K^+-ATPase β_2 isoform in crude muscle homogenates of the human vastus lateralis muscle. (A) Homogenates were treated without (lanes 1 & 2) or with (lanes 3, 4, 5 & 6) *N*-glycosidase F to remove sugars from the glycoprotein, causing a shift in the apparent molecular weight. The amount of protein loaded for lanes 1-6 was as follows; 150, 50, 50, 100, 175 and 200 μg. (B) Same as A, but at a lighter resolution.

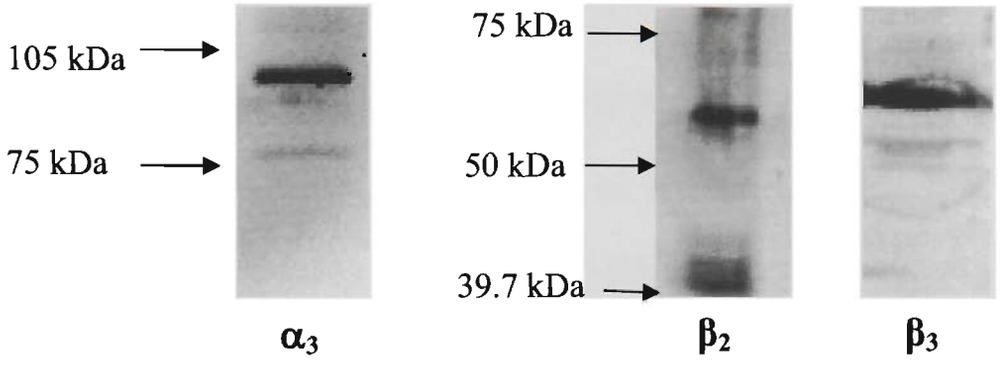


Figure 3.5 Representative immunoblots of Na^+,K^+-ATPase α_3, β_2 and β_3 isoforms in crude homogenates of the vastus lateralis muscle using additional polyclonal antibodies. Values at left indicate molecular weight of bands.

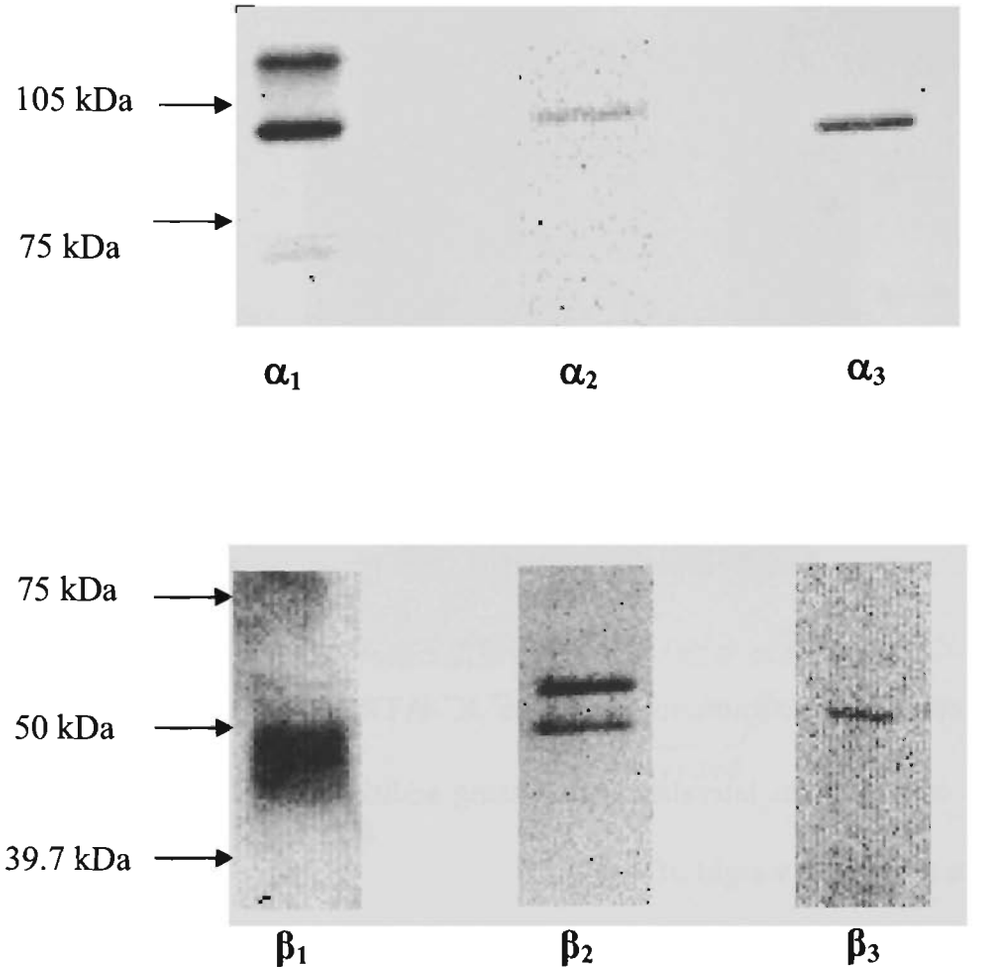


Figure 3.6 Representative immunoblots of Na^+,K^+-ATPase α_1, α_2, α_3, β_1, β_2 and β_3 isoforms in crude homogenates of primary human skeletal muscle cell cultures derived from vastus lateralis muscle. Values at left indicate molecular weight of bands.

3.3.4 Exercise effects on Na^+,K^+-ATPase mRNA and protein expression

α subunit isoforms

Exercise elevated α_1 mRNA expression by 3.5-fold at 24 h post-exercise ($P < 0.05$), but had no effect on crude muscle homogenate α_1 protein expression (Figure 3.7). The α_2 isoform mRNA expression increased by 4.5-fold at 3 h post-exercise ($P < 0.05$), then returned to resting levels by 24 h post-exercise ($P < 0.05$, Figure 3.8). In contrast, exercise had no effect on α_2 protein expression (Figure 3.8). Immediately after the cessation of exercise, α_3 mRNA expression increased by 3.4-fold ($P < 0.05$), then declined significantly by 24 h post-exercise ($P < 0.05$, Figure 3.9). A trend to elevated α_3 protein expression at 3 h post-exercise was seen ($P < 0.06$, Figure 3.9).

β subunit isoforms

Despite a tendency to increase at 3 h post-exercise for β_1 mRNA ($P < 0.08$), exercise had no significant effect on β_1 mRNA or protein expression (Figure 3.10). The β_2 isoform mRNA expression was elevated by 2.7-fold immediately after the cessation of exercise ($P < 0.05$), then returned to resting levels by 24 h post-exercise ($P < 0.05$), with no significant exercise effect on β_2 protein expression (Figure 3.11). Despite a tendency to increase at fatigue and 3 h post-exercise for β_3 mRNA ($P < 0.06$), exercise had no significant effect on β_3 mRNA or protein expression (Figure 3.12).

3.3.5 Individual time course variability in mRNA expression

When investigating mRNA expression for each individual, it became apparent that exercise induced individual variability in isoform mRNA time responsiveness. To further investigate this observation, the average post-exercise expression of the Na^+,K^+-ATPase α_1-α_3 and β_1-β_3 mRNA were determined. As demonstrated in Figure 3.13, for each of the six isoforms, exercise induced elevated mRNA expression in 10 or 12 of the 14 subjects.

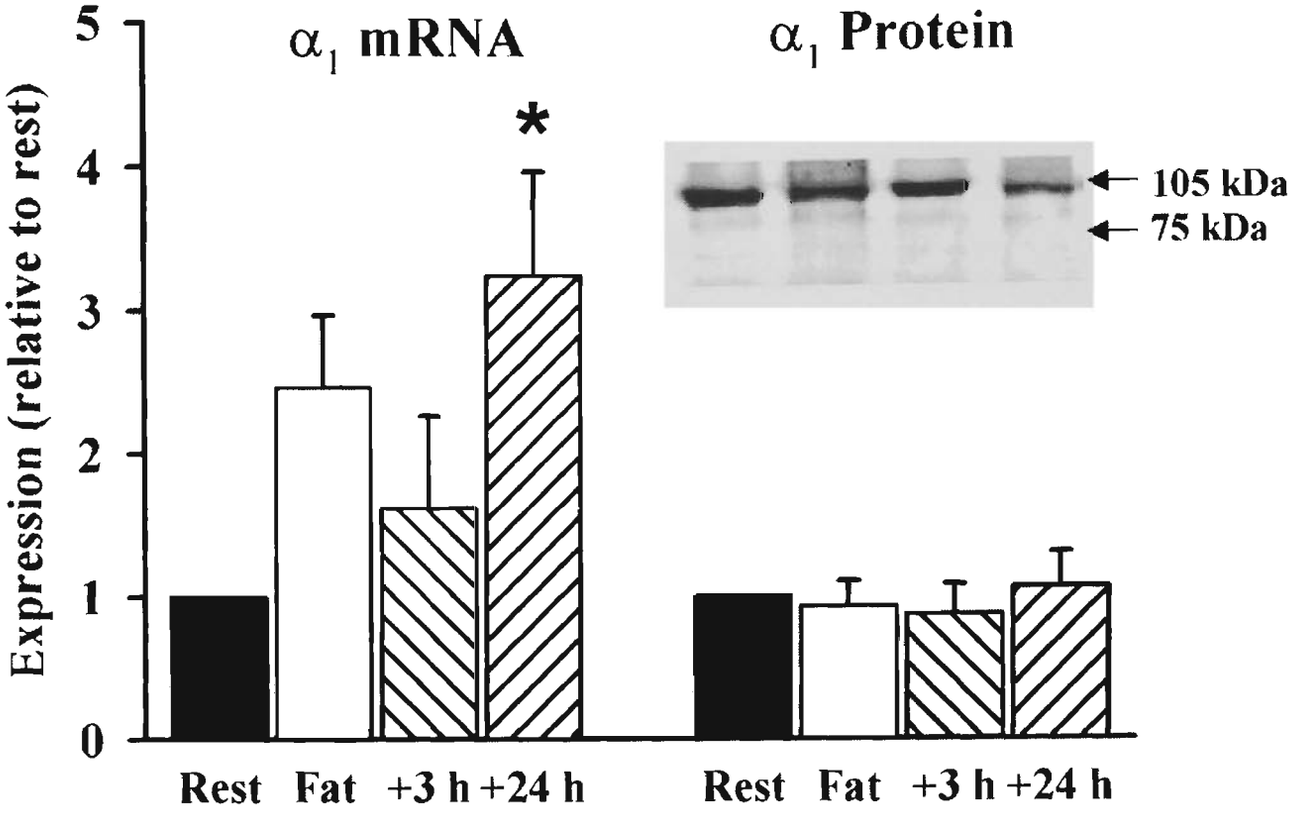


Figure 3.7 Na^+,K^+-ATPase α_1 isoform mRNA and protein expression before and after acute one-legged knee extensor exercise. Na^+,K^+-ATPase α_1 isoform mRNA and crude muscle homogenate protein expression at rest and immediately following fatigue (Fat), and 3 h (+3 h) and 24 h (+24 h) after brief intense exercise. All results were normalised against resting values. Insert shows representative immunoblot of α_1 protein expression at corresponding times. Mean ± SEM, n = 14 for mRNA, n = 15 protein. * $P < 0.05$ greater than rest.

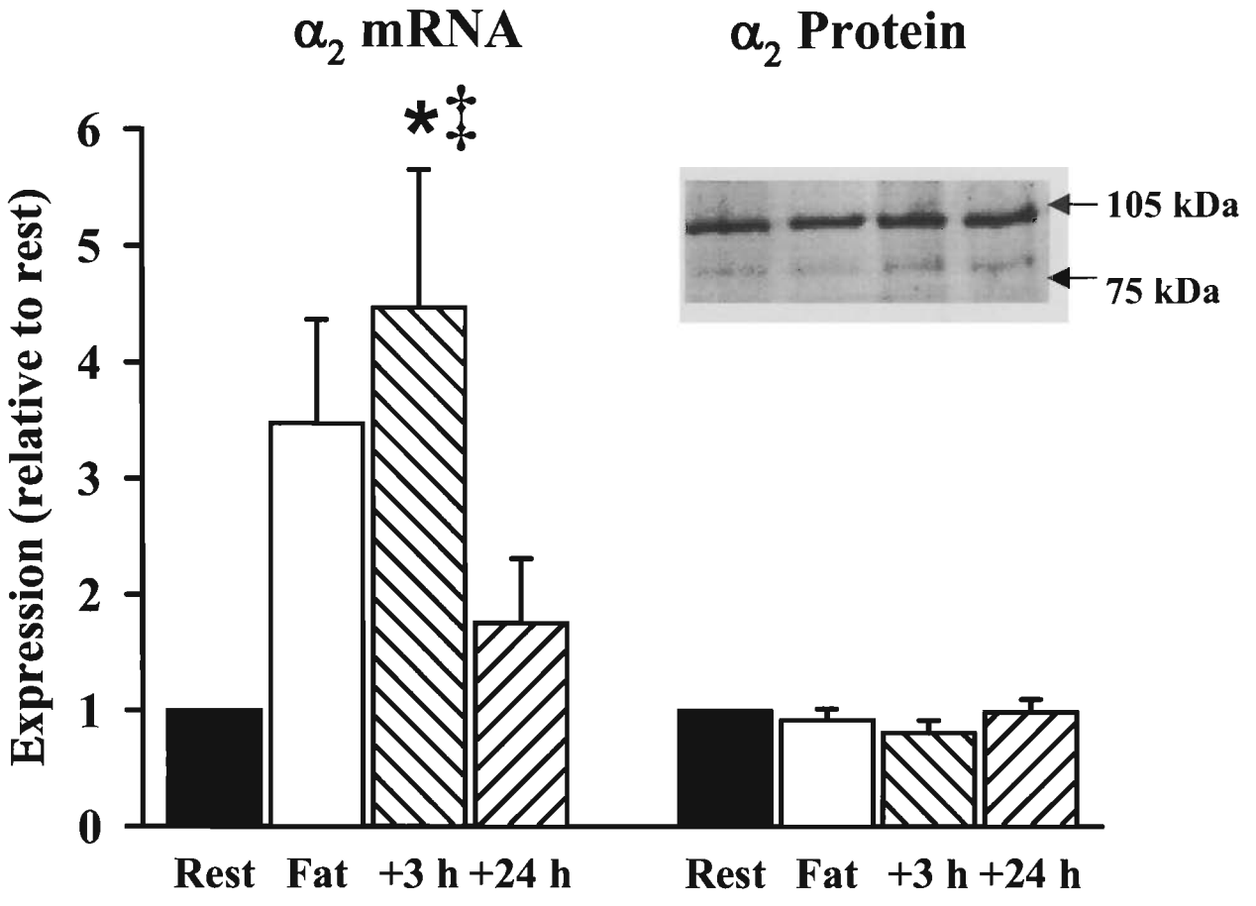


Figure 3.8 Na^+,K^+-ATPase α_2 isoform mRNA and protein expression before and after acute one-legged knee extensor exercise. Na^+,K^+-ATPase α_2 isoform mRNA and crude muscle homogenate protein expression at rest and immediately following fatigue (Fat), and 3 h (+3 h) and 24 h (+24 h) after brief intense exercise. All results were normalised against resting values. Insert shows representative immunoblot of α_2 protein expression at corresponding times. Mean ± SEM, n = 14 for mRNA, n = 15 protein. * $P < 0.05$ greater than rest; ‡ $P < 0.05$ greater than +24 h.

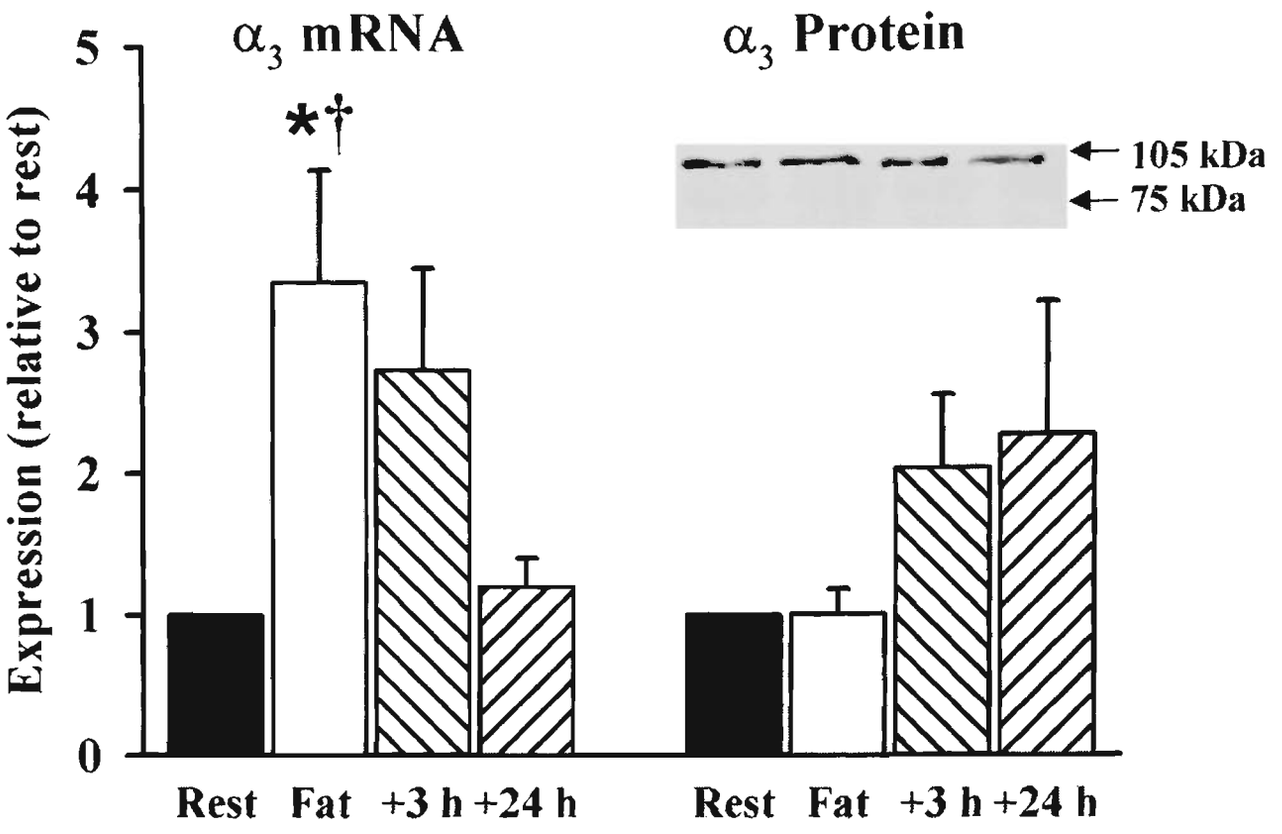


Figure 3.9 Na^+,K^+-ATPase α_3 isoform mRNA and protein expression before and after acute one-legged knee extensor exercise. Na^+,K^+-ATPase α_3 isoform mRNA and crude muscle homogenate protein expression at rest and immediately following fatigue (Fat), and 3 h (+3 h) and 24 h (+24 h) after brief intense exercise. All results were normalised against resting values. Insert shows representative immunoblot of α_3 protein expression at corresponding times. Mean ± SEM, n = 14 for mRNA, n = 15 protein. * $P < 0.05$ greater than rest; † $P < 0.05$ greater than +24 h.

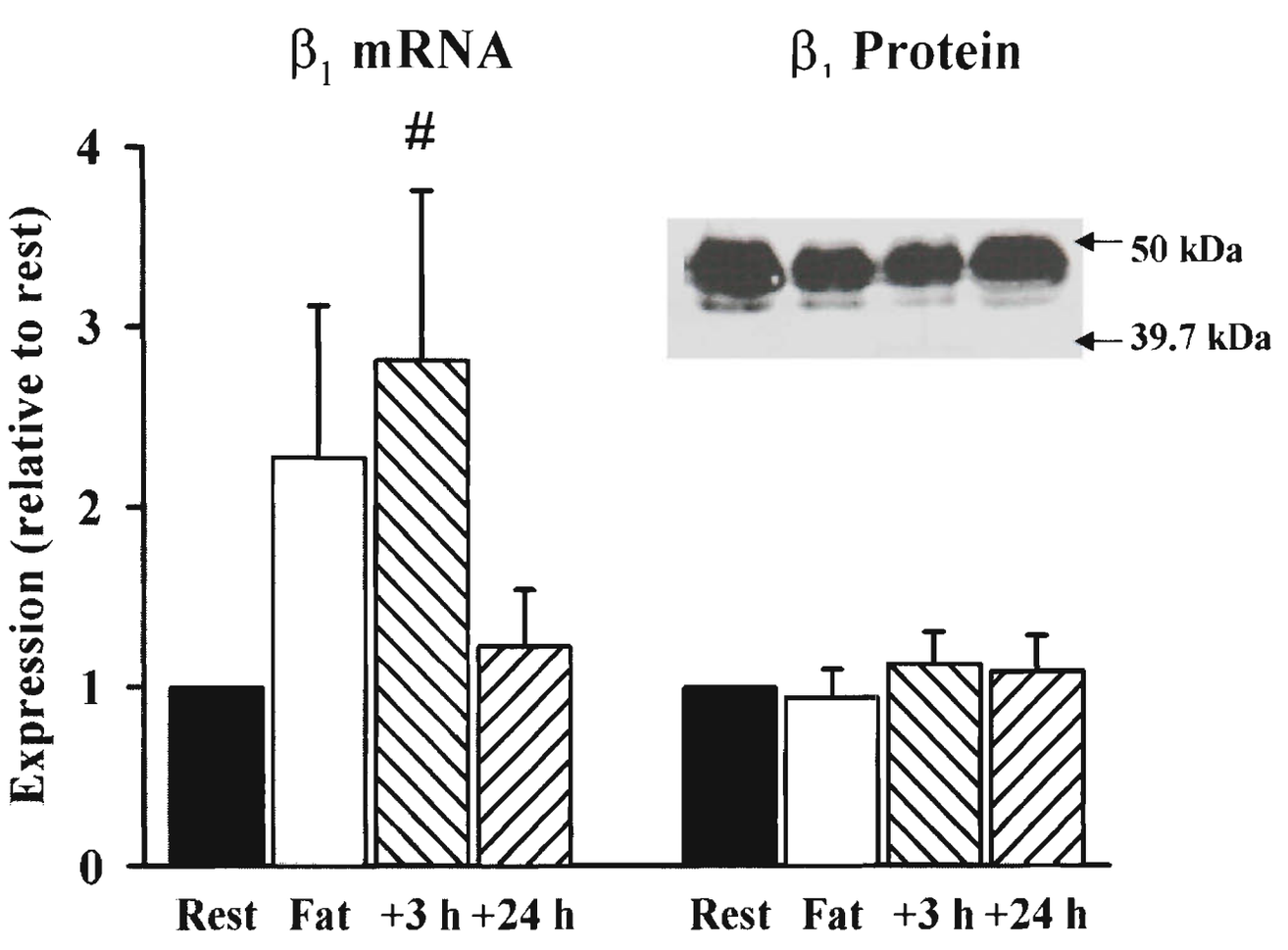


Figure 3.10 Na^+,K^+-ATPase β_1 isoform mRNA and protein expression before and after acute one-legged knee extensor exercise. Na^+,K^+-ATPase β_1 isoform mRNA and crude muscle homogenate protein expression at rest and immediately fatigue (Fat), and 3 h (+3 h) and 24 h (+24 h) after brief intense exercise. All results were normalised against resting values. Insert shows representative immunoblot of β_1 protein expression at corresponding times. Mean ± SEM, n = 14 for mRNA, n = 15 protein. # $P < 0.08$ greater than rest.

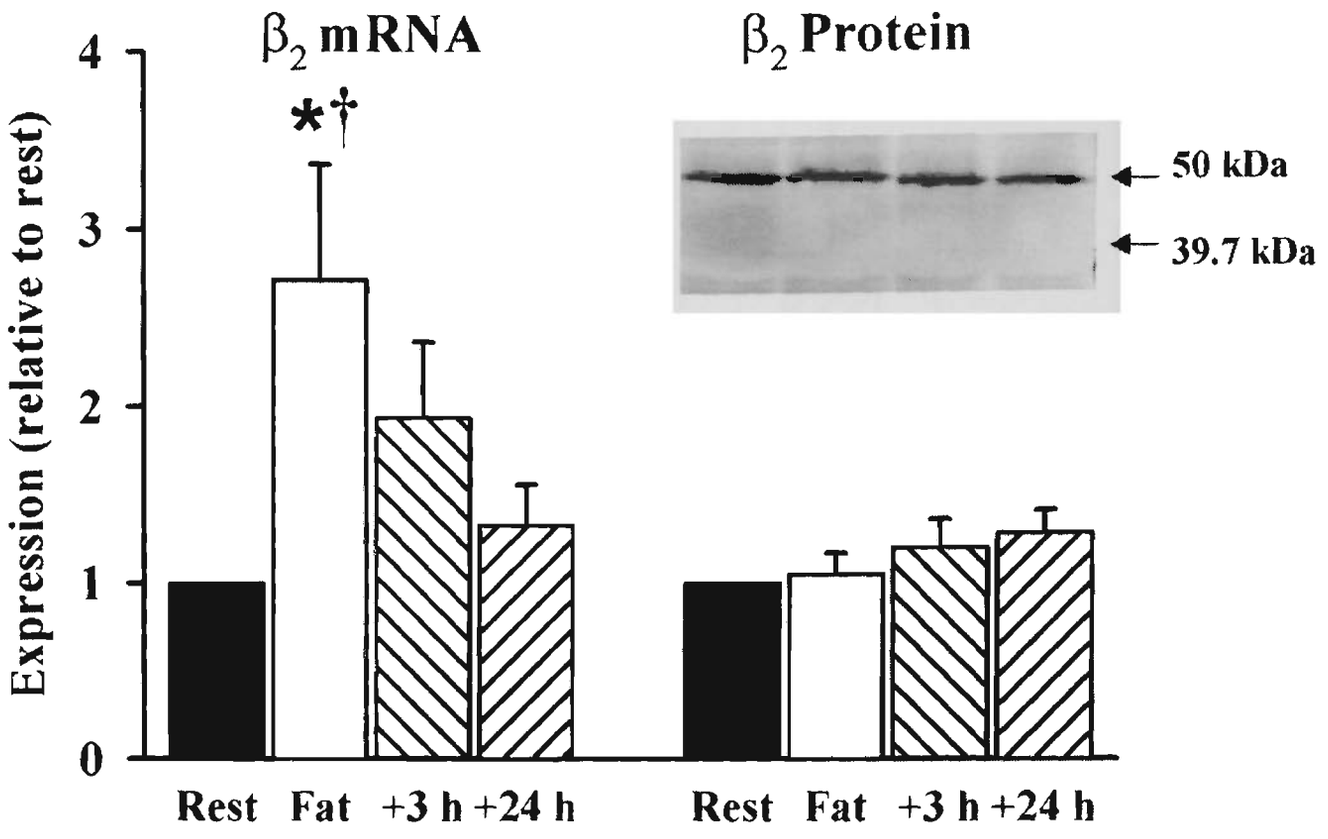


Figure 3.11 Na^+,K^+-ATPase β_2 isoform mRNA and protein expression before and after acute one-legged knee extensor exercise. Na^+,K^+-ATPase β_2 isoform mRNA and crude muscle homogenate protein expression at rest and immediately following fatigue (Fat), and 3 h (+3 h) and 24 h (+24 h) after brief intense exercise. All results were normalised against resting values. Insert shows representative immunoblot of β_2 protein expression at corresponding times. Mean ± SEM, n =14 for mRNA, n = 15 protein. * $P < 0.05$ greater than rest; † $P < 0.05$ greater than +24 h.

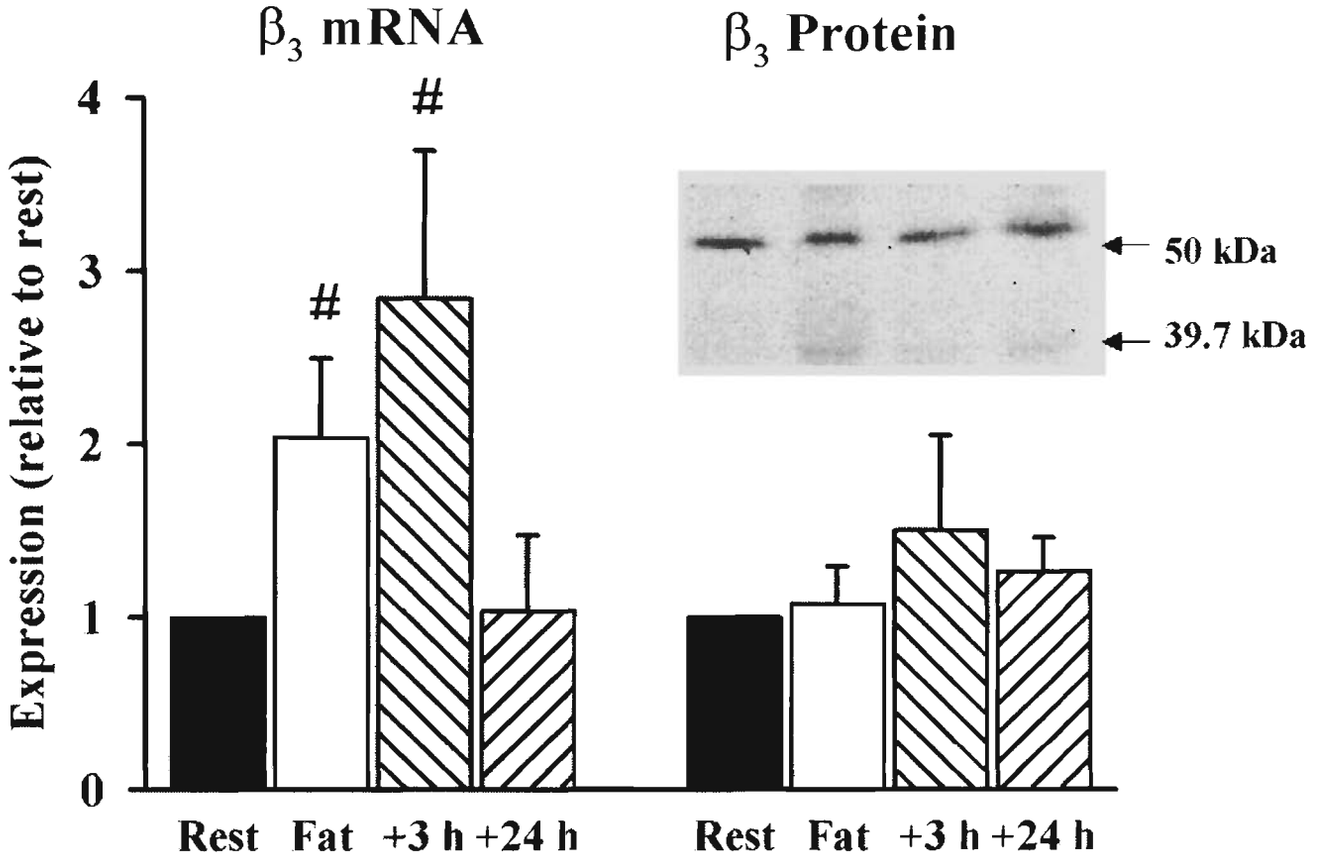


Figure 3.12 Na^+,K^+-ATPase β_3 isoform mRNA and protein expression before and after acute one-legged knee extensor exercise. Na^+,K^+-ATPase β_3 isoform mRNA and crude muscle homogenate protein expression at rest and immediately following (fatigue (Fat), and 3 h (+3 h) and 24 h (+24 h) after brief intense exercise. All results were normalised against resting values. Insert shows representative immunoblot of β_3 protein expression at corresponding times. Mean ± SEM, n = 14 for mRNA, n = 15 protein. # $P < 0.08$ greater than rest.

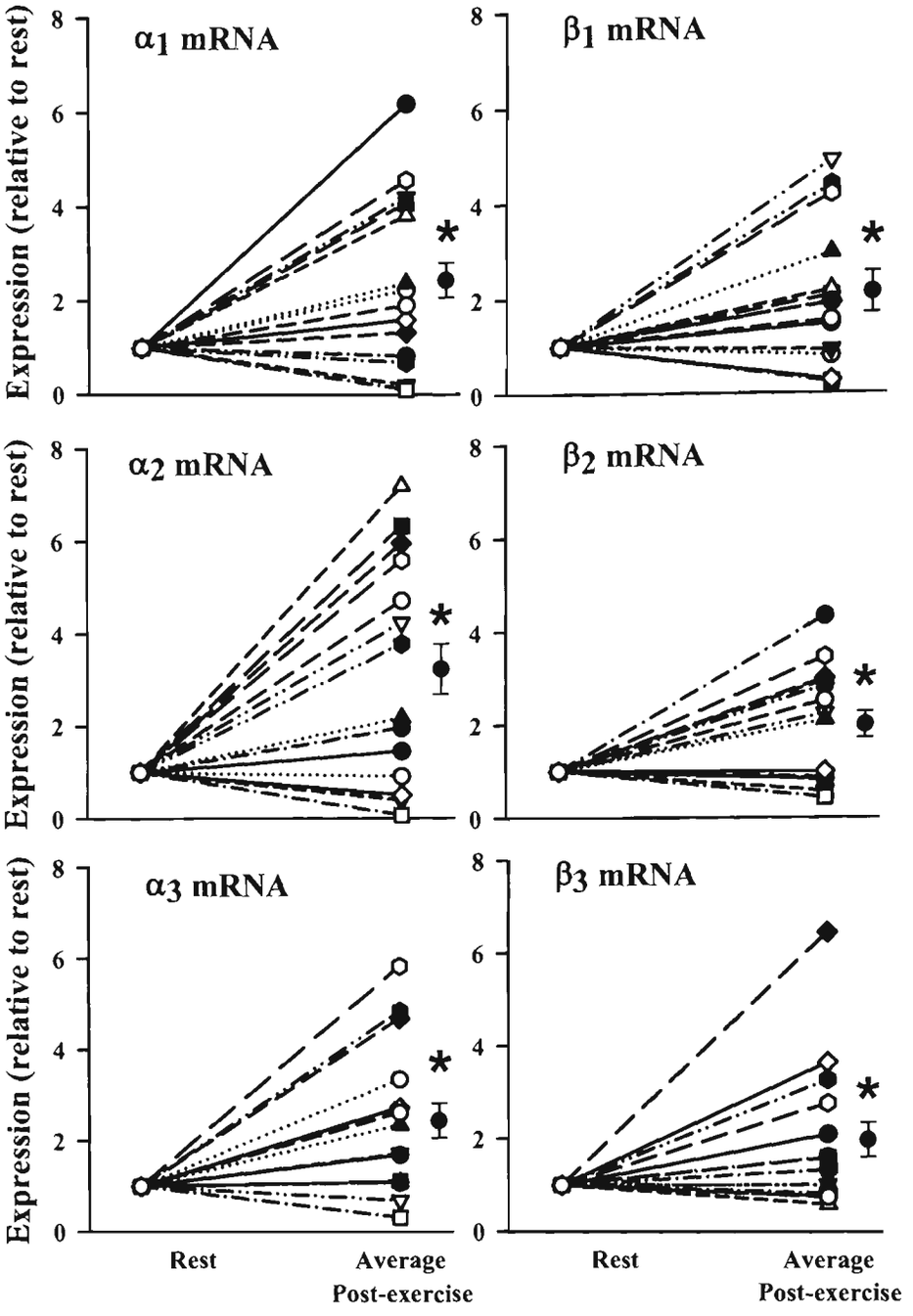


Figure 3.13 Average post-exercise response in Na^+,K^+-ATPase mRNA expression. Individual expression of α_1, α_2, α_3, β_1, β_2 and β_3 mRNA at rest and the average expression following brief intense exercise (average of fatigue, 3 h and 24 h values). All symbols joined by lines indicate individual responses, while symbol on right indicates group average post-exercise expressed as mean ± SEM. n = 14. * Mean $P < 0.05$ greater than rest.

Indeed, when the group mean results were analysed, the average post-exercise response in mRNA expression of the α_1, α_2, α_3, β_1, β_2, and β_3 isoforms was 2.4-, 3.2-, 2.4-, 2.1, 2.0- and 2.0-fold above resting values, respectively ($P < 0.05$, Figure 3.13). The relative change in mRNA expression from rest to average post-exercise expression for each of the Na^+,K^+-ATPase α_1, α_2, α_3, β_1, β_2 and β_3 isoforms were not significantly correlated with age, gender, $\dot{V}O_{2peak}$ or exercise time to fatigue (data not shown).

3.4 DISCUSSION

The first main finding of this study was that skeletal muscle obtained from healthy humans expresses each of the Na^+,K^+-ATPase α_1, α_2, α_3, β_1, β_2 and β_3 isoforms, as demonstrated at both gene transcript and protein levels. The second major finding was that even acute brief exercise provided a sufficient stimulus to increase the mRNA abundance of each of these isoforms. However, this exercise challenge did not elevate the protein abundance of any of these isoforms.

3.4.1 Presence of Na^+,K^+-ATPase α_1-α_3 and β_1-β_3 isoform gene transcripts and proteins

This study provides novel characterisation of the six Na^+,K^+-ATPase gene transcripts present in human vastus lateralis muscle. These findings demonstrate expression of the previously uninvestigated α_3 and β_2 gene transcripts, and verify expression of the α_1, α_2, β_1 and β_3 gene transcripts (Malik *et al.*, 1998; Nordsborg *et al.*, 2003a).

This is also the first study to demonstrate expression of each of these six Na^+,K^+-ATPase isoform (α_1-α_3, β_1-β_3) proteins in human skeletal muscle. The finding of three α and three β isoforms in human skeletal muscle greatly extends previous work. Whilst the presence of the α_1, α_2 and β_1 proteins is consistent with previous studies (Hundal *et al.*, 1994; Juel *et al.*, 2000a), the detection of both the α_3 gene transcript and protein resolves the apparent

conflict regarding the presence of α_3 in human muscle (Hundal *et al.*, 1994; Wang *et al.*, 2001). Detection of the α_3 isoform was also confirmed using the polyclonal antibody utilised by Hundal *et al.*, (1994), but the immunoreactive band was of poor quality. The β_2 protein had previously been undetected in human soleus and vastus lateralis muscle (Hundal *et al.*, 1994; Juel *et al.*, 2000a). However, the β_2 protein was detected in the present study using two separate antibodies, including those employed by previous studies that did not detect the β_2 isoform (Hundal *et al.*, 1994; Juel *et al.*, 2000a), and the specificity of the β_2 antibody was confirmed by the shift in apparent molecular weight with deglycosylation. Thus, a difference in antibody reactivity cannot explain these differing results. Rather, this discrepancy might be explained on the basis of β_2 isoform localisation. Importantly, the present study utilised crude muscle homogenates, which include all intracellular and sarcolemmal membranes, whilst the previous studies utilised separated membranes or membrane fractions (Hundal *et al.*, 1994; Juel *et al.*, 2000a). However, Juel *et al.*, (2000) also utilised a purified homogenate and did not detect the β_2 protein in human muscle. Whilst the β_3 protein had not been previously investigated, expression of the β_3 protein was confirmed with an additional polyclonal anti-β_3 antibody, although the reactivity was weaker than that with the initial antibody. Therefore, when isoform composition of all membranes in human skeletal muscle were investigated via a crude homogenate, it is clear that human and rat muscle similarly express the Na^+,K^+-ATPase α_1-α_3 and β_1-β_2 gene transcripts (Orlowski & Lingrel, 1988; Tsakiridis *et al.*, 1996), and the α_1-α_2 and β_1-β_3 proteins (Hundal *et al.*, 1993; Tsakiridis *et al.*, 1996; Arystarkova & Sweadner, 1997).

The specificity of the antibodies used was assessed on three accounts. First, the apparent molecular weights of the protein bands detected with antibodies specific to each of the α_1, α_2, α_3, β_1, β_2 and β_3 isoforms corresponded with those previously reported in mammalian

skeletal muscle (Hundal *et al.*, 1994; Arystarkova & Sweadner, 1997; Juel *et al.*, 2000a). Second, control samples including rat kidney and rat brain homogenates were ran on each gel and supported previous observations of isoform-specific expression (Martin-Vasallo *et al.*, 1989; Hundal *et al.*, 1994; Malik *et al.*, 1996). Third, BLAST analysis of the antigen sequence of each antibody was performed to evaluate isoform cross-reactivity. Whilst results from these assessments suggest that isoform cross-reactivity with each antibody would be unlikely, complete verification of isoform-specificity will only be achieved with sequencing of the protein bands detected with each isoform-specific antibody.

The use of muscle tissue samples collected by biopsy raises the possibility that some of the six Na^+,K^+-ATPase isoforms detected may have originated from contamination by other tissues, such as vascular and nervous tissue, adipocytes or fibrocytes. For instance, human erythrocytes and reticulocytes express the α_1, α_3, β_1, β_2 and β_3 proteins (Stengelin & Hoffman, 1997; Hoffman *et al.*, 2002), while human leukocytes express the α_1, α_3, β_1 and β_3 gene transcripts (Stengelin & Hoffman, 1997). Rat adipocytes express both α_1 and α_2 isoforms (Lytton, 1985; Voldstedlund *et al.*, 1993; Bofill *et al.*, 1994; Sargeant *et al.*, 1995), while α_1 and β_1, but not α_2 and β_2 isoforms, were detected in mouse 3T3-L1 fibroblasts (Sargeant *et al.*, 1995). Rat astrocytes and neurons express the α_1-α_2 and α_3 isoforms, respectively (Blanco & Mercer, 1998). Importantly, each muscle sample from all individuals expressed all six isoforms at mRNA and protein levels. Whilst the likelihood of contamination by nervous tissue in each biopsy sample seems very low, blood contamination will certainly be present in all samples. Na^+,K^+-ATPase isoform expression was therefore probed in cell cultures derived from human vastus lateralis muscle, being careful to minimise any contamination. In the first comprehensive assessment of Na^+,K^+-ATPase isoform expression in human skeletal muscle cell cultures, each of the α_1-α_3 and β_1-β_3 proteins were detected. These results thus extend findings from a previous study

which reported only α_1 and α_2 isoform expression in cultured human muscle cells (Al-Khalili *et al.*, 2003). Although these cell culture results do not provide unequivocal verification, they do indicate that the novel expression of the α_3, β_2 and β_3 proteins in human muscle obtained via biopsy sampling, and indeed also the other isoforms, is unlikely to originate from contamination by other cell types.

3.4.2 Exercise effects on Na^+,K^+-ATPase isoform mRNA expression

An important finding was that all six of the Na^+,K^+-ATPase gene transcripts expressed in human skeletal muscle were elevated in response to only ~6 min of exercise. Clear increases were found at specific time points for each of α_1, α_2, α_3 and β_2 mRNA, whilst the average post-exercise value was increased for both β_1 and β_3 mRNA. This differs to a recent study which reported that ~15 min of intense intermittent knee extensor exercise induced an elevation in only α_1 mRNA expression (Nordsborg *et al.*, 2003a). One reason for this discrepancy is that their study was limited by a low statistical power for α_2 and β_1 mRNA, whilst α_3, β_2 and β_3 mRNA were not probed (Nordsborg *et al.*, 2003a). Large inter- and intra-individual variability was observed for Na^+,K^+-ATPase isoform mRNA expression, similar to numerous other genes (Boivin *et al.*, 2000; Hameed *et al.*, 2003; Psilander *et al.*, 2003). The cause of such variability has not been established, and in the present study, was not significantly correlated to physical characteristics such as age or gender, or physiological factors underpinning exercise $\dot{V}O_{2peak}$, and time to fatigue.

The observed increases in isoform mRNA expression following exercise may reflect increased transcription, reduced mRNA degradation or enhanced mRNA stability. Although the present study could not evaluate these mechanisms, the rapid increase in Na^+,K^+-ATPase α_1 and β mRNA expression induced by veratridine in cultured chick skeletal muscle cells was due to elevated rates of gene transcription (Taormino & Fambrough, 1990). The mechanisms responsible for Na^+,K^+-ATPase gene activation with

exercise also remain unknown, but may involve any of elevated $[Na^+]_i$ (Rayson, 1993), $[Ca^{2+}]_i$ (Rayson, 1991) and ROS (Xie *et al.*, 1999), and/or altered $[K^+]_e$ (Pressley *et al.*, 1988).

An important limitation of the methods utilised to assess mRNA expression is that they do not discriminate between cell types. Whilst highly unlikely, contamination of my mRNA samples by cell types other than skeletal muscle is possible.

3.4.3 Exercise effects on Na^+,K^+-ATPase isoform protein expression

In contrast to the up-regulation in mRNA expression, a single bout of one-legged knee extensor exercise had no significant effect on protein expression of any of the six Na^+,K^+-ATPase isoforms expressed in human skeletal muscle homogenate. These results clearly indicate that increases in mRNA were not matched by increases in Na^+,K^+-ATPase protein abundance, highlighting the separate and possibly independent regulation of gene transcription and protein translation (Orphanides & Reinberg, 2002). Na^+,K^+-ATPase translational regulation has been shown to involve $[Na^+]_i$ in each of cultured chick ventricular cells (Kim & Smith, 1986) and chick skeletal muscle cells (Wolitzky & Fambrough, 1986). Since the intense exercise protocol in the present study would have almost certainly increased $[Na^+]_i$ (Sjøgaard *et al.*, 1985), the lack of exercise effects on isoform protein abundance suggests that either $[Na^+]_i$ was not elevated for sufficient duration to signal the cellular responses to increase isoform protein abundance, or that Na^+,K^+-ATPase translational regulation in human muscle may not involve $[Na^+]_i$. Neither proposition has been investigated.

It is known that repeated bouts of exercise induce a training effect of an increased [^{3}H]-ouabain binding, representing an increased total muscle Na^+,K^+-ATPase content (Green *et al.*, 1993; McKenna *et al.*, 1993). The transient accumulation of Na^+,K^+-ATPase mRNA induced by exercise appears to be the initial step of this adaptive response. Interestingly,

this initial response does not appear to be isoform-specific, as mRNA for all six isoforms were increased post-exercise. The lack of increase in Na^+,K^+-ATPase isoform protein observed here suggests that the exercise challenge employed was insufficient to induce Na^+,K^+-ATPase up-regulation, which may require several hours of continuous exercise (Overgaard *et al.*, 2002), or repeated exercise bouts (Green *et al.*, 1993).

Expression of three α (α_1-α_3) and three β isoforms (β_1-β_3) in human muscle raises the possibility of nine different αβ heterodimers in human skeletal muscle, with implications for diverse functions. Expression of three α isoforms in human skeletal muscle also has implications for interpretation of [^{3}H]-ouabain binding, which quantifies total muscle Na^+,K^+-ATPase content (Nørgaard *et al.*, 1984a; Clausen, 1996). In human kidney, heart and brain, all three α isoforms have similar affinities for ouabain (Wang *et al.*, 2001). If similar affinities are apparent in skeletal muscle, the findings from the present study imply that the [^{3}H]-ouabain binding assay in human muscle measures the combined content of functional Na^+,K^+-ATPase $\alpha_1\beta$, $\alpha_2\beta$ and $\alpha_3\beta$ heterodimers. Finally, expression of six Na^+,K^+-ATPase isoforms in human skeletal muscle may have important implications for the proposed isoform-specific translocation with exercise (Tsakiridis *et al.*, 1996; Juel *et al.*, 2000a; Juel *et al.*, 2001) and insulin (Hundal *et al.*, 1992; Marette *et al.*, 1993). These previous studies have failed to probe for the full complement of Na^+,K^+-ATPase isoforms, which may challenge the reported specificity of any isoform translocation. Moreover, the concept of isoform translocation has recently been challenged as being a quantitatively important means of increasing Na^+,K^+-ATPase transport in rat skeletal muscle, failing to detect an increase in [^{3}H]-ouabain binding in response to either muscle contractions or insulin (McKenna *et al.*, 2003b).

In conclusion, each of the Na^+,K^+-ATPase α_1-α_3 and β_1-β_3 isoforms are expressed in the vastus lateralis muscle of healthy humans, as evidenced at both the transcription and protein levels. Further, only ~6 min of intense exercise was sufficient to increase the mRNA expression of each of these six isoforms, evidenced by increases either at specific time points or in the average post-exercise value, suggesting a non-specific up-regulatory transcription response of Na^+,K^+-ATPase isoforms. In contrast, this exercise bout had no effect on isoform protein abundance.

CHAPTER 4

STUDY 2: PROLONGED SUBMAXIMAL EXERCISE INDUCES ISOFORM-SPECIFIC Na^+,K^+-ATPase MRNA AND PROTEIN RESPONSES IN HUMAN SKELETAL MUSCLE

4.1 INTRODUCTION

Training comprising prolonged exercise has repeatedly been shown to elevate muscle Na^+,K^+-ATPase content in humans (Green *et al.*, 1993; Green *et al.*, 1999a; Green *et al.*, 1999b; Green *et al.*, 2004). However, the mechanisms responsible for this elevation are poorly understood. In the previous chapter, each of the Na^+,K^+-ATPase α_1, α_2, α_3, β_1, β_2 and β_3 gene transcripts and proteins were found to be expressed in human skeletal muscle. These Na^+,K^+-ATPase gene transcripts appear to be very responsive to exercise, with only ~6 min of intense exercise elevating the mRNA expression of each of the α_1-α_3 and β_1-β_3 isoforms (Chapter 3). Furthermore, 15-20 min of intense intermittent exercise elevated α_1 mRNA expression (Nordsborg *et al.*, 2003a). Little is known about the adaptations in these gene transcripts in response to prolonged submaximal exercise. In rats, 1 h of treadmill running immediately increased α_1 and β_2 mRNA expression in red, oxidative and in white, glycolytic muscles, respectively (Tsakiridis *et al.*, 1996). In contrast, there was no effect of this exercise bout on α_2 and β_1 mRNA expression, while α_3 and β_3 mRNA were not measured (Tsakiridis *et al.*, 1996).

Compared to their respective gene transcripts, isoform protein abundance appears much less responsive to acute exercise. There was no effect of brief intense exercise on the crude muscle homogenate protein abundance of any of the α_1-α_3 and β_1-β_3 isoforms in one study (Chapter 3), nor on the α_1, α_2 and β_1 isoform abundance studied in another (Juel *et al.*, 2000a). In contrast, 9 and 39% increases in α_2 and β_1 protein abundances were recently

demonstrated following only 3 and 6 d of prolonged cycling training, respectively (Green *et al.*, 2004). In that study, none of α_3, β_2 and β_3 protein abundances were measured. Whether a single bout of prolonged exercise can also induce increases in protein abundance of these isoforms in human muscle, and thus provide a mechanism for the elavted muscle Na^+,K^+-ATPase content seen with repeated bouts of prolonged exercise (ie. training) is unknown and was investigated here. The first hypothesis tested was that prolonged submaximal exercise would increase mRNA expression, but not protein abundance of each of the Na^+,K^+-ATPase α_1-α_3 and β_1-β_3 isoforms.

The immediate effects of prolonged submaximal exercise on Na^+,K^+-ATPase maximal activity and content in human skeletal muscle are better understood, with maximal activity depressed by ~13-35%, and content unchanged, immediately following exercise (Fowles *et al.*, 2002b; Leppik *et al.*, 2004; Sandiford *et al.*, 2004; Aughey *et al.*, 2005). However, little is known about the effects of prolonged submaximal exercise on Na^+,K^+-ATPase maximal activity and content in the 0-24 h period following exercise, when any exercise-induced increase in Na^+,K^+-ATPase content might be expected to occur. An increase might be anticipated since physical training induces an up-regulation of content, measured by [^{3}H]-ouabain binding (Green *et al.*, 1993; McKenna *et al.*, 1993; Green *et al.*, 2004). A recovery of maximal Na^+,K^+-ATPase activity and no change in Na^+,K^+-ATPase content in the 4 h period was shown following 30 min of intermittent exercise, but their findings were inconclusive due to internally inconsistent results (Fowles *et al.*, 2002). A 13% increase in Na^+,K^+-ATPase content was found at 30 min following ~10 h of running (Overgaard *et al.*, 2002). Whether prolonged submaximal exercise of ~1 h duration is sufficient to also increase Na^+,K^+-ATPase content in the 24 h period following exercise is unknown and was therefore investigated here. A recent study found a negative correlation between the depression in maximal Na^+,K^+-ATPase activity and the increase in Na^+,K^+-ATPase mRNA

expression with brief intense exercise (Petersen *et al.*, 2005). This was interpreted by the authors to suggest a possible modulatory role of depressed Na^+,K^+-ATPase activity in muscle Na^+,K^+-ATPase gene expression. Whether a similar relationship is found between maximal Na^+,K^+-ATPase activity and Na^+,K^+-ATPase mRNA expression following prolonged submaximal is unknown and was investigated here. It was hypothesised that prolonged submaximal exercise would transiently depress maximal Na^+,K^+-ATPase activity with recovery by 3 or 24 h post-exercise, but would not elevate Na^+,K^+-ATPase content in the 24 h period following exercise. The depression in maximal Na^+,K^+-ATPase activity would also be negatively correlated with the increase in Na^+,K^+-ATPase mRNA expression.

Gene transcription and protein translation are vital to the cellular adaptations to prolonged submaximal exercise. Under most circumstances, transcription and translation are controlled at the level of initiation (Orphanides *et al.*, 1996; Gautsch *et al.*, 1998), which involves RNA polymerase II (RNAP II), and eukaryotic initiation factor-4E (eIF-4E) and 4E-binding protein 1 (4E-BP1). RNAP II directly initiates transcription by binding to the promoter region of a protein-coding gene and subsequently elongating the transcript, while initiation of translation involves binding of eIF-4E to the gene transcript and the ensuing recognition of a cap structure at the 5' termini (Orphanides & Reinberg, 2002). However, recognition only occurs when eIF-4E interacts with other members of the eukaryotic initiation factor family, eIF-4A and eIF-4G to form a heterotrimetric eIF-4F complex, and in skeletal muscle, this event is compromised by the competing interaction of 4E-BP1 (Gingras *et al.*, 1999). Since prolonged submaximal exercise increases the mRNA expression of numerous metabolic genes (Pilegaard *et al.*, 2000; Hildebrandt *et al.*, 2003), but inhibits protein synthesis (Carraro *et al.*, 1990; Gautsch *et al.*, 1998), it is conceivable that prolonged submaximal exercise would increase mRNA expression of RNAP II and

4E-BP1, but not eIF-4E. However, this has yet to be investigated. Therefore the third hypothesis tested was that prolonged submaximal exercise would increase the mRNA expression of RNAP II and 4E-BP1, but reduce mRNA expression of eIF-4E.

4.2 METHODS

4.2.1 Subjects

Eleven healthy subjects, comprising 6 males and 5 females (age, 23.9 ± 3.9 yr; height, 171.2 ± 10.4 cm; body mass, 65.0 ± 10.8 kg; mean ± SD) gave written informed consent and participated in this study. All subjects participated in regular physical activity, but none were specifically trained in any sport. All protocols and procedures were approved by the Human Research Ethics Committee at Victoria University of Technology.

4.2.2 Experimental design

Subjects performed three exercise tests cycling at 60 rev.min^{-1} on an electrically braked ergometer (Lode Excalibur, Groningen, the Netherlands), comprising an incremental exercise test, a familiarisation and then experimental prolonged submaximal exercise trial. Each test was separated by ~10 d to minimise any training effect. Subjects refrained from vigorous exercise for 48 h, and from caffeine, nicotine and alcohol consumption for 24 h, prior to each of the exercise tests.

4.2.3 Incremental exercise test

Each subject completed an incremental exercise test to determine $\dot{V}O_{2peak}$, comprising 4 min at each of 50, 80, 110 and 140 W, then 25 W increments each minute until fatigue, defined as the inability to maintain pedal cadence above 55 rev.min^{-1}. The work rate corresponding to 75% $\dot{V}O_{2peak}$ was calculated from the linear regression of submaximal $\dot{V}O_2$ versus work rate, for use in all subsequent exercise sessions. All equipment, procedures and calibration were as detailed in Chapter 3.

4.2.4 Prolonged submaximal exercise test

The prolonged submaximal submaximal exercise test was conducted at a work rate corresponding to 75% $\dot{V}O_{2peak}$, and continued until fatigue. Muscle biopsy and venous blood sampling procedures were performed with the prolonged submaximal exercise test. Pulmonary $\dot{V}O_2$, CO_2 output ($\dot{V}CO_2$) and respiratory exchange ratio (RER) were measured during the periods 5-10, 25-30 and 40-45 min. Subjects consumed standardised meals and fluid intake on the day prior to, during and the subsequent day following the trial.

4.2.5 Blood sampling

Prior to the prolonged submaximal exercise trial, a Teflon catheter (20G, Jelco) was inserted into a superficial dorsal hand vein. The hand was placed in a waterproof plastic glove and was heated in a 45 °C water bath throughout the test to allow sampling of arterialised venous blood. Samples were taken at rest, in the final 10 s of each 15 min during exercise, immediately following exercise, and at 1, 2, 5 and 10 min in recovery. The catheter was kept patent by regular infusions of sterile heparinised saline.

4.2.6 Blood processing

Two blood samples were taken at each sampling time point. The first sample was taken using a blood gas syringe containing lithium heparin. Air bubbles were immediately expelled from the syringe, which was then capped, mixed well and placed on ice for immediate triplicate analyses of plasma [K^+] by use of an automated analyser (Ciba Corning 865, Bayer, MA, USA). The second sample was transferred to a tube containing lithium heparin, mixed well and taken for duplicate analyses of [Hb] and Hct using an automated Hematology Analyser (Sysmex K-800, Kobe, Japan). Analysers were calibrated immediately before and during the analyses with precision standards.

4.2.7 Muscle biopsy sampling

A muscle biopsy was taken from the same leg at rest, immediately following prolonged submaximal exercise, and at 3 and 24 h post-exercise. A local anaesthetic (1% Xylocaine) was injected into the skin and subcutaneous tissue above the vastus lateralis muscle. A small incision was then made through the skin and fascia, and a muscle sample of approximately 120 mg was excised. The muscle sample was blotted on filter paper to remove blood, and immediately frozen in liquid N_2 and stored at –80°C until assayed later for Na^+,K^+-ATPase isoform mRNA and protein expression, maximal 3-*O*-MFPase activity, [^{3}H]-ouabain binding site content and RNAP II, eIF-4E and 4E-BP1 mRNA expression.

4.2.8 Real-Time RT-PCR measurement of mRNA

Muscle was analysed for Na^+,K^+-ATPase α_1, α_2, α_3, β_1, β_2 and β_3 mRNA expression as described in Chapter 3. The ratio of absorbance at 260 and 280 nm (260/280) was 2.04 ± 0.06. Primer sequences were also designed for the Na^+,K^+-ATPase α_4, and RNAP II, eIF-4E and 4E-BP1 genes from published sequences (Table 4.1). However, the Na^+,K^+-ATPase α_4 gene was undetected by RT-PCR. Exercise had no significant effect (P = 0.211) on the mRNA expression of CYC, when expressed in the linear (2^{-C_T}) form (data not shown) and was therefore used as a control (housekeeping gene) to account for any variations in the amount of input RNA and the efficiency of reverse transcription. Gene expression was quantified using a cycle threshold (C_T) method, whereby the relative expression of the genes compared with resting samples was made using the expression, $2^{-\Delta\Delta C_T}$, in which the expression of each gene was normalised for input cDNA using the housekeeping gene, CYC. The intra-assay coefficient of variation for each target gene was <15.0% for 2^{-C_T} (Table 4.2).

4.2.9 Western Blotting

Immunoblotting and antibodies used were as described in Chapter 3.

Table 4.1 Na^+,K^+-ATPase α_4 and RNAP II, eIF-4E and 4E-BP1 gene primer sequences used for mRNA analyses

Gene	GenBank Accession Number	Identity	Sense Primer (5'-3')	Antisense Primer (5'-3')
α_4	NM_144699	ATP1A4	CAACTGTTCGGAGGCTTCTC	CTCCCAACACCACCTCTTGT
RNAP	NM_000937	POLR2A	CGCTTAAGCCTTCCAACAA	GAGGACGACCTTGCTGTCTC
eIF-4E	NM_001968	EIF4E	CTGTCCGCTTTCAGGAAGA	CACGCCAACAGAACAAGAGA
4E-BP1	NM_004095	EIF4EBP1	GATACCTCCTTGTGCCTCCA	GAAGGGTTCGTTCTTGTCCA

Primer sequences were designed using Primer Express software (Applied Biosystems) from gene sequences obtained from GeneBank. Primer specificity was determined using a BLAST search.

Table 4.2 Intra-assay variability of 2^{-C_T} values

Gene	2^{-C_T} CV (%)
α_1	12.4
α_2	8.9
α_3	14.7
β_1	9.4
β_2	11.2
β_3	12.3
RNAP II	13.0
eIF-4E	9.9
4E-BP1	13.0
CYC	8.8

Each sample was run in triplicate wells in the same Real-Time PCR run. n = 42. CV, coefficient of variation; C_T, cycle threshold.

4.2.10 Maximal *in-vitro* 3-O-MFPase activity

Maximal *in-vitro* 3-*O*-MFPase activity was measured using the K^+-stimulated 3-*O*-methylfluorescein phosphatase (3-*O*-MFPase) activity assay (Fraser & McKenna, 1998; Fraser *et al.*, 2002; Aughey *et al.*, 2005). Five to fifteen mg of muscle was weighed, then homogenised (5% wt/vol) on ice for 2 x 20 s at 20,000 rpm (Omni 1000, Omni International, Marietta, Georgia, USA) in homogenising buffer (10 mM Tris, 2 mM EDTA, 250 mM sucrose; pH 7.4). Muscle homogenates were then exposed to one freeze-thaw cycle, following which homogenates were diluted one in five with ice-cold homogenising buffer. Diluted muscle homogenates were then freeze-thawed a further 3 times. Thirty µl of the diluted homogenate was incubated in 2.5 ml of assay medium (5 mM $MgCl_2$, 1.25 mM EDTA, 100 mM Tris, and an 80 nM 3-*O*-methylfluorescein standard; pH 7.4) for 5 min at 37 °C, before the addition of 40 µl of 10 mM 3-*O*-MFP to initiate the reaction. After 80 s, 10 µl 2.58 M KCl (final concentration of 10 mM) was added to stimulate K^+-dependent phosphatase activity. The K^+-stimulated 3-*O*-MFPase activity assay was performed at 37 °C, with continuous stirring, on a spectrofluorometer (Aminco Bowman AB2 SLM, Urbana, IL, USA), using 4 nm slit widths, and excitation and emission wavelengths of 475 and 515 nm, respectively. The K^+-stimulated 3-*O*-MFPase activity was calculated by subtracting the initial un-specific ATPase activity from the activity obtained after the addition of 10 mM KCl. The muscle 3-*O*-MFPase activity intra-assay coefficient of variation was 16.7% (n = 43).

4.2.11 Muscle [^{3}H]-ouabain binding site content

Muscle Na^+,K^+-ATPase content was determined using vanadate-facilitated [^{3}H]-ouabain binding site content (Nørgaard *et al.*, 1984a). Muscle samples (3-5 mg) were washed for 2 x 10 min at 37 °C in vanadate buffer (250 mM sucrose, 10 mM Tris-HCl, 3 mM $MgSO_4$, 1 mM $NaVO_4$; pH 7.3). Muscle samples were then incubated for 2 h at 37 °C in vanadate

buffer with the addition of [^{3}H]-ouabain (10^{-6} M, 2.0 μCi.ml^{-1}, Amersham Pharmacia Biotech, Castle Hill, NSW, Australia). Following incubation, the muscle samples were washed for 4 x 30 min in ice-cold vanadate buffer to remove an unbound [^{3}H]-ouabain, blotted on filter paper and weighed before being soaked overnight in vials containing 500 μl 5% trichloroacetic acid (TCA) and 0.1 mM ouabain. The following morning, 2.5 ml of scintillation cocktail (Opti-Fluor, Packard) was added prior to liquid scintillation counting of the [^{3}H] activity. The content of [^{3}H]-ouabain binding sites was calculated on the basis of the sample wet weight and the specific activity of the incubation medium and samples, and was expressed as pmol (g wet wt)$^{-1}$. The final [^{3}H]-ouabain binding site content was then calculated by subtracting the non-specific [^{3}H]-ouabain uptake (2.5%) (Nørgaard *et al.*, 1984a) and multiplying by a correction factor of 1.13 to allow for impurity of the [^{3}H]-ouabain (1.05; measured by supplier), loss of specifically bound [^{3}H]-ouabain during washout (1.05) (Nørgaard *et al.*, 1984a) and incomplete saturation (1.025) (T. Clausen, personal communication). The muscle [^{3}H]-ouabain binding site content intra-assay coefficient of variation was 18.4% (n = 41).

4.2.12 Calculations

Based on the observation that the Na^+,K^+-ATPase also acts as a signal transducer in cardiac muscle, (for review, see Xie & Askari, 2002), a significant correlation was recently found between the percentage change in maximal 3-*O*-MFPase activity from rest to fatigue and each of α_1 and α_2 mRNA expression at fatigue, and average post-exercise α_2 mRNA expression following brief intense exercise in human skeletal muscle (Petersen *et al.*, 2005). In the present study, the correlation between the percentage change in maximal 3-*O*-MFPase activity from rest to fatigue and the percentage changes in both mRNA at fatigue, and the average post-exercise mRNA were determined.

4.2.13 Statistical analyses

All data are presented as mean ± SEM, except population statistics where mean ± SD are reported. Respiratory, blood and muscle data were analysed using a one-way repeated measures ANOVA, with Newman-Kuels *post-hoc* analyses. To account for individual variability in time responsiveness of mRNA to exercise, the average post-exercise mRNA was also calculated as the mean of the fatigue, 3 h and 24 h recovery samples for each individual (Chapter 3). This average was contrasted against the resting value and analysed using Student's *t* test for paired-samples. Correlations were determined by least squares linear regression. Significance was accepted at $P < 0.05$.

4.3 RESULTS

4.3.1 Exercise results

Incremental exercise $\dot{V}O_{2peak}$ was 4.05 ± 0.40 L.min^{-1} and $\dot{V}O_2$, $\dot{V}CO_2$ and RER during prolonged submaximal exercise was 3.05 ± 0.31 L.min^{-1} (75.5 ± 1.5% $\dot{V}O_{2peak}$), 2.94 ± 0.30 L.min^{-1} and 0.95 ± 0.02, respectively (all n = 11). Work performed and time to fatigue during prolonged submaximal exercise was 691.2 ± 121.3 kJ and 54.5 ± 6.1 min, respectively (all n = 11).

4.3.2 Exercise effects on plasma [K^+]

Arterialised venous plasma [K^+] increased ($P < 0.03$) after 1 min of exercise and remained above rest at fatigue ($P < 0.03$, Figure 4.1). In recovery, plasma [K^+] fell rapidly, reaching values not significantly different to those at rest by 1 min post-exercise and thereafter (Figure 4.1).

4.3.3 Exercise effects on muscle Na^+,K^+-ATPase mRNA and protein expression

Effects of exercise differed for isoform gene transcripts and protein abundance.

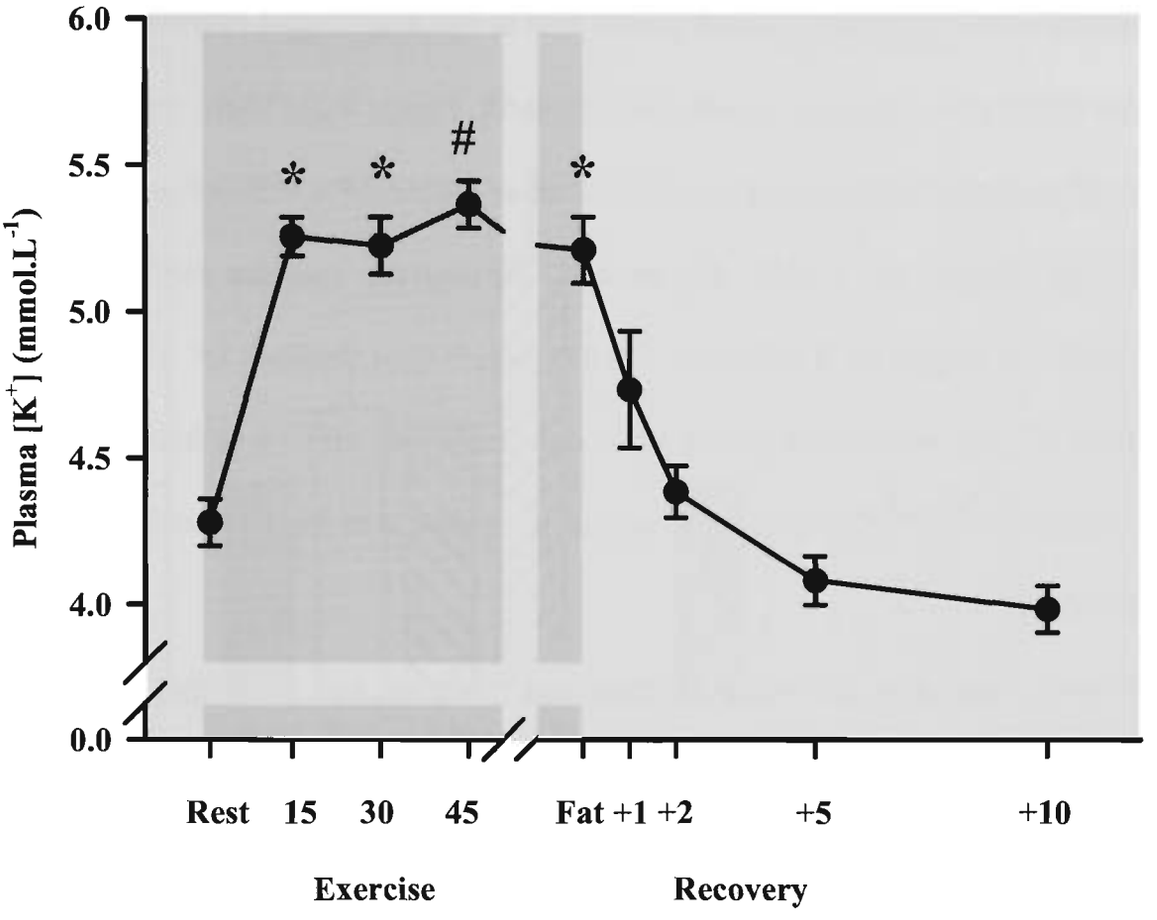


Figure 4.1 Arterialised venous plasma [K^+] at rest, during prolonged submaximal cycling continued to fatigue (Fat), and for 10 min recovery. Shaded bar represents exercise bout. Data are mean ± SEM; n = 11, except during exercise at 30 min (n = 10), 45 min and in recovery at +1 min (n = 8). * $P < 0.03$ greater than rest; # $P < 0.06$ greater than rest.

α subunit isoforms. Prolonged submaximal exercise induced a delayed up-regulation of α_1 mRNA, reaching values 2.0-fold greater than at rest at 24 h post-exercise ($P < 0.04$), but had no effect on α_1 protein abundance ($P < 0.63$, Figure 4.2). There was no significant effect of prolonged submaximal exercise on either mRNA ($P < 0.50$) or protein expression of the α_2 isoform ($P < 0.55$, Figure 4.3). In contrast, exercise elevated α_3 mRNA expression at fatigue by 2.2-fold ($P < 0.05$), which then declined ($P < 0.02$) to resting levels by 3 h post-exercise (Figure 4.4). A significant time effect was found for α_3 protein expression ($P < 0.05$), but post-hoc analyses were unable to locate significant differences (Figure 4.4).

β subunit isoforms. Prolonged submaximal exercise had no effect on β_1 mRNA expression ($P < 0.16$), but tended to increase β_1 protein abundance ($P < 0.06$, Figure 4.5). Exercise elevated β_2 mRNA expression at fatigue by 1.9-fold ($P < 0.05$), which then declined ($P < 0.05$) to resting levels by 24 h post-exercise (Figure 4.6). In contrast, there was no effect of exercise on β_2 protein abundance ($P < 0.86$, Figure 4.6). Exercise had no significant effect on the mRNA ($P < 0.18$) or protein expression of the β_3 isoform ($P < 0.31$, Figure 4.7).

4.3.4 Individual time course variability in mRNA expression

Since brief intense exercise induced individual variability in isoform mRNA time responsiveness (Chapter 3), the average post-exercise mRNA expression of each of the Na^+,K^+-ATPase isoforms was also investigated. A tendency ($P < 0.06$) was observed for elevated average post-exercise mRNA expression only for α_1 (Rest, 1.00 ± 0.00; Average post-exercise, 1.52 ± 0.24 expression relative to rest), with no significant effect of exercise on mRNA expression of any of the other Na^+,K^+-ATPase isoforms (α_2, 1.30 ± 0.18, $P <$

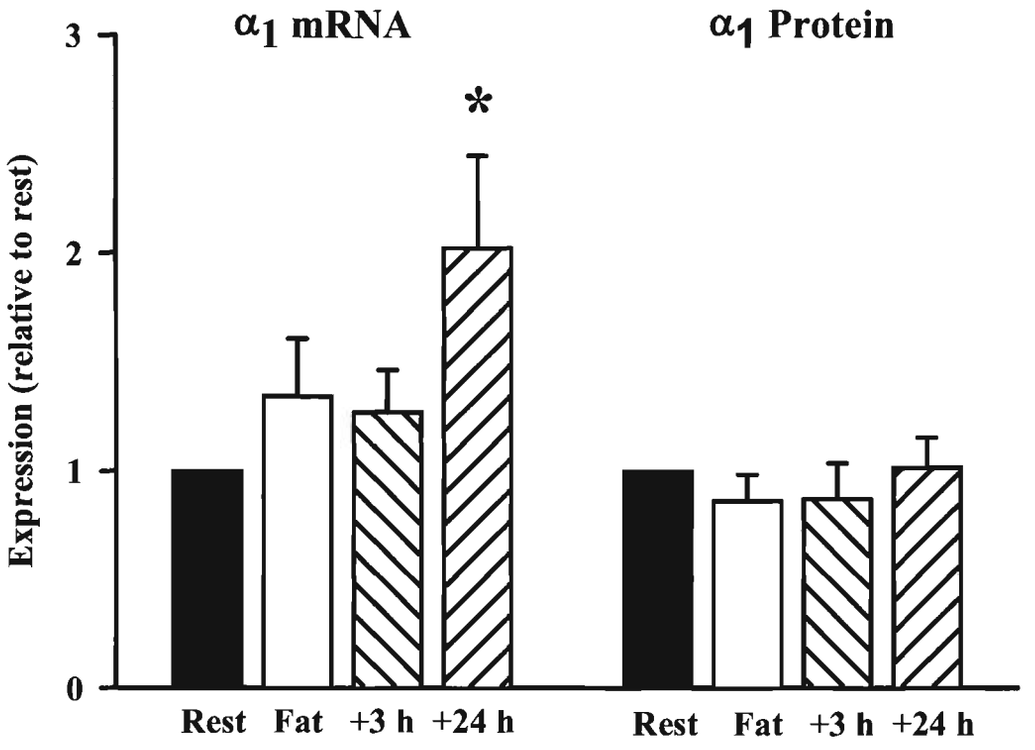


Figure 4.2 Muscle Na^+,K^+-ATPase α_1 mRNA and protein expression at rest and following prolonged submaximal exercise. Samples were collected at rest, fatigue (Fat), 3 h (+3 h) and 24 h post-exercise (+24 h). Samples were normalised against resting values. Data are mean ± SEM; n = 11. * $P < 0.04$ vs. rest.

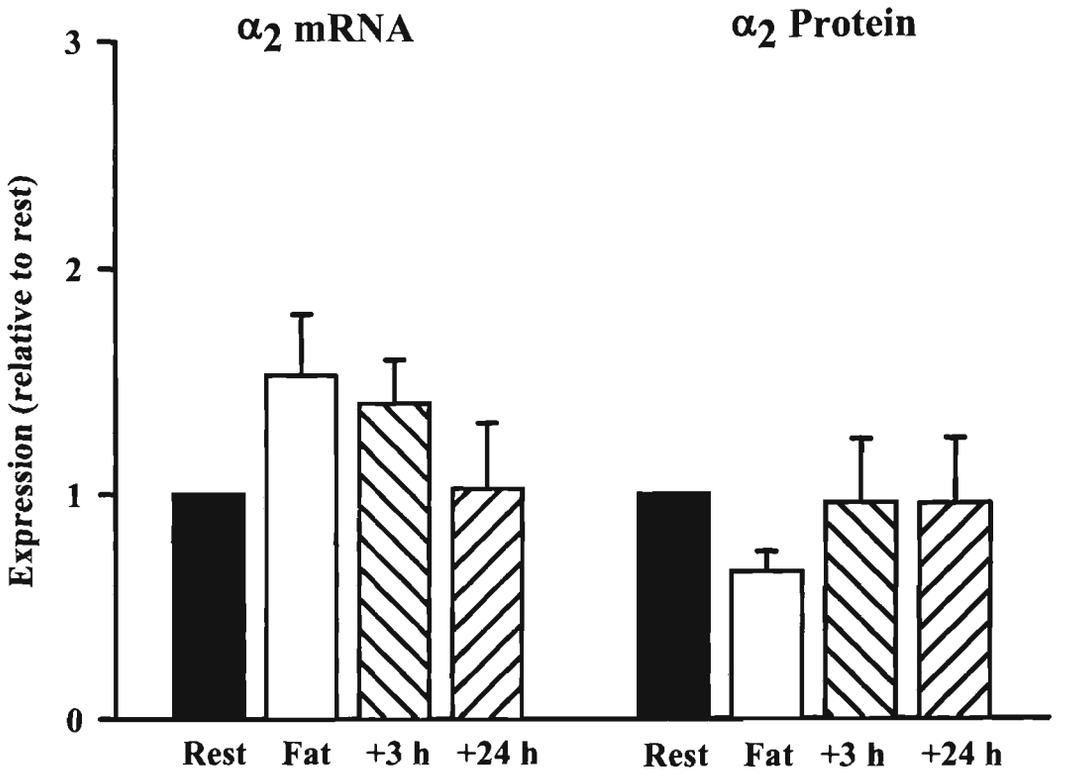


Figure 4.3 Muscle Na^+,K^+-ATPase α_2 mRNA and protein expression at rest and following prolonged submaximal exercise. Samples were collected at rest, fatigue (Fat), 3 h (+3 h) and 24 h post-exercise (+24 h). All results were normalised against resting values. Data are mean ± SEM; n = 11.

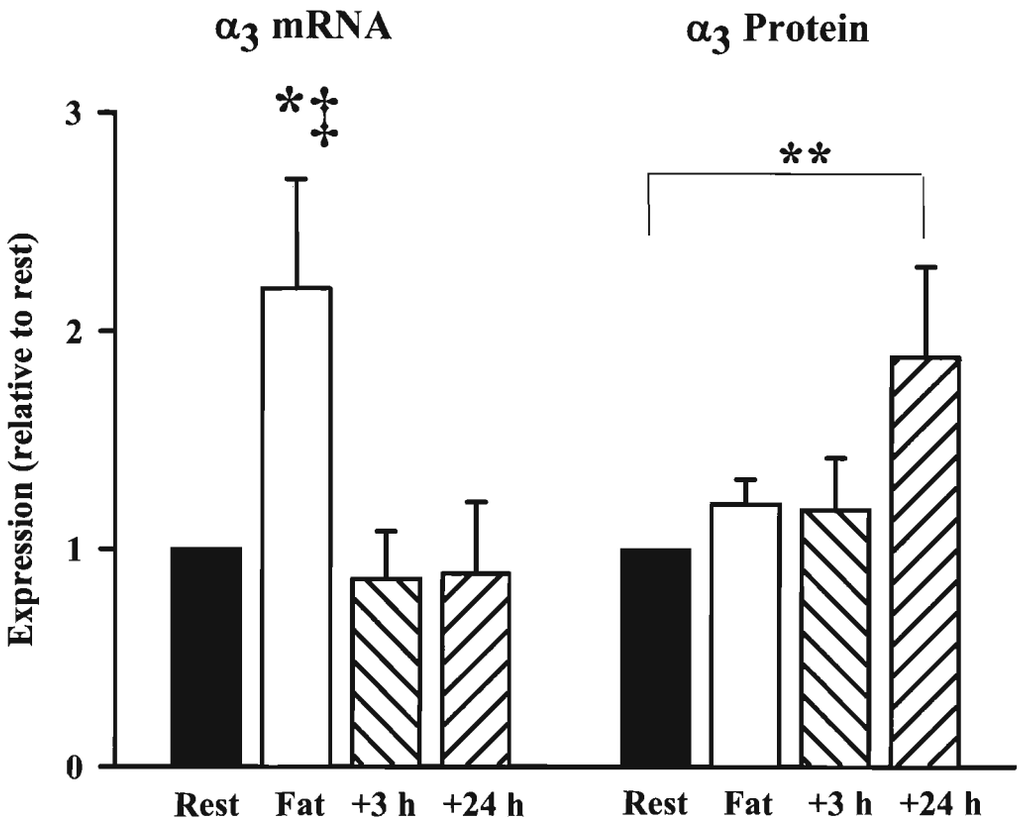


Figure 4.4 Muscle Na^+,K^+-ATPase α_3 mRNA and protein expression at rest and following prolonged submaximal exercise. Samples were collected at rest, fatigue (Fat), 3 h (+3 h) and 24 h post-exercise (+24 h). Samples were normalised against resting values. Data are mean ± SEM; n = 11. * $P < 0.05$ vs. rest; ‡ $P < 0.02$ vs. + 3 h; ** $P < 0.05$ time main effect.

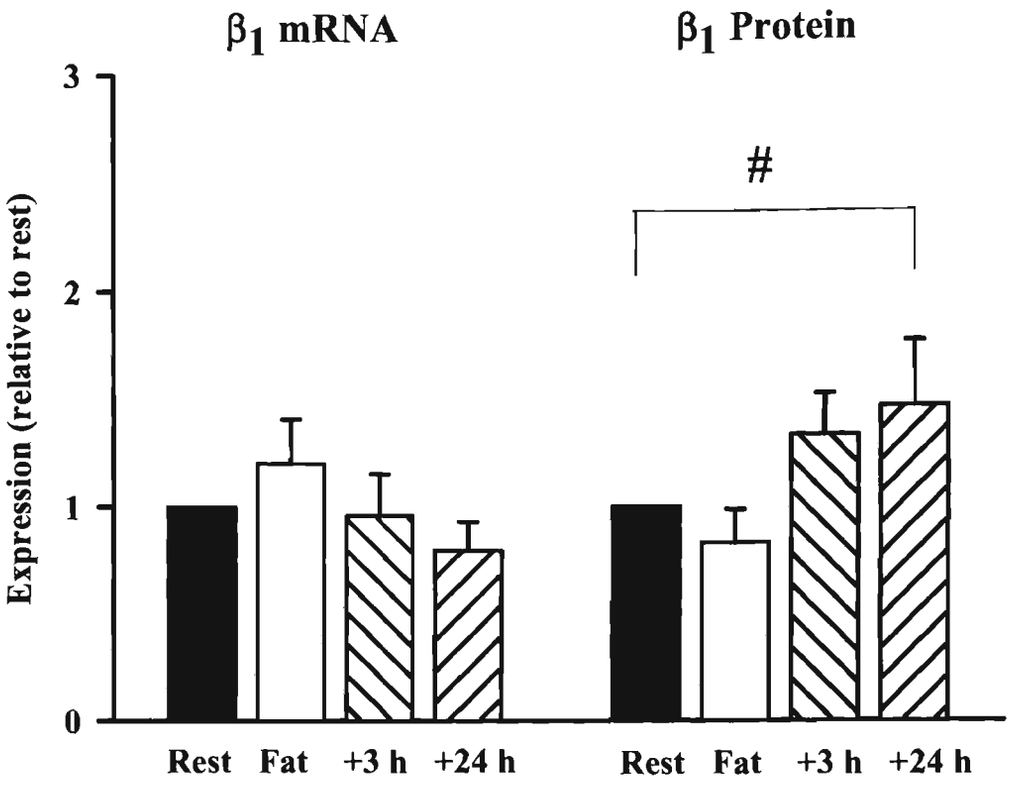


Figure 4.5 Muscle Na^+,K^+-ATPase β_1 mRNA and protein expression at rest and following prolonged submaximal exercise. Samples were collected at rest, fatigue (Fat), 3 h (+3 h) and 24 h post-exercise (+24 h). Samples were normalised against resting values. Data are mean ± SEM; n = 11. # $P < 0.06$ time main effect.

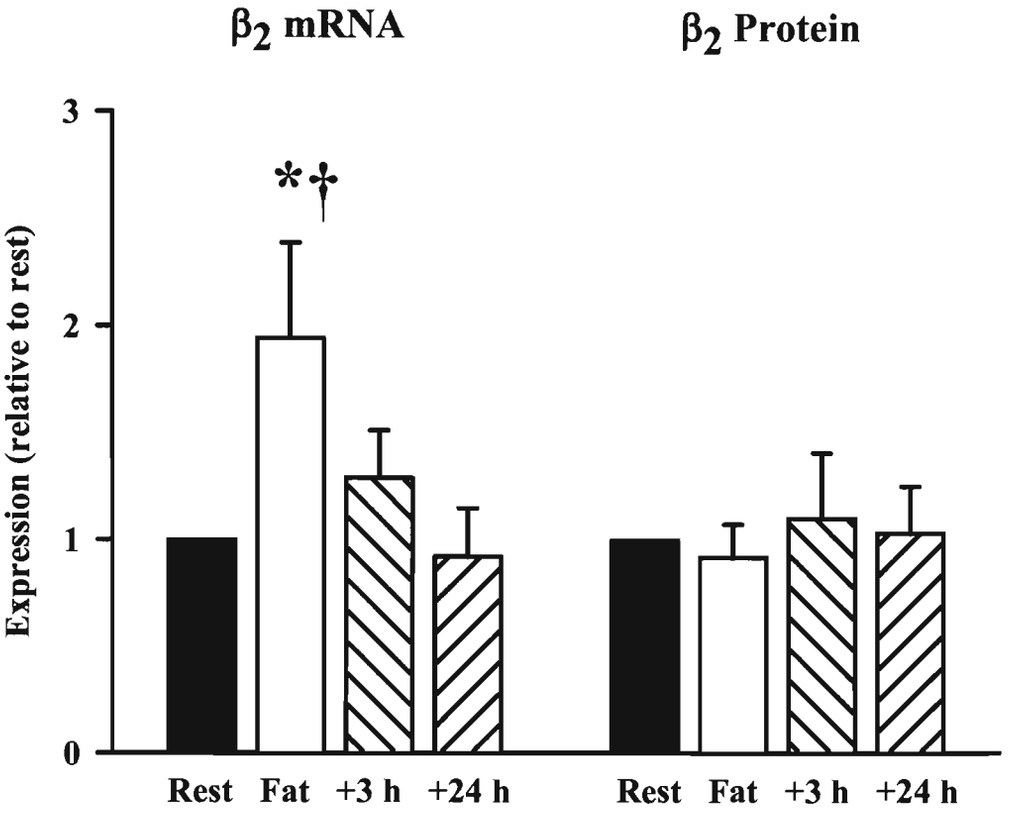


Figure 4.6 Muscle Na^+,K^+-ATPase β_2 mRNA and protein expression at rest and following prolonged submaximal exercise. Samples were collected at rest, fatigue (Fat), 3 h (+3 h) and 24 h post-exercise (+24 h). Samples were normalised against resting values. Data are mean ± SEM; n = 11. * $P < 0.05$ vs. rest; † $P < 0.05$ vs. +24 h.

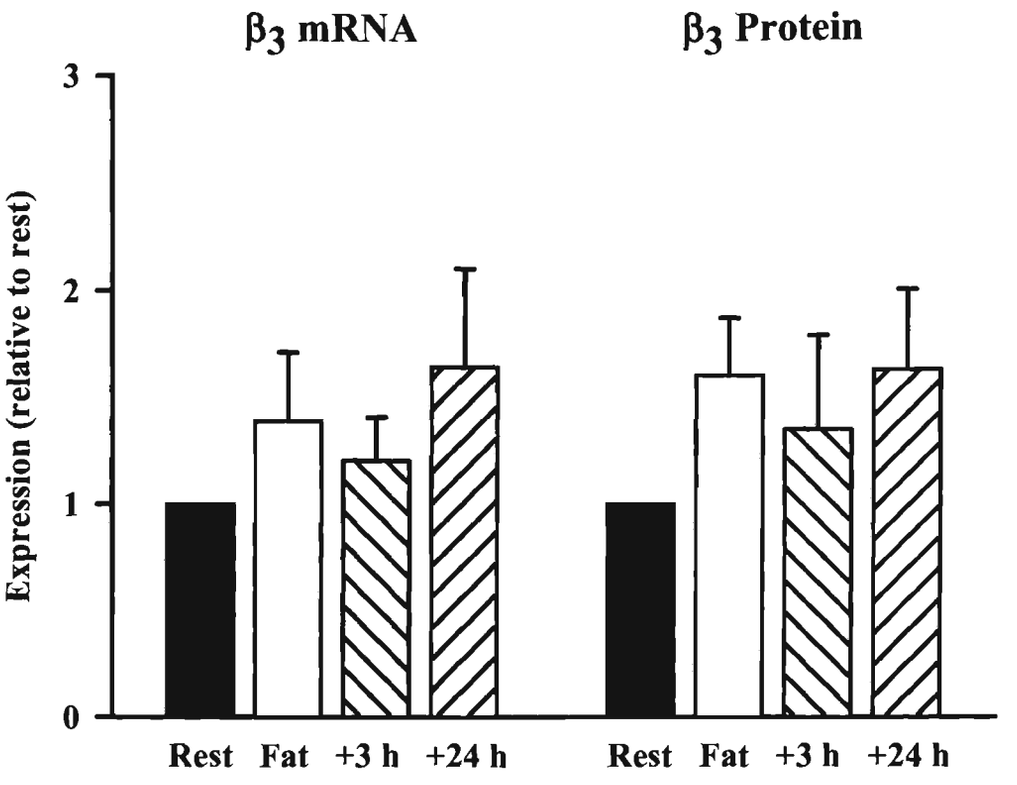


Figure 4.7 Muscle Na^+,K^+-ATPase β_3 mRNA and protein expression at rest and following prolonged submaximal exercise. Samples were collected at rest, fatigue (Fat), 3 h (+3 h) and 24 h post-exercise (+24 h). Samples were normalised against resting values. Data are mean ± SEM; n = 11.

0.14; α_3, 1.32 ± 0.30, $P < 0.65$; β_1, 0.96 ± 0.13, $P < 0.77$; β_2, 1.38 ± 0.20, $P < 0.09$; β_3, 1.38 ± 0.24, $P < 0.15$).

4.3.5 Exercise effects on muscle maximal *in-vitro* 3-*O*-MFPase activity and [^{3}H]-ouabain binding site content

Maximal 3-*O*-MFPase activity was decreased by 18.9 ± 4.7% at fatigue ($P < 0.01$), but did not differ significantly from rest at 3 h and 24 h post-exercise (Figure 4.8A). There was no effect of exercise or 24 h recovery on muscle [^{3}H]-ouabain binding site content ($P < 0.80$, Figure 4.8B).

There was no significant correlation between the percentage change in maximal 3-*O*-MFPase activity from rest to fatigue with the percentage changes in both mRNA at fatigue, or the average post-exercise mRNA, for any of the α_1-α_3 and β_1-β_3 isoforms (data not shown).

4.3.6 Exercise effects on muscle RNAP II, eIF-4E and 4E-BP1 mRNA expression

The mRNA expression of the transcription initiator gene, RNAP II was elevated at fatigue by 2.6-fold ($P < 0.02$), with expression then declining ($P < 0.05$) to resting levels at 24 h post-exercise (Figure 4.9). In contrast, there was no effect of exercise on the mRNA expression of key genes involved in protein translation, eIF-4E ($P < 0.52$) and 4E-BP1 ($P < 0.28$, Figure 4.9).

Average post-exercise mRNA expression of RNAP II was increased by 1.8-fold (1.79 ± 0.27, $P < 0.02$) compared to rest (1.00), while there was no difference in average post-exercise mRNA expression of eIF-4E (0.87 ± 0.11, $P < 0.29$) and 4E-BP1 (1.15 ± 0.17, $P < 0.40$).

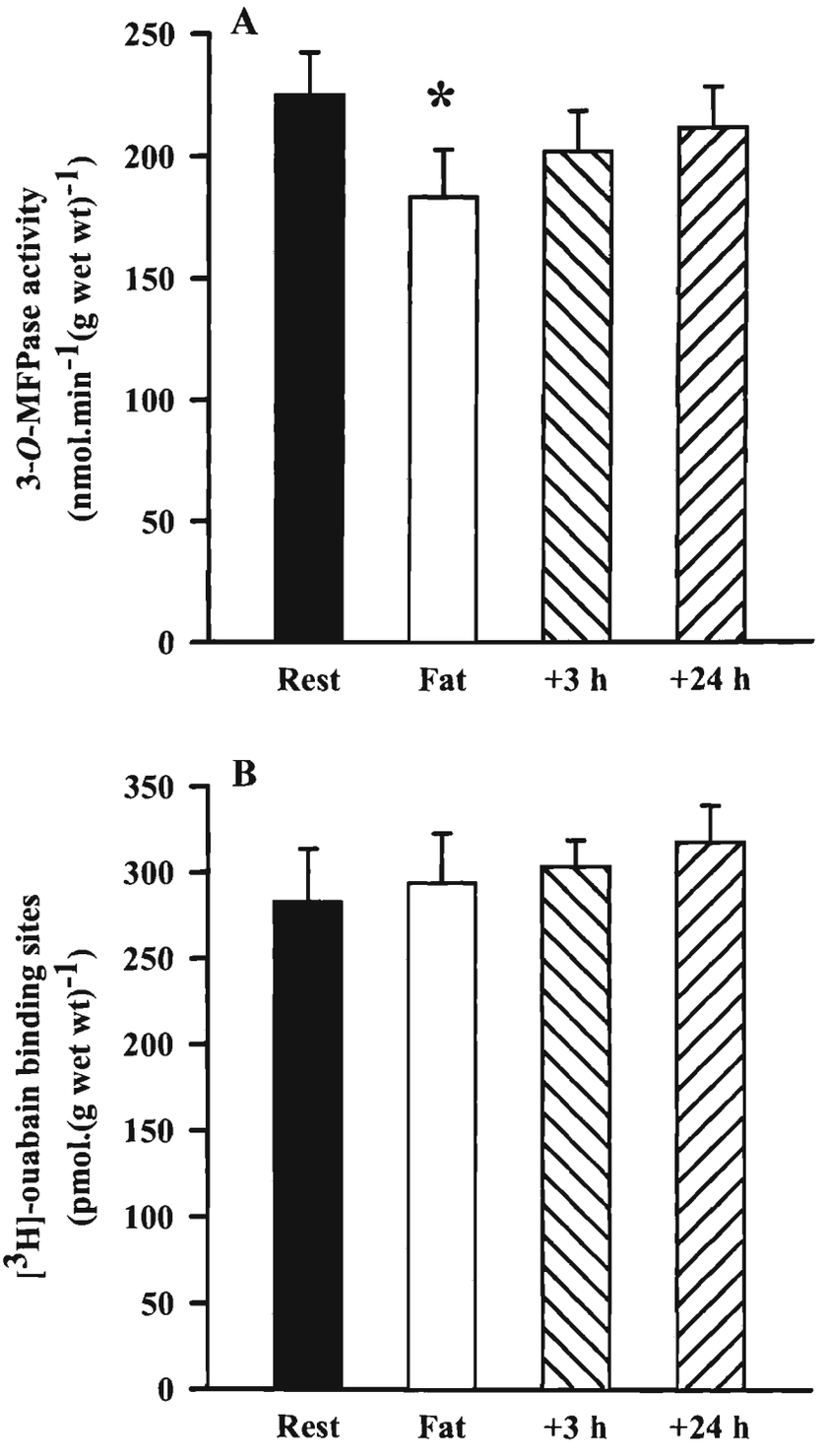


Figure 4.8 Muscle maximal K^+-stimulated 3-*O*-methylfluorescein phosphatase (3-*O*-MFPase) activity (A) and [^{3}H]-ouabain binding site content (B) at rest and following prolonged submaximal exercise. Samples were collected at rest, fatigue (Fat), 3 h (+3 h) and 24 h post-exercise (+24 h). Data are mean ± SEM; n = 11. * $P < 0.01$ vs. rest.

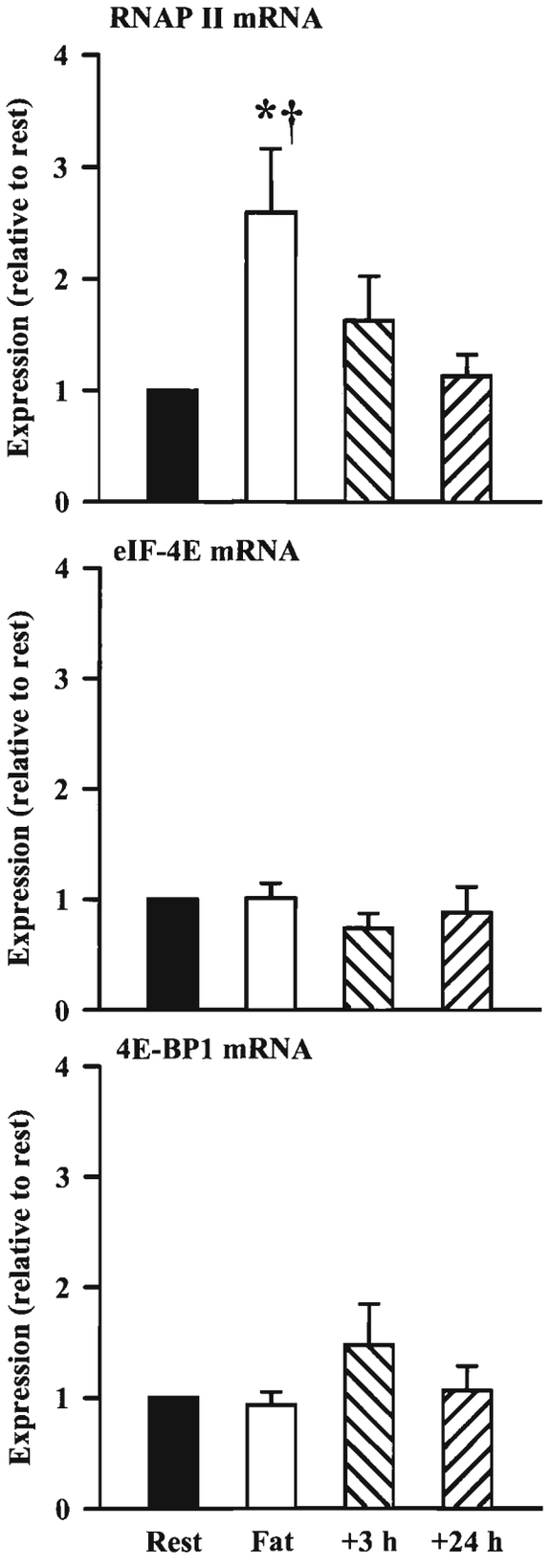


Figure 4.9 Muscle mRNA expression of RNAP II, eIF-4E and 4E-BP1 at rest and following prolonged submaximal exercise. Samples were collected at rest, fatigue (Fat), 3 h (+3 h) and 24 h post-exercise (+24 h). Samples were normalised against resting values. Data are mean ± SEM, n = 11. * $P < 0.02$ vs. rest; † $P < 0.05$ vs. +24 h.

4.4 DISCUSSION

This study investigated the effects of prolonged submaximal exercise on mRNA and protein expression of the full complement of Na^+,K^+-ATPase isoforms expressed in human skeletal muscle. The first main finding was that prolonged submaximal exercise induced isoform-specific Na^+,K^+-ATPase mRNA and protein regulation, with α_1, α_3 and β_2 isoform mRNA expression increased, together with α_3 protein abundance. These findings provide evidence for exercise-specific effects, as they strongly contrast those of Chapter 3, where brief intense exercise up-regulated each of the six Na^+,K^+-ATPase isoform gene transcripts, but did not change the protein abundance of any isoform. The second main finding was that prolonged submaximal exercise only transiently depressed maximal Na^+,K^+-ATPase activity, and did not alter Na^+,K^+-ATPase content, for up to 24 h following exercise. The third main finding was that prolonged submaximal exercise increased the mRNA expression of the transcription initiator gene, RNAP II, but not of key genes involved in translation, eIF-4E and 4E-BP1.

4.4.1 Prolonged submaximal exercise induces isoform-specific increases in Na^+,K^+-ATPase isoform mRNA expression

This is the first study to investigate prolonged exercise effects on mRNA expression of all of the six Na^+,K^+-ATPase isoforms expressed in human skeletal muscle. An isoform-specific effect was observed, with an immediate post-exercise increase in α_3 and β_2 mRNA, and a more delayed increase in α_1 mRNA, detected at 24 h post-exercise. Interestingly, the time course of increases for each of these α_1, α_3 and β_2 isoforms was similar to that seen following brief intense exercise (Chapter 1). These findings of an isoform-specific increase in Na^+,K^+-ATPase mRNA expression with prolonged submaximal exercise is of considerable interest since they contrast with Chapter 3, where

brief intense knee extensor exercise lasting ~6 min increased mRNA expression of all six isoforms.

Furthermore, acute prolonged exercise had no effect on the average post-exercise mRNA expression of the Na^+,K^+-ATPase isoforms, which was elevated after brief intense exercise for each of the α_1-α_3 and β_1-β_3 isoforms (Chapter 3). Thus isoform mRNA adaptation to exercise appears to be exercise intensity and/or duration specific. This is consistent with the finding of elevated α_1, but not α_2 or β_1, mRNA expression after 15 min of high-intensity knee extensor exercise (Nordsborg *et al.*, 2003a). In that study, exercise effects on α_3, β_2 and β_3 were not measured. Only one other study has investigated prolonged exercise effects on skeletal muscle Na^+,K^+-ATPase mRNA expression and this was performed in rats after 1 h of treadmill running (Tsakiridis *et al.*, 1996). They reported increased α_1 mRNA expression in red, oxidative but not white, glycolytic muscles, and β_2 mRNA expression in white, glycolytic muscles in which it was exclusively detected (Tsakiridis *et al.*, 1996). There was no effect of exercise on α_2 and β_1 mRNA expression in red, oxidative muscles in which they were exclusively detected, whilst α_3 and β_3 mRNA were not measured (Tsakiridis *et al.*, 1996).

The consistent α_1 and β_2 mRNA up-regulation following exercise in this and three other studies (Chapter 3; Tsakiridis *et al.*, 1996; Nordsborg *et al.*, 2003a) raises speculation regarding the physiological significance of increased mRNA expression of each of these isoforms. The α_1 isoform was recently shown to play a direct role in maintaining membrane potential and Na^+ and K^+ equilibrium gradients (Radzyukevich *et al.*, 2004). A specific function of the β_2 isoform in skeletal muscle is not known, although the β subunit facilitates correct processing, assembly and membrane insertion of the Na^+,K^+-ATPase (Ackermann & Geering, 1990; Noguchi *et al.*, 1990; Hundal *et al.*, 1994). However, since there was no corresponding up-regulation of α_1 and β_2 protein, the significance of these

isoform-specific increases in mRNA are uncertain. Presumably repeated exercise bouts are required to induce protein up-regulation.

The physiological mechanisms contributing to the elevated α_1, α_3 and β_2 mRNA expression with prolonged submaximal exercise are unknown. However, these may involve raised muscle intracellular Na^+ and/or Ca^{2+}, both of which occur in human muscle with submaximal exercise (Sjøgaard *et al.*, 1985; Overgaard *et al.*, 2004), and which have each been associated with increased Na^+,K^+-ATPase mRNA expression, in cultured canine kidney (Bowen & McDonough, 1987), rat kidney (Pressley *et al.*, 1988; Rayson, 1993) and chick skeletal muscle cells (Taormino & Fambrough, 1990).

It was recently found with brief intense exercise, that there were significant inverse correlations between the percentage change from rest to fatigue in maximal 3-*O*-MFPase activity and the mRNA expression at fatigue for α_1 ($y = -0.60$, $P < 0.05$) and α_2 ($r = -0.60$, $P = 0.05$) (Petersen *et al.*, 2005). The reduced mRNA responsiveness found with prolonged submaximal exercise may explain the lack of correlation between maximal 3-*O*-MFPase activity and mRNA in the present study, or that different stimuli are involved with the two different exercise regimes. Alternatively, the lack of correlation may indicate that the previous finding was coincidental.

4.4.2 Prolonged submaximal exercise induces an α_3-specific increase in Na^+,K^+-ATPase isoform protein abundance

It was previously shown that brief intense exercise had no effect on protein abundance of any of the Na^+,K^+-ATPase α_1-α_3 and β_1-β_3 isoforms in crude muscle homogenates (Chapter 3). In contrast, prolonged submaximal exercise had isoform-specific effects, increasing α_3 protein abundance, with a strong tendency toward elevated β_1 protein abundance ($P < 0.06$). The lack of effect of prolonged exercise on α_1, α_2, β_2 and β_3 protein abundance is therefore consistent with the findings with brief intense exercise (Chapter 3).

Although a specific role of the α_3 isoform has yet to be elucidated in skeletal muscle, the preferential increase in protein abundance of the α_3 isoform following prolonged submaximal exercise may reflect the substrate properties of the isoform. In HeLa cells expressed with the rat α_1, α_2 and α_3 isoforms, the α_3 isoform had a 2-3 fold lower apparent affinity for Na^+, as well as a 1.3-2.0 fold greater affinity for ATP compared to the α_1 and α_2 isoforms (Jewell & Lingrel, 1991). In *Xenopus* oocytes, the human α_3 isoform also showed a low affinity for intracellular Na^+ (Crambert *et al.*, 2000). These relative affinities imply specific activation of the α_3 isoform when intracellular concentrations of Na^+ are elevated and ATP is reduced (Blanco & Mercer, 1998). Increases in intracellular Na^+ (Sjøgaard *et al.*, 1985) and a reduction in intracellular ATP (Sahlin *et al.*, 1990) have been reported following prolonged submaximal exercise. However, since the relative protein expression of the α_3 isoform in human muscle is likely to be low, the quantitative importance of an increased α_3 protein abundance is uncertain.

The observed increase in crude muscle homogenate α_3 protein abundance following exercise may reflect increased protein synthesis, reduced protein degradation or a combination of both. Although the present study could not directly evaluate these mechanisms, exercise also elevated α_3 mRNA expression, strongly suggesting involvement of increased protein synthesis. In cultured chick skeletal muscle, the increase in Na^+,K^+-ATPase β subunit protein abundance with 36 h of veratridine treatment was initially due to a progressive increase in synthesis, followed by an attenuation of degradation over the remaining time (Wolitzky & Fambrough, 1986).

4.4.3 Functional effects of prolonged submaximal exercise on Na^+,K^+-ATPase

The present study confirms that prolonged submaximal exercise immediately depresses maximal *in-vitro* 3-*O*-MFPase (Na^+,K^+-ATPase) activity, but has no effect on [^{3}H] ouabain binding site (Na^+,K^+-ATPase) content (Leppik *et al.*, 2004). Additionally, it was

demonstrated that Na^+,K^+-ATPase content remained unchanged from resting values at 3 and 24 h post-exercise. This finding extends the lack of change in Na^+,K^+-ATPase content found previously immediately following (Leppik *et al.*, 2004; Aughey *et al.*, 2005) and up to 24 h following acute exercise in humans (Fowles *et al.*, 2002b), and with electrical stimulation in rat muscle (McKenna *et al.*, 2003b). Thus, ~55 min of exhaustive exercise with a 24 h recovery period was insufficient to increase Na^+,K^+-ATPase content, which may require ultra-endurance exercise (Overgaard *et al.*, 2002), or repeated bouts of exercise (Green *et al.*, 1993; McKenna *et al.*, 1993; Green *et al.*, 2004).

This study also showed confirmed that the depression in maximal *in-vitro* Na^+,K^+-ATPase activity with prolonged submaximal exercise was transient, returning to levels not significantly different from those at rest at 3 and 24 h post-exercise. The transient depression in maximal activity was also seen following 30 min of intermittent isometric exercise (Fowles *et al.*, 2002b), however, their findings were inconclusive due to internally inconsistent results. This was due to comparisons being made between non-exercised and exercised legs, and also within the exercised leg but only in the period following exercise, as well as a potentially insufficient recovery period of only 4 h duration.

A limitation in interpreting the importance of this finding is that depressed maximal *in-vitro* activity does not directly reflect the functionally important *in-vivo* activity, as discussed previously (Fraser *et al.*, 2002; Leppik *et al.*, 2004; Aughey *et al.*, 2005). The magnitude and the time-dependent pattern of depression in maximal Na^+,K^+-ATPase activity might also differ from that observed *in-vitro* due to additional effects of any localised decline in glycogen, phosphocreatine, ATP and build up of other metabolites. Nonetheless, the marked K^+ fluxes in contracting muscle are consistent with a possible depressed maximal Na^+,K^+-ATPase activity also occurring *in-vivo*. The depressed activity may augment the reduction in $[K^+]_i$ (Sjøgaard *et al.*, 1985) and the increase in interstitial

$[K^+]$ (Green *et al.*, 2000; Nordsborg *et al.*, 2003b) that occur with exercise. Although there was only a modest rise in plasma $[K^+]$ to ~5.6 $mmol.L^{-1}$ with prolonged submaximal exercise, a greater rise in muscle interstitial $[K^+]$ is probable. Thus, impaired maximal Na^+,K^+-ATPase activity may contribute to a transient loss of K^+ homeostasis, reducing the transmembrane $[K^+]$ gradient and membrane excitability (Sjøgaard *et al.*, 1985; Renaud & Light, 1992) and subsequently, impairing the development of force (Renaud & Light, 1992). Thus the relatively modest ~19% depression in maximal *in-vitro* Na^+,K^+-ATPase activity immediately following prolonged submaximal exercise may have important implications for the development of muscle fatigue.

4.4.4 Prolonged submaximal exercise increases RNAP II mRNA expression

This study demonstrate for the first time that a single bout of prolonged submaximal exercise elevated the mRNA expression of the non-specific transcription initiator gene, RNAP II in human muscle. This is consistent with up-regulated mRNA expression of numerous other genes in human skeletal muscle with acute prolonged exercise, including those involved in metabolism (Pilegaard *et al.*, 2000) and oxidative stress (Pilegaard *et al.*, 2000).

4.4.5 Prolonged submaximal exercise has no effect on genes involved in protein translation

A single bout of prolonged submaximal exercise had no effect on mRNA expression of the key genes involved in protein translation, eIF-4E and 4E-BP1. The lack of change in the average post-exercise mRNA expression confirmed these findings. The effects of acute prolonged exercise on eIF-4E and 4E-BP1 have previously only been investigated at the protein level in rat skeletal muscle (Gautsch *et al.*, 1998). In that study, measured 1 h following 2 h of treadmill running, there was a 250% increase in the amount of eIF-4E associated with 4E-BP1, and consequently a 26% reduction in net protein synthesis

(Gautsch *et al.*, 1998). Unchanged eIF-4E and 4E-BP1 mRNA expression in the present study suggests that the elevated association of eIF-4E and 4E-BP1 was not due to an increased abundance of either protein, but rather may reflect a reduction in phosphorylation of 4E-BP1 (Bolster *et al.*, 2003). These mechanisms responsible for the subsequent inhibitory effect on net protein synthesis in mammalian muscle remain inconclusive and require further investigation.

In conclusion, a single bout of prolonged submaximal exercise induced isoform-specific Na^+,K^+-ATPase mRNA and protein regulation in muscle, with increased mRNA expression of the α_1, α_3 and β_2 isoforms, and increased protein abundance of the α_3 isoform. Furthermore, prolonged exercise transiently depressed maximal Na^+,K^+-ATPase activity, but was insufficient to up-regulate Na^+,K^+-ATPase content in the 24 h period following exercise. Prolonged submaximal exercise also elevated mRNA expression of the transcription initiator gene RNAP II, but had no effect on mRNA expression of key genes involved in protein translation, eIF-4E and 4E-BP1.

CHAPTER 5

STUDY 3: EFFECTS OF HIGH-INTENSITY, INTERMITTENT EXERCISE AND TRAINING ON SKELETAL MUSCLE Na^+,K^+-ATPase mRNA AND PROTEIN EXPRESSION IN ALREADY WELL-TRAINED CYCLISTS

5.1 INTRODUCTION

Results from the two preceding chapters suggest an effect of exercise intensity and/or duration on the transcriptional and translational regulation of the Na^+,K^+-ATPase in human skeletal muscle. Brief intense exercise increased mRNA expression of each of the Na^+,K^+-ATPase α_1, α_2, α_3, β_1, β_2 and β_3 isoforms, but had no effect on the protein abundance of any of these isoforms (Chapter 3). In contrast, prolonged submaximal exercise increased mRNA expression of the α_1, α_3 and β_2 isoforms, but also increased α_3 protein abundance (Chapter 4). The effects of high-intensity, intermittent exercise on Na^+,K^+-ATPase isoform mRNA and protein expression are not well established. Fifteen min of high-intensity, intermittent knee extensor exercise increased α_1 mRNA expression by 3.0-fold at each of 0, 1 and 3 h post-exercise (Nordsborg *et al.*, 2003a). There was no exercise effect on either α_2 or β_1 mRNA expression, whilst the α_3, β_2 and β_3 gene transcripts were not probed (Nordsborg *et al.*, 2003a). The effects of high-intensity, intermittent exercise on Na^+,K^+-ATPase isoform protein abundance have yet to be investigated. The first aim of this study was to therefore investigate the effects of high-intensity, intermittent exercise on the mRNA and protein expression of each of the six Na^+,K^+-ATPase isoforms present in human skeletal muscle. The hypothesis tested was that a single bout of high-intensity, intermittent exercise would increase the mRNA expression, but not protein abundance of each of the Na^+,K^+-ATPase α_1-α_3 and β_1-β_3 isoforms, consistent with Study 1.

High-intensity intermittent training (HIT) is commonly used by competitive cyclists to improve their performance. The benefits of HIT include improvements in performance measures (Westgarth-Taylor *et al.*, 1997; Weston *et al.*, 1997; Stepto *et al.*, 1999), as well as physiological measures, including increased skeletal muscle buffering capacity (Weston *et al.*, 1997), reduced carbohydrate oxidation and plasma lactate concentration at submaximal work rates (Westgarth-Taylor *et al.*, 1997). Whether skeletal muscle Na^+,K^+-ATPase regulation is improved with HIT is unclear. In previously untrained subjects, 5.5 wks of HIT had no significant effect on resting mRNA expression of the α_1, α_2 and β_1 isoforms, and abolished the exercise-induced increase in α_1 mRNA that was evident prior to HIT (Nordsborg *et al.*, 2003a). Conversely, 7 wks of HIT increased the protein abundance of the α_1 and α_2 isoforms by 29 and 15%, respectively, but had no significant effect on β_1 protein abundance in habitually active subjects (Nielsen *et al.*, 2004a). The effects of HIT on α_3, β_2 and β_3 mRNA and protein expression are unknown. Furthermore, whether the effects of HIT on Na^+,K^+-ATPase mRNA and protein expression are similar in already well-trained athletes have yet to be investigated. The second aim of this study was to therefore investigate the effects of short-term HIT on the mRNA and protein expression of each of the Na^+,K^+-ATPase isoforms expressed in skeletal muscle of already well-trained athletes. The hypotheses tested were that short-term HIT would increase resting mRNA and protein expression of each of the Na^+,K^+-ATPase α_1-α_3 and β_1-β_3 isoforms, but HIT would not significantly alter the response of Na^+,K^+-ATPase isoform mRNA and protein expression to high-intensity, intermittent exercise.

5.2 METHODS

5.2.1 Subjects

Twelve healthy, well-trained endurance trained male cyclists/triathletes (age, 31.4 ± 3.0 yrs; height, 177.7 ± 6.4 cm; Pre-HIT body mass, 75.4 ± 3.0 kg; Post-HIT body mass, 75.2 ± 3.0 kg; yrs of training 6.8 ± 4.3; mean ± SD) gave written informed consent and participated in this study, which was approved by the Human Research Ethics Committees of Victoria University of Technology and of RMIT University. Only subjects who were cycling >350 $km.wk^{-1}$, and who had not undertaken any high-intensity, intermittent training in the 4 wk prior to the beginning of this study were recruited. This study utilized trained athletes since untrained subjects demonstrate much greater variability between repeated tests of performance than trained athletes, and the experimental design of this study involved repeated performance tests. Thus, the use of trained subjects would minimise the likelihood of any changes observed with training being due to within-subject variability (Hopkins *et al.*, 2001). Furthermore, untrained subjects cannot undertake such intensified training (Clark *et al.*, 2004). All subjects refrained from vigorous exercise, caffeine and alcohol consumption, and consumed a controlled diet for 24 h prior to each of the exercise tests.

5.2.2 Experimental design

Subjects acted as their own control. Each subject underwent a resting muscle biopsy, and performed an incremental exercise test to assess peak power output (PPO) and $\dot{V}O_{2peak}$ (Baseline). Subjects then maintained their normal endurance training for a further 4 wks, before undergoing pre-training performance testing (Pre-HIT), followed by the high-intensity, intermittent training program (HIT), and Post-HIT performance testing (Post-HIT). An untrained control group could not be included in this study as untrained subjects are unable to ride for greater than 15 min (3 x 15 min) at the same relative intensity

sustained by the well-trained subjects used in this study. Furthermore, untrained subjects may also have different mechanisms facilitating gene transcription (Yu *et al.*, 2003).

Each subject performed three separate incremental exercise tests; being at Baseline, Pre-HIT and Post-HIT. Both PPO and $\dot{V}O_{2peak}$ were measured during each test, which was performed on the same electromagnetically braked cycle ergometer (Lode, Groningen, The Netherlands).

5.2.3 Incremental exercise test

The incremental exercise test involved cycling at a work rate equivalent to lactate threshold, as previously defined (Clark *et al.*, 2004), followed by 25 W increments every 150 s, until volitional exhaustion, which was defined as the inability to maintain pedal cadence above 60 rev.min^{-1}. PPO was determined as the final completed work rate, plus the fraction of the next completed work rate, calculated as $PPO = W_f + (T_i / 150) \times 25$; where, W_f = final completed work rate; T_i = time at incomplete work rate; 150 = time spent at each work rate; and 25 is the size of the work rate increment. The calculated PPO was used to determine the subsequently described HIT sessions. Throughout the test, subjects inspired air through a two-way Hans-Rudolph valve (model R2700, Kansas City, KS), with expirate directed to a CardioO_2 Cardiorespiratory Diagnostic Systems metabolic cart (Medical Graphics, St. Paul, MN). Expired gas was passed through a flowmeter, an O_2 analyser, and a CO_2 analyser. The flowmeter was calibrated with a 3 L Hans-Rudolph syringe. The gas analysers were calibrated with gases of known concentrations. The flowmeter and gas analysers were connected to a computer, which calculated minute ventilation ($\dot{V}_E$), O_2 consumption ($\dot{V}O_2$), and CO_2 output ($\dot{V}CO_2$). Some expired gas data was retained in the laboratory of RMIT and was unfortunately misplaced, and is therefore presented for 5 subjects.

5.2.4 High-intensity, intermittent exercise

Each exercise session involved a 20 min warm up at a work rate corresponding to 58% PPO, followed by eight 5-min intervals at a work rate corresponding to 80% PPO, equivalent to 85% $\dot{V}O_{2peak}$ (Stepto *et al.*, 2001). Each interval was separated by 1 min recovery cycling at 100 W.

5.2.5 High-intensity, intermittent training

HIT involved the high-intensity, intermittent exercise session described above being repeated on a further 6 occasions in a 21-d period, with 2 sessions performed in wk 1, 3 sessions performed in wk 2 and 2 sessions performed in wk 3. The PPO was redetermined during the fourth session to enable percentage workloads for HIT to be adjusted for sessions 5 and 6. This HIT regime has been previously shown to improve performance in well-trained subjects (Lindsay *et al.*, 1996; Westgarth-Taylor *et al.*, 1997; Weston *et al.*, 1997; Stepto *et al.*, 1999; Stepto *et al.*, 2001). Identical matched work rates were used for sessions 1 (Pre-HIT) and 7 (Post-HIT) to ensure the same amount of exercise was completed pre- and Post-HIT. This enabled us to investigate whether training modified the exercise-induced changes in Na^+,K^+-ATPase mRNA and protein expression using a standard exercise bout. Use of matched work bouts are essential to properly compare training effects on markers of muscle ion regulation (Harmer *et al.*, 2000).

5.2.6 Muscle biopsy sampling

An initial muscle biopsy was taken at rest at Baseline, with a further four biopsies taken at rest (Rest) and immediately post-exercise (Ex) at both Pre-HIT and Post-HIT. The needle biopsy sample was taken from the vastus lateralis muscle under local anaesthesia (1% Xylocaine), with suction applied to the needle. The post-exercise sample was taken immediately after the cessation of the final 5 min interval of the exercise bout, with the subject lying supported on the cycle ergometer. Biopsies were taken from separate

incisions in the same leg, with the exercise sample taken from an incision ~1.5 cm proximal to the rest sample. An experienced medical examiner took all biopsies at approximately constant depth. Muscle samples were removed, and rapidly frozen and stored in liquid N_2 for subsequent analysis of Na^+,K^+-ATPase isoform mRNA and protein expression. Muscle isoform mRNA and protein data are presented for 12 and 5 subjects, respectively, due to insufficient sample procurement.

5.2.7 Real-Time RT-PCR measurement of mRNA

Muscle was analysed for Na^+,K^+-ATPase α_1, α_2, α_3, β_1, β_2 and β_3 mRNA expression as described in Chapter 3. The ratio of absorbance at 260 and 280 nm (260/280) was 1.92 ± 0.04. Cyclophilin (CYC) mRNA expression was unchanged with either high-intensity, intermittent exercise ($P < 0.77$) or HIT ($P < 0.77$), when expressed in the linear (2^{-C_T}) form (data not shown), and was therefore used as a control (housekeeping gene) to account for any variations in the amount of input RNA and the efficiency of reverse transcription. Gene expression was quantified using a cycle threshold (C_T) method, whereby the relative expression of the genes compared with the Pre-HIT or Post-HIT resting sample was made using the expression, $2^{-\Delta\Delta C_T}$, in which the expression of each gene was normalised for input cDNA using the housekeeping gene, CYC. The intra-assay coefficient of variation for each target gene was <14.0% for 2^{-C_T} (Table 5.1).

5.2.8 Western blotting

Immunoblotting and antibodies used were as described in Chapter 3.

5.2.9 Statistical Analyses

All data are presented as mean ± SEM, except population statistics where mean ± SD are reported. Differences between Baseline and Pre-HIT for performance and resting muscle data, and training effects on exercise performance was analysed using a paired samples student *t*-test. Differences between Rest and Ex at both Pre-HIT and Post-HIT for isoform mRNA and protein expression were analysed using a paired samples student *t*-test as

Table 5.1 Intra-assay variability of 2^{-CT} values

Gene	2^{-C_T} CV (%)
α_1	9.9
α_2	13.8
α_3	12.7
β_1	10.3
β_2	12.2
β_3	11.5
CYC	8.2

Each sample was run in triplicate wells in the same Real-Time PCR run. n = 45. CV, coefficient of variation; C_T, cycle threshold.

uneven numbers for the Pre-HIT and Post-HIT trials precluded use of ANOVA. Differences between Pre-HIT and Post-HIT for the exercise-induced change in isoform mRNA and protein expression were analysed using a paired samples student *t*-test. Differences were located with a Student-Newman-Kuels *post hoc* test. Significance was accepted at $P < 0.05$.

5.3 RESULTS

5.3.1 Baseline stability

There was no significant difference between Baseline and Pre-HIT for the performance measures PPO and $\dot{V}O_{2peak}$, for muscle Na^+,K^+-ATPase α_1, α_2, α_3, β_1, β_2 and β_3 and CYC mRNA expression, or for muscle Na^+,K^+-ATPase α_1, α_2, α_3, β_1, β_2 and β_3 protein abundance (Table 5.2). These findings therefore demonstrate the stability of these measurements during the subjects' pre-HIT training regime. However, there was a tendency toward increased β_2 mRNA ($P < 0.07$) and reduced α_2 protein ($P < 0.08$) at Baseline (Table 5.2).

5.3.2 High-intensity exercise effects on muscle Na^+,K^+-ATPase isoform mRNA and protein expression

mRNA expression. At Pre-HIT, high-intensity exercise immediately increased the mRNA expression of the α_1, α_2 and α_3 isoforms by 2.0- ($P < 0.01$), 2.4- ($P < 0.01$) and 4.0-fold, respectively ($P < 0.05$, Figure 5.1A). Exercise also induced a tendency toward elevated β_2 mRNA expression ($P < 0.09$, β_2 mRNA elevated in 10 of 12 subjects), but had no significant effect on β_1 ($P < 0.11$) or β_3 mRNA expression ($P < 0.12$, Figure 5.1A).

Protein expression. In contrast to the elevated α mRNA, high-intensity exercise had no significant effect on the protein abundance of any of the Na^+,K^+-ATPase α_1-α_3 or β_1-β_3 isoforms (Figure 5.1B).

Table 5.2 Baseline versus Pre-HIT comparison of performance and muscle measures

	n	Baseline	Pre-HIT	*P* value
Performance				
PPO (W)	10	368 ± 12	371 ± 10	0.311
$\dot{V}O_{2peak}$ ($ml.kg^{-1}.min^{-1}$)	5	65.5 ± 2.1	64.2 ± 1.8	0.328
Muscle				
Isoform mRNA expression				
α_1	12	1.25 ± 0.27	1.00 ± 0.00	0.666
α_2	12	0.65 ± 0.40	1.00 ± 0.00	0.966
α_3	12	1.01 ± 0.22	1.00 ± 0.00	0.133
β_1	12	1.20 ± 0.13	1.00 ± 0.00	0.369
β_2	12	2.16 ± 0.66	1.00 ± 0.00	0.061
β_3	12	1.01 ± 0.26	1.00 ± 0.00	0.298
CYC	12	$1.41x10^{-8} \pm 0.15x10^{-8}$	$1.81x10^{-8} \pm 0.27x10^{-8}$	0.194
Isoform protein expression				
α_1	11	0.86 ± 0.13	1.00 ± 0.00	0.306
α_2	11	0.80 ± 0.10	1.00 ± 0.00	0.077
α_3	11	1.38 ± 0.29	1.00 ± 0.00	0.223
β_1	11	1.17 ± 0.24	1.00 ± 0.00	0.486
β_2	11	1.03 ± 0.19	1.00 ± 0.00	0.862
β_3	11	1.11 ± 0.25	1.00 ± 0.00	0.663

Data are mean ± SEM. PPO, peak power output; $\dot{V}O_{2peak}$, peak O_2 consumption; CYC, cyclophilin. Na^+,K^+-ATPase α_1, α_2, α_3, β_1, β_2 and β_3 mRNA and protein data are expressed relative to Pre-HIT values. CYC is expressed in the linear (2^{-C_T}) form.

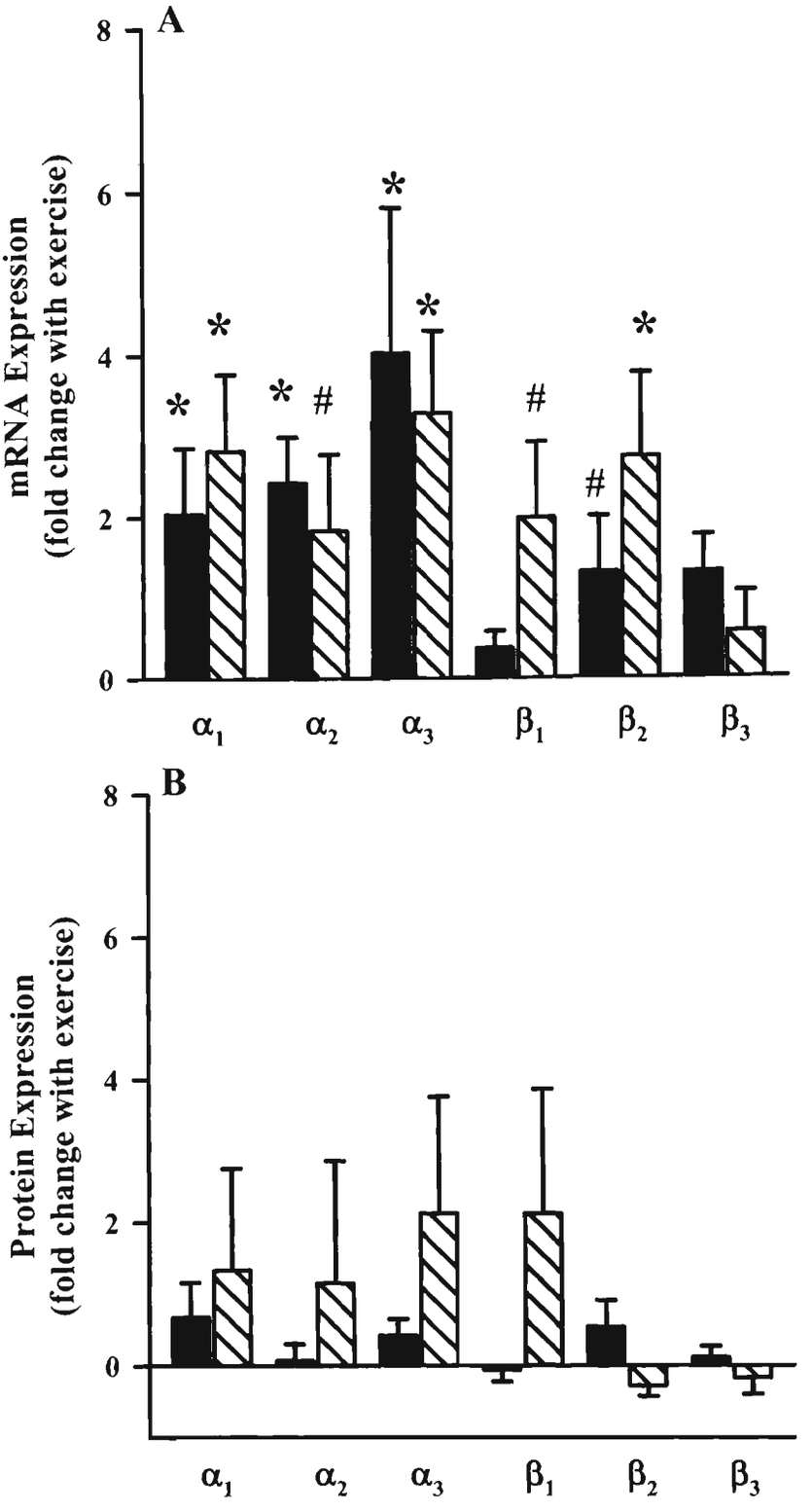


Figure 5.1 High-intensity exercise-induced change in Na^+,K^+-ATPase α_1, α_2, α_3, β_1, β_2 and β_3 mRNA (A) and protein expression (B) conducted before (Pre-HIT, filled bars) and after (Post-HIT, hatched bars) 3 weeks of HIT. All results were normalised to their respective resting value (1.00 ± 0.00). Data are mean ± SEM; n = 12 for mRNA, n = 11 Pre-HIT and n = 5 post-HIT protein. * $P < 0.05$ greater than resting value, # $P < 0.09$ greater than resting value.

5.3.3 Effect of training on exercise performance and on muscle Na^+,K^+-ATPase isoform mRNA and protein expression

5.3.3.1 Exercise performance

Seven sessions of HIT significantly increased PPO by 2.7 ± 0.1% (Pre-HIT, 369 ± 9; Post-HIT, 379 ± 14 W, $P = 0.042$), but had no significant effect on $\dot{V}O_{2peak}$ in these well-trained athletes (Pre-HIT, 63.8 ± 1.4; Post-HIT, 65.3 ± 1.7 $ml.kg^{-1}.min^{-1}$).

5.3.3.2 Resting muscle isoform mRNA and protein expression

mRNA expression. Three wks of HIT elevated the resting mRNA expression of the α_3 and β_3 isoforms by 4.6- and 2.5-fold, respectively ($P < 0.03$), but had no significant effect on the mRNA expression of the other Na^+,K^+-ATPase isoforms (Figure 5.2A).

Protein expression. There was no effect of HIT on the resting protein abundance of any of the Na^+,K^+-ATPase α_1-α_3 or β_1-β_3 isoforms (Figure 5.2B).

5.3.3.3 High-intensity exercise effects on muscle isoform mRNA and protein expression

mRNA expression. Following 3 wks of HIT, the up-regulatory effect of high-intensity, intermittent exercise on α_1 ($P < 0.02$) and α_3 mRNA expression ($P < 0.01$) was still present (Figure 5.1A), whilst exercise tended to increase α_2 mRNA ($P < 0.08$, α_2 mRNA elevated in 10 of 12 subjects). Exercise also increased β_2 mRNA expression by 2.7-fold ($P < 0.03$), and tended to increase β_1 mRNA expression ($P < 0.06$, β_1 mRNA increased in 10 of 12 subjects), with no significant change in β_3 mRNA expression ($P < 0.29$, Figure 5.1A).

There was no significant difference between Pre-HIT and Post-HIT in the exercise effect on the mRNA expression of any of the α_1 ($P < 0.62$), α_2 ($P < 0.51$), α_3 ($P < 0.75$), β_1 ($P < 0.11$), β_2 ($P < 0.24$) or β_3 isoforms ($P > 0.36$, Figure 5.1A).

Protein expression. HIT had no effect on the high-intensity exercise effect on the protein abundance of any of the α_1 ($P < 0.67$), α_2 ($P < 0.46$), α_3 ($P < 0.32$), β_1 ($P < 0.34$), β_2 ($P < 0.26$) or β_3 isoforms ($P > 0.20$, Figure 5.1B).

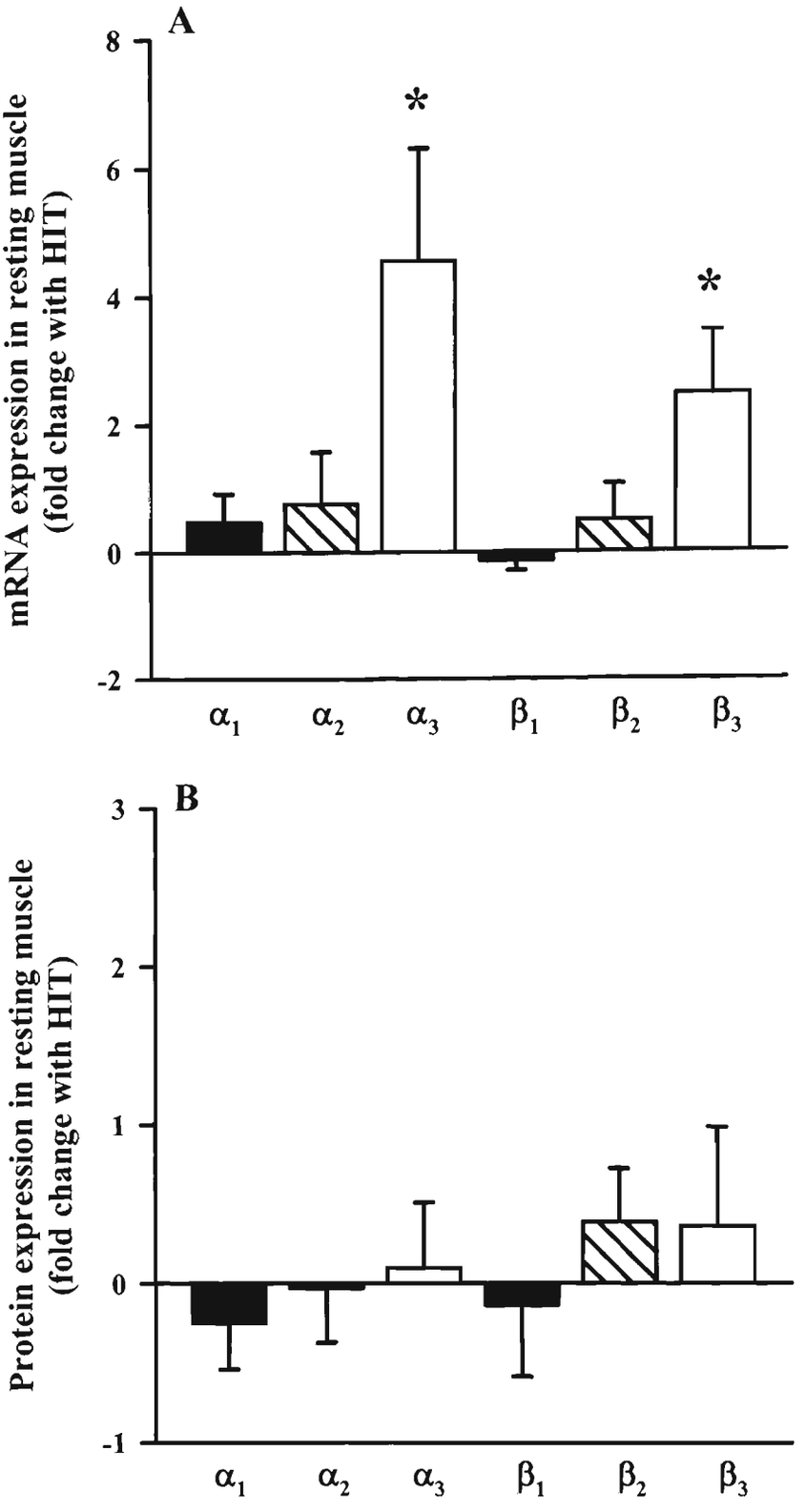


Figure 5.2 Effect of 3 wks of high-intensity intermittent training on resting Na^+,K^+-ATPase α_1, α_2, α_3, β_1, β_2 and β_3 mRNA (A) and protein expression (B). All results were normalised against Pre-HIT resting values and expressed as % change compared to Pre-HIT for that isoform (zero means no change). Data are mean ± SEM; n = 12 for mRNA, n = 5 for protein * $P < 0.03$ greater than Pre-HIT resting value.

5.4 DISCUSSION

The first major finding from this study was that a single bout of high-intensity, intermittent exercise in already well-trained endurance athletes induced isoform-specific Na^+,K^+-ATPase transcriptional regulation, by increasing α_1, α_2 and α_3 mRNA expression Pre-HIT and also β_2 mRNA expression Post-HIT. However, this did not translate into isoform-specific translational regulation, with high-intensity, intermittent exercise having no effect on the protein abundance of any of the α_1, α_2, α_3, β_1, β_2 and β_3 isoforms. The second major finding was that only 3 wks of high-intensity, intermittent training (HIT) was sufficient to elevate resting Na^+,K^+-ATPase α_3 and β_3 mRNA expression, but not isoform protein abundance. The third main finding was that an increase in peak power output with 3 wks of HIT was not associated with any significant improvements in the exercise-induced change in skeletal muscle Na^+,K^+-ATPase isoform mRNA and protein expression in already well-trained endurance athletes.

5.4.1 High-intensity, intermittent exercise induced isoform-specific increases in Na^+,K^+-ATPase mRNA expression

This study investigated for the first time the effects of a single session of high-intensity, intermittent exercise on the mRNA expression of each of six Na^+,K^+-ATPase isoforms present in human skeletal muscle. An immediate post-exercise increase in α_1, α_2 and α_3 mRNA were found Pre-HIT. The β_2 mRNA was also increased Post-HIT and tended to be increased Pre-HIT ($P < 0.09$). Since measurements were only made immediately post-exercise in the present study due to restriction of the number of muscle biopsies, and chapters from this thesis (Chapters 3 and 4) and studies by others (Pilegaard *et al.*, 2000) have shown that a higher post-exercise mRNA response may occur in the recovery period following high-intensity exercise, it is also possible that any other significant increases in mRNA expression may have been missed. These findings contrast the isoform-specific up-

regulation of only α_1 mRNA previously shown following high-intensity, intermittent exercise (Nordsborg *et al.*, 2003a). The reasons for such a discrepancy are unknown, but may involve differences in the total duration of the exercise protocol, as this study utilised intermittent exercise (8 x 5 min) totalling 40 min in duration, whilst Nordsborg *et al.*, (2003a) utilised intermittent exercise (15 x 1 min) totalling 15 min in duration.

The up-regulation of each of the Na^+,K^+-ATPase α genes with high-intensity, intermittent exercise may reflect the catalytic nature of the α isoforms. Indeed, the α isoforms are regulated by $[Na^+]_i$ and $[K^+]_e$ (Jewell & Lingrel, 1991; Crambert *et al.*, 2000), as well as ATP (Jewell & Lingrel, 1991). Both muscle $[Na^+]_i$ and $[K^+]_e$ were increased with fatiguing repeated muscle contractions (Balog & Fitts, 1996; Nordsborg *et al.*, 2003b), while muscle ATP was reduced following fatiguing cycling (Sahlin *et al.*, 1990).

5.4.2. High-intensity, intermittent exercise had no effect on muscle isoform protein abundance

This study demonstrated for the first time that a single bout of high-intensity, intermittent exercise had no immediate effect on the protein abundance of any of the Na^+,K^+-ATPase isoforms present in skeletal muscle of already well-trained athletes. These results contrast the increase in α_1, α_2, α_3 and β_2 mRNA with high-intensity, intermittent exercise, demonstrating an additional instance of differential Na^+,K^+-ATPase transcriptional and translational regulation in human skeletal muscle, consistent with findings in Chapters 3 and 4. When investigating isoform protein abundance for each individual, there was no obvious consistent effect of exercise. Thus, the lack of significant effect of high-intensity, intermittent exercise on isoform protein abundance seems unlikely to reflect a type II error. This lack of high-intensity, intermittent exercise effect on isoform protein abundance was also found following ~6 min of intense exercise (Chapter 3), but contrasts the elevation in α_3 protein abundance found following ~55 min of submaximal exercise (Chapter 4).

Therefore, duration may be important for determining exercise-induced isoform protein responsiveness. Indeed, elevations in muscle Na^+,K^+-ATPase content appears to be dependent on exercise duration as muscle Na^+,K^+-ATPase content was unchanged following up to 75 min of exercise (Chapter 4) (Fowles *et al.*, 2002b; Leppik *et al.*, 2004), but was significantly elevated by 13% following ~10 h of running (Overgaard *et al.*, 2002). Conversely, it remains possible that exercise in fact did not induce differential Na^+,K^+-ATPase transcriptional and translational regulation, since any exercise-induced alterations in protein synthesis and/or degradation may not have been measurable until the recovery period, which was not investigated in the present study. It is also possible that any increases in isoform protein abundance with a single exercise bout were too small to be detected, and that repeated exercise bouts are required to induce detectable increases in isoform protein abundance (Green *et al.*, 2004).

5.4.3 HIT increased resting Na^+,K^+-ATPase α_3 and β_3 mRNA but not isoform protein expression

Three wks of HIT was a sufficient stimulus to significantly elevate the resting mRNA expression of the Na^+,K^+-ATPase α_3 and β_3 isoforms. There was no effect of HIT on resting α_1, α_2, β_1 or β_2 mRNA, supporting the lack of change in α_1, α_2 and β_1 mRNA found previously following 5.5 wks of HIT (Nordsborg *et al.*, 2003a). The increase in α_3 and β_3 mRNA expression with training might reflect transient increases in the mRNA expression of these isoforms with each exercise bout (Williams & Neufer, 1996). On the other hand, the data from Chapters 3 and 4 suggest that mRNA expression of these isoforms returns to levels not significantly different to those at rest by 24 h post-exercise.

The specific up-regulation of α_3 and β_3 mRNA may reflect distinct physiological roles of each isoform. The α_3 isoform has the lowest apparent affinity for $[Na^+]_i$ (Jewell & Lingrel, 1991; Crambert *et al.*, 2000) as well as the highest affinity for ATP (Jewell & Lingrel,

1991), compared to the α_1 and α_2 isoforms. These relative affinities imply specific activation of the α_3 isoform in conditions of elevated $[Na^+]_i$ and reduced ATP, such as that occurring with high-intensity exercise (Jacobs *et al.*, 1982; Sjøgaard *et al.*, 1985). Although isoform-specific properties have not been differentiated for the β isoforms, the β subunit facilitates correct processing, assembly and membrane insertion of the Na^+,K^+-ATPase (Ackermann & Geering, 1990; Noguchi *et al.*, 1990).

In comparison to the increase in α_3 and β_3 mRNA, 3 wks of HIT had no effect on resting protein abundance of any of the α_1-α_3 or β_1-β_3 isoforms. These findings contrast the elevations in resting α_1 and α_2 protein abundance found following 7 wks of HIT in previously habitually active subjects (Nielsen *et al.*, 2004a). Neither the α_3, β_2 or β_3 proteins were measured in that study. These differences might be explained by differences in the duration of the training protocols employed (ie. 3 wks vs. 7 wks). However, this seems unlikely since increases in α_2 and β_1 protein abundance were evident after only 3 and 6 d of prolonged submaximal training, respectively (Green *et al.*, 2004). Conversely, these differences may be explained by variances in the fitness status of the subjects utilised, as this study investigated well-trained endurance athletes, whilst Nielsen *et al.*, (2004a) utilised habitually active subjects. Indeed, at the mRNA level, the exercise-induced mRNA responsiveness of vascular endothelial growth factor (VEGF) was dampened with endurance training (Jensen *et al.*, 2004). Therefore, previous endurance training may have blunted the Na^+,K^+-ATPase isoform protein response to high-intensity, intermittent training.

5.4.4 HIT had no effect on exercise-induced changes in muscle Na^+,K^+-ATPase isoform mRNA and protein expression

There was no significant change in the Na^+,K^+-ATPase isoform mRNA and protein response to high-intensity exercise with HIT. These results contrast the attenuation of the

exercise-induced increase in α_1 mRNA found in a previous study (Nordsborg *et al.*, 2003a). The reasons for this discrepancy are unknown. The effects of short-term training on exercise-induced changes in isoform protein abundance have not been previously investigated. In contrast to the lack of change in isoform protein abundance, HIT was sufficient to modestly increase peak power output in already well-trained athletes. Taken together, these results suggest that the physiological adaptations occurring with HIT that enable an increase in peak power output may not be associated with any measurable changes in Na^+,K^+-ATPase isoform mRNA and protein expression with exercise.

In conclusion, a single bout of high-intensity, intermittent exercise was sufficient to immediately increase the mRNA expression of the α_1, α_2 and α_3 isoforms Pre-HIT, and also of the β_2 isoform Post-HIT, providing evidence for Na^+,K^+-ATPase isoform-specific transcriptional regulation with high-intensity exercise. However, the high-intensity exercise stimulus was insufficient to immediately increase isoform protein abundance. Furthermore, 3 wks of high-intensity, intermittent training increased resting mRNA expression of the α_3 and β_3 isoforms, but had no effect on isoform protein abundance. Finally, short-term high-intensity, intermittent training induced modest improvements in peak power output in already well-trained endurance athletes, but was not associated with any significant effect on the exercise-induced changes in Na^+,K^+-ATPase isoform mRNA and protein expression.

CHAPTER 6

STUDY 4: CHRONIC ENDURANCE TRAINING BUT NOT GENDER MODULATE Na^+,K^+-ATPase mRNA EXPRESSION, MAXIMAL ACTIVITY AND CONTENT IN HUMAN SKELETAL MUSCLE

6.1 INTRODUCTION

Despite considerable research, several fundamental gaps exist in our understanding of the extent to which how chronic training modulates Na^+,K^+-ATPase, and whether gender affects Na^+,K^+-ATPase in human skeletal muscle.

The previous three studies in this thesis showed that each of brief intense (Chapter 3), prolonged submaximal (Chapter 4) and high-intensity, intermittent exercise (Chapter 5) increased Na^+,K^+-ATPase mRNA expression of some isoforms in human vastus lateralis muscle. Furthermore, only 3 wks of high-intensity, intermittent training increased resting α_3 and β_3 mRNA expression in already well-trained endurance athletes (Chapter 5). It is therefore conceivable that long-term repeated bouts of endurance exercise (ie. training) might also result in chronic elevations in resting isoform mRNA expression. Indeed, skeletal muscle from chronically trained athletes demonstrated elevated mRNA expression of genes involved in lipid and glucose metabolism (Andersen *et al.*, 1993; Schmitt *et al.*, 2003). However, the effects of chronic endurance training on mRNA expression of each of the Na^+,K^+-ATPase isoforms are unknown and were therefore investigated here. It was hypothesised that chronic endurance trained athletes would demonstrate higher mRNA expression than recreationally active subjects for each of the six Na^+,K^+-ATPase isoforms.

Human longitudinal training studies have shown an increase in muscle Na^+,K^+-ATPase content, as measured by [^{3}H]-ouabain binding site content, of 14-18% with endurance training ranging in length from 6 d to 5 mo (Green *et al.*, 1993; Madsen *et al.*, 1994;

Evertsen *et al.*, 1997; Green *et al.*, 1999b). Studies sampling muscle at various periods throughout their training regime showed that initial increases in Na^+,K^+-ATPase content with 3 d (Green *et al.*, 2004) and 3 wks (Green *et al.*, 1999a) of training were not significantly augmented with an additional 3 d and 8 wks of training, respectively. Furthermore, in a cross-sectional study, muscle Na^+,K^+-ATPase content was only 18% higher in young endurance trained adults (age, 26.4 ± 3.1 yr) who had been training continuously for at least 2 yrs, than young untrained adults (26.4 ± 3.9 yr) (Fraser *et al.*, 2002). These findings suggest that endurance training may cause an early (~6 d) increase in muscle Na^+,K^+-ATPase content (~14-18%), that is not further augmented with additional training. However, in an early cross-sectional study, muscle Na^+,K^+-ATPase content was 30-32% higher in older endurance trained adults (~69-70 yr) who had been training continuously for the previous 12-17 yrs, than older untrained adults (~68-69 yr) (Klitgaard & Clausen, 1989). Whether the considerably greater increase in muscle Na^+,K^+-ATPase content shown in that study was due to a further increase in Na^+,K^+-ATPase content that occurs with extended training is unknown and was therefore investigated here. The hypothesis tested was that muscle Na^+,K^+-ATPase content in chronically endurance trained athletes would not be significantly correlated with years of training.

Few studies have investigated the effects of endurance training on maximal Na^+,K^+-ATPase activity. One recent study showed that only 6 d of prolonged cycling training increased maximal Na^+,K^+-ATPase activity, as well as Na^+,K^+-ATPase content in human muscle (Green *et al.*, 2004). Whilst cross-sectional studies have shown higher muscle Na^+,K^+-ATPase content in endurance trained athletes (Klitgaard & Clausen, 1989; Fraser *et al.*, 2002), it is not conclusively established whether higher maximal Na^+,K^+-ATPase activity persists in chronically endurance trained athletes compared to untrained subjects (Fraser *et al.*, 2002). Endurance trained athletes demonstrated a tendency (20%) toward a

higher muscle maximal Na^+,K^+-ATPase activity than untrained subjects, with the lack of statistical significance of this result possibly due to the considerable inter-individual variability of results, as well as the small sample size. (Fraser *et al.*, 2002). The third aim of this study was therefore to investigate whether chronic endurance training is associated with increased maximal activity of the Na^+,K^+-ATPase in a large cohort of well-trained endurance athletes. It was hypothesised that chronically endurance trained athletes would demonstrate higher skeletal muscle maximal Na^+,K^+-ATPase activity than recreationally active subjects.

Possible gender effects on muscle Na^+,K^+-ATPase remain unclear, with no study directly investigating the effect of sex hormones on muscle Na^+,K^+-ATPase. A recent study in cattle found no difference between heifers (females) and steers (males) for semitendinosus muscle Na^+,K^+-ATPase content (Veeneklaas *et al.*, 2004). In humans, three studies have contrasted the muscle Na^+,K^+-ATPase content between males and females, but with conflicting findings (Nørgaard, 1986; Evertsen *et al.*, 1997; Green *et al.*, 2001). There was no gender difference found in 25 to 80 yr old subjects (Nørgaard, 1986), or in older (53-61 yr), sedentary subjects (Green *et al.*, 2001). In contrast, in well-trained cross-country skiers, muscle Na^+,K^+-ATPase content was 18% higher in young adult males than females (Evertsen *et al.*, 1997). It is unclear whether these varying findings between studies reflect differences in aerobic fitness of their subjects, as indicated by $\dot{V}O_{2peak}$. Whether a gender difference exists for Na^+,K^+-ATPase isoform mRNA expression and maximal activity remains to be investigated. The fourth aim of this study was therefore to investigate whether any differences exist between genders for Na^+,K^+-ATPase isoform mRNA expression and maximal activity, and to clarify possible differences in muscle Na^+,K^+-ATPase content. The hypothesis was that there would be no gender difference for these variables where no gender difference was also found for $\dot{V}O_{2peak}$.

6.2 METHODS

6.2.1 Subjects

Forty-five healthy, chronic endurance trained males (ETM), eleven healthy, recreationally active males (RAM), and nine healthy, recreationally active females (RAF) gave written informed consent and participated in this study. Subjects were drawn from five separate studies investigating skeletal muscle Na^+,K^+-ATPase and exercise (Chapters 3-5; Aughey *et al.*, 2005; Aughey *et al.*, unpublished observations). As such, many of the measures for muscle [^{3}H]-ouabain binding site content and maximal 3-*O*-MFPase activity were performed by Rob Aughey and by Aaron Petersen. All other measurements of muscle [^{3}H]-ouabain binding site content and maximal 3-*O*-MFPase activity, and all of the mRNA measures were performed by myself. All analyses were performed in the same laboratory with identical procedures for each of Real-Time RT-PCR, [^{3}H]-ouabain binding site content and maximal 3-*O*-MFPase activity assays. Physical characteristics are detailed in Table 6.1. All RAM and RAF participated in regular recreational physical activity, but none were specifically trained in any sport. Each subject performed a maximal incremental exercise test ($\dot{V}O_{2peak}$ test) as previously described (Chapters 3-5; Aughey *et al.*, 2005; Clark *et al.*, 2004). ETM had been training continuously for 7 ± 1 yrs (mean ± SEM) (range, 2-15 yrs; median, 6 yrs), were cycling 514 ± 65 km.wk^{-1}, and had a $\dot{V}O_{2peak}$ greater than 60 ml.kg^{-1}.min^{-1} (Table 6.1). Each subject refrained from vigorous exercise, caffeine, nicotine and alcohol consumption for 24 h prior to the exercise test and muscle biopsy sampling. All protocols and procedures were approved by the Human Research Ethics Committee of Victoria University of Technology and of RMIT University.

6.2.2 Muscle biopsy sampling

At rest, a local anaesthetic (1% Xylocaine) was injected into the skin and subcutaneous tissue above the vastus lateralis muscle. A small incision was then made through the skin

Table 6.1 Group physical characteristics

Group	Description	n	Age (yr)	Height (cm)	Body Mass (kg)	$\dot{V}O_{2pcak}$ ($ml.kg^{-1}.min^{-1}$)
ETM	Endurance trained males	45	27.2 ± 4.8*	178.9 ± 6.6†	72.4 ± 6.7†	67.6 ± 4.9*†
RAM	Recreationally active males	11	23.9 ± 3.9	176.7 ± 6.3‡	74.6 ± 11.2‡	55.7 ± 6.2
RAF	Recreationally active females	9	24.6 ± 6.2	165.5 ± 8.6	60.2 ± 11.2	51.2 ± 6.7

Values are mean ± SD; n, number of subjects. ETM, endurance trained males; RAM, recreationally active males; RAF, recreationally active females. * $P < 0.03$ ETM greater than RAM; † $P < 0.03$ ETM greater than RAF; ‡ $P < 0.01$ RAM greater than RAF.

and fascia, and a muscle sample of approximately 120 mg was excised. The muscle sample was blotted on filter paper to remove blood, then immediately frozen in liquid N_2 and stored at –80°C until assayed later for Na^+,K^+-ATPase mRNA expression, [^{3}H]-ouabain binding site content and maximal 3-*O*-MFPase activity.

6.2.3 Real-Time RT-PCR measurement of mRNA

Muscle was analysed for Na^+,K^+-ATPase α_1, α_2, α_3, β_1, β_2 and β_3 mRNA expression as described in Chapter 3. The ratio of absorbance at 260 and 280 nm (260/280) was 1.98 ± 0.06. Cyclophilin (CYC) mRNA was used as a control to account for any variations in the amount of input RNA and the efficiency of reverse transcription. Gene expression was quantified using a cycle threshold (C_T) method, and a ΔC_T value was calculated by subtracting the CYC C_T from the gene C_T. The relative gene expression was then calculated using the expression, $2^{-\Delta CT}$. The intra-assay coefficient of variation for each target gene was <14.0% for 2^{-C_T} (Table 6.2). Muscle for mRNA analyses were only available for 14 ETM, 5 RAM and 9 RAF.

6.2.4 Muscle [^{3}H]-ouabain binding site content

Muscle Na^+,K^+-ATPase content was determined using vanadate-facilitated [^{3}H]-ouabain binding site content (Chapter 4), with an intra-assay coefficient of variation of 16.3% (n = 58). Due to insufficient sample, muscle [^{3}H]-ouabain binding site content are presented for 41 ETM, 11 RAM and 9 RAF.

6.2.5 Maximal 3-O-MFPase activity

Maximal *in-vitro* 3-*O*-MFPase activity was measured using K^+-stimulated 3-*O*-methylfluorescein phosphatase (3-*O*-MFPase) activity (Chapter 4), with an intra-assay coefficient of variation of 10.5% (n = 58). Due to insufficient sample, muscle maximal 3-*O*-MFPase activity are presented for 43 ETM, 11 RAM and 9 RAF.

Table 6.2 Intra-assay variability of 2^{-C_T} values

Gene	2^{-C_T} CV (%)
α_1	11.1
α_2	11.9
α_3	13.5
β_1	11.2
β_2	11.5
β_3	11.5
CYC	9.6

Each sample was run in triplicate wells in the same Real-Time PCR run. n = 29. CV, coefficient of variation; C_T, cycle threshold.

6.2.6 Correlations

Results for all subjects were pooled to investigate the potential relationship between $\dot{V}O_{2peak}$ and each of Na^+,K^+-ATPase α_1-α_3 and β_1-β_3 mRNA expression (n = 28), muscle [^{3}H]-ouabain binding site content (n = 58), and maximal 3-*O*-MFPase activity (n = 58), as well as between muscle [^{3}H]-ouabain binding site content and maximal 3-*O*-MFPase activity (n = 58). The potential relationship between years of endurance training and each of muscle [^{3}H]-ouabain binding site content (n = 35) and maximal 3-*O*-MFPase activity for ETM (n = 33) was also investigated.

6.2.7 Statistical Analysis

All data are presented as mean ± SEM, except population statistics where mean ± SD are reported. Differences in the muscle analyses between groups was analysed using one-way ANOVA. Post-hoc analyses used the Least Squares Differences test. Correlations were determined by least squares linear regression. Significance was accepted at $P < 0.05$.

6.3 RESULTS

6.3.1 Cyclophilin as an endogenous control for mRNA expression

Cyclophilin (CYC) mRNA expression, when expressed in the linear (2^{-C_T}) form, did not differ ($P < 0.97$) between ETM, RAM and RAF groups (ETM, $1.33 \times 10^{-8} \pm 0.31 \times 10^{-8}$; RAM, $1.45 \times 10^{-8} \pm 0.42 \times 10^{-8}$; RAF, $1.33 \times 10^{-8} \pm 0.31 \times 10^{-8}$ C_T, respectively), thereby validating the use of CYC as an endogenous control for mRNA expression.

6.3.2 Muscle Na^+,K^+-ATPase isoform mRNA expression

6.3.2.1 Chronic endurance trained athletes vs. recreationally active subjects

ETM demonstrated lower mRNA expression of the α_1 (0.7-fold, $P < 0.03$), α_3 (0.6-fold, $P < 0.03$), β_2 (0.7-fold, $P < 0.02$) and β_3 (0.8-fold, $P < 0.01$) isoforms compared to RAM (Figure 6.1). There was also a tendency toward lower α_2 mRNA expression for ETM

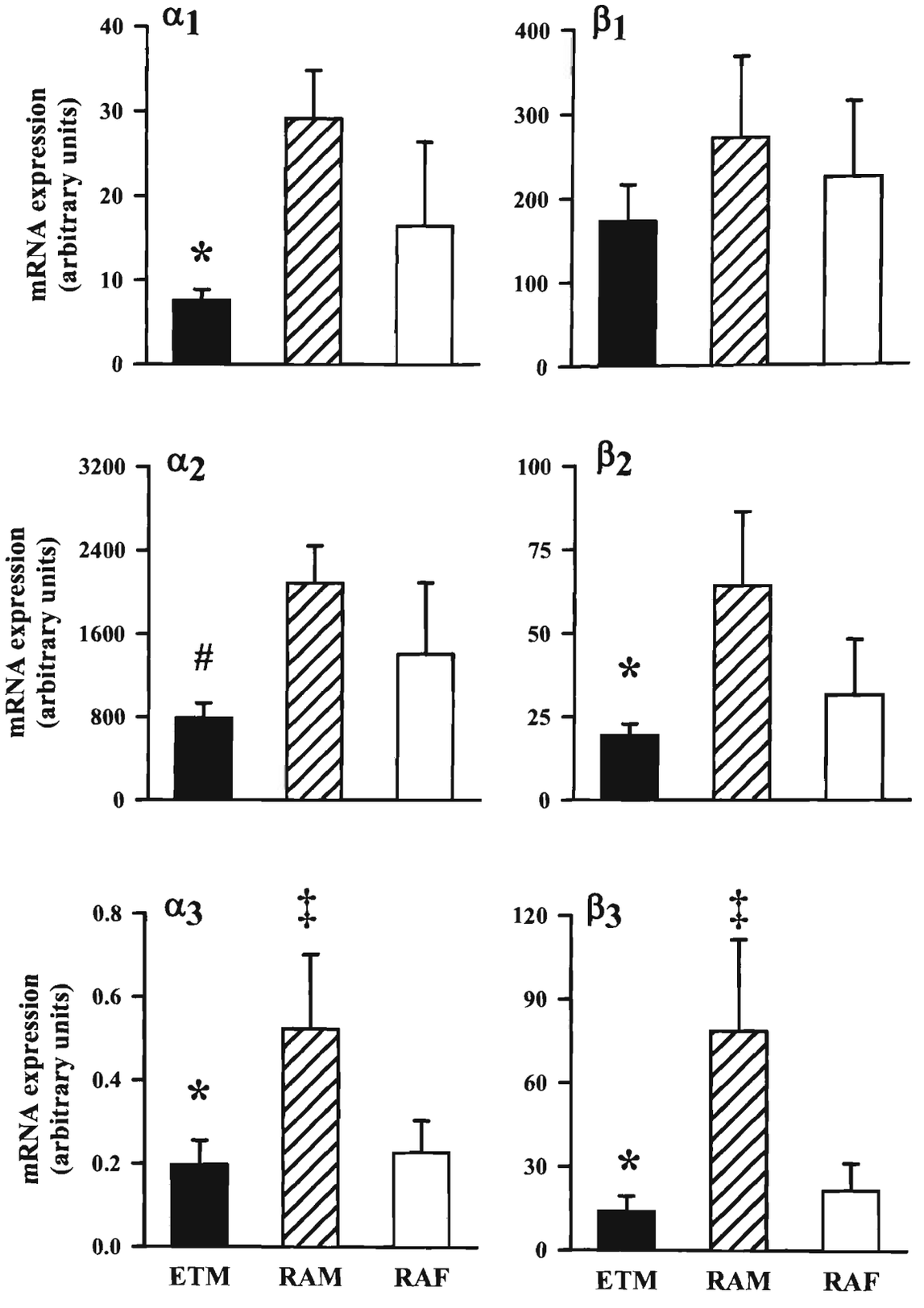


Figure 6.1 Muscle Na^+,K^+-ATPase α_1, α_2, α_3, β_1, β_2 and β_3 mRNA expression in ETM, RAM and RAF. ETM, endurance trained males; RAM, recreationally active males; RAF, recreationally active females. All values are normalised to CYC mRNA. Data are mean ± SEM; n = 14 for ETM, n = 5 for RAM, n = 9 for RAF. * $P < 0.03$ ETM less than RAM; # $P < 0.06$ ETM less than RAM; ‡ $P < 0.05$ RAM greater than RAF.

compared to RAM ($P < 0.06$, Figure 6.1). There was no significant difference between ETM and RAF for the mRNA expression of any other isoform (Figure 6.1).

6.3.2.2 Males vs. females

RAM demonstrated a higher mRNA expression of the α_3 (2.3-fold, $P < 0.05$) and β_3 (3.6-fold, $P < 0.01$) isoforms than RAF (Figure 6.1). There were no difference between RAM and RAF for α_1 ($P < 0.23$), α_2 ($P < 0.35$), β_1 ($P < 0.69$) or β_2 mRNA expression ($P < 0.12$; Figure 6.1).

6.3.2.3 Correlations between Na^+,K^+-ATPase isoform mRNA expression and training status

When the results for all subjects were pooled (n = 28), there were significant negative correlations between $\dot{V}O_{2peak}$ and mRNA expression for each of the α_1 (r = -0.46, $P < 0.01$), α_2 (r = -0.42, $P < 0.03$), α_3 (r = -0.39, $P < 0.04$) and β_2 (r = -0.38, $P < 0.05$) isoforms (Figure 6.2). No significant correlation was found between $\dot{V}O_{2peak}$ and mRNA expression for the β_1 (r = -0.29, $P < 0.15$) and β_3 (r = -0.21, $P < 0.29$ isoforms, data not shown).

6.3.3 Muscle [^{3}H]-ouabain binding site content

6.3.3.1 Chronic endurance trained athletes vs. recreationally active subjects

Muscle [^{3}H]-ouabain binding site content was higher in ETM than in both RAM and RAF, by 16.1% ($P < 0.03$) and 19.0% ($P < 0.01$), respectively (Figure 6.3A).

6.3.3.2 Males vs. females

There was no significant difference between RAM and RAF for muscle [^{3}H]-ouabain binding site content ($P < 0.52$, Figure 6.3A).

6.3.3.3 Correlation between muscle Na^+,K^+-ATPase content and training status

When the results for all subjects were pooled (n = 58), there was a weak correlation between $\dot{V}O_{2peak}$ and muscle [^{3}H]-ouabain binding site content (r = 0.31, $P < 0.01$, Figure 6.4A).

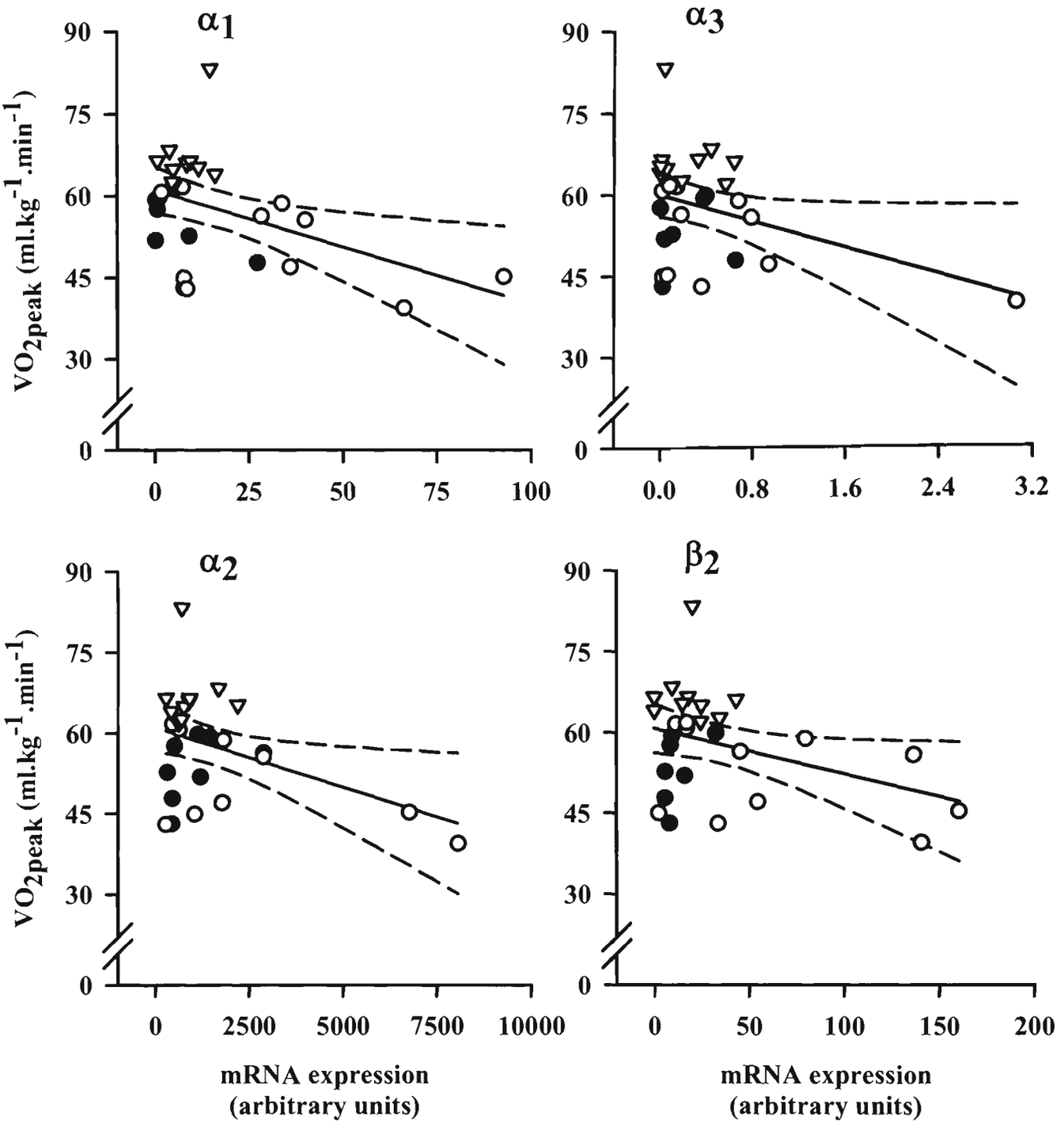


Figure 6.2 Negative relationship between peak O_2 consumption ($\dot{V}O_{2peak}$) and Na^+,K^+-ATPase α_1, α_2, α_3 and β_2 mRNA expression for pooled subjects. ETM (▽), RAM (○) and RAF (●). Solid line represents regression line; dashed lines represent 95% confidence interval. Regression equations; α_1, y = -0.2x + 61.1, r = -0.46, $P < 0.01$; α_2, y = -2.2x + 61.0, r = -0.42, $P < 0.03$; α_3, y = -6.3x + 60.0, r = -0.39, $P < 0.04$; β_2, y = -0.1x + 60.7, r = -0.38, $P < 0.05$, all n = 28.

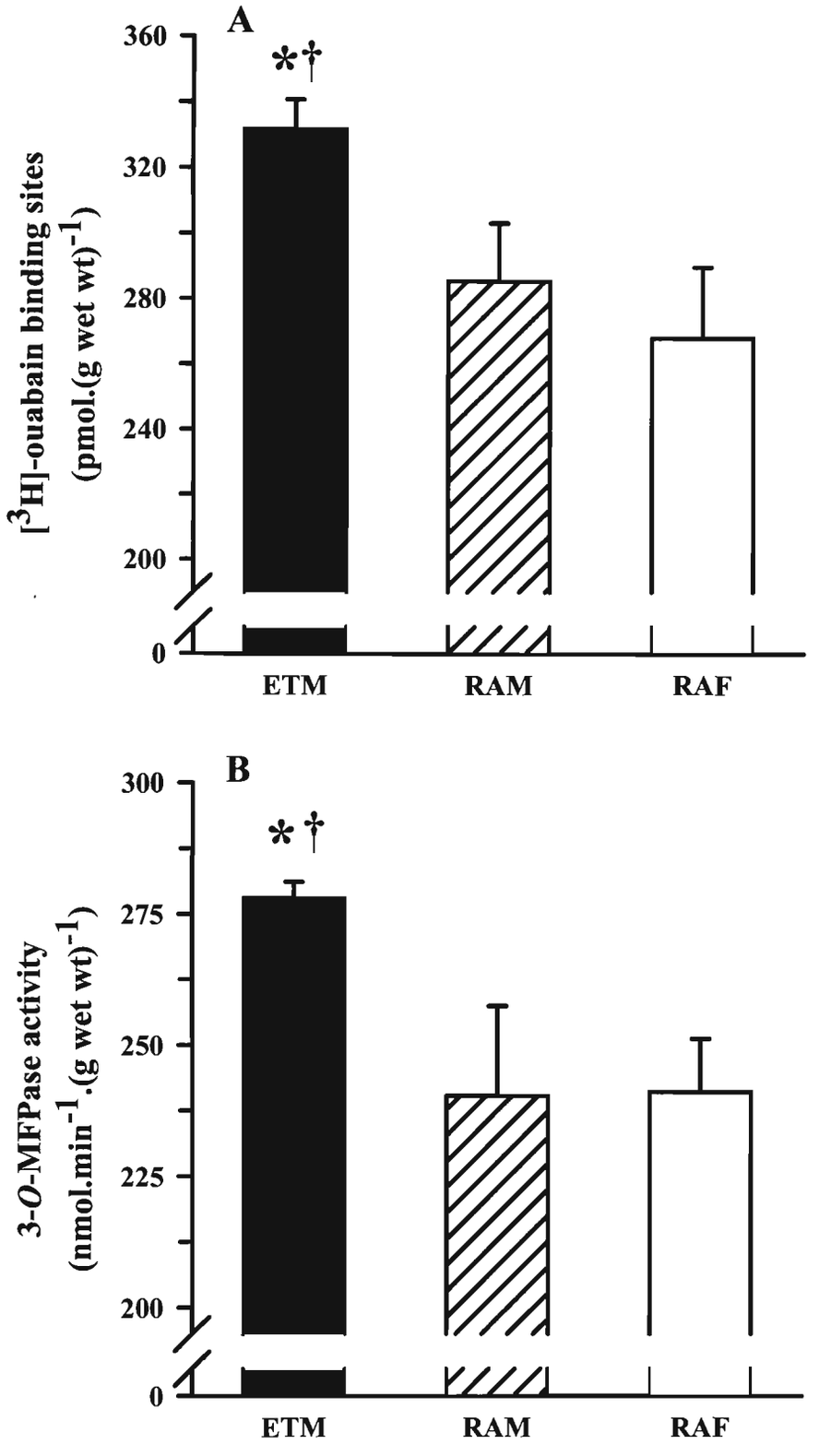


Figure 6.3 Higher muscle [^{3}H]-ouabain binding site content (A) and maximal K^+-stimulated 3-*O*-methylfluorescein phosphatase (3-*O*-MFPase) activity (B) in ETM than in RAM and RAF. ETM, endurance trained males; RAM, recreationally active males; RAF, recreationally active females. Data are mean ± SEM; n = 41 for ETM, n = 11 for RAM, n = 9 for RAF. * $P < 0.03$ ETM greater than RAM; † $P < 0.01$ ETM greater than RAF.

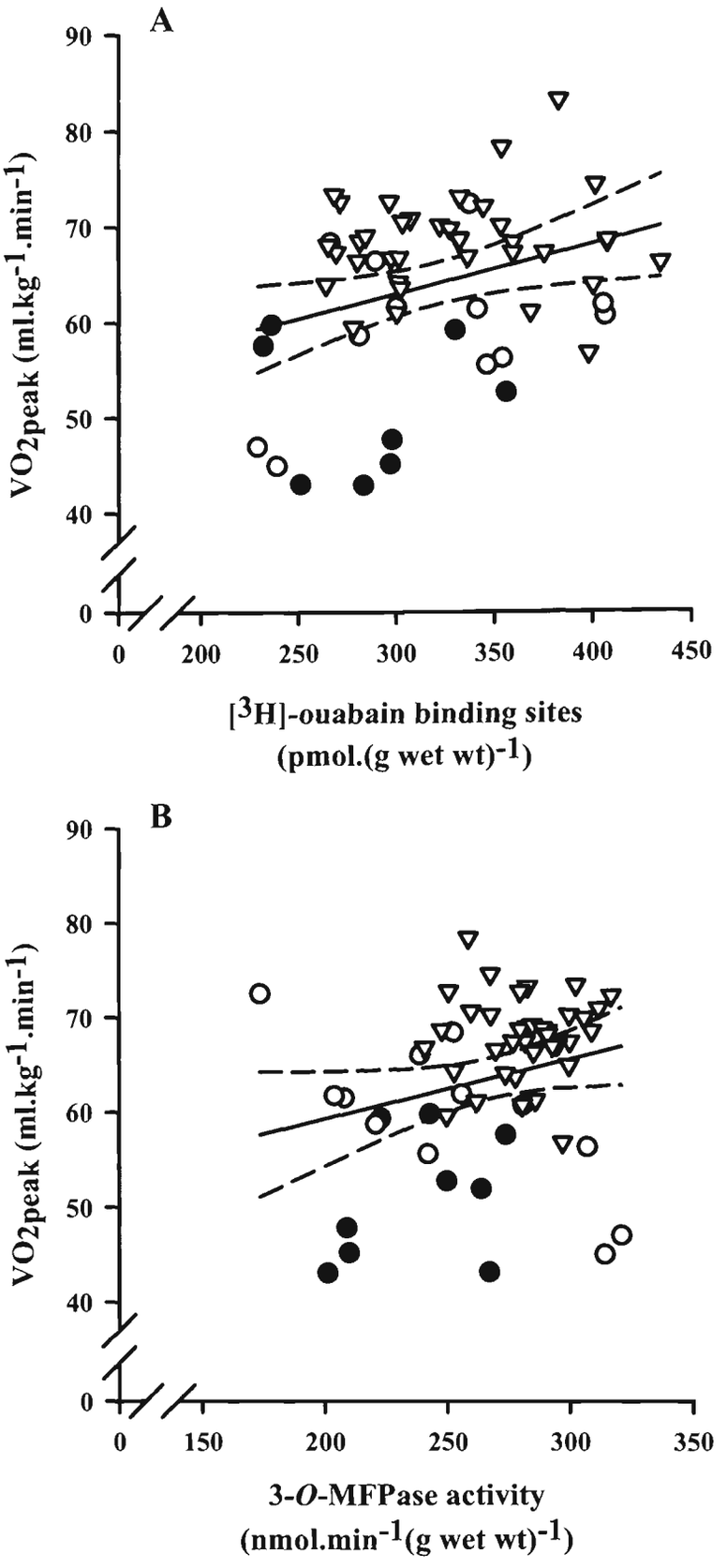


Figure 6.4 Relationship between incremental exercise peak O_2 consumption ($\dot{V}O_{2peak}$) and muscle [^{3}H]-ouabain binding site content (A) and maximal 3-*O*-MFPase activity (B) for pooled subjects. ETM (▽), RAM (○) and RAF (•) subjects. Solid line represents regression line; dashed lines represent 95% confidence interval. Regression equations; A, y = 0.05x + 47.4, r = 0.31, $P < 0.01$; B, y = 0.06x + 46.7, r = 0.25, $P < 0.02$, all n = 58.

6.3.3.4 Correlation between years of training and muscle Na^+,K^+-ATPase content

No significant correlation was found between years of endurance training and muscle [^{3}H]-ouabain binding site content in ETM (r = -0.19, $P < 0.30$, Figure 6.5A).

6.3.4 Muscle maximal 3-*O*-MFPase activity

6.3.4.1 Chronic endurance trained athletes vs. recreationally active subjects

Muscle maximal 3-*O*-MFPase activity was higher in ETM than in both RAM and RAF, by 15.7% ($P < 0.01$) and 15.4%, respectively ($P < 0.01$, Figure 6.3B).

6.3.4.2 Males vs. females

There was no significant difference between RAM and RAF for maximal 3-*O*-MFPase activity ($P < 0.97$ Figure 6.3B).

6.3.4.3 Correlation between maximal Na^+,K^+-ATPase activity and training status

When the results for all subjects (n = 58) were pooled, there was a weak correlation between $\dot{V}O_{2peak}$ and maximal 3-*O*-MFPase activity (r = 0.25, $P < 0.02$, Figure 6.4B).

6.3.4.4 Correlation between years of training and maximal Na^+,K^+-ATPase activity

No significant correlation was found between years of endurance training and maximal 3-*O*-MFPase activity in ETM (r = -0.17, $P < 0.36$, Figure 6.5B).

6.3.4.5 Correlation between muscle Na^+,K^+-ATPase content and maximal activity

When the results for all subjects were pooled (n = 58), no correlation was found between muscle [^{3}H]-ouabain binding site content and maximal 3-*O*-MFPase activity (y = 0.10x + 284.17, r = 0.06, $P < 0.67$).

6.3.5 Anthropometric measurements and Na^+,K^+-ATPase characteristics

No significant correlations were observed between age, height or body mass against any of muscle maximal 3-*O*-MFPase activity, [^{3}H]-ouabain binding site content or relative mRNA expression of the Na^+,K^+-ATPase α_1, α_2, α_3, β_1, β_2 or β_3 isoforms (data not shown, all $P >$ 0.21).

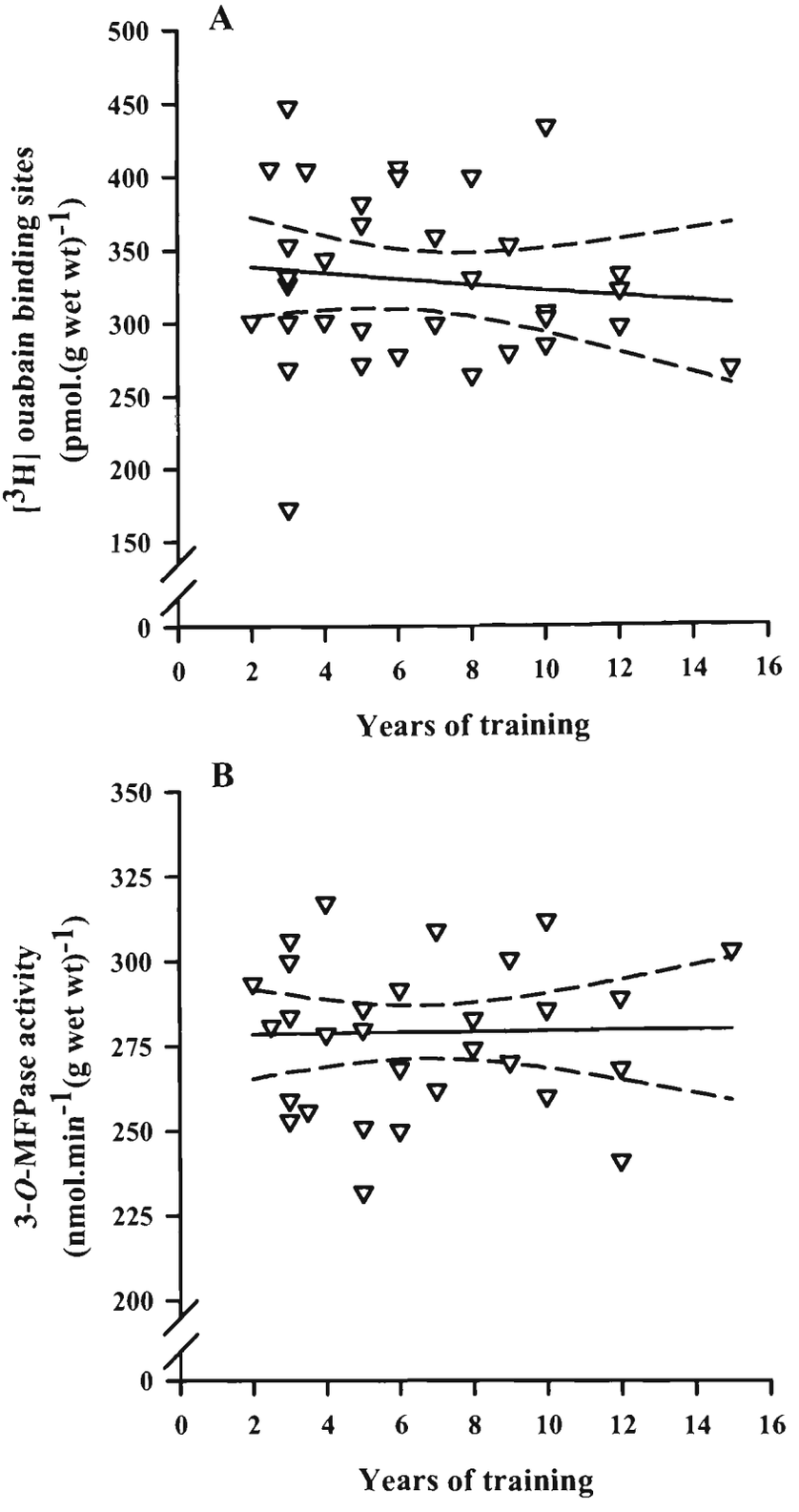


Figure 6.5 No relationship between years of endurance training and muscle [^{3}H]-ouabain binding site content (A) and maximal 3-*O*-MFPase activity (B) for ETM. Solid line represents regression line; dashed lines represent 95% confidence interval. Regression equation; A, y = -2.1x + 342.8, r = -0.19, $P < 0.30$; B, y = 0.1x + 278.1, r = -0.17, $P < 0.36$, n = 35 for A, n = 33 for B.

6.4 DISCUSSION

This study demonstrates a striking contrast between the strong effect in chronically endurance trained males (ETM) towards reduced muscle Na^+,K^+-ATPase α_1, α_3, β_2 and β_3 mRNA expression, but elevated muscle Na^+,K^+-ATPase content and maximal activity compared to recreationally active males (RAM). There was also no correlation between the number of years of endurance training and either muscle Na^+,K^+-ATPase content or maximal activity in ETM. An interesting gender difference was also found in that RAM demonstrated higher α_3 and β_3 mRNA expression, but no difference for either muscle Na^+,K^+-ATPase content or maximal activity than recreationally active females (RAF).

6.4.1 Chronic endurance trained athletes demonstrate lower muscle Na^+,K^+-ATPase mRNA expression

A surprising finding was the lower mRNA expression of the Na^+,K^+-ATPase α_1, α_2 ($P < 0.06$), α_3, β_2 and β_3 isoforms in skeletal muscle of well-trained male athletes, compared to recreationally active males. These findings were supported by the significant inverse correlation found between incremental exercise $\dot{V}O_{2peak}$ and each of Na^+,K^+-ATPase α_1, α_2, α_3 and β_2 mRNA expression. However, the relationship between $\dot{V}O_{2peak}$ and each of Na^+,K^+-ATPase α_1, α_2, α_3 and β_2 mRNA expression is likely to have been influenced by the outlying results of three subjects.

The lower mRNA expression of Na^+,K^+-ATPase isoforms in ETM is in sharp contrast to the elevation in mRNA expression following acute exercise. Each of the α_1-α_3 and β_1-β_3 isoforms were increased following brief intense exercise (Chapter 3), α_1, α_3 and β_2 mRNA increased following prolonged submaximal exercise (Chapter 4), and α_1, α_2, α_3 and β_2 (Post-HIT) mRNA were increased following 40 min of high-intensity, intermittent exercise (Chapter 5). The lower isoform mRNA expression in ETM also contrasts the increase in

resting α_3 and β_3 mRNA expression following 3 wks of high-intensity, intermittent training (Chapter 5). Thus mRNA expression of numerous Na^+,K^+-ATPase isoforms are elevated with acute exercise and short-term training, but appears to be chronically depressed with chronic endurance training. This differential transcriptional regulation by acute and chronic exercise has also been observed for numerous metabolic genes (Pilegaard *et al.*, 2000; Tunstall *et al.*, 2002; Nordsborg *et al.*, 2003a).

It was not possible to compare isoform protein abundance between ETM and RAM due to the inter-assay variability found with immunoblotting. Therefore considerable error would have been involved in comparing absolute isoform protein expression between assays. It is unclear which isoforms are increased to account for the elevation in muscle Na^+,K^+-ATPase content with endurance training. However, Green *et al.*, (2004) recently found an increase in α_2 and β_1 protein abundance following only 6 d of endurance training in previously untrained subjects.

The lack of difference in β_1 mRNA between ETM and RAM was consistent with the lack of significant correlation found between β_1 mRNA expression and incremental exercise $\dot{V}O_{2peak}$. These findings suggest that this gene transcript may not necessarily be regulated by the same stimuli as the other Na^+,K^+-ATPase gene transcripts. Indeed, it was proposed in Chapter 4 that different stimuli may regulate each of the Na^+,K^+-ATPase gene transcripts, since acute exercise induced isoform-specific transcriptional regulation (Chapters 3-5), and since brief intense, prolonged submaximal and high-intensity, intermittent exercise induced different effects on isoform mRNA expression (Chapters 3-5). While such stimuli remain unknown, increased $[Na^+]_i$ (Taormino & Fambrough, 1990; Rayson, 1993), $[Ca^{2+}]_i$ (Rayson, 1991; Rayson, 1993), ROS (Xie *et al.*, 1999) and altered $[K^+]_e$ (Bowen & McDonough, 1987; Pressley *et al.*, 1988) have each been previously implicated in inducing Na^+,K^+-ATPase gene transcription. Little is known about the effect

of endurance training on resting muscle $[Na^+]_i$, $[Ca^{2+}]_i$, ROS and $[K^+]_e$. Endurance training reduced resting muscle intracellular Na^+ content in rats in one study (Schleusing & Noecker, 1960), but not in another (Kjeldsen *et al.*, 1986b), nor had a significant effect on resting muscle intracellular Na^+ content in dogs (Knochel *et al.*, 1985). In contrast, only 5 d of endurance training was sufficient to increase diaphragm antioxidant capacity in rats (Vincent *et al.*, 2000), which might suggest increased ROS scavenging after training. The effects of endurance training on $[Ca^{2+}]_i$ and $[K^+]_e$ are not known. Thus further research is required to determine intracellular signals responsible for the lower isoform mRNA expression in ETM.

6.4.2 Chronic endurance trained athletes demonstrate higher skeletal muscle Na^+,K^+-ATPase content and maximal activity

In contrast to their lower Na^+,K^+-ATPase isoform mRNA expression, ETM also demonstrated a 16% higher Na^+,K^+-ATPase content and maximal activity compared to RAM. This difference in maximal activity is similar to a previous study where a non-significant (20%) higher maximal Na^+,K^+-ATPase activity was found in eight ET athletes compared to eight untrained (UT) subjects (Fraser *et al.*, 2002). The greater sample size in the present study enabled the detection of a statistically significant difference in maximal 3-*O*-MFPase activity between ETM and RAM groups. This result is also consistent with findings of increased maximal Na^+,K^+-ATPase activity after only 6 d of prolonged cycling training (Green *et al.*, 2004). Therefore, maximal Na^+,K^+-ATPase activity is elevated in the skeletal muscle of humans that have performed both short- and long-term training.

In Chapter 4, a single bout of fatiguing exercise induced an immediate depression in maximal Na^+,K^+-ATPase activity in skeletal muscle from untrained subjects, that was recovered by 3 and 24 h post-exercise. This depression in maximal Na^+,K^+-ATPase activity following fatiguing exercise does not appear to reflect the training status of

subjects, since a single bout of incremental cycling was recently shown to depress maximal Na^+,K^+-ATPase activity in the skeletal muscle of well-trained endurance athletes (Fraser *et al.*, 2002; Aughey *et al.*, 2005). Thus, the finding of an increased maximal Na^+,K^+-ATPase activity in the skeletal muscle of ETM in the present study does not appear to reflect inhibition of the depressing effects of acute exercise for maximal Na^+,K^+-ATPase activity, but rather the increase in muscle Na^+,K^+-ATPase content. Thus, a greater number of functional Na^+,K^+-ATPase enzymes in the skeletal muscle of ETM enhances the capacity for Na^+,K^+-ATPase activity. Indeed, muscle Na^+,K^+-ATPase content and maximal Na^+,K^+-ATPase activity were higher by the same percentage (16%) in ETM than RAM.

The lower isoform mRNA expression, but higher muscle Na^+,K^+-ATPase content, in skeletal muscle of ETM compared to RAM subjects, provokes speculation regarding the mechanisms responsible for this surprising finding. Since isoform mRNA expression is reduced, the increase in the number of functional Na^+,K^+-ATPase enzymes suggests an improvement in translational efficiency and/or a reduction in degradation of the enzyme in the skeletal muscle of ETM. On the other hand, these differences may not necessarily reflect changes induced with chronic endurance training. It is possible that these differences may simply reflect inherent genetic factors that predispose ETM for endurance exercise and training.

6.4.3 Relationship between muscle Na^+,K^+-ATPase and peak O_2 consumption

A weak positive correlation was found between $\dot{V}O_{2peak}$ and both muscle Na^+,K^+-ATPase content and maximal activity, supporting previous studies in which smaller sample sizes were used (Evertsen *et al.*, 1997; Fraser *et al.*, 2002). The weak nature of these correlations is not surprising given that a multiplicity of factors contribute to determining $\dot{V}O_{2peak}$, including cardiac output and arterial-venous O_2 difference, the latter of which is influenced by muscle performance. Muscle performance is determined by numerous factors

(Westerblad *et al.*, 1991), including membrane excitability. Muscle Na^+,K^+-ATPase activity is therefore only one of several factors affecting the development of muscle fatigue and hence performance. Thus whilst the findings from the present study confirm a relationship between muscle Na^+,K^+-ATPase content, maximal activity and aerobic power, it is clear that Na^+,K^+-ATPase content and maximal activity is only a small component of the multifactorial processes determining $\dot{V}O_{2peak}$.

6.4.4 No relationship between years of endurance training and muscle Na^+,K^+-ATPase

ETM demonstrated higher muscle Na^+,K^+-ATPase content than RAM, but since no correlation was found between the years of training and either Na^+,K^+-ATPase content or maximal activity, this likely reflects an initial increase in muscle Na^+,K^+-ATPase content within days to months of training (Green *et al.*, 1993; McKenna *et al.*, 1993; Madsen *et al.*, 1994; Evertsen *et al.*, 1997; Green *et al.*, 1999b). Thus, the up-regulatory response of endurance training for muscle Na^+,K^+-ATPase content was not dependent on the length of endurance training performed. This finding also suggests strongly that the up-regulatory response of training for muscle Na^+,K^+-ATPase content is constrained to within a narrow range (~14-18%) in human muscle (Green *et al.*, 1993; Madsen *et al.*, 1994; Evertsen *et al.*, 1997; Green *et al.*, 1999b). In contrast, one cross-sectional study showed a 30-32% higher muscle Na^+,K^+-ATPase content in older ET adults (~69-70 yr), that had been training for the previous 12-17 yrs, compared to older UT (~68-69 yr) (Klitgaard & Clausen, 1989). The reasons for the considerably larger Na^+,K^+-ATPase up-regulation evident in that study are unknown.

The suggestion of a constraint on the magnitude of the up-regulatory response of training for muscle Na^+,K^+-ATPase content, to within ~14-18%, in human muscle contrasts sharply with the much larger increases in Na^+,K^+-ATPase content (25-46%) observed with training

in rat skeletal muscle (Kjeldsen *et al.*, 1986b; Kjeldsen *et al.*, 1988). Furthermore, other skeletal muscle enzymes, such as citrate synthase, a marker of oxidative potential, may be up-regulated by ~55-75% with training (Kjeldsen *et al.*, 1986b; Jansson & Kaijser, 1987). This contrast points to an important regulatory significance for the modest increases in Na^+,K^+-ATPase content and maximal activity in human muscle with endurance training. This may involve the maintenance of plasma $[K^+]$ to within a small range, since too large an up-regulation of Na^+,K^+-ATPase with training could result in severe post-exercise hypokalemia, whereas insufficient Na^+,K^+-ATPase up-regulation with training could lead to more pronounced exercise-induced hyperkalemia. Both conditions could induce cardiac arrhythmias or cardiac arrest (Lindinger, 1995; Paterson, 1996), while severe hyperkalemia has been associated with an accelerated development of muscle fatigue (Lindinger & Heigenhauser, 1988; Renaud & Light, 1992). Thus, the modest increase in Na^+,K^+-ATPase content with chronic endurance training may act as an important protective mechanism for K^+ regulation. Furthermore, there is accumulating evidence in human training studies that such relatively modest changes in Na^+,K^+-ATPase content are physiologically important. Seven wks of sprint training increased muscle Na^+,K^+-ATPase content by 13%, which was also associated with a 19% reduction in the exercise-induced rise in plasma $[K^+]$ and a 27% reduction in the ratio between the rise in plasma $[K^+]$ and work output (McKenna *et al.*, 1993). Furthermore, 6 d of prolonged cycling increased muscle Na^+,K^+-ATPase content by 14%, and reduced plasma $[K^+]$ during exercise (Green *et al.*, 1993).

6.4.5 No relationship between muscle Na^+,K^+-ATPase content and maximal *in-vitro* activity

A very surprising finding was the lack of correlation found between muscle $[^3H]$-ouabain binding site content and maximal 3-*O*-MFPase activity. Thus, the number of functional Na^+,K^+-ATPase enzymes does not appear to dictate the capacity of the *in-vitro* Na^+,K^+-

ATPase activity. This finding contrasts the modest, but significant, correlation previously found for [^{3}H]-ouabain binding site content and maximal 3-*O*-MFPase activity in human muscle (Fowles *et al.*, 2002b; Fraser *et al.*, 2002). The reasons for this discrepancy are unknown. The lack of significant correlation between these variables is difficult to interpret but may suggest an inherent problem with the maximal *in-vitro* 3-*O*-MFPase activity assay. It is not possible to state that phosphatase activity measured enzymatically directly reflects complete functional pump activity. Nonetheless, the 3-*O*-MFPase assay is specific for Na^+,K^+-ATPase, as shown by ouabain inhibition (Nørgaard *et al.*, 1984b; Fraser & McKenna, 1998). This finding may also suggest that the 3-*O*-MFPase or [^{3}H]-ouabain binding assays, especially the 3-*O*-MFPase assay, is too variable. Conversely, a physiological reason may exist to explain the lack of significant correlation between these variables, however, this is unknown and requires further investigation.

6.4.6 Gender influences Na^+,K^+-ATPase mRNA expression, but not content or maximal activity

An interesting and novel finding was that recreationally active males demonstrated higher muscle Na^+,K^+-ATPase α_3 and β_3 mRNA expression than recreationally active females, whereas there was no difference in the mRNA expression of any of the α_1, α_2, β_1 or β_2 isoforms between genders. The importance of the differing α_3 and β_3 mRNA expression between genders is unknown, but suggests that the transcriptional regulation of these isoforms may be affected by sex hormones.

No gender difference was also found for muscle Na^+,K^+-ATPase content, supporting two previous studies, which compared eleven female and nine male subjects aged 25 to 80 yrs (Nørgaard, 1986), and nine female and ten male sedentary subjects aged 53 to 61 yrs (Green *et al.*, 2001). This finding contrasts the 18% higher Na^+,K^+-ATPase content reported in male compared to female well trained cross-country skiers (Evertsen *et al.*,

1997). However, the higher muscle Na^+,K^+-ATPase content found for the males in that study may simply reflect the 26% higher $\dot{V}O_{2peak}$ compared to females (Evertsen *et al.*, 1997), since Na^+,K^+-ATPase content was positively correlated to $\dot{V}O_{2peak}$ in this and two other studies (Evertsen *et al.*, 1997; Fraser *et al.*, 2002). Where no gender difference for $\dot{V}O_{2peak}$ was found in this (8.8%, NS) and another study (3.6%, NS) (Green *et al.*, 2001), no gender difference was also found for Na^+,K^+-ATPase content. Furthermore, a recent study in cattle found no significant difference between heifers (females) and steers (males) for semitendinosus muscle [^{3}H]-ouabain binding site content (Veeneklaas *et al.*, 2004). Thus our results do not support a gender difference for Na^+,K^+-ATPase content in human muscle.

Maximal Na^+,K^+-ATPase activity also did not differ between genders. No study had previously contrasted maximal Na^+,K^+-ATPase activity between genders. Whilst there are studies measuring maximal Na^+,K^+-ATPase activity in different genders (Nørgaard *et al.*, 1984b; Fowles *et al.*, 2004), these used different assay conditions and consequently, their results cannot be compared.

In conclusion, chronic endurance trained athletes demonstrated lower muscle Na^+,K^+-ATPase α_1, α_3, β_2 and β_3 mRNA expression, than recreationally active subjects. However, in a striking comparison, endurance trained athletes also demonstrated elevated Na^+,K^+-ATPase content and maximal activity. This higher Na^+,K^+-ATPase content and maximal activity appears to be independent of the years of training performed. This suggests that the skeletal muscle adaptations occurring with chronic endurance training may involve pre- and post-translational regulation of the Na^+,K^+-ATPase. Males also demonstrated higher Na^+,K^+-ATPase α_3 and β_3 mRNA expression compared to females, but there was no

difference between genders for Na^+,K^+-ATPase α_1, α_2, β_1 or β_2 mRNA expression, maximal activity or content in human skeletal muscle.

CHAPTER 7

STUDY 5: MUSCLE FIBRE-TYPE, ELECTRICAL STIMULATION AND INTRACELLULAR EFFECTS ON Na^+,K^+-ATPase mRNA EXPRESSION IN RAT SKELETAL MUSCLE

7.1 INTRODUCTION

Findings from the preceding chapters have shown that brief intense exercise increased the mRNA expression of all six Na^+,K^+-ATPase isoforms (Chapter 3), prolonged submaximal exercise increased the mRNA expression of three Na^+,K^+-ATPase isoforms (α_1, α_3, β_2; Chapter 4), while high-intensity, intermittent exercise increased the mRNA expression of four Na^+,K^+-ATPase isoforms in human skeletal muscle (α_1, α_2, α_3, β_2 (Post-HIT); Chapter 5). Furthermore, chronic endurance trained athletes demonstrated lower mRNA expression of four Na^+,K^+-ATPase isoforms than recreationally active subjects (α_1, α_3, β_2, β_3; Chapter 6). The factors underlying these effects remain largely unknown. Specifically, the effect of differences in muscle fibre-type composition, and the intracellular signals involved in Na^+,K^+-ATPase isoform transcription in skeletal muscle are largely unknown. Possible differences between muscles of varying fibre-type composition in the relative mRNA expression of each of the Na^+,K^+-ATPase isoforms remain inconclusive. In rats, α_1 mRNA expression was similar in the soleus and EDL muscles, whereas α_2 and β_1 mRNA were 4.0- and 3.0-fold more abundant in the soleus than the EDL, respectively, whilst β_2 mRNA was 8.0-fold more abundant in the EDL (Hundal *et al.*, 1993). The accuracy of these differences between muscles is uncertain since the mRNA analyses were performed using Northern Blotting, which is less sensitive than Real-Time RT-PCR (Bustin, 2000). Furthermore, whether differences in α_3 and β_3 mRNA expression exist between muscles of different fibre-type composition are unknown. The first aim of this study was therefore to

clarify the relative expression of each of the Na^+,K^+-ATPase isoform gene transcripts between muscles of differing fibre-type composition, measured by Real-Time RT-PCR. Based on the findings by Hundal *et al.*, (1993), it was hypothesised that the relative expression of the α_2 and β_1 gene transcripts would be greater in the soleus than the EDL, while in contrast β_2 mRNA expression would be greater in the EDL than the soleus. There would be no difference between muscles in α_1, α_3 or β_3 mRNA expression.

Acute exercise has been shown to elevate mRNA expression of each Na^+,K^+-ATPase isoform in both human (Chapters 3, 4 and 5, and Nordsborg *et al.*, 2003) and rat muscle (Tsakiridis *et al.*, 1996). Whether electrical stimulation exerts similar effects is unknown. In this thesis chapter, it was therefore hypothesised that three bouts of high-frequency electrical stimulation of isolated rat soleus and EDL muscle would increase the mRNA expression of each of the Na^+,K^+-ATPase α_1, α_2, α_3, β_1, β_2 and β_3 isoforms.

The intracellular signals involved in Na^+,K^+-ATPase transcriptional regulation in skeletal muscle are unclear. Since acute exercise increases Na^+,K^+-ATPase isoform mRNA expression in mammalian muscle (Chapters 3, 4, 5 and Nordsborg *et al.*, 2003, Tsakiridis *et al.*, 1996), it is likely that one or several of the transmembrane ionic fluxes and subsequent intracellular ionic concentration changes that occur with exercise may be involved in signalling pathways inducing isoform mRNA transcription. Furthermore, since both acute and chronic exercise were found to induce isoform-specific transcriptional regulation (Chapters 4, 5 and 6), it seems highly probable that different intracellular signals regulate different Na^+,K^+-ATPase isoforms.

Repeated muscle contractions induce elevations in $[Na^+]_i$ in human (Sjøgaard *et al.*, 1985) and in rat muscle (Lindinger & Heigenhauser, 1988). This elevation is transient, due to activation of the Na^+,K^+-ATPase, induced in part via the elevation in $[Na^+]_i$ (Everts & Clausen, 1992; Nielsen & Clausen, 1997). However, it is possible that the transient rise in

$[Na^+]_i$ with repeated muscle contractions is involved in increasing Na^+,K^+-ATPase isoform mRNA expression in skeletal muscle. Two interventions have been used to induce increases in $[Na^+]_i$ for investigation of possible elevations in Na^+,K^+-ATPase mRNA expression in cultured rat kidney (Rayson, 1993) or chick skeletal muscle cells (Taormino & Fambrough, 1990). These include ouabain, an inhibitor of the Na^+,K^+-ATPase (Clausen & Hansen, 1974) and veratridine, an activator of the voltage-gated Na^+-channels (Sutro, 1986). Ouabain inhibited Na^+,K^+-ATPase activity (Clausen & Everts, 1991) and increased intracellular Na^+ content by 194% in isolated rat soleus muscle (Gissel & Clausen, 1999), and also induced an ~2.0-fold increase in each of Na^+,K^+-ATPase α_1 and β_1 mRNA expression in rat kidney cells (Rayson, 1993). Interestingly, the effect of ouabain on Na^+,K^+-ATPase mRNA expression may be tissue- and/or isoform-specific, since in rat cardiac myocytes, α_3 mRNA expression was reduced, but β_1 mRNA expression was increased (Huang *et al.*, 1997; Xie *et al.*, 1999; Kometiani *et al.*, 2000). Veratridine increased ouabain-suppressible K^+ uptake (Everts & Clausen, 1992) and elevated Na^+ content by 21% in isolated rat EDL muscle (Gissel, H. & Clausen, T. personal communication), and also increased non-isoform specific α and β mRNA expression by 1.7- and 2.5-fold, respectively, in chick skeletal muscle cells (Taormino & Fambrough, 1990). Monensin, a specific Na^+-ionophore (Pressman & Fahim, 1982) has also been used to elevate the Na^+/K^+ ratio in rat skeletal muscle (Everts & Clausen, 1992), but the effects of monensin on Na^+,K^+-ATPase mRNA expression are unknown. It was therefore hypothesised that elevated $[Na^+]_i$, induced by ouabain, veratridine and monensin would increase Na^+,K^+-ATPase α_1, α_2, α_3, β_1, β_2 and β_3 mRNA expression in rat skeletal muscle.

Baseline $[Ca^{2+}]_{cyto}$ increased during repeated muscle contractions in isolated mammalian muscle (Westerblad *et al.*, 1993; Chin & Allen, 1996; Lunde *et al.*, 2001). An increase in $[Ca^{2+}]_{cyto}$ is also known to activate differential signalling pathways, including

phosphorylase kinase, protein kinase C, protein kinase A and protein phosphatase 2B activity (Williams & Neufer, 1996). Thus an elevation in $[Ca^{2+}]_{cyto}$ has been shown to alter the expression of several genes, including the gene encoding for the nicotinic acetylcholine receptor in rat soleus muscle (Walke *et al.*, 1994). In rat kidney cells, high $[Ca^{2+}]_i$ (1.0 μM) induced a 4.0-fold increase in both Na^+,K^+-ATPase α_1 and β_1 mRNA expression, with the α_1 and β_1 mRNA degradation rates related to the $[Ca^{2+}]_i$ (Rayson, 1991). Furthermore, the magnitude of the ouabain-induced increases in Na^+,K^+-ATPase α_1 and β_1 mRNA expression in cultured rat kidney cells depended on the $[Ca^{2+}]_i$ (Rayson, 1993). Whether increased $[Ca^{2+}]_i$ or $[Ca^{2+}]_{cyto}$ exerts similar effects on the other Na^+,K^+-ATPase genes is unknown. Caffeine elevates $[Ca^{2+}]_{cyto}$ in rat EDL muscle (Fryer & Neering, 1989; Batkai *et al.*, 1999), by activation of both sarcoplasmic reticulum (SR) Ca^{2+} release (Lamb *et al.*, 2001) and of the sarcolemmal L-type Ca^{2+} channels (Ortega *et al.*, 1997). Based on the findings by Rayson (1993), it was hypothesised that elevated $[Ca^{2+}]_{cyto}$ induced by caffeine would therefore elevate each of α_1, α_2, α_3, β_1, β_2 and β_3 mRNA expression in rat skeletal muscle.

Repeated muscle contractions induce a reduction in $[K^+]_i$ and a subsequent increase in muscle $[K^+]_e$, leading to membrane depolarisation (see Section 2.2.1 for references). Muscle $[K^+]_e$ can reach as high as ~12 mM in humans during intense exercise (Nordsborg *et al.*, 2003b). A $[K^+]_e$ of 13 mM induced a 26 mV membrane depolarisation in rat EDL muscle (Hansen, A.K., Clausen, T. & Nielsen, O.B., unpublished observations). No study has elevated muscle $[K^+]_e$ to investigate Na^+,K^+-ATPase isoform mRNA expression. However, several have incubated rat liver (Pressley *et al.*, 1988) and canine kidney cell cultures (Bowen & McDonough, 1987) in low $[K^+]_e$ (0.25 – 0.65 mM). In these studies, low $[K^+]_e$ induced a 3.5-fold increase in non-isoform specific Na^+,K^+-ATPase α mRNA expression in one study (Pressley *et al.*, 1988), and a 3.0-fold increase in both non-isoform

specific Na^+,K^+-ATPase α and β mRNA expression in another (Bowen & McDonough, 1987). Furthermore, restoration of $[K^+]_e$ from 0.25 mM to control levels (5.5 mM) for 6 h completely recovered α and β mRNA expression to control values, in cultured pig kidney cells (Lescale-Matys *et al.*, 1993b). It was therefore hypothesised that membrane depolarisation induced by high $[K^+]_e$, to replicate repeated muscle contractions, would increase each of α_1, α_2, α_3, β_1, β_2 and β_3 mRNA expression in rat skeletal muscle.

Repeated muscle contractions also increase ROS in rat (Jackson *et al.*, 1985; Reid *et al.*, 1992) and in human muscle (Jackson *et al.*, 1985; Bailey *et al.*, 2003). There is accumulating evidence that increased ROS may alter the expression of numerous genes, by activation of transcription factors (Storz *et al.*, 1990; Schreck *et al.*, 1991; Toledano & Leonard, 1991), and by acting as a second messenger in several signalling pathways involved in transcription (Xie *et al.*, 1999). An increase in ROS in skeletal muscle may be involved in Na^+,K^+-ATPase isoform transcriptional regulation, as in rat cardiac myocytes, the non-specific antioxidant compound, NAC, inhibited the ouabain-induced decrease in α_3 mRNA expression (Xie *et al.*, 1999). It was therefore hypothesised that scavenging of ROS, via incubation of muscles with NAC, would attenuate the stimulation-induced increase in α_1, α_2, α_3, β_1, β_2 and β_3 mRNA expression in rat skeletal muscle.

7.2 METHODS

7.2.1 Animals and preparation of muscles

Experiments were carried out using 4 wk old female or male Wistar rats, weighing approximately 60-70 g. Rats of this age were used since their relatively small size reduces the diffusion issues that are often raised when performing intact muscle experiments. All handling and use of animals complied with Danish animal welfare regulations. The animals were fed *ad libitum* and were maintained in a temperature-controlled environment

(21 °C) with constant day length (12 h). The animals were killed by cervical dislocation, followed by decapitation, with intact soleus and EDL muscles dissected out as previously described (Nielsen & Clausen, 1996). Sample size (n) values given represents the number of muscles used.

Muscles were equilibrated for 30 min at 30 °C in standard Krebs-Ringer bicarbonate buffer (KR) (pH 7.4), containing the following (in mM): 122.1 NaCl, 25.1 $NaHCO_3$, 2.8 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 1.3 $CaCl_2$ and 5.0 D-glucose, and were bubbled continuously with a mixture of 95% O_2 and 5% CO_2. In buffer with high $[K^+]$, an equivalent amount of Na^+ was omitted to maintain iso-osmolarity.

7.2.2 Electrical stimulation

Intact soleus and EDL muscles were mounted for isometric contractions in thermostated chambers containing standard KR, were adjusted to optimal length for force production and checked for contractile activity (Soleus, 2 s trains, 12 V pulses, at each of 2 Hz, 30 Hz, 100 Hz; EDL, 0.5 ms, 12 V pulses at each of 2 Hz, 60 Hz, 90 Hz). Following standard equilibrium in KR, muscles were exposed to field stimulation across the central region through platinum electrodes, using 2 s (soleus) or 0.5 s (EDL) trains of 0.2 ms, 12 V pulses at 60 Hz. The effects of three bouts of high-frequency electrical stimulation on muscle Na^+,K^+-ATPase mRNA expression was measured using three stimulation bouts, comprising 10 s of stimulation given every 10 min (Section 7.2.3). The effects of NAC on muscle Na^+,K^+-ATPase mRNA expression was measured using a single stimulation bout, lasting 90 s (Section 7.2.5.3). The effects of caffeine on twitch force was measured using stimulations of 0.5 sec duration, elicited at a frequency of 2 Hz. Force (mN) was measured using force displacement transducers and recorded with a chart recorder and/or digitally on a computer. For experiments using NAC, mean tetanic force output was calculated as the average of the force produced by each tetani. Additionally, the percent force decline was

calculated from the force produced in the first tetani to the final tetani. Following stimulation, muscles were rapidly removed, well blotted with the tendons cut off, divided in half and immediately frozen in liquid N_2 for analyses of Na^+,K^+-ATPase isoform mRNA expression.

7.2.3 Effects of electrical stimulation and subsequent recovery on Na^+,K^+-ATPase mRNA expression

As detailed in the preceding section, following standard equilibrium in KR, soleus and EDL muscles were subject to three bouts of high-intensity, electrical stimulation. Following the final stimulation bout, muscles were either immediately removed or allowed to recover for a further 3 h, since studies in this thesis (Chapters 3-5) and by others (Pilegaard *et al.*, 2000) have demonstrated increased mRNA expression in the 2-4 h period following exercise.

7.2.4 Effects of ions and of membrane depolarisation on Na^+,K^+-ATPase mRNA expression

Experiments were performed to investigate the effects of increased $[Na^+]_i$, $[Ca^{2+}]_{cyto}$ as well as membrane depolarisation on Na^+,K^+-ATPase isoform mRNA expression. Due to thesis time constraints, the signals involved in Na^+,K^+-ATPase isoform transcriptional regulation were studied only in EDL muscle. In each experiment, EDL muscles were placed in polyethylene baskets and following standard equilibrium in KR, were incubated in the appropriate buffer for the indicated duration. Muscles were then allowed to recover for a further 3 h in standard KR, after which the tendons were removed and the muscle frozen in liquid N_2 for measurement of Na^+,K^+-ATPase isoform mRNA expression. Muscle handling was as detailed above.

7.2.4.1 Increased $[Na^+]_i$ induced by ouabain, veratridine and monensin

Three interventions were used to increase $[Na^+]_i$; ouabain, a specific inhibitor of the Na^+,K^+-ATPase (Clausen & Hansen, 1974), veratridine, an activator of the voltage-gated Na^+ channels (Sutro, 1986) and monensin, a Na^+ ionophore (Pressman & Fahim, 1982). Following standard equilibrium in KR, experimental muscles were incubated for 120 min with ouabain (10^{-3} M); for 30 min with veratridine (10^{-4} M); or for 30 min with monensin (10^{-4} M). In rat soleus muscle, ouabain (120 min, 10^{-3} M) increased intracellular Na^+ content by 194% (Gissel & Clausen, 1999). Although not known for the EDL, this increase is likely to be larger due to its higher Na^+,K^+-ATPase and Na^+-channel contents, by 23% and 70%, respectively, than the soleus (Gissel & Clausen, 2000; Clausen *et al.*, 2004). In rat EDL muscle, veratridine (15 min, 10^{-4} M) increased intracellular Na^+ content by 21% (Gissel, H. & Clausen, T., personal communication), while monensin (30 min, 10^{-4} M) elevated the Na^+/K^+ ratio by 273% (Everts & Clausen, 1992). Control muscles were incubated in only KR for durations matching their respective experimental muscles. Muscles were then either immediately removed and measured for muscle Na^+ content, or allowed to recover for a further 3 h in standard KR, after which the tendons were removed and the muscle frozen in liquid N_2 for measurement of Na^+,K^+-ATPase isoform mRNA expression.

7.2.4.2 Increased $[Ca^{2+}]_{cyto}$ induced by caffeine

Increased $[Ca^{2+}]_{cyto}$ was induced by incubating EDL muscles in caffeine, a known activator of SR Ca^{2+} release (Lamb *et al.*, 2001). Caffeine may also elevate $[Ca^{2+}]_{cyto}$ by increasing the open probability of the sarcolemmal L-type Ca^{2+} channels (Ortega *et al.*, 1997). Following standard equilibrium in KR, muscles were incubated for 30 min with or without (control) caffeine (5 mM), before being allowed to recover for 3 h in standard KR, after which the tendons were removed and the muscle frozen in liquid N_2 for measurement of

Na^+,K^+-ATPase isoform mRNA expression. Previously, this concentration of caffeine has been shown to increase baseline $[Ca^{2+}]_{cyto}$ by ~900%, and twitch force by ~60% in isolated single fibres of rat EDL muscle (Fryer & Neering, 1989). The effects of caffeine on baseline tension (Section 7.2.2) were assessed to ensure that the concentration of caffeine used was not sufficient to develop contracture. Twitch force was also measured with 5 mM caffeine, as increased twitch force would indicate an elevation in $[Ca^{2+}]_{cyto}$.

7.2.4.3 Membrane depolarisation induced by high $[K^+]_e$

Membrane depolarisation was induced by incubating EDL muscles in KR containing a high $[K^+]_e$. Following equilibration, muscles were incubated for 60 min in KR containing 4mM K^+ (control) or 13 mM K^+, before being allowed to recover for 3 h in standard (4 mM K^+) KR. This concentration of K^+ (13 mM) was chosen since it was recently found to depolarise isolated rat EDL muscle from –82 mV to –56 mV (Hansen, A.K., Clausen, T. & Nielsen, O.B., unpublished observations), and is close to the physiological range (11-12 mM) found following intense exercise in human muscle (Green *et al.*, 2000; Nordsborg *et al.*, 2003b) and electrical stimulation in rabbit skeletal muscle (Hnik *et al.*, 1976).

7.2.5 Incubation of muscles for investigation of ROS production

7.2.5.1 NAC

The effects of ROS on Na^+,K^+-ATPase isoform mRNA expression were investigated using NAC (Shindoh *et al.*, 1990). NAC scavenges numerous ROS, such as hypochlorous acid, hydroxyl radical and hydrogen peroxide (Aruoma *et al.*, 1989). NAC also increased the content of the total and reduced forms of the endogenous antioxidant, glutathione, in muscle *in-vivo* during fatiguing exercise (Medved *et al.*, 2004b). Furthermore, NAC was found to be membrane permeable in skeletal muscle (Medved *et al.*, 2004b). ROS production increases with elevated muscle temperatures (van der Poel & Stephenson,

2002). However, the experiments in the present study were performed at 30 °C due to loss of muscle integrity at higher temperatures.

7.2.5.2 Pilot experiment to determine working NAC concentration

An initial pilot experiment was performed to determine the optimal working [NAC] for rat EDL muscle. EDL muscles were mounted for isometric contractions, stretched to their optimal length and checked for contractile activity, as detailed in Section 7.2.2. Following a 30 min equilibration in normal KR, muscles were checked for initial tetanic force production (60 Hz, 0.2 ms, 0.5 s). Muscles were then sequentially incubated for 30 min in each of 2, 5, 10 and 25 mM NAC, with tetanic force production (as initial) determined after each incubation period. The time of the tetani to rise from 10-90% of peak height ($T_{rise,\ 10\text{-}90}$) and to fall from 90-10% of peak height ($T_{fall,\ 90\text{-}10}$) were also determined. Increasing the [NAC] through 2, 5, 10 and 25 mM had no effect on either tetanic force production, or on the $T_{fall,\ 90\text{-}10}$ (Appendix 6.1). The 25 mM NAC $T_{rise,\ 10\text{-}90}$ was longer (Appendix 6.1). Therefore, 30 min incubation in 10 mM NAC was used in all subsequent experiments.

7.2.5.3 Effect of NAC on Na^+,K^+-ATPase mRNA expression in resting and stimulated muscles

Following standard equilibrium in KR, EDL muscles were stimulated for tetanic force production (60 Hz, 0.2 ms, 0.5 sec). One group of muscles were then incubated for 30 min in normal KR and either allowed to rest for 90 s (Con), or were electrically stimulated for 90 s (60 Hz, 0.2 ms; Stim). The second group of muscles were incubated for 30 min in KR containing 10 mM NAC and either allowed to rest for 90 s (NAC), or were electrically stimulated for 90 s (60 Hz, 0.2 ms; Stim+NAC). Immediately following the incubation period, muscles were again stimulated to determine tetanic force production (as above), and subsequently allowed to recover for 20 min, before the 90 s resting or stimulation

period. All muscles were then allowed to recover for a further 3 h in standard KR, after which the tendons were removed and the muscle frozen in liquid N_2 for measurement of Na^+,K^+-ATPase isoform mRNA expression.

7.2.6 Measurement of Na^+,K^+-ATPase mRNA expression

Total RNA was extracted from ~10 mg of muscle using the FastRNA reagents (BIO 101, Vista, CA, USA) using methods previously described (Murphy *et al.*, 2001). The resulting RNA pellet was dissolved in EDTA-treated water and total RNA concentration was determined spectrophotometrically at 260 nm. The ratio of absorbance at 260 and 280 nm (260/280) was 2.06 ± 0.04. RNA (1 μg) was transcribed into cDNA using the Promega AMV Reverse Transcription Kit (Promega, Madison, Wisconsin, USA), with the resulting cDNA stored at –20°C for further analysis. Real-Time PCR (GeneAmp 5700 Sequence Detection System) was run for 1 cycle (50°C for 2 min, 95°C for 10 min) and 40 cycles (95°C for 15 s, 60°C for 60 s). Primer sequences were designed for the rat Na^+,K^+-ATPase α_1, α_2, α_3, α_4, β_1, β_2 and β_3 genes from published sequences (Table 7.1). All samples were run in triplicate and measurements included a no-template control (no cDNA), as well as a rat skeletal muscle sample endogenous control. Primer sequences for the housekeeping gene, cyclophilin (CYC), were also designed from published sequences (Table 7.1) and CYC mRNA was used as a control to account for any variations in the amount of input RNA and the efficiency of reverse transcription. Gene expression was quantified from fluorescence emission using a cycle threshold (C_T) method. For comparison of isoform mRNA expression between fibre-types, a ΔC_T value was calculated by subtracting the CYC C_T from the gene C_T. The relative gene expression was then calculated using the expression, $2^{-\Delta CT}$. For all other comparisons, the relative expression of the genes compared with control sample was made using the expression, $2^{-\Delta\Delta CT}$, in which the expression of each gene was normalised for input cDNA using the housekeeping gene CYC. Gene expression

Table 7.1 Rat Na^+,K^+-ATPase gene α_1-α_4 and β_1-β_3, and CYC primer sequences used for mRNA analyses

Gene	GenBank Accession Number	Sense Primer (5'-3')	Antisense Primer (5'-3')
α_1	NM_012504	CAGTGTTTCAGGCTAACCAAGAAA	CGCCGACTCGGAAGCAT
α_2	NM_012505	GCTAGGAGCAGCATAGTTAGTTTCAA	AATTAGCCTATGCACTTCCTGATTC
α_3	M90659	GGGAGTCTGTGAGGTGGTGT	GCTCAAAAACCAGCAGAAGG
α_4	NM_022848	TTTTGCTCCAGTTTCCTGCT	GGCACTTGCTAACAGCATCA
β_1	NM_013113	TCCAAACGTCCTACCTGTCC	CGGATTTCAGTGTCCAAGGT
β_2	D90048	AGGAGCCAGTGGAACTGAGA	CCCCCTTAGAAGCTCAAACC
β_3	XM_213132	AATCGAGTTCGTCCCTGATG	TTCCATCAATTTGGCACTCA
CYC	M19533	CTGATGGCGAGCCCTTG	TCTGCTGTCTTTGGAACTTTGTC

Primer sequences were designed using Primer Express software (Applied Biosystems) from gene sequences obtained from GeneBank. Primer specificity was determined using a BLAST search. CYC, cyclophilin.

of CYC was not significantly altered with any intervention (data not shown). The intra-assay coefficient of variation for each target gene was <13.0% for 2^{-C_T} (Table 7.2). The validity of each rat primer was established as detailed in Chapter 3. For each primer, a linear relationship was found between the C_T values and the logarithm of the cDNA template concentration (Appendix 6.2), and the mean efficiency of each primer was –1.0 to –1.1 (Appendix 6.2).

7.2.7 Muscle Na^+ content

For measurement of muscle Na^+ content, muscles that were cut in half had their wet weight determined, and were soaked overnight in 0.3 M trichloroacetic (TCA) acid to give complete extraction of Na^+ (Everts *et al.*, 1993). The Na^+ content in the TCA extract was measured by flame photometry (FLM3, Radiometer, Copenhagen, Denmark). The muscle Na^+ content measurement reflects both intra and extracellular Na^+ contents.

7.2.8 Chemicals

All chemicals were of analytical grade. Monensin, veratridine, ouabain, caffeine, NAC, and D-glucamine were purchased from Sigma Chemicals (St. Louis, MO).

7.2.9 Statistical analysis

All data are presented as mean ± SEM. Statistical differences between two groups in the relative mRNA expression, force production or % force decline were analysed using an independent-samples student *t*-test. The statistical difference in the relative mRNA expression between three or more groups was analysed using a one-way ANOVA. A two-way ANOVA with measures for treatment (Control vs. NAC) and stimulation (Rest vs. Stim) was used to test for main and interaction effects for muscle Na^+,K^+-ATPase isoform mRNA expression. Differences were located with a Student-Newman-Kuels *post hoc* test. Significance was accepted at $P < 0.05$.

Table 7.2 Intra-assay variability of 2^{-CT} values

Gene	2^{-C_T} CV (%)
α_1	11.2
α_2	11.0
α_3	9.8
β_1	12.2
β_2	11.6
β_3	11.1
CYC	10.0

Each sample was run in triplicate wells in the same Real-Time PCR run. n = 241. CV, coefficient of variation; C_T, cycle threshold.

7.3 RESULTS

7.3.1 Relative Na^+,K^+-ATPase isoform mRNA expression in rat soleus and EDL muscle

In both rat soleus and EDL muscle, gene transcripts were detected for each of the Na^+,K^+-ATPase α_1, α_2, α_3, β_1, β_2 and β_3 isoforms. The α_4 gene transcript could not be detected for either soleus or EDL. The relative expression of each of the Na^+,K^+-ATPase α_1, α_2, α_3, β_1, β_2 and β_3 gene transcripts, normalised to CYC, were compared between rat soleus (n = 127) and EDL (n = 114) muscles (Figure 7.1). Only resting muscles that were only incubated in standard KR were compared. There was no difference in CYC mRNA expression between muscles (Soleus, $1.12 \times 10^{-7} \pm 0.09 \times 10^{-7}$; EDL, $1.12 \times 10^{-7} \pm 0.11 \times 10^{-7}$ C_T). Gene expression of the α_2 and β_3 isoforms was higher in the EDL than the soleus, by 1.5- ($P < 0.01$) and 1.7-fold ($P < 0.04$), respectively (Figure 7.1). In contrast, β_1 mRNA expression was 0.3-fold lower in the EDL than the soleus ($P < 0.03$, Figure 7.1). There were no significant differences between the soleus and EDL muscles for α_1, α_3 or β_2 mRNA expression (Figure 7.1).

7.3.2 Effect of electrical stimulation on muscle Na^+,K^+-ATPase mRNA expression

7.3.2.1 EDL muscle

Three bouts of high-frequency electrical stimulation had no significant immediate effect on the mRNA expression of any of the α_1, α_2 or α_3 isoforms in rat EDL (Figure 7.2). These were, however, increased at 3 h post-stimulation, by 3.2- ($P < 0.02$), 7.2- ($P < 0.01$) and 9.9-fold ($P < 0.01$), respectively (Figure 7.2). Gene expression of the α_2 and α_3 isoforms at 3 h post-stimulation was also higher than that immediately following electrical stimulation, by 2.3- ($P < 0.02$) and 2.6-fold ($P < 0.01$), respectively (Figure 7.2). However, electrical stimulation had no significant effect on β_1, β_2 or β_3 mRNA expression, either immediately or at 3 h following stimulation (Figure 7.2).

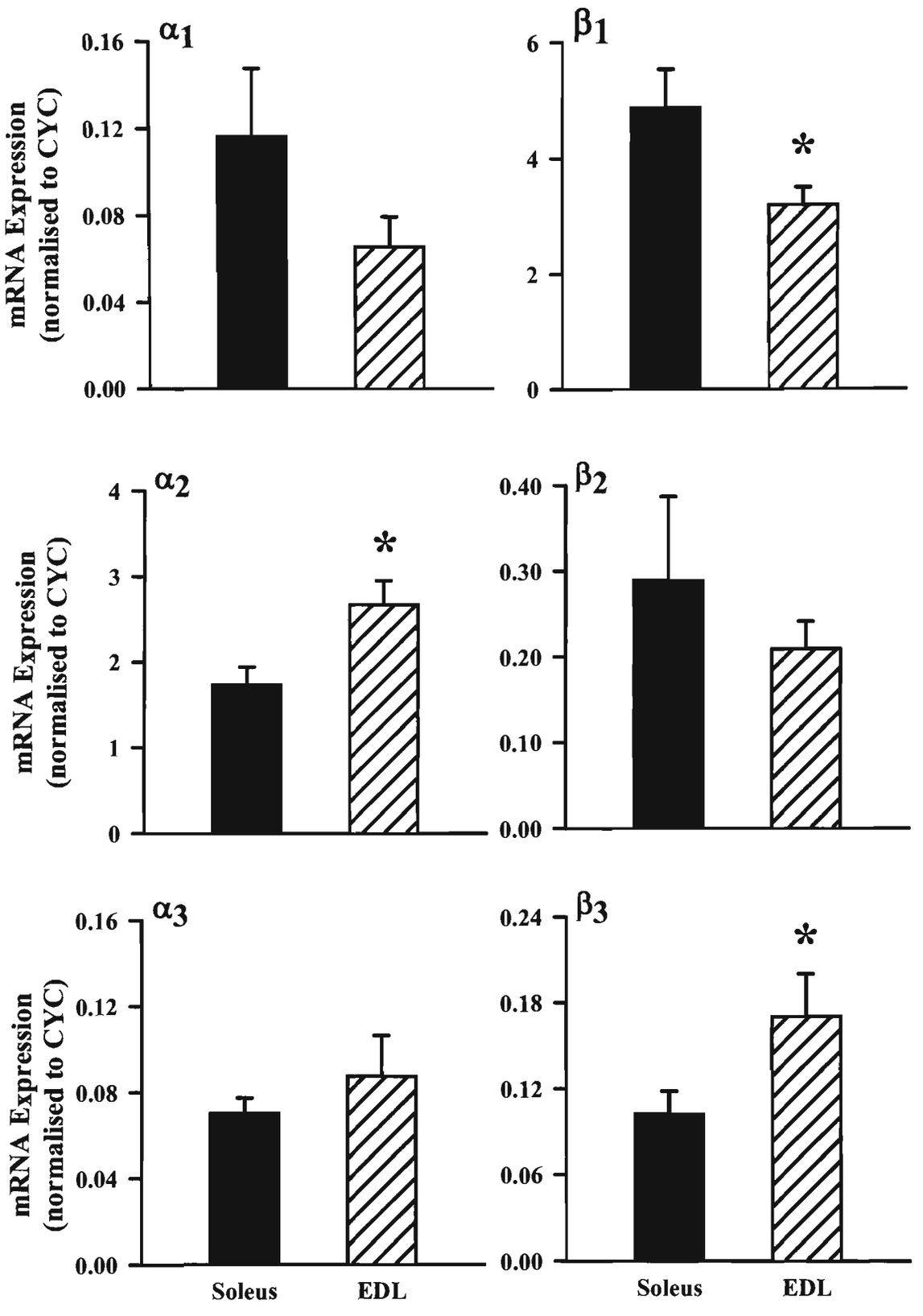


Figure 7.1 Relative Na^+,K^+-ATPase α_1, α_2, α_3, β_1, β_2 and β_3 mRNA expression in rat soleus and EDL muscle. Values are normalised to cyclophilin (CYC) mRNA expression. Data are mean ± SEM; n = 127 for soleus, n = 114 for EDL. * $P < 0.04$ vs. Soleus.

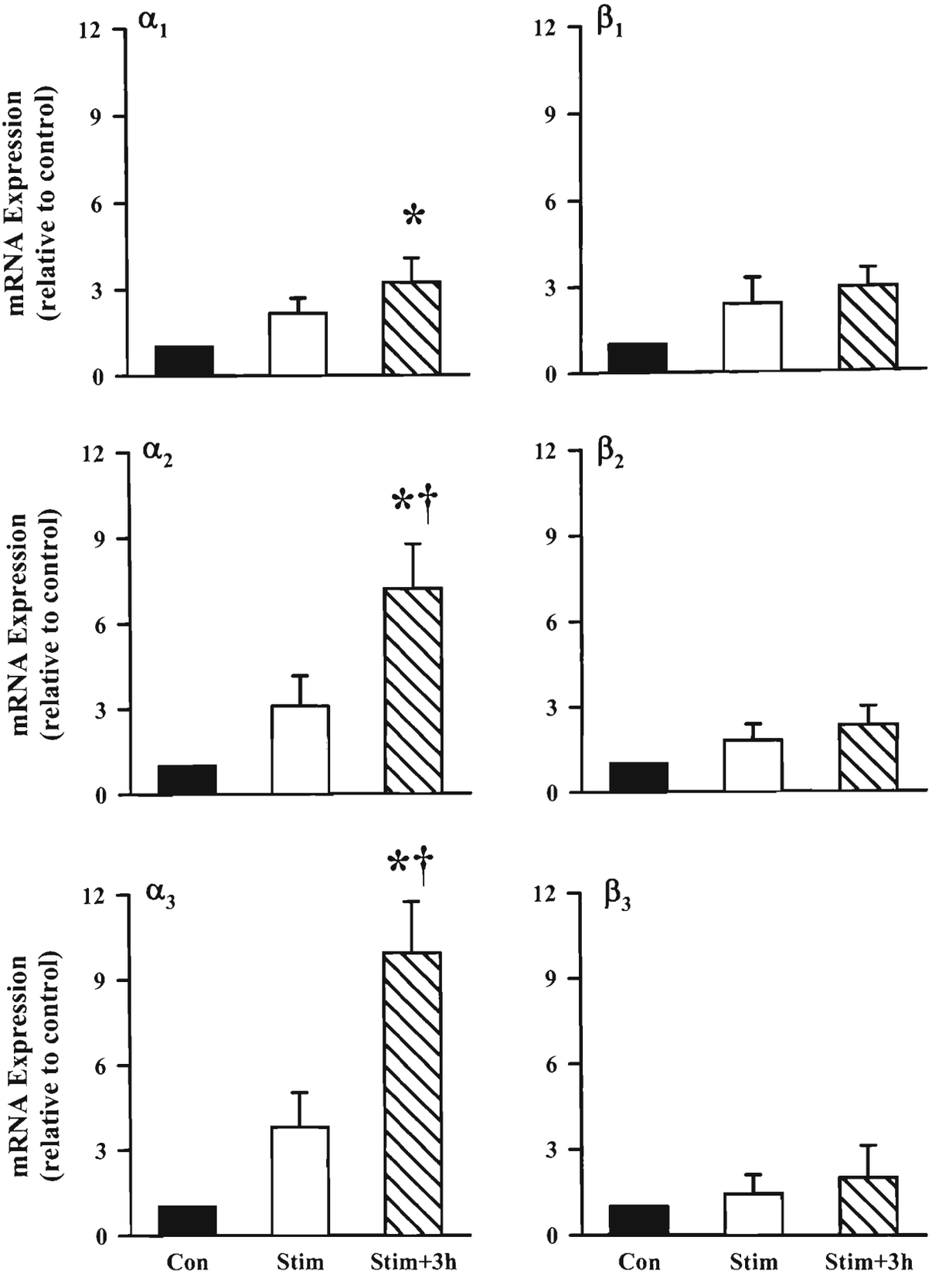


Figure 7.2 Effects of three bouts of high-frequency electrical stimulation and subsequent 3 h recovery on Na^+,K^+-ATPase α_1, α_2, α_3, β_1, β_2 and β_3 mRNA expression in rat EDL muscle. Data are mean ± SEM; n = 8 except Stim where n = 6. * $P < 0.02$ greater than Con, † $P < 0.02$ greater than Stim.

7.3.2.2 Soleus muscle

There was no effect of three bouts of high-frequency electrical stimulation on the mRNA expression in rat soleus muscle for any of the α_1, α_2, α_3, β_1, β_2 or β_3 isoforms, either immediately or at 3 h following stimulation (Figure 7.3).

7.3.3 Effects of ouabain, veratridine and monensin on muscle Na^+ content and Na^+,K^+-ATPase mRNA expression in rat EDL muscle

Muscle Na^+ content was 90% higher with ouabain (10^{-3} M, $P < 0.01$), but was not significantly elevated with either veratridine (10^{-3} M) or monensin (10^{-4} M), compared to control (Figure 7.4).

Ouabain reduced β_2 and β_3 mRNA expression, by 0.8- ($P < 0.05$) and 0.9-fold ($P < 0.01$), respectively, but had no significant effect on the mRNA expression of any of the α_1, α_2, α_3 or β_1 isoforms (Figure 7.5). Both veratridine and monensin reduced β_3 mRNA expression by 0.9- ($P < 0.02$) and 0.9-fold ($P < 0.02$), respectively, but had no significant effect on the mRNA expression of any of the α_1, α_2, α_3, β_1 or β_2 isoforms (Figure 7.5).

7.3.4 Effects of caffeine on baseline tension, twitch force and Na^+,K^+-ATPase mRNA expression in rat EDL muscle

Caffeine (5 mM) did not alter baseline tension (Control, 4.4 ± 0.2; Caffeine, 4.2 ± 0.1 mN, n = 4, $P < 0.37$), but did potentiate twitch force (2 Hz) by 80% (Control, 128.2 ± 16.6; Caffeine, 230.2 ± 53.7 mN, n = 4, $P < 0.04$).

Caffeine increased the mRNA expression of each of the α_1, α_2 and β_1 isoforms, by 2.6- ($P < 0.02$), 2.8- ($P < 0.02$) and 2.7-fold ($P < 0.03$), respectively, but did not affect any of α_3, β_2 or β_3 mRNA expression (Figure 7.6).

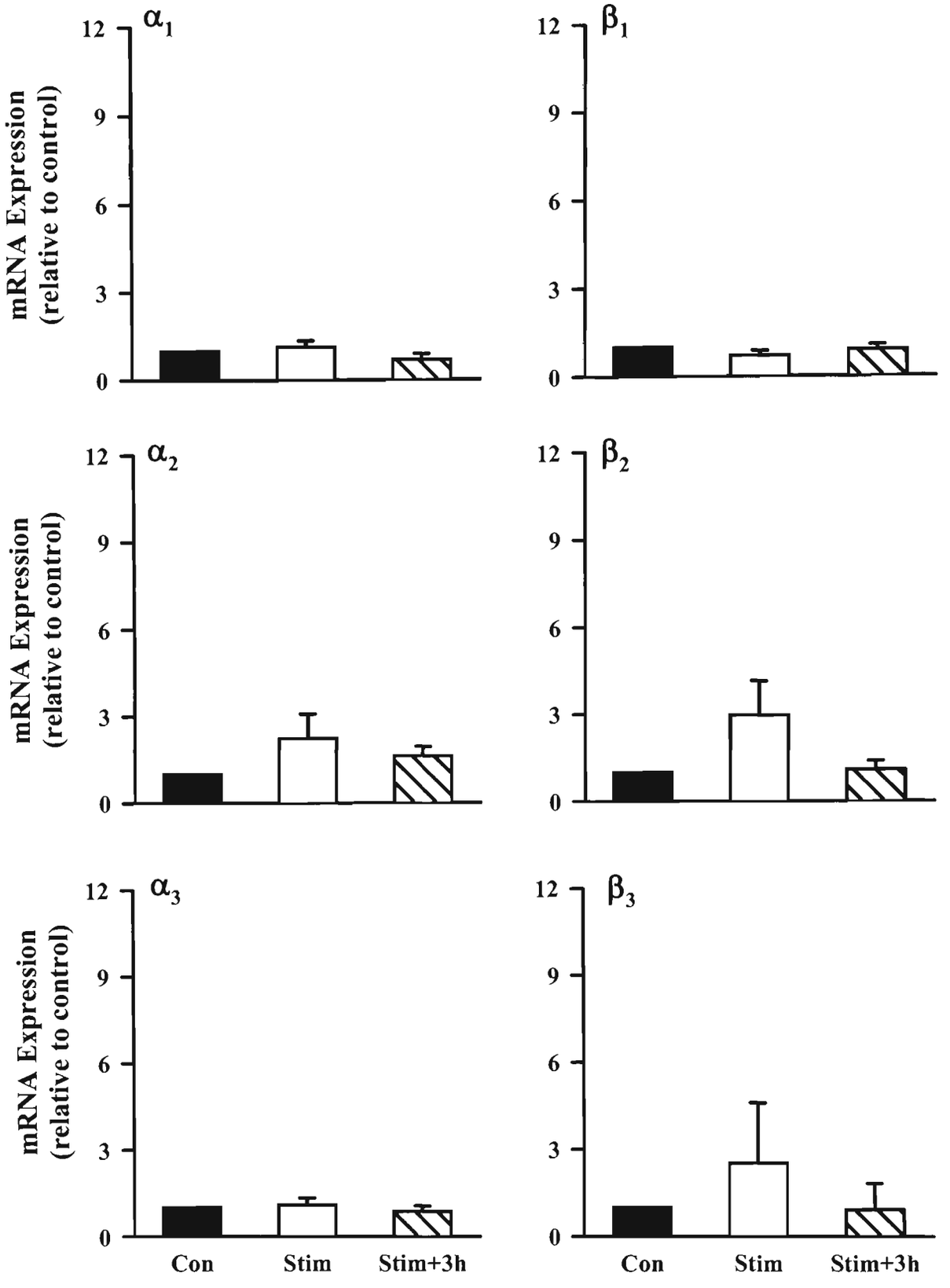


Figure 7.3 No effect of three bouts of high-frequency electrical stimulation and subsequent 3 h recovery on Na^+,K^+-ATPase α_1, α_2, α_3, β_1, β_2 and β_3 mRNA expression in rat soleus muscle. Data are mean ± SEM; n = 6.

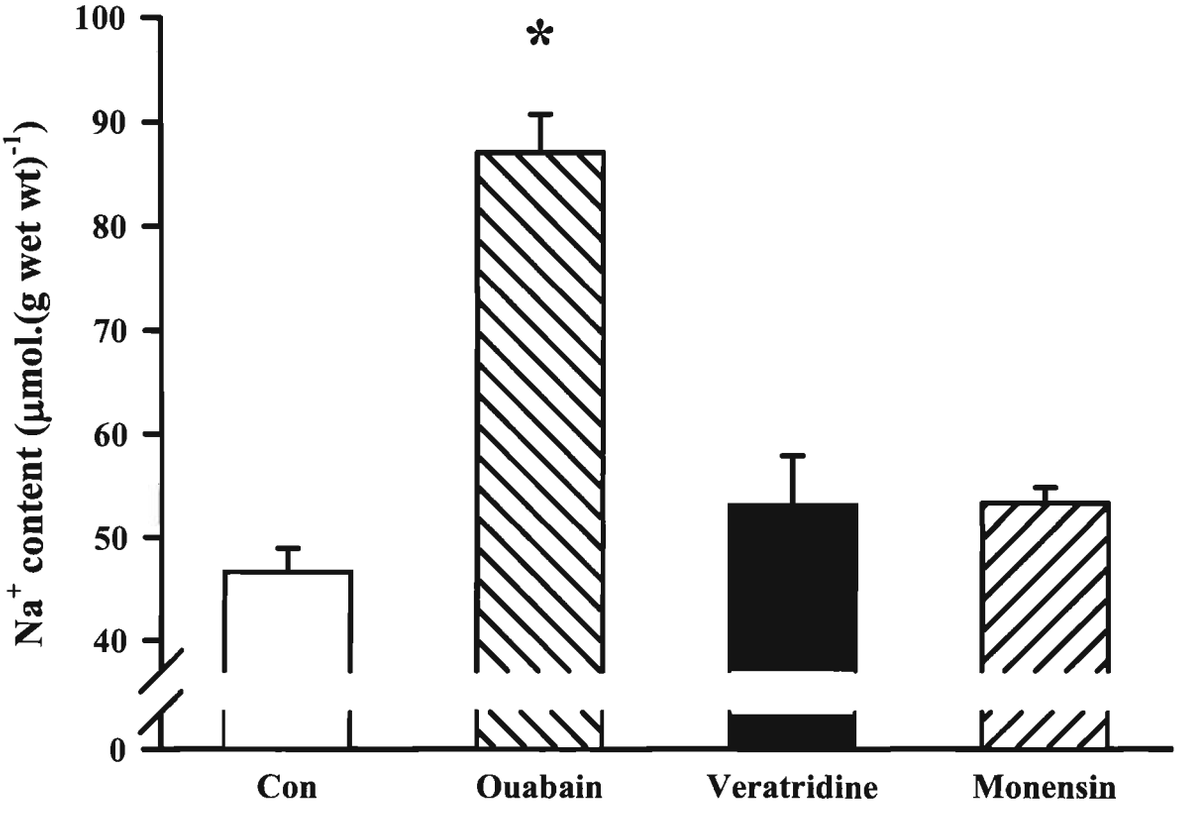


Figure 7.4 Effects of ouabain, veratridine and monensin on muscle Na^+ content in rat EDL. EDL muscles were incubated with ouabain (10^{-3} M), veratridine (10^{-4} M) or monensin (10^{-4} M) before being blotted, weighed and analysed for Na^+ content. Data are mean ± SEM; n = 6, except Monen where n = 3. * $P < 0.01$ greater than Con.

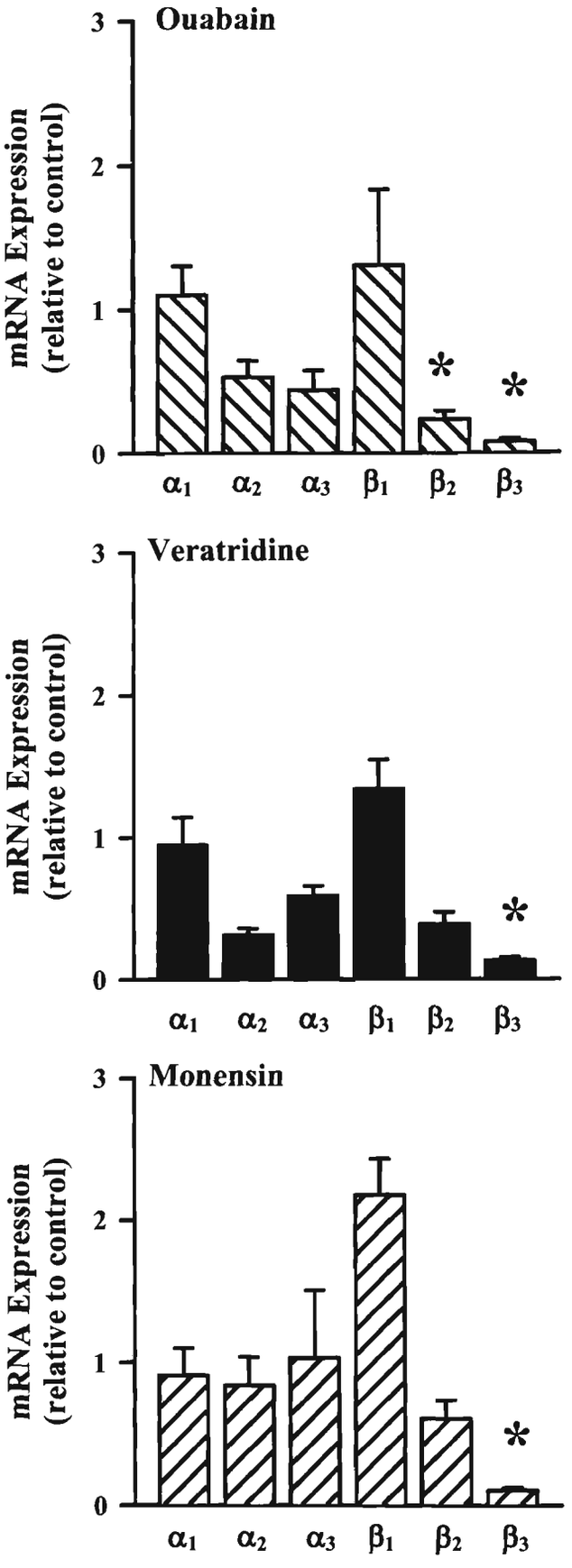


Figure 7.5 Effects of ouabain, veratridine and monensin on Na^+,K^+-ATPase α_1, α_2, α_3, β_1, β_2 and β_3 mRNA expression in rat EDL muscle. Isoform mRNA expression is expressed relative to control (1.00). Muscles were incubated with ouabain (Ouab, 10^{-3} M), veratridine (Verat, 10^{-4} M) and monensin (Monen, 10^{-4} M) before being allowed to recover in normal KR buffer for a further 3 h. Data are mean ± SEM; n = 6. * $P < 0.05$ less than Con.

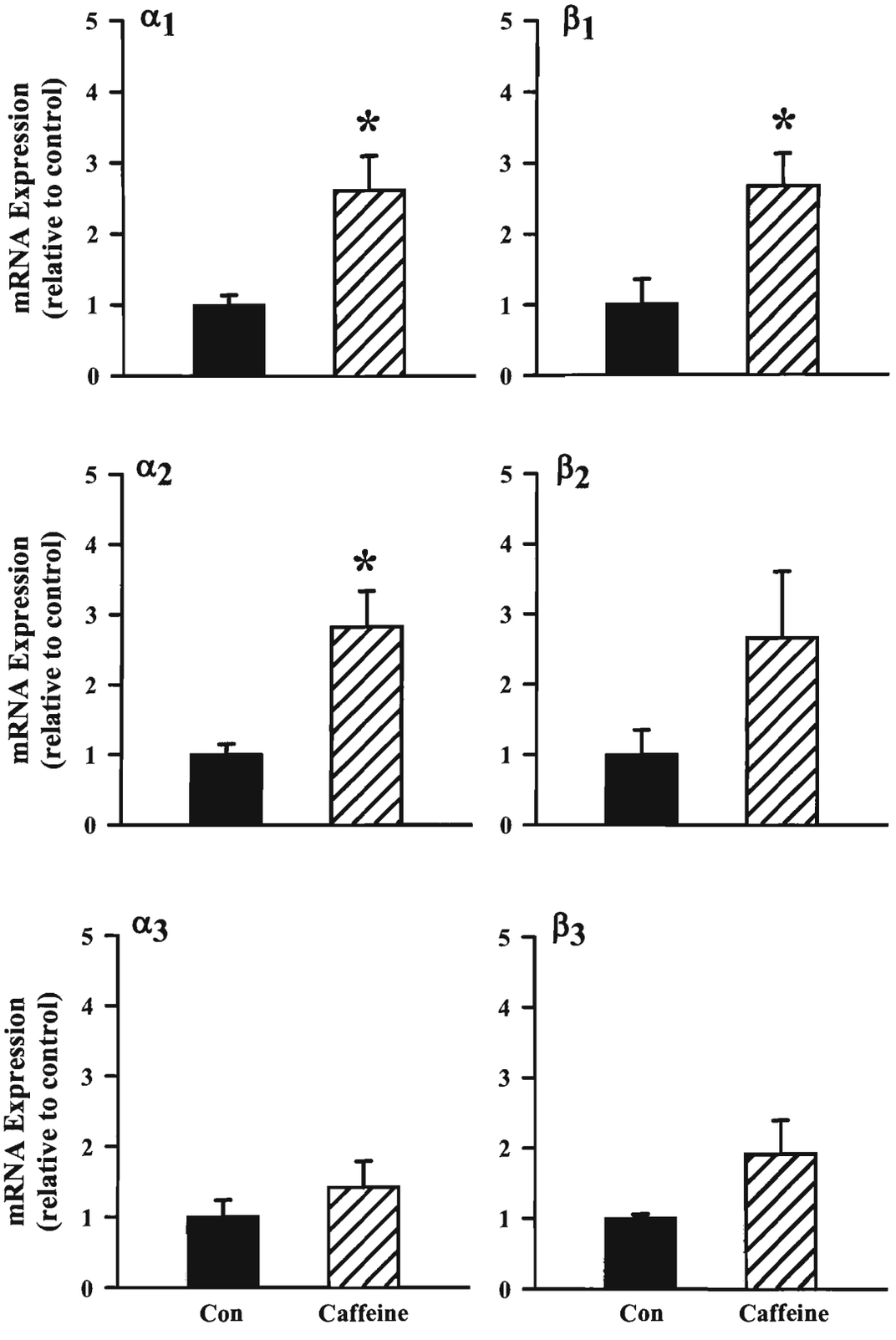


Figure 7.6 Effects of caffeine on Na^+,K^+-ATPase α_1, α_2, α_3, β_1, β_2 and β_3 mRNA expression in rat EDL muscle. Muscles were incubated with caffeine (5 mM), before being allowed to recover in normal KR buffer for a further 3 h. Data are mean ± SEM; n = 4. * $P < 0.03$ greater than Con.

7.3.5 Effects of high $[K^+]_e$ on Na^+,K^+-ATPase mRNA expression in rat EDL muscle

High $[K^+]_e$ (13 mM) had no significant effect on the mRNA expression of any of the Na^+,K^+-ATPase α_1, α_2, α_3, β_1, β_2 or β_3 isoforms (Figure 7.7).

7.3.6 Effects of NAC on tetanic force production and Na^+,K^+-ATPase mRNA expression in rat EDL muscle

There was no significant difference in tetanic force production before (initial) and following incubation with the antioxidant NAC (Table 7.3), nor was there any effect of NAC on initial force production, mean force production and the percentage decline in force production, during 90 s of electrical stimulation (Table 7.3).

In resting muscles, NAC increased α_2 and β_1 mRNA expression by 4.6- ($P < 0.01$) and 4.1-fold, respectively ($P < 0.01$, Figure 7.8, interaction effect).

In muscles incubated without NAC, 90 s of high-frequency (60 Hz) electrical stimulation increased α_1, α_2 and α_3 mRNA expression, by 3.7- ($P < 0.01$), 5.3- ($P < 0.01$) and 24.0-fold ($P < 0.01$), respectively, compared to control, with no significant effect for any of β_1, β_2 or β_3 mRNA (Figure 7.8, interaction effects). In contrast, in muscles incubated with NAC, 90 s of high-frequency electrical stimulation decreased α_2, β_1 and β_2 mRNA expression, by 3.3- ($P < 0.01$), 4.1- ($P < 0.01$) and 2.2-fold ($P < 0.01$), respectively, compared to Rest+NAC (Figure 7.8, interaction effects).

The β_3 mRNA expression was lower with NAC treated muscles ($P < 0.02$, Figure 7.8, treatment main effect).

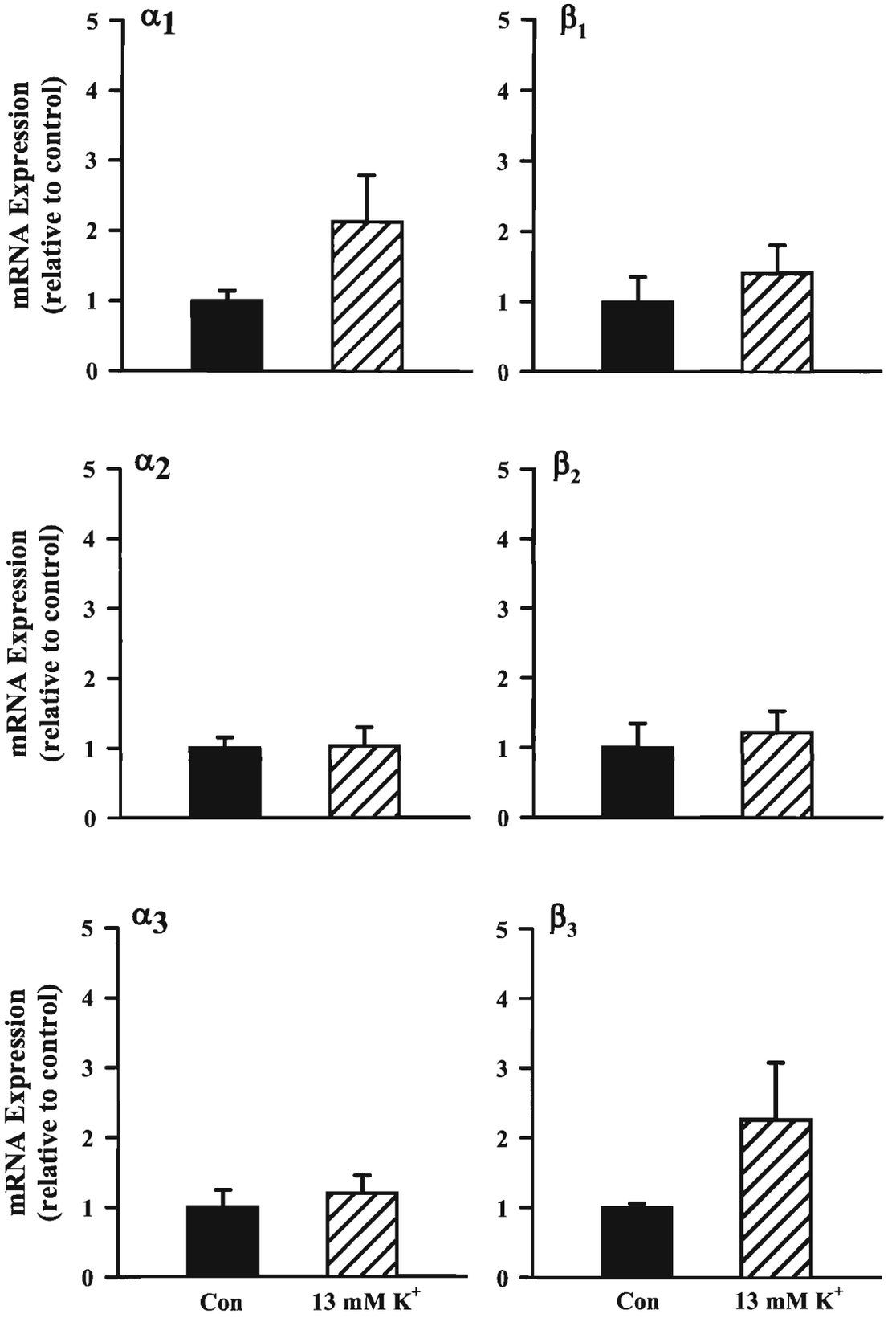


Figure 7.7 No effect of high $[K^+]_e$ on Na^+,K^+-ATPase α_1, α_2, α_3, β_1, β_2 and β_3 mRNA expression in rat EDL muscle. Muscles were incubated in high K^+ (13 mM), before being allowed to recover in normal KR buffer (4 mM K^+) for a further 3 h. Data are mean ± SEM; n = 9.

Table 7.3 No effect of NAC on tetanic force in rat EDL muscle

			Stimulation at 60 Hz for 90 s		
	Pre-incubation tetanic force (mN)	Post-incubation tetanic force (mN)	Initial force production (mN)	Mean force production (mN)	Force decline (%)
Stimulated	359 ± 22	354 ± 8	360 ± 22	225 ± 13	23 ± 1
Stimulated + NAC	385 ± 37	374 ± 2	379 ± 31	225 ± 15	22 ± 1

EDL muscles were incubated for 30 min in Krebs-Ringer buffer with (Stimulated + NAC) or without (Stimulated) 10 mM NAC, before being stimulated directly at 60 Hz for 90 s. Prior to (pre-incubation), and immediately following (post-incubation) incubation, muscles were checked for tetanic force production (60 Hz, 0.2 ms, 0.5 sec). No statistical significance was observed between stimulated and stimulated + NAC incubated muscles. Data are mean ± SEM; n = 4.

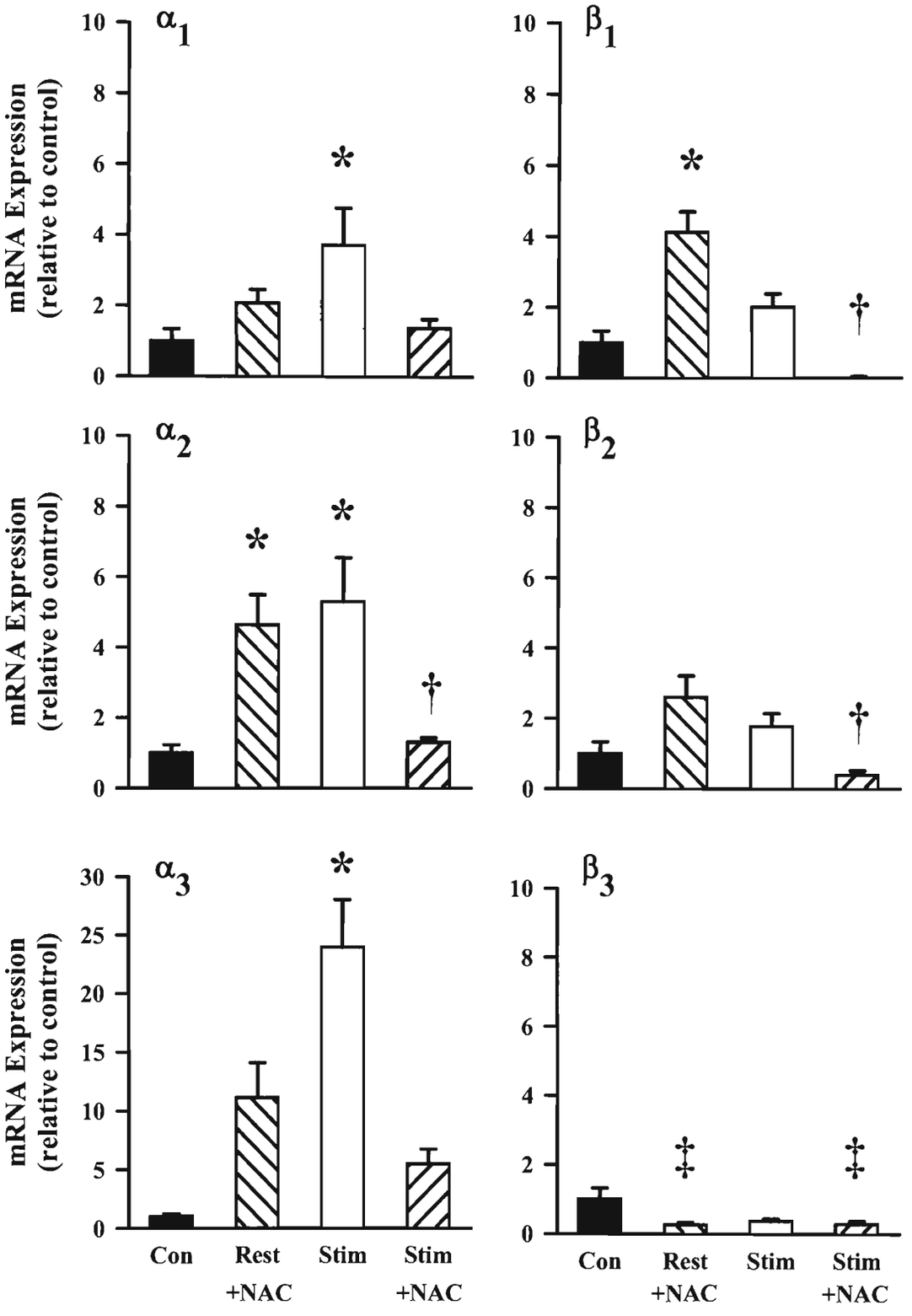


Figure 7.8 Effect of NAC on Na^+,K^+-ATPase α_1, α_2, α_3, β_1, β_2 and β_3 mRNA expression in rat EDL muscle. Muscles were either incubated in normal KR buffer and allowed to rest for 90 s (Con), were electrically stimulated (60 Hz, 90 s; Stim), or rest for 90 s whilst incubated in 10 mM NAC (Rest+NAC) or were incubated in 10 mM NAC and electrically stimulated (60 Hz, 90 s; Stim+NAC). All muscles were then allowed to rest for 3 h. Data are mean ± SEM; n = 4, except Rest+NAC where n = 6. Note the different y axis for α_3. * $P < 0.01$ greater than Con, † $P < 0.01$ less than Rest+NAC, ‡ $P < 0.02$ treatment main effect Con vs. NAC.

7.4 DISCUSSION

This study investigated the effects of muscle fibre-type and electrical stimulation on Na^+,K^+-ATPase isoform mRNA expression in rat skeletal muscle. Factors regulating Na^+,K^+-ATPase transcription in rat EDL muscle were also explored, using interventions designed to induce each of increased $[Na^+]_i$ and $[Ca^{2+}]_{cyto}$, membrane depolarisation and scavenging of ROS. The first main finding was that both EDL and soleus muscle from rats expressed gene transcripts for each of the Na^+,K^+-ATPase α_1, α_2, α_3, β_1, β_2 and β_3 isoforms, but not the α_4 isoform. The second main finding was that the relative mRNA expression differed between EDL and soleus muscles for 3 isoforms, with the EDL containing a greater α_2 and β_3 mRNA expression, but a lower β_1 mRNA expression than the soleus muscle. The third main finding was that three bouts of high-frequency electrical stimulation induced isoform- and muscle-specific Na^+,K^+-ATPase transcriptional regulation. Each of α_1, α_2 and α_3 isoform mRNA expression were increased with stimulation in the EDL, whereas no change was found in any isoform mRNA expression in the soleus. The fourth main finding was that ouabain increased muscle Na^+ content, but did not increase mRNA expression for any isoform, in fact reducing β_2 and β_3 mRNA expression. In contrast, caffeine, which is known to elevate $[Ca^{2+}]_{cyto}$, increased the mRNA expression of each of the α_1, α_2 and β_1 isoforms. *N*-acetylcysteine (NAC), a scavenger of ROS, had complex effects, increasing α_2 and β_1 mRNA expression in resting muscle, but abolishing the rise in α_1, α_2 and α_3 mRNA expression with electrical stimulation. Thus, the six Na^+,K^+-ATPase isoform gene transcripts expressed in rat EDL muscle may be regulated by different intracellular stimuli.

7.4.1 Presence of the Na^+,K^+-ATPase α_1-α_3 and β_1-β_3 gene transcripts in rat EDL and soleus muscle

This is the first study to probe for the full complement of Na^+,K^+-ATPase gene transcripts in rat skeletal muscle, detecting each of the α_1, α_2, α_3, β_1, β_2 and β_3 gene transcripts, but not the α_4 gene transcript, in both the soleus and EDL muscles. Detection of the α_3 gene transcript has previously only been reported in skeletal muscle of un-specified origin (Orlowski & Lingrel, 1988). This appears to be the first study to probe for the α_4 gene transcript in rat skeletal muscle and its absence is consistent with findings in human vastus lateralis muscle (Chapter 4). Another study detected the α_4 gene transcript in human skeletal muscle of unspecified origin (Keryanov & Gardner, 2002). The reason for this discrepancy is unknown, but the α_4 gene transcript detected in the latter study may have originated from contamination of other tissues or from cross-reactivity of other gene transcripts. The detection of both the β_1 and β_2 gene transcripts in EDL and soleus muscles in the present chapter clearly differs from other studies where the β_1 and β_2 gene transcripts were detected exclusively in muscles predominantly containing type I or type II fibres, respectively (Hundal *et al.*, 1993; Tsakiridis *et al.*, 1996; Thompson *et al.*, 1999). This discrepancy may be explained by the use of Real-Time RT-PCR in the present study, which is much more sensitive (Bustin, 2000) than the Northern Blotting technique utilised in each of the other studies (Hundal & Klip, 1993; Tsakiridis *et al.*, 1996; Thompson *et al.*, 1999). Detection of the β_3 gene transcript in rat skeletal muscle is a novel finding.

7.4.2 Rat EDL muscle contains higher α_2 and β_3, but lower β_1 mRNA expression than the soleus

The relative mRNA expression of the full complement of Na^+,K^+-ATPase gene transcripts were compared between rat muscles of different fibre-type composition. The EDL muscle demonstrated a 1.5-fold higher α_2 and a 1.7-fold higher β_3 mRNA expression, but a 0.3-

fold lower β_1 mRNA expression, compared to the soleus. The EDL and soleus muscles were selected since they vary considerably in their fibre-type composition (Delp & Duan, 1996). In both young (5 wk old) and older adult (18-20 wk old) rats, the EDL is predominantly comprised of type II fibres (10-20% IIa, 38-40% IId/x, 35-38% IIb), whereas the soleus is predominantly comprised of type I fibres (75-84%) (Delp & Duan, 1996; Suwa *et al.*, 2003). Thus, the relative expression of the rat α_2, β_1 and β_3 gene transcripts appears to depend on fibre-type composition. It is important to note that these differences in relative isoform mRNA expression between muscles of different fibre-type composition may not necessarily reflect the relative protein expression of the Na^+,K^+-ATPase isoforms.

The only other comparison of Na^+,K^+-ATPase isoform mRNA expression between muscles of different fibre-type composition found no difference in α_1 mRNA expression between the soleus and EDL muscles. The relative mRNA expression was 4.0-fold higher for the α_2 and 3.0-fold higher for the β_1 isoforms, but 8.0-fold lower for the β_2 isoform in the soleus than the EDL, (Hundal *et al.*, 1993). Neither the α_3 or β_3 gene transcripts were probed in that study. The findings from the present study clearly differed regarding α_2 and β_2 mRNA expression between muscles. The reason for the variance between studies is unknown. One possibility involves differences in the age of the rats utilised, since isoform mRNA expression has been shown to age-dependent (Orlowski & Lingrel, 1988). Since Hundal *et al.*, (1993) did not state the age or weight of the rats utilised this possibility cannot be evaluated. Nonetheless, the results from the present study are given further credence by the use of a large sample size (n = 127 soleus, n = 114 EDL) and the application of the more sensitive Real-Time RT-PCR technique for mRNA measurements.

7.4.3 The effects of three bouts of high-frequency electrical stimulation on isoform mRNA expression differ between EDL and soleus muscles

The effects of electrical stimulation on skeletal muscle Na^+,K^+-ATPase isoform mRNA expression were investigated for the first time. Findings were an increase in α_1, α_2 and α_3 mRNA, but not β_1, β_2 or β_3 mRNA with stimulation in rat EDL muscle, but with no effect on any of the six Na^+,K^+-ATPase isoforms in rat soleus muscle. Thus the effects of three bouts of high-frequency electrical stimulation on Na^+,K^+-ATPase isoform mRNA expression clearly differs between EDL and soleus muscles.

The stimulatory effect of electrical stimulation on α mRNA expression was not evident until 3 h post-stimulation. This is consistent with the higher mRNA response of metabolic genes in the 2-4 h period following exercise (Pilegaard *et al.*, 2000). The increase in α mRNA, but not β mRNA in the EDL with three bouts of high-frequency electrical stimulation was also seen with 90 s of high-frequency stimulation in the experiment investigating the effects of NAC on isoform mRNA expression. Thus it appears that an α subunit-specific increase in mRNA may be an obligatory response to intense muscle contractions in rat EDL muscle.

In striking contrast to the elevations in α mRNA in the EDL, three bouts of high-frequency electrical stimulation had no significant effect on the mRNA expression of any of the Na^+,K^+-ATPase isoforms in rat soleus muscle, including in the 3 h period following stimulation. The stimulation frequency used (60 Hz) is within the range of *in-vivo* motor unit firing frequency in freely moving adult rats in the EDL (40-111 Hz), but is higher than that in the soleus (12-29 Hz) (Hennig & Lomo, 1985). However, it is likely that the soleus was recruited to at least the same extent as the EDL with the electrical stimulation protocol used in this study. Force-frequency curves demonstrate that at 60 Hz, the soleus muscle produces maximal tetanic force (Claflin & Faulkner, *J Physiol,* 411: 627-637, 1989). In

contrast, the EDL muscle is not completely fused at 60 Hz and only reaches maximal tetanic force at a stimulation frequency of 90 Hz (McGuire *et al.*, Chest 123, 875-881, 2003). It is unknown whether stimulation protocols of lower frequencies would elicit any effect on Na^+,K^+-ATPase mRNA expression in soleus muscle.

The different responses to electrical stimulation on Na^+,K^+-ATPase isoform mRNA expression between EDL and soleus muscles may reflect differences in the extent of evoked intracellular changes. During electrical stimulation, the EDL demonstrated a significantly greater Ca^{2+} uptake (Gissel & Clausen, 2000) and SR Ca^{2+} release (Baylor & Hollingworth, 2003) than the soleus, suggesting greater increases in $[Ca^{2+}]_{cyto}$. Furthermore, stimulation-induced increases in ROS may be greater in the EDL than the soleus, since muscles predominantly containing type I fibres demonstrated higher antioxidant capacity compared to muscles primarily containing type II fibres (Ji *et al.*, 1992). EDL muscles also demonstrated a greater Na^+ uptake, and K^+ loss during high-frequency electrical stimulation than the soleus (Clausen *et al.*, 2004). However, results from the present study suggest that no relationship is present between either elevated $[Na^+]_i$ or $[K^+]_e$ and Na^+,K^+-ATPase mRNA expression in rat skeletal muscle. Thus, the increases in isoform mRNA with electrical stimulation in the EDL, but not the soleus, might be explained by greater increases in $[Ca^{2+}]_{cyto}$ and/or ROS, but this remains to be tested.

7.4.4 Limitations of study

There were several limitations to this study that need to be acknowledged regarding the experiments investigating the possible intracellular factors involved in Na^+,K^+-ATPase transcriptional regulation in rat muscle.

First, no measurements were made of the effects of ouabain, veratridine and monensin on $[Na^+]_i$, or of caffeine effects on $[Ca^{2+}]_{cyto}$. This was due to the fact that the laboratory in which these experiments were performed, was not equipped for these measurements. The

measurement of muscle Na^+ content was made, but this measurement includes possible Na^+ contamination from the extracellular space. Thus any increase in $[Na^+]_i$ may have been missed due to the Na^+ contamination from other cellular compartments. As such, previous studies in which measurements of intracellular Na^+ content or $[Ca^{2+}]_{cyto}$ were made have been cited. Intracellular Na^+ content was cited since the effects of ouabain or veratridine on $[Na^+]_i$ do not appear to have been investigated . For monensin, muscle Na^+/K^+ fluxes was cited since I am unaware of any study measuring the effect of monensin on either $[Na^+]_i$ or intracellular Na^+ content. It is anticipated that similar effects to those cited would have been present in the muscle preparations performed in this study. However, further work is clearly required to confirm this.

Second, due to the time constraints of completing this thesis, relatively small samples sizes (4-9) were used. It is therefore possible that any small changes in isoform mRNA expression, or muscle Na^+ content, were undetected. Future work includes performing additional experiments to minimise the possibility of a type II error for some of these measures.

7.4.5 Ouabain increased muscle Na^+ content, but did not increase Na^+,K^+-ATPase isoform mRNA expression

There was a clear increase in muscle Na^+ content with ouabain, and as such, a likely elevation in $[Na^+]_i$, however, there was no corresponding increase in Na^+,K^+-ATPase isoform mRNA expression. These findings contrast those in cultured rat kidney cells, where ouabain (10^{-4} M) induced an ~2.0-fold increase in each of Na^+,K^+-ATPase α_1 and β_1 mRNA expression (Rayson, 1993). Furthermore, in cultured chick skeletal muscle cells, elevated $[Na^+]_i$ induced with veratridine (10 μM), increased non-isoform specific Na^+,K^+-ATPase α and β mRNA expression by 1.7- and 2.5-fold, respectively (Taormino & Fambrough, 1990). Thus, it appears that elevated $[Na^+]_i$ induces an increase in Na^+,K^+-

ATPase isoform mRNA expression in cultured muscles cells, but not in isolated rat skeletal muscle. In fact, ouabain reduced β_2 and β_3 mRNA expression in isolated rat EDL muscle. These findings compare with ouabain effects in rat cardiac myocytes, where α_3 mRNA expression was reduced (Huang *et al.*, 1997; Xie *et al.*, 1999), but contrast the increase in β_1 mRNA expression (Kometiani *et al.*, 2000). Thus the depressive effect of ouabain for Na^+,K^+-ATPase mRNA expression may be tissue and isoform-specific. The mechanisms responsible for this effect are unclear, but may involve Na^+,K^+-ATPase inhibition (Clausen & Everts, 1991), with likely membrane depolarisation. However, since high $[K^+]_e$ and presumably the resulting membrane depolarisation, had no effect on Na^+,K^+-ATPase isoform mRNA expression in the present study, the electrogenic effect of the Na^+,K^+-ATPase appears unlikely to be important for β_2 and β_3 mRNA expression in isolated rat skeletal muscle.

7.4.6 Veratridine and monensin did not increase muscle Na^+ content or Na^+,K^+-ATPase isoform mRNA expression

Surprisingly, neither veratridine nor monensin significantly increased muscle Na^+ content in the present study. However, there was an apparent small increase (~14%) in muscle Na^+ content with both veratridine and monensin, with the lack of significance possibly reflecting a type II error. Furthermore, a likely increase in $[Na^+]_i$ may have been missed due to Na^+ contamination from other cellular compartments.

Neither veratridine nor monensin increased mRNA expression of any of the Na^+,K^+-ATPase isoforms. Since muscle Na^+ content was not significantly elevated with either veratridine or monensin, these findings cannot support or refute the suggestion with ouabain that elevated $[Na^+]_i$ does not increase Na^+,K^+-ATPase mRNA expression in isolated rat skeletal muscle. However, both veratridine and monensin actually reduced β_3 mRNA expression and demonstrated tendencies towards reducing β_2 mRNA expression.

These findings are consistent with the reductions in β_2 and β_3 mRNA with ouabain, where muscle Na^+ content was significantly elevated. It therefore appears that the reduction in β_2 and β_3 mRNA expression with ouabain, veratridine and monensin may not be dependent on $[Na^+]_i$. In contrast, since there was a tendency for increased Na^+ content with veratridine and monensin, it appears more likely that β_2 and β_3 mRNA expression may in fact be negatively related to $[Na^+]_i$. Nonetheless, the cellular changes involved in reducing β_2 and β_3 mRNA expression are unclear and clearly require further investigation.

7.4.7 Caffeine increased Na^+,K^+-ATPase α_1, α_2 and β_1 mRNA expression

Caffeine was used to induce an elevation in $[Ca^{2+}]_{cyto}$. Caffeine stimulates SR Ca^{2+} release via the RyR (Lamb *et al.*, 2001), by increasing the sensitivity of the RyR to activation by Ca^{2+} and ATP (Rousseau *et al.*, 1988). Caffeine also activates the sarcolemmal L-type Ca^{2+} channels (Ortega *et al.*, 1997). As a consequence of both of these actions, $[Ca^{2+}]_{cyto}$ is increased. $[Ca^{2+}]_{cyto}$ was unable to be measured in the present study, but is likely to have increased, since the concentration of caffeine used in the present study (5 mM) has previously been shown to elevate $[Ca^{2+}]_{cyto}$ in single fibres from rat EDL muscle (Fryer & Neering, 1989). $[Ca^{2+}]_{cyto}$ was also increased with 5 mM caffeine in rat epitrochlearis muscle (Terada *et al.*, 2003), which is also predominantly composed of type II fibres (80-85%) (Nesher *et al.*, 1980). Some evidence for an increase in $[Ca^{2+}]_{cyto}$ with caffeine in the present study was the increase in twitch force. Furthermore, the effects of caffeine on Na^+,K^+-ATPase isoform mRNA expression were not due to any caffeine-induced contracture since there was no increase in baseline tension with application of caffeine.

Caffeine induced an elevation in α_1, α_2 and β_1 isoform mRNA expression, but had no significant effect on any of α_3, β_2 or β_3 mRNA expression in isolated rat EDL muscle. As discussed above, caffeine-induced increases in Na^+,K^+-ATPase isoform mRNA expression are likely to involve an increase in $[Ca^{2+}]_{cyto}$. This would be consistent with the 4.0-fold

elevations in both α_1 and β_1 mRNA expression found in rat kidney cells found after 1 h incubation in solution containing 1.0 μM Ca^{2+} compared to that containing 0.1 μM (Rayson, 1991). The increases in α_1 and β_1 mRNA expression in rat kidney cells were transient, returning to control levels (in a solution containing 0.1 μM Ca^{2+}) after 3 h incubation in 1.0 μM Ca^{2+} (Rayson, 1991). Since measurements from this study were made at 3 h following 30 min incubation in caffeine, it is possible that even greater elevations in isoform mRNA expression may have occurred in the 0-3 h period following incubation.

If the mechanisms responsible for the caffeine-induced increases in Na^+,K^+-ATPase isoform mRNA expression involves an increase in $[Ca^{2+}]_{cyto}$, the pathways by which elevated $[Ca^{2+}]_{cyto}$ may regulate Na^+,K^+-ATPase isoform mRNA expression remain to be investigated. A prime candidate may be the Ca^{2+}-calmodulin complex, the inhibitor of which completely blocked the caffeine-induced increases in the transcription factors MEF2A and MEF2D in L6 myocytes (Ojuka *et al.*, 2002). Indeed, in cardiac myocytes, the Ca^{2+}-calmodulin complex was involved in the ouabain-induced changes in Na^+,K^+-ATPase α_3 and β_1 mRNA expression (Huang *et al.*, 1997; Kometiani *et al.*, 2000). A second possibility may involve the inhibition of phosphodiesterase activity with caffeine, leading to an increase in cyclic AMP (cAMP) (Lindinger *et al.*, 1996). cAMP has been shown to enhance the transcriptional activation of specific genes, including the cAMP response element-binding protein (CREB) (Matthews *et al.*, 1994; Sun *et al.*, 1994). Furthermore, Na^+,K^+-ATPase stimulation induced by β_2-agonists such as salbutamol (Buchanan *et al.*, 2002) and CGRP (Andersen & Clausen, 1993) appears to be mediated by cAMP pathways (Clausen, 1986). Nonetheless, further research is clearly required to identify the exact mechanisms and subsequent pathways by which caffeine is involved in Na^+,K^+-ATPase isoform transcriptional regulation in rat skeletal muscle.

7.4.8 High $[K^+]_e$ had no effect on isoform mRNA expression

This study demonstrated that when the elevated $[K^+]_e$ conditions that occur during exercise and electrical stimulation were mimicked in isolated muscles, there was no significant effect on Na^+,K^+-ATPase α_1-α_3 or β_1-β_3 mRNA expression. Importantly, the $[K^+]_e$ of 13 mM used in the present study is close to the physiological range (11-12 mM) found in skeletal muscle following intense exercise in humans (Green *et al.*, 2000; Nordsborg *et al.*, 2003b) and electrical stimulation in rabbit skeletal muscle (Hnik *et al.*, 1976). This $[K^+]$ was specifically used as this concentration has recently been shown to depolarise rat EDL muscle from –82 mV to –56 mV (Hansen, A.K., Clausen, T. & Nielsen, O.B., unpublished observations). In isolated rat EDL, Na^+,K^+-ATPase activity with 13 mM K^+ was not significantly different to that with 5.9 mM K^+ (307 ± 23 vs. 301 ± 45 nmol.(g wet wt)$^{-1}$) (Everts & Clausen, 1992; Hansen, A.K., Clausen, T. & Nielsen, O.B., unpublished observations). Thus the lack of effect of high $[K^+]_e$ on Na^+,K^+-ATPase mRNA expression in the present study is consistent with the lack of effect of high $[K^+]_e$ on Na^+,K^+-ATPase activity.

Low $[K^+]_e$ (0.65 mM) increased non-isoform specific Na^+,K^+-ATPase α mRNA expression by 3.5-fold in rat liver cells (Pressley *et al.*, 1988), while an even lower $[K^+]$ (0.25 mM) induced a 3.0-fold increase in each of α and β mRNA expression in canine kidney cells (Bowen & McDonough, 1987). However, with such a low $[K^+]_e$, the membrane potential would actually have become hyperpolarized (Khuri *et al.*, 1992) rather than depolarised, which occurs with repeated muscle contractions (Balog *et al.*, 1994). Thus, hyper- but not hypo-polarisation may influence muscle Na^+,K^+-ATPase mRNA expression.

7.4.9 NAC had opposing effects on isoform mRNA expression in resting and stimulated muscle

In a striking finding, this study demonstrated that 90 s of high-frequency electrical stimulation increased α_1, α_2 and α_3 mRNA expression, but that these increases were abolished with application of NAC. This finding is particularly intriguing given that ROS, which are scavenged by NAC, induces differential effects of muscle force development at rest and during repeated muscle contractions. ROS at low levels has been shown to be necessary for optimal contractile function at rest, since endogenous antioxidants reduced twitch characteristics in rat diaphragm muscle (Reid *et al.*, 1993). However, during electrical stimulation, ROS accelerate the development of muscle fatigue (Jackson *et al.*, 1985; Reid *et al.*, 1992).

The cellular processes involved in the opposing effects of NAC on Na^+,K^+-ATPase isoform transcriptional regulation in resting and stimulated muscles are not clear. Effects of NAC on muscle during repeated muscle contractions, include scavenging of ROS (Aruoma *et al.*, 1989), increasing intracellular cysteine and glutathione levels (Medved *et al.*, 2004b), and maintaining the cellular environment in a reduced state. The latter finding was implied by the elevation in the reduced forms of muscle cysteine and glutathione predominantly accounting for the elevation in total muscle cysteine and glutathione with NAC (Medved *et al.*, 2004b). Consequently any of these may be involved. Since the effects of NAC on isoform mRNA expression were opposing in resting and stimulated muscles, these findings suggest that either none of these effects appear to be involved in isoform transcriptional regulation, or that that separate and possibly independent mechanisms are involved in the transcriptional regulation of the Na^+,K^+-ATPase isoforms by NAC in resting and stimulated muscles.

It is unlikely that scavenging of ROS was involved in the increase in isoform mRNA expression with NAC at rest. This is because the effects of NAC on ROS would be minimal due to the low levels of ROS at rest (Reid *et al.*, 1993). A more likely explanation is the more reduced cellular environment that is thought to result with NAC treatment (Medved *et al.*, 2004b). In resting humans, intravenous infusion of NAC increased the concentrations of the reduced forms of NAC and cysteine in each of muscle, whole blood, plasma and red blood cells (Medved *et al.*, 2003; Medved *et al.*, 2004a; Medved *et al.*, 2004b). The effects of NAC on the redox environment in resting muscle are unknown. However these findings suggest that at least in blood, NAC induces a more reduced environment. The Na^+,K^+-ATPase has been suggested to be under redox modulation in skeletal muscle derived L6 cells, since 25 μM tert-butylhydroperoxide (TBOOH) increased ouabain-suppressible K^+-uptake, but 75 and 200 mM TBOOH inhibited ouabain-suppressible K^+-uptake (Sen *et al.*, 1995). It is therefore likely that cellular redox environment may be involved in Na^+,K^+-ATPase isoform transcriptional regulation in resting muscle. Indeed, several redox-sensitive genes have been identified, such as c-fos and c-jun, early growth factor response 1 and nuclear factor kB, and each display reduced DNA binding in the oxidised state (Abate *et al.*, 1990; Toledano & Leonard, 1991; Huang & Adamson, 1993).

A more reduced cellular environment does not appear to be involved in Na^+,K^+-ATPase transcriptional regulation during electrical stimulation, since electrical stimulation actually decreased isoform mRNA expression in NAC treated muscles. Instead, scavenging of ROS may be involved since NAC would have had a greater effect on scavenging ROS during electrical stimulation than at rest, due to ROS levels being much greater with repeated muscle contractions (Jackson *et al.*, 1985; Reid *et al.*, 1992; Bailey *et al.*, 2003). Thus, the depressing effect of electrical stimulation for isoform mRNA expression in NAC treated

muscles may have reflected enhanced scavenging of ROS. Further studies are clearly required to investigate these possibilities, as well as to determine the physiological significance of the contrasting effects of NAC in resting and stimulated muscles.

In conclusion, the relative expression of the rat α_2, β_1 and β_3 gene transcripts differed between EDL and soleus muscles, while the effects of three bouts of high-frequency electrical stimulation on Na^+,K^+-ATPase isoform mRNA expression was both isoform- and muscle-specific, with increases in α isoform mRNA only in the EDL muscle. Furthermore, the transcription of the Na^+,K^+-ATPase isoforms appear to be regulated by different stimuli in rat skeletal muscle, including the cellular changes associated with each of caffeine (such as elevated $[Ca^{2+}]_{cyto}$) and NAC.

CHAPTER 8: GENERAL DISCUSSION

8.1 Summary of major findings

This thesis provides several novel findings regarding the expression, the modulatory effects of acute and chronic exercise, as well as the electrical stimulation effects on the Na^+,K^+-ATPase isoforms in skeletal muscle. Furthermore, the thesis explored several factors regulating Na^+,K^+-ATPase isoform transcription and translation in skeletal muscle. A summary of the findings in human skeletal muscle is shown in Table 8.1, and for rat skeletal muscle, in Table 8.2. First, human skeletal muscle expressed three α and three β Na^+,K^+-ATPase isoforms (Study 1). Second, studies with brief intense, prolonged submaximal and high-intensity exercise showed that the acute exercise effects on Na^+,K^+-ATPase isoform mRNA and protein expression were isoform-specific and depended on the exercise stimulus (Studies 1-3). Third, contrasting effects of long-term training on Na^+,K^+-ATPase regulation were seen, with lower isoform mRNA expression, but higher Na^+,K^+-ATPase content and maximal activity (Study 4). Fourth, genders differed in isoform-specific Na^+,K^+-ATPase mRNA expression, but not in Na^+,K^+-ATPase content or maximal activity (Study 4). Fifth, both rat EDL and soleus muscle expressed six Na^+,K^+-ATPase isoform gene transcripts (Study 5). Sixth, the relative expression of three Na^+,K^+-ATPase isoform gene transcripts varied between EDL and soleus muscles (Study 5). Seventh, the effects of electrical stimulation on Na^+,K^+-ATPase isoform mRNA expression were both isoform- and muscle-specific (Study 5). Eighth, Na^+,K^+-ATPase transcription appear to be regulated by different stimuli in rat skeletal muscle, including the cellular changes associated with each of caffeine (such as elevated $[Ca^{2+}]_{cyto}$) and ROS (Study 5). These major findings are now discussed.

Table 8.1 Expression of, and acute and chronic exercise effects on Na^+,K^+-ATPase isoform mRNA and protein expression in human skeletal muscle

	Expression		Acute exercise effects						Chronic exercise effects			
			6 min intense		55 min submaximal		40 min intense		3 wks intense		3-15 yr endurance	
	Transcript	Protein	Study 1		Study 2		Study 3		Study 3		Study 4	
Isoform	detected	detected	mRNA	Protein	mRNA	Protein	mRNA	Protein	mRNA	Protein	mRNA	
α_1	Yes	Yes	↑ (↑)	NS	↑ (NS)	NS	↑	NS	NS	NS	↓	
α_2	Yes	Yes	↑ (↑)	NS	NS (NS)	NS	↑	NS	NS	NS	NS	
α_3	Yes	Yes	↑ (↑)	NS	↑ (NS)	↑	↑	NS	↑	NS	↓	
α_4	No											
β_1	Yes	Yes	NS (↑)	NS	NS (NS)	NS	NS	NS	NS	NS	NS	
β_2	Yes	Yes	↑ (↑)	NS	↑ (NS)	NS	↑	NS	NS	NS	↓	
β_3	Yes	Yes	NS (↑)	NS	NS (NS)	NS	NS	NS	↑	NS	↓	

For exercise effect on mRNA for Study 1 and 2, data are immediate response (average post-exercise response). Blank, not measured; NS, no significant change.

Table 8.2 Expression of, and electrical stimulation and intervention effects on Na^+,K^+-ATPase isoform mRNA expression in rat skeletal muscle

		Relative mRNA expression		Electrical stimulation effects			Effects of different interventions on mRNA expression in EDL muscle					
				10x10s, 60Hz		90s, 60Hz						
Isoform	Transcript detection	EDL	Soleus	Sol	EDL	EDL	Ouabain	Veratridine	Monensin	Caffeine	High $[K^+]_e$	NAC + Stim
α_1	Yes	0.07±0.01	0.12±0.03	NS	↑	↑	NS	NS	NS	↑	NS	NS
α_2	Yes	2.67±0.28	1.73±0.21	NS	↑	↑	NS	NS	NS	↑	NS	↓
α_3	Yes	0.09±0.02	0.07±0.01	NS	↑	↑	NS	NS	NS	NS	NS	NS
α_4	No											
β_1	Yes	3.20±0.03	4.87±0.66	NS	NS	NS	NS	NS	NS	↑	NS	↓
β_2	Yes	0.21±0.03	0.29±0.10	NS	NS	NS	↓	NS	NS	NS	NS	↓
β_3	Yes	0.17±0.03	0.10±0.02	NS	NS	NS	↓	↓	↓	NS	NS	NS

Relative mRNA expression is normalised to CYC mRNA expression, and expressed in arbitrary units. Sol, Soleus; EDL, Extensor digitorum longus; Blank, not measured; NS, no significant change. NAC effect is compared to NAC + Rest.

8.2 Na^+,K^+-ATPase isoform expression in human and rat skeletal muscle

The human vastus lateralis, which is a mixed muscle comprised of relatively similar proportions of type I and type II fibres (Adams *et al.*, 1993), expressed each of the Na^+,K^+-ATPase α_1-α_3 and β_1-β_3 isoforms, as demonstrated at both gene transcript and protein levels (Studies 1-3). These findings were extended in Study 5, where expression of the Na^+,K^+-ATPase gene transcripts was investigated in rat EDL and soleus muscles, which are predominantly composed of type II and type I fibres, respectively (Delp & Duan, 1996; Bortolotto *et al.*, 2000). Each of the Na^+,K^+-ATPase α_1-α_3 and β_1-β_3 gene transcripts were also detected in both rat EDL and soleus muscles (Study 5). Thus, each of the Na^+,K^+-ATPase α_1-α_3 and β_1-β_3 gene transcripts are likely to also be expressed in both type I and type II fibres in human muscle, although this clearly requires further investigation.

The finding of three α isoforms and three β isoforms in human skeletal muscle raises the possibility of nine different combinations of $\alpha\beta$ heterodimers. Since the facilities for co-immunoprecitation were not available, it was unable to be confirmed in this thesis whether all nine different heterodimers do in fact exist. As such, it is not possible to speculate on whether the lack of increase in protein abundance of a given α subunit (except for in Chapter 4 where α_3 protein abundance was increased) was due to a lack of increase in the protein abundance of a given β subunit.

The Na^+,K^+-ATPase α_4 gene transcript was not detected in either human vastus lateralis (Study 2), or rat EDL or soleus muscles (Study 5). These results provide good evidence for the absence of the α_4 gene transcript in mammalian skeletal muscle. The α_4 gene transcript was previously reported in human skeletal muscle of unspecified origin (Keryanov & Gardner, 2002). However, that study used a less sensitive measurement for mRNA

analysis, suggesting that the detected transcript may have originated from cross-reactivity of other gene transcripts.

8.3 Acute effects of repeated muscle contractions on muscle Na^+,K^+-ATPase

8.3.1 Isoform mRNA expression

8.3.1.1 Exercise effects

The Na^+,K^+-ATPase gene transcripts appear very responsive to exercise, with increases in each of α_1, α_2, α_3 and β_2 mRNA with brief intense exercise (Study 1), α_1, α_3 and β_2 mRNA with prolonged submaximal exercise (Study 2), and α_1, α_2, α_3 (Pre-HIT) and β_2 mRNA (Post-HIT) with high-intensity, intermittent exercise (Study 3). Furthermore, intense exercise elevated the average post-exercise mRNA expression of all six Na^+,K^+-ATPase isoforms present in human muscle (Study 1). In contrast, there was no effect of submaximal exercise on the average post-exercise mRNA expression of any of the Na^+,K^+-ATPase isoforms (Study 2). Thus, Na^+,K^+-ATPase isoform mRNA responsiveness appears to be less with submaximal exercise than intense exercise. To explore this possibility further, the average post-exercise mRNA expression following intense exercise (Study 1) was contrasted to that following submaximal exercise (Study 2, Figure 8.1). The average post-exercise mRNA expression of each of the α_1, α_2, α_3 and β_1 isoforms was significantly less ($P < 0.04$) after submaximal exercise. Different sample groups could not explain the variation in mRNA expression since the average post-exercise mRNA expression was not significantly correlated to physical characteristics or physiological factors underpinning exercise $\dot{V}O_{2peak}$ or time to fatigue, when results were pooled for both studies. Exercise intensity may therefore be an important determinant for Na^+,K^+-ATPase isoform mRNA responsiveness in human skeletal muscle. Whilst this clearly requires further investigation, exercise intensity has been previously shown to be important for the mRNA

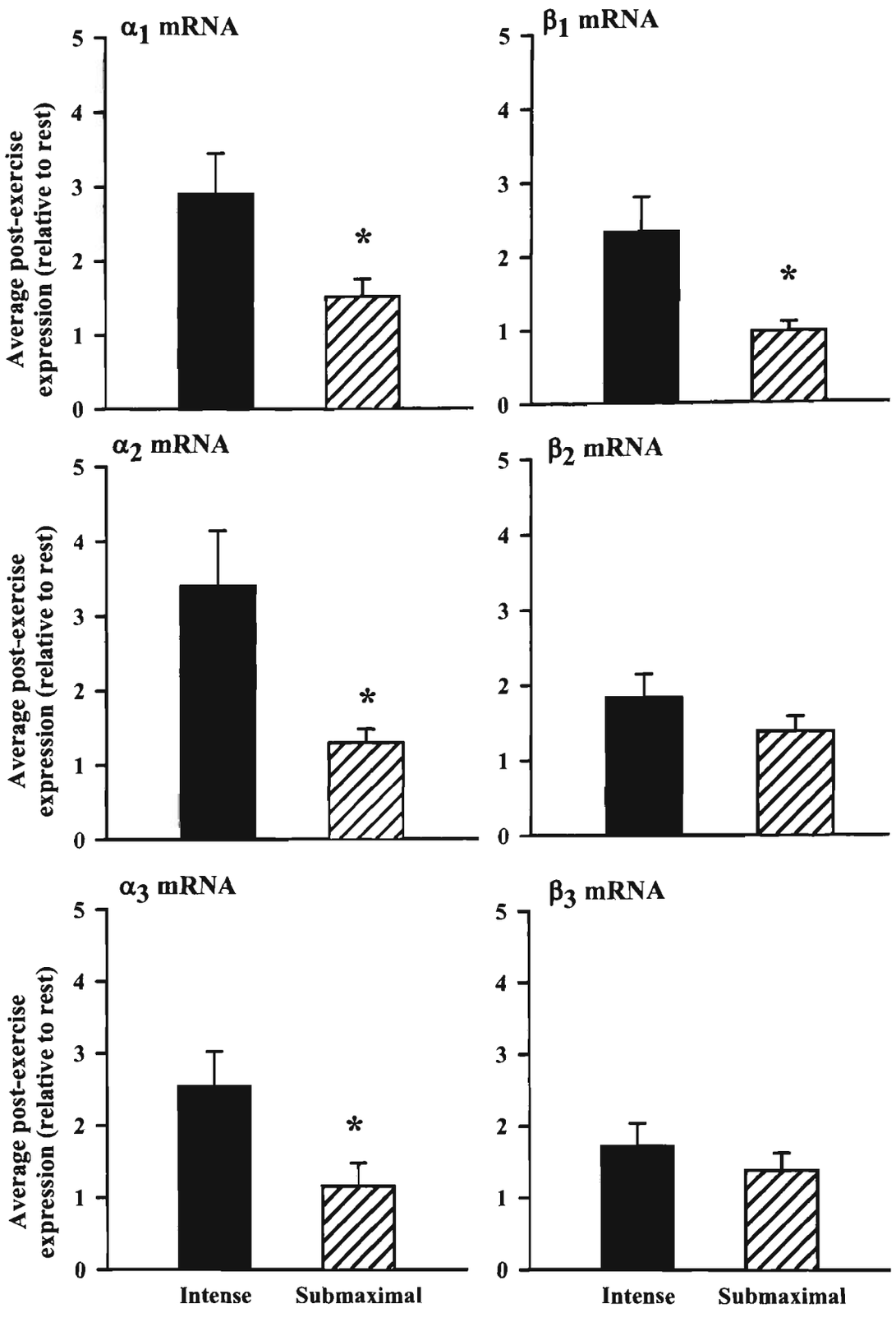


Figure 8.1 Comparison of average post-exercise response in Na^+,K^+-ATPase α_1, α_2, α_3, β_1, β_2 and β_3 mRNA expression following either intense or submaximal exercise. Data relative to the resting mRNA expression (1.00). Data are mean ± SEM, n = 14 for intense, n = 11 for submaximal. * $P < 0.04$ lower than intense.

responsiveness to exercise of heat shock proteins (Liu *et al.*, 2004) and of numerous metabolic genes (Hildebrandt *et al.*, 2003).

8.3.1.2 Electrical stimulation effects

The effects of three bouts (10 s every 10 min) of high-intensity (60 Hz) electrical stimulation on Na^+,K^+-ATPase mRNA expression were isoform- and muscle-specific, with increases in α_1, α_2 and α_3 mRNA in rat EDL muscle, and no effect on the mRNA expression of any of the isoforms in rat soleus muscle (Study 5). An increase in α_1-α_3 mRNA, but not β mRNA, was also seen following 90 s of high-intensity (60 Hz) electrical stimulation in rat EDL muscle (Study 5).

Thus, expression of each of the Na^+,K^+-ATPase α (α_1, α_2 and α_3) gene transcripts was elevated (with the exception of α_2 mRNA following prolonged submaximal exercise) in response to each of the three acute exercise and two electrical stimulation protocols used in this thesis. The consistent adaptability of these α gene transcripts to muscle contraction may reflect their catalytic nature, predisposing them to respond to fluctuations in unknown transcription factors.

8.3.2 Isoform protein abundance

There was no effect of either ~6 min or 40 min of intense exercise on isoform protein abundance (Studies 1 and 3), however, ~55 min of submaximal exercise increased α_3, and tended to increase β_1 protein abundance (Study 2). These studies suggest that the responsiveness of the Na^+,K^+-ATPase proteins to acute exercise may depend on exercise duration. The greater isoform protein response to ~55 min of exercise does not reflect a larger exercise stimulus, since total work performed was actually greater with 40 min of intermittent exercise (709.1 ± 18.4 kJ) than with ~55 min of continuous exercise (691.2 ± 121.3 kJ).

Although expression of the Na^+,K^+-ATPase isoform gene transcripts are elevated with exercise, isoform protein expression is much less responsive, suggesting an important role for Na^+,K^+-ATPase translational regulation.

8.3.3 Na^+,K^+-ATPase content

The acute exercise effects on muscle Na^+,K^+-ATPase content and maximal activity were only presented in this thesis for Study 2. Measurements of Na^+,K^+-ATPase content and maximal activity were also made for Study 1 and Study 3 by fellow colleagues and are presented in the Appendices (Study 1, Appendix 2.12 and 2.13; Study 3, Appendix 4.7 and 4.8). Their results are cited in this General Discussion.

There was no effect of either ~6 or 55 min of exercise on muscle Na^+,K^+-ATPase content, including in the 24 h period following exercise, as measured by [^{3}H]-ouabain binding (Studies 1-3), consistent with previous studies with exercise ranging from ~20-75 min (Fowles *et al.*, 2002b; Leppik *et al.*, 2004; Aughey *et al.*, 2005). These findings therefore suggest that exercise of up to 1 h in duration does not affect Na^+,K^+-ATPase content in human muscle. In contrast, ~10 h of running increased muscle Na^+,K^+-ATPase content by 13% (Overgaard *et al.*, 2002), suggesting that only acute exercise of ultra-duration is sufficient to increase Na^+,K^+-ATPase content in human muscle.

8.3.4 Maximal Na^+,K^+-ATPase activity

The magnitude of the depression in maximal Na^+,K^+-ATPase activity, as measured by maximal 3-*O*-MFPase activity, immediately following acute exercise might be dependent on the duration of exercise performed, with ~6, 40 and 55 min of exercise depressing maximal Na^+,K^+-ATPase activity by ~10, 13 and 19%, respectively (Studies 1-3). These findings are consistent with a recent study showing a progressive decline in maximal Na^+,K^+-ATPase activity during fatiguing submaximal exercise (Leppik *et al.*, 2004). In that study, the decline in maximal Na^+,K^+-ATPase activity was greater at fatigue (~72 min)

than at 45 min, which in turn was greater than at 10 min of exercise (Leppik *et al.*, 2004). In contrast, another study found no difference between maximal Na^+,K^+-ATPase activity at 30 and 90 min of submaximal exercise (Sandiford *et al.*, 2004). The reason for these discrepancies between studies is unknown. Further research is therefore required to determine whether exercise duration may be important for the magnitude of the decline in maximal Na^+,K^+-ATPase activity with acute exercise.

8.4 Training effects on muscle Na^+,K^+-ATPase

8.4.1 Isoform mRNA expression

Three wks of high-intensity, intermittent training (HIT) significantly elevated resting Na^+,K^+-ATPase α_3 and β_3 mRNA expression (Study 3). The lack of effect for the α_1, α_2 and β_1 isoforms was consistent with that found following 5.5 wks of high-intensity, intermittent training (Nordsborg *et al.*, 2003a). However, in perhaps the most surprising finding of this thesis, chronically endurance trained athletes demonstrated lower skeletal muscle α_1, α_3, β_2 and β_3 mRNA expression than recreationally active subjects. Thus, Na^+,K^+-ATPase isoform mRNA expression is elevated with short-term intense training, but appears to be chronically depressed in individuals that have undergone 7 ± 1 yrs of endurance training. The reasons for these contrasting findings for isoform mRNA expression between individuals that have undergone either short- or long-term training are unknown and require further investigation.

8.4.2 Isoform protein abundance

There was no effect of 3 wks of HIT on isoform protein abundance in the skeletal muscle of already well-trained athletes (Study 3). This finding contrasts the increase in α_1 and α_2 protein abundance evident following 7 wks of HIT in previously habitually active subjects (Nielsen *et al.*, 2004a). These differences may be explained by variances in the fitness

status of the subjects utilised. Endurance training has been shown to dampen the exercise-induced mRNA responsiveness of vascular endothelial growth factor (VEGF) (Jensen *et al.*, 2004) and uncoupling protein-3 (UCP-3) (Noland *et al.*, 2003). Thus, previous endurance training may have blunted the responsiveness of the Na^+,K^+-ATPase isoform proteins to high-intensity, intermittent training.

8.4.3 Na^+,K^+-ATPase content

It is well established that physical training, ranging in length from 6 d to 5 mo, increases Na^+,K^+-ATPase content in previously untrained human muscle (Green *et al.*, 1993; McKenna *et al.*, 1993; Green *et al.*, 1999a; Green *et al.*, 2004). However, 3 wks of HIT had no significant effect on muscle Na^+,K^+-ATPase content in already well-trained endurance athletes (Appendix 4.7). This lack of change may be explained by the finding in this thesis that muscle Na^+,K^+-ATPase content in chronically endurance trained athletes is not significantly correlated to the years of training. This suggests that training induces an initial increase in muscle Na^+,K^+-ATPase content, that is not further augmented with additional training. Thus, muscle Na^+,K^+-ATPase content was likely to have already been elevated in these athletes prior to HIT, and was therefore not responsive to additional short-term high-intensity training.

8.4.4 Maximal Na^+,K^+-ATPase activity

Chronically endurance trained athletes demonstrated higher maximal Na^+,K^+-ATPase activity than recreationally active subjects, but this was also not significantly correlated with years of endurance training (Study 4). This suggested that maximal Na^+,K^+-ATPase activity was not further increased with additional training. In contrast, 3 wks of HIT increased maximal Na^+,K^+-ATPase activity in already well-trained athletes, who were likely to already have increased muscle maximal activity (Appendix 4.8). The reasons for this discrepancy are unknown, but may reflect the intense nature of the HIT. Thus,

maximal Na^+,K^+-ATPase activity in endurance trained athletes may not be further increased with additional endurance training, but may be responsive to intense training.

In Study 2, a single bout of fatiguing exercise induced an immediate depression in maximal Na^+,K^+-ATPase activity in skeletal muscle from untrained subjects, that was recovered by 3 and 24 h post-exercise. This depression in maximal Na^+,K^+-ATPase activity following fatiguing exercise does not appear to reflect the training status of subjects, since a single bout of incremental cycling was recently shown to depress maximal Na^+,K^+-ATPase activity in the skeletal muscle of well-trained endurance athletes (Fraser *et al.*, 2002; Aughey *et al.*, 2005). Thus, the finding of an increased maximal Na^+,K^+-ATPase activity in the skeletal muscle of ETM in the present study does not appear to reflect inhibition of the depressing effects of acute exercise for maximal Na^+,K^+-ATPase activity, but rather the increase in muscle Na^+,K^+-ATPase content. Thus, a greater number of functional Na^+,K^+-ATPase enzymes in the skeletal muscle of ETM enhances the capacity for Na^+,K^+-ATPase activity. Indeed, muscle Na^+,K^+-ATPase content and maximal Na^+,K^+-ATPase activity were higher by the same percentage (16%) in ETM than RAM.

8.5 Intracellular signals involved in Na^+,K^+-ATPase transcriptional regulation in rat EDL muscle

Na^+,K^+-ATPase isoform transcriptional regulation in rat EDL muscle appears to involve the cellular changes associated with caffeine (such as elevated $[Ca^{2+}]_{cyto}$) and NAC incubation (such as ROS scavenging), but not with elevated $[Na^+]_i$, or with membrane depolarisation induced by increased $[K^+]_e$ (Figure 8.2). Furthermore, the transcriptional regulation imposed by NAC appears to be different at rest and during electrical stimulation and may involve the cellular redox environment and scavenging of ROS, respectively.

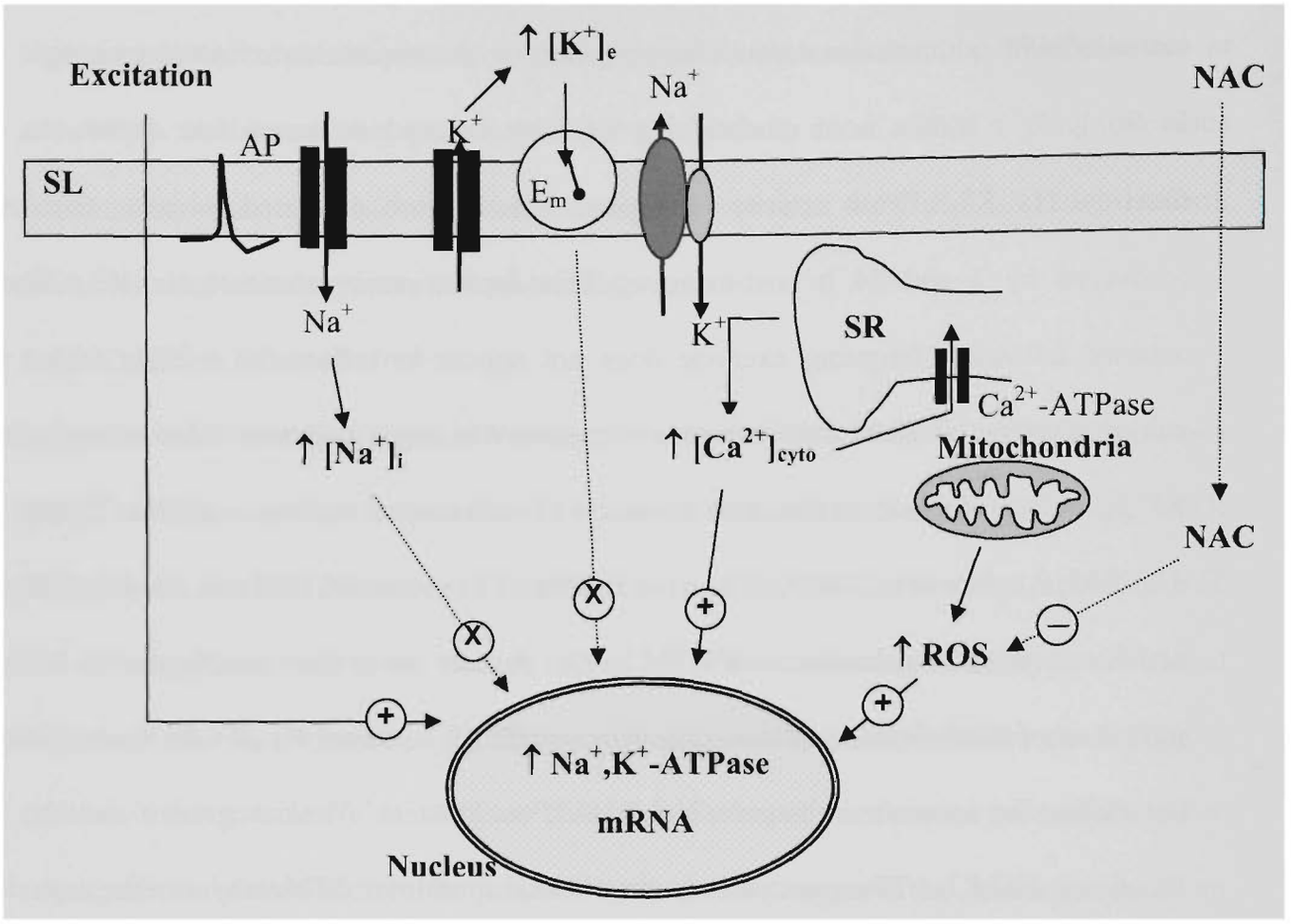


Figure 8.2 Excitation and consequent intracellular effects on Na^+,K^+-ATPase isoform mRNA expression in rat EDL muscle. SL, sarcolemmal membrane; AP, action potential; SR, sarcoplasmic reticulum; $[K^+]_e$, extracellular $[K^+]$; $[Na^+]_i$, intracellular $[Na^+]$; $[Ca^{2+}]_{cyto}$, cytosolic $[Ca^{2+}]$; ROS, Reactive oxygen species; NAC, *N*-acetylcysteine. ⊕, stimulatory effect; ⊖, inhibitory effect; ⊗, no significant increase; E_m, membrane potential. \, membrane depolarisation.

These findings suggest that the intracellular signals responsible for the increase in Na^+,K^+-ATPase isoform mRNA expression with high-frequency electrical stimulation in rat EDL muscle (Study 5) may involve an increase in $[Ca^{2+}]_{cyto}$ and/or ROS. Indeed, high-frequency electrical stimulation has previously been shown to induce an elevation in both $[Ca^{2+}]_{cyto}$ in isolated mouse single fibres from flexor brevis muscle (Westerblad *et al.*, 1993) and in ROS in rat diaphragm muscle (Reid *et al.*, 1992).

It is important to note that other factors not explored in this study may be responsible for inducing the increased mRNA expression of the other Na^+,K^+-ATPase isoforms observed after exercise (Studies 1-3). This may include hormones such as thyroid hormone, since levels of plasma T_4 increase during exercise (O'Connell *et al.*, 1979) and T_3 treatment has been shown to increase the mRNA expression of the α_2 and β_2 isoforms in rat hindlimb muscle (Azuma *et al.*, 1993).

CHAPTER 9: CONCLUSIONS

A number of conclusions can be drawn from the results of this thesis:

Study 1, Chapter 3

i. Human vastus lateralis muscle obtained from healthy individuals expressed each of the Na^+,K^+-ATPase α_1, α_2, α_3, β_1, β_2 and β_3 isoforms, at both gene transcript and protein levels. This raises the possibility of nine different $\alpha\beta$ heterodimers in human skeletal muscle.

ii. Brief intense exercise increased mRNA expression of each of these six Na^+,K^+-ATPase isoforms, but was insufficient to elevate isoform protein abundance within a 24 h post-exercise period.

Study 2, Chapter 4

i. Prolonged submaximal exercise induced isoform-specific increases in Na^+,K^+-ATPase α_1, α_3 and β_2 mRNA expression and was a sufficient stimulus to elevate α_3 protein abundance.

ii. Prolonged submaximal exercise transiently depressed maximal Na^+,K^+-ATPase activity, but was insufficient to up-regulate Na^+,K^+-ATPase content, even in the 24 h period following exercise.

iii. This exercise bout also increased the mRNA expression of the transcription initiator gene RNAP II, but had no effect on the mRNA expression of key genes involved in protein translation, eIF-4E and 4E-BP1.

Study 3, Chapter 5

i. In already well-trained endurance athletes, a single bout of high-intensity, intermittent exercise increased α_1, α_2 and α_3 mRNA expression at Pre-HIT, and

also β_2 mRNA expression at Post-HIT, but had no effect on protein abundance of any of the Na^+,K^+-ATPase isoforms.

ii. Short-term high-intensity, intermittent training induced isoform-specific increases in resting Na^+,K^+-ATPase α_3 and β_3 mRNA expression, but had no effect on resting isoform protein abundance, in already well-trained endurance athletes. Furthermore, the improvements in peak power output with short-term high-intensity intermittent training were not associated with any significant alterations in the effect of acute exercise on mRNA and protein expression of the Na^+,K^+-ATPase isoforms.

Study 4, Chapter 6

i. In striking contrast, muscle Na^+,K^+-ATPase isoform α_1, α_3, β_2 and β_3 mRNA expression was much lower in chronic endurance trained athletes, compared to recreationally active subjects.

ii. Chronically endurance trained athletes demonstrated increased muscle Na^+,K^+-ATPase content, compared to recreationally active subjects. Importantly, this up-regulation was independent of years of training.

iii. Chronically endurance trained athletes demonstrated increased maximal Na^+,K^+-ATPase activity, compared to recreationally active subjects.

iv. Genders differed in isoform-specific Na^+,K^+-ATPase α_3 and β_3 mRNA expression, but not in Na^+,K^+-ATPase content or maximal activity.

Study 5, Chapter 7

i. In rats, the relative expression of three of the Na^+,K^+-ATPase gene transcripts (α_2, β_1, β_3) varied between soleus and EDL muscles.

ii. The effects of high-frequency, intermittent electrical stimulation on Na^+,K^+-ATPase isoform mRNA expression were both isoform- and muscle-specific, with increases in α_1, α_2 and α_3 mRNA expression in the EDL, but not in the soleus.

iii. mRNA expression of the Na^+,K^+-ATPase isoforms appeared to be regulated by different stimuli in rat skeletal muscle, including the cellular changes associated with caffeine (such as elevated $[Ca^{2+}]_{cyto}$) and NAC (such as altered redox state and scavenging of ROS).

CHAPTER 10: DIRECTIONS FOR FUTURE RESEARCH

In this thesis, characterisation of the Na^+,K^+-ATPase isoforms present in human skeletal muscle was limited to qualitative observations of relative expression/abundance. To extend these observations, future work needs to include semi-quantitative comparison of the relative abundance of each Na^+,K^+-ATPase gene transcript and protein to determine the importance of any exercise-induced changes in isoform expression. This could be achieved by the use of standard curves of known quantities of RNA or protein on each PCR run or western gel, respectively[a].

This thesis compared the relative abundance of the Na^+,K^+-ATPase gene transcripts between muscles predominantly composed of type I or type II fibres in rats. However, the relative abundance of both the Na^+,K^+-ATPase gene transcripts and proteins between muscles of different fibre-type in human skeletal muscle are unknown and warrant further investigation. Importantly, this information may improve our understanding of the physiological factors responsible for the lower mRNA expression observed in the vastus lateralis muscle of chronic endurance trained subjects (Study 4), as well as for the considerable inter-individual variability seen for Na^+,K^+-ATPase mRNA expression.

---

[a]In the week before this thesis was submitted for examination, the following paper was published, that quantified the relative abundance of the Na^+,K^+-ATPase isoform gene transcripts in human skeletal muscle: Nordsborg, N., Thomassen, M., Lundby, C., Pilegaard, H. & Bangsbo, J. (2005). Contraction induced increases in Na^+, K^+ -ATPase mRNA levels in human skeletal muscle are not amplified by activation of additional muscle mass. *Am J Physiol*, 00771.02004.

Exercise induced isoform-specific changes in both Na^+,K^+-ATPase mRNA and protein expression, suggesting specific physiological roles of the Na^+,K^+-ATPase isoforms. However, these roles have yet to be elucidated, which is required if we are to further our understanding of both the physiological adaptations occurring during acute and chronic exercise, as well as the rationale for these changes. The recent use of gene modified mice in determining roles for the α_1 and α_2 isoforms in muscle excitability and contractility (He *et al.*, 2001; Radzyukevich *et al.*, 2004) provides a good example of the techniques that are currently available for such an investigation in animal muscle.

In study 4 of this thesis, an apparent paradox was observed for chronically endurance trained subjects who demonstrated an increased content of Na^+,K^+-ATPase enzymes, but a lower mRNA expression of the Na^+,K^+-ATPase isoforms compared to recreationally active subjects. To further our understanding of the physiological mechanisms responsible for this apparent inconsistency, the relative protein abundance of the Na^+,K^+-ATPase isoforms between chronically endurance trained and recreationally active subjects needs to be determined.

In study 5, caffeine and NAC resulted in increased isoform mRNA expression in rat skeletal muscle. However, the exact physiological changes resulting from these interventions and involved in isoform transcriptional regulation are unknown. The effects of caffeine were likely to reflect increases in $[Ca^{2+}]_{cyto}$, however this assumption requires confirmation. This may include the use of specific Ca^{2+}-ionophores and measurement of $[Ca^{2+}]_{cyto}$ using specific Ca^{2+} dyes, such as Indo-1 (Lunde *et al.*, 2001) or Fura-2 (Westerblad *et al.*, 1990). Furthermore, the pathways of any Ca^{2+}-induced isoform transcriptional regulation need to be investigated, and may involve the use of inhibitors to block the formation of any Ca^{2+}-calmodulin complexes or to block phosphodiesterase activity. As discussed in Study 5, the differing effects of NAC on isoform mRNA

expression in resting and stimulated muscles were postulated to reflect the effects of NAC on cellular redox state and scavenging of ROS, respectively. Clearly, this area needs further investigation to confirm or refute these postulations, as well as to determine the subsequent pathways leading to Na^+,K^+-ATPase isoform transcriptional regulation.

The three interventions designed to increase $[Na^+]_i$; ouabain, veratridine and monensin did not increase the mRNA expression of any of the Na^+,K^+-ATPase isoforms. These findings suggested that Na^+,K^+-ATPase transcriptional regulation was independent of $[Na^+]_i$. However, a limitation to the study was that $[Na^+]_i$ was unable to be measured with the techniques available. Thus, future studies measuring the effects of each of these interventions on $[Na^+]_i$ are clearly required to verify that increased $[Na^+]_i$ has no effect on Na^+,K^+-ATPase mRNA expression in skeletal muscle.

REFERENCES

Abate C, Patel L, Rauscher FJ, 3rd & Curran T. (1990). Redox regulation of fos and jun DNA-binding activity in vitro. *Science* **249,** 1157-1161.

Ackermann U & Geering K. (1990). Mutual dependence of Na,K-ATPase α- and β-subunits for correct posttranslational processing and intracellular transport. *FEBS Letters* **269,** 105-108.

Adams GR, Hather BM, Baldwin KM & Dudley GA. (1993). Skeletal muscle myosin heavy chain composition and resistance training. *J Appl Physiol* **74,** 911-915.

Al-Khalili L, Yu M & Chibalin AV. (2003). Na^+,K^+-ATPase trafficking in skeletal muscle: insulin stimulates translocation of both α_1- and α_2-subunit isoforms. *FEBS Letters* **536,** 198-202.

Aledo J & Hundal H. (1995). Sedimentation and immunological analyses of GLUT4 and α2-Na,K-ATPase subunit-containing vesicles from rat skeletal muscle: evidence for segregation. *FEBS Letters* **376,** 211-215.

Andersen PH, Lund S, Schmitz O, Junker S, Kahn BB & Pedersen O. (1993). Increased insulin-stimulated glucose uptake in athletes: the importance of GLUT4 mRNA, GLUT4 protein and fibre type composition of skeletal muscle. *Acta Physiol Scand* **149,** 393-404.

Andersen SL & Clausen T. (1993). Calcitonin gene-related peptide stimulates active Na^+-K^+ transport in rat soleus muscle. *Am J Physiol* **264,** C419-429.

Armstrong CM. (1981). Sodium channels and gating currents. *Physiol Rev* **61,** 644-683.

Aruoma OI, Halliwell B, Hoey BM & Butler J. (1989). The antioxidant action of *N*-acetylcysteine: its reaction with hydrogen peroxide, hydroxyl radical, superoxide, and hypochlorous acid. *Free Radic Biol Med* **6,** 593-597.

Arystarkhova E, Gasparian M, Modyanov N & Sweadner K. (1992). Na,K-ATPase extracellular surface probed with a monoclonal antibody that enhances ouabain binding. *J Biol Chem* **267,** 13694-13701.

Arystarkhova E, Wetzel RK, Asinovski NK & Sweadner KJ. (1999). The γ subunit modulates Na^+ and K^+ affinity of the renal Na,K-ATPase. *J Biol Chem* **274,** 33183-33185.

Arystarkova E & Sweadner K. (1997). Tissue-specific expression of the Na,K-ATPase β3 subunit. *J Biol Chem* **272,** 22405 - 22408.

Arystarkova E & Sweadner KJ. (1996). Isoform-specific monoclonal antibodies to Na,K-ATPase α subunits. *J Biol Chem* **271,** 23407 - 23417.

Asano S, Tega, Y., Konishi, K., Fujioka, M. & Tageguchi, N. (1996). Functional expression of gastric H^+,K^+-ATPase and site-directed mutagenesis of the putative cation binding site and the catalytic center. *Journal of Biological Chemistry* **271,** 2740 - 2745.

Aughey RJ, Gore CJ, Hahn AG, Garnham AP, Clark SA, Petersen AC, Roberts AD & McKenna MJ. (2005). Chronic intermittent hypoxia and incremental cycling exercise independently depress muscle *in vitro* maximal Na^+-K^+-ATPase activity in well-trained athletes. *J Appl Physiol* **98,** 186-192.

Azuma KK, Hensley CB, Tang MJ & McDonough AA. (1993). Thyroid hormone specifically regulates skeletal muscle Na^+-K^+-ATPase α_2- and β_2-isoforms. *Am J Physiol Regul Integr Comp Physiol* **265,** C680-687.

Bailey DM, Davies B, Young IS, Jackson MJ, Davison GW, Isaacson R & Richardson RS. (2003). EPR spectroscopic detection of free radical outflow from an isolated muscle bed in exercising humans. *J Appl Physiol* **94,** 1714-1718.

Balog EM & Fitts RH. (1996). Effects of fatiguing stimulation on intracellular Na^+ and K^+ in frog skeletal muscle. *J Appl Physiol* **81,** 679-685.

Balog EM, Thompson LV & Fitts RH. (1994). Role of sarcolemma action potentials and excitability in muscle fatigue. *J Appl Physiol* **76,** 2157 - 2162.

Batkai S, Racz IB, Ivanics T, Toth A, Hamar J, Slaaf DW, Reneman RS & Ligeti L. (1999). An in vivo model for studying the dynamics of intracellular free calcium changes in slow- and fast-twitch muscle fibres. *Pflugers Arch* **438,** 665-670.

Baylor SM & Hollingworth S. (2003). Sarcoplasmic reticulum calcium release compared in slow-twitch and fast-twitch fibres of mouse muscle. *J Physiol* **551,** 125-138.

Beggah AT, Jaunin P & Geering K. (1997). Role of glycosylation and disulfide bond formation in the beta subunit in the folding and functional expression of Na,K-ATPase. *J Biol Chem* **272,** 10318-10326.

Beguin P, Crambert G, Monnet-Tschudi F, Uldry M, Horisberger JD, Garty H & Geering K. (2002). FXYD7 is a brain-specific regulator of Na,K-ATPase alpha 1-beta isozymes. *EMBO J* **21,** 3264-3273.

Beguin P, Hasler U, Beggah A, Horisberger JD & Geering K. (1998). Membrane integration of Na,K-ATPase α-subunits and β-subunit assembly. *J Biol Chem* **273,** 24921-24931.

Beguin P, Wang X, Firsov D, Puoti A, Claeys D, Horisberger JD & Geering K. (1997). The gamma subunit is a specific component of the Na,K-ATPase and modulates its transport function. *EMBO J* **16,** 4250-4260.

Belin D, Bost S, Vassalli JD & Strub K. (1996). A two-step recognition of signal sequences determines the translocation efficiency of proteins. *EMBO J* **15,** 468-478.

Bellemare F & Garzaniti N. (1988). Failure of neuromuscular propagation during human maximal voluntary contraction. *J Appl Physiol* **64,** 1084-1093.

Bezanilla F, Caputo C, Gonzalez-Serratos H & Venosa RA. (1972). Sodium dependence of the inward spread of activation in isolated twitch muscle fibres of the frog. *J Physiol* **223,** 507-523.

Bigland-Ritchie B, Kukulka CG, Lippold OC & Woods JJ. (1982). The absence of neuromuscular transmission failure in sustained maximal voluntary contractions. *J Physiol* **330,** 265-278.

Blanco G & Mercer R. (1998). Isozymes of the Na-K-ATPase: heterogeneity in structure, diversity in function. *Am J Physiol* **275,** F633-650.

Bofill P, Goecke IA, Bonilla S, Alvo M & Marusic ET. (1994). Tissue-specific modulation of Na, K-ATPase α-subunit gene expression in uremic rats. *Kidney Int* **45,** 672-678.

Boivin M, Camirand A, Carli F, Hoffer LJ & Silva JE. (2000). Uncoupling protein-2 and -3 messenger ribonucleic acids in adipose tissue and skeletal muscle of healthy males: variability, factors affecting expression, and relation to measures of metabolic rate. *J Clin Endocrinol Metab* **85,** 1975-1983.

Bolster DR, Kubica N, Crozier SJ, Williamson DL, Farrell PA, Kimball SR & Jefferson LS. (2003). Immediate response of mammalian target of rapamycin (mTOR)-

mediated signalling following acute resistance exercise in rat skeletal muscle. *J Physiol* **553,** 213-220.

Bortolotto SK, Cellini M, Stephenson DG & Stephenson GM. (2000). MHC isoform composition and Ca^{2+}- or Sr^{2+}-activation properties of rat skeletal muscle fibers. *Am J Physiol* **279,** C1564-1577.

Bouclin R, Charbonneau E & Renaud JM. (1995). Na^+ and K^+ effect on contractility of frog sartorius muscle: implication for the mechanism of fatigue. *Am J Physiol* **268,** C1528-1536.

Bovia F & Strub K. (1996). The signal recognition particle and related small cytoplasmic ribonucleoprotein particles. *J Cell Sci* **109,** 2601-2608.

Bowen JW & McDonough A. (1987). Pretranslational regulation of Na-K-ATPase in cultured canine kidney cells by low K^+. *Am J Physiol* **252,** C179-189.

Buchanan R, Nielsen OB & Clausen T. (2002). Excitation- and β2-agonist-induced activation of the Na^+-K^+ pump in rat soleus muscle. *J Physiol* **545,** 229-240.

Bundgaard H & Kjeldsen K. (2002). Potassium depletion increases potassium clearance capacity in skeletal muscles in vivo during acute repletion. *Am J Physiol* **283,** C1163-1170.

Bustin SA. (2000). Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *J Mol Endocrinol* **25,** 169-193.

Cairns SP, Buller SJ, Loiselle DS & Renaud JM. (2003). Changes of action potentials and force at lowered $[Na^+]_o$ in mouse skeletal muscle: implications for fatigue. *Am J Physiol* **285,** C1131-1141.

Cairns SP, Flatman JA & Clausen T. (1995). Relation between extracellular $[K^+]$, membrane potential and contraction in rat soleus muscle: modulation by the Na^+-K^+ pump. *Pflugers Arch* **430,** 909-915.

Cairns SP, Hing WA, Slack JR, Mills RG & Loiselle DS. (1997). Different effects of raised $[K^+]_o$ on membrane potential and contraction in mouse fast- and slow-twitch muscle. *Am J Physiol* **273,** C598-611.

Caplan M, Forbush B, 3d, Palade G & Jamieson J. (1990). Biosynthesis of the Na,K-ATPase in madin-darby canine kidney cells. Activation and cell surface delivery. *J Biol Chem* **265,** 3528-3534.

Carraro F, Stuart CA, Hartl WH, Rosenblatt J & Wolfe RR. (1990). Effect of exercise and recovery on muscle protein synthesis in human subjects. *Am J Physiol* **259,** E470-476.

Chin ER & Allen DG. (1996). The role of elevations in intracellular [Ca^{2+}] in the development of low frequency fatigue in mouse single muscle fibres. *J Physiol* **491,** 813-824.

Chin ER & Green HJ. (1993). Na^+-K^+ ATPase concentration in different adult rat skeletal muscles is related to oxidative potential. *Can J Physiol Pharmacol* **71,** 615-618.

Chow D & Forte J. (1995). Functional significance of the β-subunit for heterodimeric P-type ATPases. *J Exp Biol* **198,** 1-17.

Clark SA, Chen Z-P, Murphy KT, Aughey RJ, McKenna MJ, Kemp BE & Hawley JA. (2004). Intensified exercise training does not alter AMPK signaling in human skeletal muscle. *Am J Physiol* **286,** E737-743.

Clausen T. (1986). Regulation of active Na^+-K^+ transport in skeletal muscle. *Physiol Rev* **66,** 542-580.

Clausen T. (1990). Significance of Na^+,K^+-pump regulation in skeletal muscle. *NIPS* **5,** 148-151.

Clausen T. (1996). The Na^+, K^+ pump in skeletal muscle: quantification, regulation and functional significance. *Acta Physiol Scand* **156,** 227-235.

Clausen T. (2003). Na^+-K^+ pump regulation and skeletal muscle contractility. *Physiol Rev* **83,** 1269-1324.

Clausen T, Andersen SL & Flatman JA. (1993). Na^+-K^+ pump stimulation elicits recovery of contractility in K^+-paralysed rat muscle. *J Physiol* **472,** 521-536.

Clausen T & Everts ME. (1991). K^+-induced inhibition of contractile force in rat skeletal muscle: role of active Na^+-K^+ transport. *Am J Physiol* **261,** C799-807.

Clausen T & Flatman JA. (1987). Effects of insulin and epinephrine on Na^+-K^+ and glucose transport in soleus muscle. *Am J Physiol* **252,** E492-499.

Clausen T & Hansen O. (1974). Ouabain binding and Na^+,K^+ transport in rat muscle cells and adipocytes. *Biochim Biophys Acta* **356,** 387-404.

Clausen T & Hansen O. (1977). Active Na-K transport and the rate of ouabain binding. The effect of insulin and other stimuli on skeletal muscle and adipocytes. *J Physiol* **270,** 415-430.

Clausen T & Overgaard K. (2000). The role of K^+ channels in the force recovery elicited by Na^+-K^+ pump stimulation in Ba^{2+}-paralysed rat skeletal muscle. *J Physiol* **527,** 325-332.

Clausen T, Overgaard K & Nielsen OB. (2004). Evidence that the Na^+-K^+ leak/pump ratio contributes to the difference in endurance between fast- and slow-twitch muscles. *Acta Physiol Scand* **180,** 209-216.

Costantin LL & Podolsky RJ. (1967). Depolarization of the internal membrane system in the activation of frog skeletal muscle. *J Gen Physiol* **50,** 1101-1124.

Cougnon MH, Moseley AE, Radzyukevich TL, Lingrel JB & Heiny JA. (2002). Na,K-ATPase α- and β-isoform expression in developing skeletal muscles: α_2 correlates with t-tubule formation. *Pflugers Arch* **445,** 123-131.

Crambert G, Fuzesi M, Garty H, Karlish S & Geering K. (2002). Phospholemman (FXYD1) associates with Na,K-ATPase and regulates its transport properties. *Proc Natl Acad Sci U S A* **99,** 11476-11481.

Crambert G, Hasler U, Beggah AT, Yu C, Modyanov NN, Horisberger JD, Lelievre L & Geering K. (2000). Transport and pharmacological properties of nine different human Na, K-ATPase isozymes. *J Biol Chem* **275,** 1976-1986.

Dean R. (1941). Theories of electrolyte equilibrium in muscle. *Biol Symp* **3,** 331-348.

Dela F, Holten M & Juel C. (2003). Effect of resistance training on Na,K pump and Na^+/H^+ exchange protein densities in muscle from control and patients with type 2 diabetes. *Pflugers Arch* **447,** 928-933.

Delp MD & Duan C. (1996). Composition and size of type I, IIA, IID/X, and IIB fibers and citrate synthase activity of rat muscle. *J Appl Physiol* **80,** 261-270.

Dever TE. (2002). Gene-specific regulation by general translation factors. *Cell* **108,** 545-556.

Dombrowski L, Roy D, Marcotte B & Marette A. (1996). A new procedure for the isolation of plasma membranes, T tubules, and internal membranes from skeletal muscle. *Am J Physiol* **270,** E667-676.

Douen AG, Burdett E, Ramlal T, Rastogi S, Vranic M & Klip A. (1991). Characterization of glucose transporter-enriched membranes from rat skeletal muscle: assessment of endothelial cell contamination and presence of sarcoplasmic reticulum and transverse tubules. *Endocrinology* **128,** 611-616.

Dørup I & Clausen T. (1997). Effects of adrenal steroids on the concentration of Na^+-K^+ pumps in rat skeletal muscle. *J Endocrinol* **152,** 49-57.

Dørup I, Skajaa K & Clausen T. (1988). A simple and rapid method for the determination of the concentrations of magnesium, sodium, potassium and sodium, potassium pumps in human skeletal muscle. *Clin Sci (Lond)* **74,** 241-248.

Edelman IS. (1974). Thyroid thermogenesis. *N Engl J Med* **290,** 1303-1308.

Everts ME & Clausen T. (1988). Effects of thyroid hormone on Na^+-K^+ transport in resting and stimulated rat skeletal muscle. *Am J Physiol* **255,** E604-612.

Everts ME & Clausen T. (1992). Activation of the Na-K pump by intracellular Na in rat slow- and fast-twitch muscle. *Acta Physiol Scand* **145,** 353-362.

Everts ME & Clausen T. (1994). Excitation-induced activation of the Na^+-K^+ pump in rat skeletal muscle. *Am J Physiol* **266,** C925-934.

Everts ME, Lomo T & Clausen T. (1993). Changes in K^+, Na^+ and calcium contents during in vivo stimulation of rat skeletal muscle. *Acta Physiol Scand* **147,** 357-368.

Everts ME, Ording H, Hansen O & Nielsen PA. (1992). Ca^{2+}-ATPase and Na^+-K^+-ATPase content in skeletal muscle from malignant hyperthermia patients. *Muscle Nerve* **15,** 162-167.

Everts ME, Retterstol K & Clausen T. (1988). Effects of adrenaline on excitation-induced stimulation of the sodium-potassium pump in rat skeletal muscle. *Acta Physiol Scand* **134,** 189-198.

Evertsen F, Medbø JI, Jebens E & Nicolaysen K. (1997). Hard training for 5 mo increases Na^+-K^+ pump concentration in skeletal muscle of cross-country skiers. *Am J Physiol* **272,** R1417-1424.

Ewart HS & Klip A. (1995). Hormonal regulation of the Na^+-K^+-ATPase: mechanisms underlying rapid and sustained changes in pump activity. *Am J Physiol* **269,** C295-311.

Fambrough DM, Wolitzky BA, Taormino JP, Tamkun MM, Takeyasu K, Somerville D, Renaud KJ, Lemas MV, Lebovitz RM, Kone BC, Hamrick M, Rome J, Inman EM & Barnstein A. (1991). A cell biologist's perspective on sites of Na,K-ATPase regulation. In *The sodium pump: structure, mechanism and regulation*, pp. 17-30. The Rockefeller University Press.

Fenn WO. (1936). Electrolyte changes in muscle during activity. *Am J Physiol* **115,** 345-356.

Ferrannini E, Taddei S, Santoro D, Natali A, Boni C, Del Chiaro D & Buzzigoli G. (1988). Independent stimulation of glucose metabolism and Na^+-K^+ exchange by insulin in the human forearm. *Am J Physiol* **255,** E953-958.

Flatman JA & Clausen T. (1979). Combined effects of adrenaline and insulin on active electrogenic Na^+-K^+ transport in rat soleus muscle. *Nature* **281,** 580-581.

Forbush B, Kaplan JH & Hoffman JF. (1978). Characterization of a new photoaffinity derivative of ouabain: labeling of the large polypeptide and of a proteolipid component of the Na, K-ATPase. *Biochemistry* **17,** 3667-3676.

Fowles J, Green H & Ouyang J. (2004). Na^+-K^+-ATPase in rat skeletal muscle: content, isoform and activity characteristics. *J Appl Physiol* **96,** 316-326.

Fowles JR, Green HJ, Schertzer JD & Tupling AR. (2002a). Reduced activity of muscle Na^+-K^+-ATPase after prolonged running in rats. *J Appl Physiol* **93,** 1703-1708.

Fowles JR, Green HJ, Tupling R, O'Brien S & Roy BD. (2002b). Human neuromuscular fatigue is associated with altered Na^+-K^+-ATPase activity following isometric exercise. *J Appl Physiol* **92,** 1585-1593.

Fraser SF, Li JL, Carey MF, Wang XN, Sangkabutra T, Sostaric S, Selig SE, Kjeldsen K & McKenna MJ. (2002). Fatigue depresses maximal in vitro skeletal muscle Na^+-K^+-ATPase activity in untrained and trained individuals. *J Appl Physiol* **93,** 1650-1659.

Fraser SF & McKenna MJ. (1998). Measurement of Na^+,K^+-ATPase activity in human skeletal muscle. *Anal Biochem* **258,** 63-67.

Fryer MW & Neering IR. (1989). Actions of caffeine on fast- and slow-twitch muscles of the rat. *J Physiol* **416,** 435-454.

Gallant EM. (1983). Barium-treated mammalian skeletal muscle: similarities to hypokalaemic periodic paralysis. *J Physiol* **335,** 577-590.

Garcia MC, Gonzalez-Serratos H, Morgan JP, Perreault CL & Rozycka M. (1991). Differential activation of myofibrils during fatigue in phasic skeletal muscle cells. *J Muscle Res Cell Motil* **12,** 412-424.

Gautsch TA, Anthony JC, Kimball SR, Paul GL, Layman DK & Jefferson LS. (1998). Availability of eIF4E regulates skeletal muscle protein synthesis during recovery from exercise. *Am J Physiol* **274,** C406-414.

Geering K. (1991). The functional role of the β-subunit in the maturation and intracellular transport of Na,K-ATPase. *FEBS Letters* **285,** 189-193.

Gingras AC, Raught B & Sonenberg N. (1999). eIF4 initiation factors: effectors of mRNA recruitment to ribosomes and regulators of translation. *Annu Rev Biochem* **68,** 913-963.

Gissel H & Clausen T. (1999). Excitation-induced Ca^{2+} uptake in rat skeletal muscle. *Am J Physiol* **276,** R331-339.

Gissel H & Clausen T. (2000). Excitation-induced Ca^{2+} influx in rat soleus and EDL muscle: mechanisms and effects on cellular integrity. *Am J Physiol* **279,** R917-924.

Green H, Dahly A, Shoemaker K, Goreham C, Bombardier E & Ball-Burnett M. (1999a). Serial effects of high-resistance and prolonged endurance training on Na^+-K^+ pump concentration and enzymatic activities in human vastus lateralis. *Acta Physiol Scand* **165,** 177-184.

Green H, MacDougall J, Tarnopolsky M & Melissa NL. (1999b). Downregulation of Na^+-K^+-ATPase pumps in skeletal muscle with training in normobaric hypoxia. *J Appl Physiol* **86,** 1745-1748.

Green HJ, Barr DJ, Fowles JR, Sandiford SD & Ouyang J. (2004). Malleability of human skeletal muscle Na^+-K^+-ATPase pump with short-term training. *J Appl Physiol* **97,** 143-148.

Green HJ, Chin ER, Ball-Burnett M & Ranney D. (1993). Increases in human skeletal muscle Na^+-K^+-ATPase concentration with short-term training. *Am J Physiol* **264,** C1538-1541.

Green HJ, Duscha BD, Sullivan MJ, Keteyian SJ & Kraus WE. (2001). Normal skeletal muscle Na^+-K^+ pump concentration in patients with chronic heart failure. *Muscle & Nerve* **24,** 69-76.

Green S, Langberg H, Skovgaard D, Bulow J & Kjaer M. (2000). Interstitial and arterial-venous $[K^+]$ in human calf muscle during dynamic exercise: effect of ischaemia and relation to muscle pain. *J Physiol* **529,** 849-861.

Grinstein S & Erlij D. (1974). Insulin unmasks latent sodium pump sites in frog muscle. *Nature* **251,** 57-58.

Hallen J, Gullestad L & Sejersted OM. (1994). K^+ shifts of skeletal muscle during stepwise bicycle exercise with and without β-adrenoceptor blockade. *J Physiol* **477,** 149-159.

Hallen J, Saltin B & Sejersted OM. (1996). K^+ balance during exercise and role of β-adrenergic stimulation. *Am J Physiol* **270,** R1347-1354.

Hamada T, Sale DG, MacDougall JD & Tarnopolsky MA. (2003). Interaction of fibre type, potentiation and fatigue in human knee extensor muscles. *Acta Physiol Scand* **178,** 165-173.

Hameed M, Orrell RW, Cobbold M, Goldspink G & Harridge SDR. (2003). Expression of IGF-I splice variants in young and old human skeletal muscle after high resistance exercise. *J Physiol* **547,** 247-254.

Hansen O. (1984). Interaction of cardiac glycosides with (Na+ + K+)-activated ATPase. A biochemical link to digitalis-induced inotropy. *Pharmacol Rev* **36,** 143-163.

Hansen O & Clausen T. (1988). Quantitative determination of Na^+-K^+-ATPase and other sarcolemmal components in muscle cells. *Am J Physiol* **254,** C1-7.

Hansen O & Clausen T. (1996). Studies on sarcolemma components may be misleading due to inadequate recovery. *FEBS Lett* **384,** 203-205.

Harmer AR, McKenna MJ, Sutton JR, Snow RJ, Ruell PA, Booth J, Thompson MW, Mackay NA, Stathis CG, Crameri RM, Carey MF & Eager DM. (2000). Skeletal muscle metabolic and ionic adaptations during intense exercise following sprint training in humans. *J Appl Physiol* **89,** 1793-1803.

Harrison AP & Flatman JA. (1999). Measurement of force and both surface and deep M wave properties in isolated rat soleus muscles. *Am J Physiol* **277,** R1646-1653.

He S, Shelly DA, Moseley AE, James PF, James JH, Paul RJ & Lingrel JB. (2001). The α_1- and α_2-isoforms of Na-K-ATPase play different roles in skeletal muscle contractility. *Am J Physiol* **281,** R917-925.

Hennig R & Lomo T. (1985). Firing patterns of motor units in normal rats. *Nature* **314,** 164-166.

Hiatt A, McDonough AA & Edelman IS. (1984). Assembly of the (Na^+ + K^+)-adenosine triphosphatase. Post-translational membrane integration of the α subunit. *J Biol Chem* **259,** 2629-2635.

Hildebrandt AL, Pilegaard H & Neufer PD. (2003). Differential transcriptional activation of select metabolic genes in response to variations in exercise intensity and duration. *Am J Physiol* **285,** E1021-1027.

Hnik P, Holas M, Krekule I, Kuriz N, Mejsnar J, Smiesko V, Ujec E & Vyskocil F. (1976). Work-induced potassium changes in skeletal muscle and effluent venous blood assessed by liquid ion-exchanger microelectrodes. *Pflugers Arch* **362,** 85-94.

Hocherman SD & Bezanilla F. (1996). A patch-clamp study of delayed rectifier currents in skeletal muscle of control and mdx mice. *J Physiol* **493,** 113-128.

Hoffman JF, Wickrema A, Potapova O, Milanick M & Yingst DR. (2002). Na pump isoforms in human erythroid progenitor cells and mature erythrocytes. *Proc Natl Acad Sci U S A* **99,** 14572-14577.

Homareda H, Kawakami K, Nagano K & Matsui H. (1989). Location of signal sequences for membrane insertion of the Na^+,K^+-ATPase α subunit. *Mol Cell Biol* **9,** 5742-5745.

Hopkins WG, Schabort EJ & Hawley JA. (2001). Reliability of power in physical performance tests. *Sports Med* **31,** 211-234.

Huang L, Kometiani P & Xie Z. (1997). Differential regulation of Na/K-ATPase α-subunit isoform gene expressions in cardiac myocytes by ouabain and other hypertrophic stimuli. *J Mol Cell Cardiol* **29,** 3157-3167.

Huang RP & Adamson ED. (1993). Characterization of the DNA-binding properties of the early growth response-1 (Egr-1) transcription factor: evidence for modulation by a redox mechanism. *DNA Cell Biol* **12,** 265-273.

Hundal H, Marette A, Mitsumoto Y, Ramlal T, Blostein R & Klip A. (1992). Insulin induces translocation of the α2 and β1 subunits of the Na^+/K^+-ATPase from intracellular compartments to the plasma membrane in mammalian skeletal muscle. *J Biol Chem* **267,** 5040-5043.

Hundal HS & Klip A. (1993). Regulation of glucose transporters and the Na/K-ATPase by insulin in skeletal muscle. *Adv Exp Med Biol* **334,** 63-78.

Hundal HS, Marette A, Ramlal T, Liu Z & Klip A. (1993). Expression of β subunit isoforms of the Na^+,K^+-ATPase is muscle type-specific. *FEBS Letters* **328,** 253-258.

Hundal HS, Maxwell DL, Ahmed A, Darakhshan F, Mitsumoto Y & Klip A. (1994). Subcellular distribution and immunocytochemical localization of Na,K-ATPase subunit isoforms in human skeletal muscle. *Mol Membr Biol* **11,** 255-262.

Huxley AF. (2000). Cross-bridge action: present views, prospects, and unknowns. *J Biomech* **33,** 1189-1195.

Ismail-Beigi F & Edelman IS. (1971). The mechanism of the calorigenic action of thyroid hormone. Stimulation of Na plus + K plus-activated adenosinetriphosphatase activity. *J Gen Physiol* **57,** 710-722.

Izmail-Beigi F & Edelman IS. (1970). Mechanism of thyroid calorigenesis: role of active sodium transport. *Proc Natl Acad Sci U S A* **67,** 1071-1078.

Jackson MJ, Edwards RH & Symons MC. (1985). Electron spin resonance studies of intact mammalian skeletal muscle. *Biochim Biophys Acta* **847,** 185-190.

Jacobs I, Bar-Or O, Karlsson J, Dotan R, Tesch P, Kaiser P & Inbar O. (1982). Changes in muscle metabolites in females with 30-s exhaustive exercise. *Med Sci Sports Exerc* **14,** 457-460.

Jaimovich E, Donoso P, Liberona JL & Hidalgo C. (1986). Ion pathways in transverse tubules. Quantification of receptors in membranes isolated from frog and rabbit skeletal muscle. *Biochim Biophys Acta* **855,** 89-98.

James JH, Wagner KR, King JK, Leffler RE, Upputuri RK, Balasubramaniam A, Friend LA, Shelly DA, Paul RJ & Fischer JE. (1999a). Stimulation of both aerobic glycolysis and Na^+-K^+-ATPase activity in skeletal muscle by epinephrine or amylin. *Am J Physiol* **277,** E176-186.

James PF, Grupp IL, Grupp G, Woo AL, Askew GR, Croyle ML, Walsh RA & Lingrel JB. (1999b). Identification of a specific role for the Na,K-ATPase α2 isoform as a regulator of calcium in the heart. *Molecular Cell* **3,** 555-563.

Jansson E & Kaijser L. (1987). Substrate utilization and enzymes in skeletal muscle of extremely endurance-trained men. *J Appl Physiol* **62,** 999-1005.

Jebens E, Steen H, Fjeld TO, Bye E & Sejersted OM. (1995). Changes in Na^+, K^+-adenosinetriphosphatase, citrate synthase and K^+ in sheep skeletal muscle during immobilization and remobilization. *Eur J Appl Physiol Occup Physiol* **71,** 386-395.

Jenkins RR. (1988). Free radical chemistry. Relationship to exercise. *Sports Med* **5,** 156-170.

Jensen L, Pilegaard H, Neufer PD & Hellsten Y. (2004). Effect of acute exercise and exercise training on VEGF splice variants in human skeletal muscle. *Am J Physiol* **287,** R397-402.

Jewell EA & Lingrel JB. (1991). Comparison of the substrate dependence properties of the rat Na,K-ATPase α1, α2, and α3 isoforms expressed in HeLa cells. *J Biol Chem* **266,** 16925-16930.

Ji LL, Fu R & Mitchell EW. (1992). Glutathione and antioxidant enzymes in skeletal muscle: effects of fiber type and exercise intensity. *J Appl Physiol* **73,** 1854-1859.

Jones DH, Davies TC & Kidder GM. (1997). Embryonic expression of the putative γ subunit of the sodium pump is required for acquisition of fluid transport capacity during mouse blastocyst development. *J Cell Biol* **139,** 1545-1552.

Juel C. (1986). Potassium and sodium shifts during in vitro isometric muscle contraction, and the time course of the ion-gradient recovery. *Pflugers Arch* **406,** 458-463.

Juel C. (1988). Muscle action potential propagation velocity changes during activity. *Muscle Nerve* **11,** 714-719.

Juel C, Grunnet L, Holse M, Kenworthy S, Sommer V & Wulff T. (2001). Reversibility of exercise-induced translocation of Na^+-K^+ pump subunits to the plasma membrane in rat skeletal muscle. *Pflugers Arch* **443,** 212-217.

Juel C, Nielsen JJ & Bangsbo J. (2000a). Exercise-induced translocation of Na^+-K^+ pump subunits to the plasma membrane in human skeletal muscle. *Am J Physiol* **278,** R1107-1110.

Juel C, Pilegaard H, Nielsen JJ & Bangsbo J. (2000b). Interstitial K^+ in human skeletal muscle during and after dynamic graded exercise determined by microdialysis. *Am J Physiol* **278,** R400-406.

Jungnickel B & Rapoport TA. (1995). A posttargeting signal sequence recognition event in the endoplasmic reticulum membrane. *Cell* **82,** 261-270.

Katz A, Sahlin K & Juhlin-Dannfelt A. (1985). Effect of β-adrenoceptor blockade on H^+ and K^+ flux in exercising humans. *J Appl Physiol* **59,** 336-341.

Kawakami K & Nagano K. (1988). The transmembrane segment of the human Na,K-ATPase β-subunit acts as the membrane incorporation signal. *J Biochem (Tokyo)* **103,** 54-60.

Keryanov S & Gardner KL. (2002). Physical mapping and characterization of the human Na,K-ATPase isoform, ATP1A4. *Gene* **292,** 151-166.

Khuri RN, Agulian SK, Abdulnour-Nakhoul S & Nakhoul NL. (1992). Electrochemical potentials of potassium in skeletal muscle under different metabolic states. *J Cell Physiol* **153,** 534-538.

Kim D & Smith TW. (1986). Effect of growth in low-Na^+ medium on transport sites in cultured heart cells. *Am J Physiol* **250,** C32-39.

Kirley T. (1989). Determination of three disulfide bonds and one free sulfhydryl in the β subunit of (Na,K)-ATPase. *J Biol Chem* **264,** 7185-7192.

Kjeldsen K, Bjerregaard P, Richter EA, Thomsen PE & Nørgaard A. (1988). Na^+,K^+-ATPase concentration in rodent and human heart and skeletal muscle: apparent relation to muscle performance. *Cardiovasc Res* **22,** 95-100.

Kjeldsen K, Everts ME & Clausen T. (1986a). The effects of thyroid hormones on ^{3}H-ouabain binding site concentration, Na,K-contents and ^{86}Rb-efflux in rat skeletal muscle. *Pflugers Arch* **406,** 529-535.

Kjeldsen K & Gron P. (1989). Skeletal muscle Na,K-pump concentration in children and its relationship to cardiac glycoside distribution. *J Pharmacol Exp Ther* **250,** 721-725.

Kjeldsen K, Nørgaard A & Clausen T. (1982). Age-dependent changes in the number of [^{3}H]ouabain-binding sites in rat soleus muscle. *Biochim Biophys Acta* **686,** 253-256.

Kjeldsen K, Nørgaard A & Clausen T. (1984a). The age-dependent changes in the number of ^{3}H-ouabain binding sites in mammalian skeletal muscle. *Pflugers Arch* **402,** 100-108.

Kjeldsen K, Nørgaard A, Gotzsche CO, Thomassen A & Clausen T. (1984b). Effect of thyroid function on number of Na-K pumps in human skeletal muscle. *Lancet* **2,** 8-10.

Kjeldsen K, Richter EA, Galbo H, Lortie G & Clausen T. (1986b). Training increases the concentration of [^{3}H]ouabain-binding sites in rat skeletal muscle. *Biochim Biophys Acta* **860,** 708-712.

Klitgaard H & Clausen T. (1989). Increased total concentration of Na-K pumps in vastus lateralis muscle of old trained human subjects. *J Appl Physiol* **67,** 2491-2494.

Knochel JP, Blachley JD, Johnson JH & Carter NW. (1985). Muscle cell electrical hyperpolarization and reduced exercise hyperkalemia in physically conditioned dogs. *J Clin Invest* **75,** 740-745.

Koenderink JB, Hermsen HPH, Swarts HGP, Willems PHGM & De Pont JJHHM. (2000). High-affinity ouabain binding by a chimeric gastric H^+,K^+-ATPase containing transmembrane hairpins M3-M4 and M5-M6 of the α_1-subunit of rat Na^+,K^+-ATPase. *PNAS* **97,** 11209-11214.

Kometiani P, Tian J, Li J, Nabih Z, Gick G & Xie Z. (2000). Regulation of Na/K-ATPase β_1-subunit gene expression by ouabain and other hypertrophic stimuli in neonatal rat cardiac myocytes. *Mol Cell Biochem* **215,** 65-72.

Kuster B, Shainskaya A, Pu HX, Goldshleger R, Blostein R, Mann M & Karlish SJ. (2000). A new variant of the γ subunit of renal Na,K-ATPase. Identification by mass spectrometry, antibody binding, and expression in cultured cells. *J Biol Chem* **275,** 18441-18446.

Lamb GD, Cellini MA & Stephenson DG. (2001). Different Ca^{2+} releasing action of caffeine and depolarisation in skeletal muscle fibres of the rat. *J Physiol* **531,** 715-728.

Lamb GD & Stephenson DG. (1996). Effects of FK506 and rapamycin on excitation-contraction coupling in skeletal muscle fibres of the rat. *J Physiol* **494,** 569-576.

Lau YH, Caswell AH, Garcia M & Letellier L. (1979). Ouabain binding and coupled sodium, potassium, and chloride transport in isolated transverse tubules of skeletal muscle. *J Gen Physiol* **74,** 335-349.

Lavoie L, He L, Ramlal T, Ackerley C, Marette A & Klip A. (1995). The GLUT4 glucose transporter and the α_2 subunit of the Na^+,K^+-ATPase do not localize to the same intracellular vesicles in rat skeletal muscle. *FEBS Letters* **366,** 109-114.

Lavoie L, Roy D, Ramlal T, Dombrowski L, Martin-Vasallo P, Marette A, Carpentier JL & Klip A. (1996). Insulin-induced translocation of Na^+-K^+-ATPase subunits to the plasma membrane is muscle fiber type specific. *Am J Physiol* **270,** C1421-1429.

Lee CO & Vassalle M. (1983). Modulation of intracellular Na^+ activity and cardiac force by norepinephrine and Ca^{2+}. *Am J Physiol* **244,** C110-114.

Leivseth G, Clausen T, Everts ME & Bjordal E. (1992). Effects of reduced joint mobility and training on Na,K-ATPase and Ca-ATPase in skeletal muscle. *Muscle Nerve* **15,** 843-849.

Lekanne Deprez RH, Fijnvandraat AC, Ruijter JM & Moorman AF. (2002). Sensitivity and accuracy of quantitative real-time polymerase chain reaction using SYBR green I depends on cDNA synthesis conditions. *Anal Biochem* **307,** 63-69.

Leppik JA, Aughey RJ, Medved I, Fairweather I, Carey MF & McKenna MJ. (2004). Prolonged exercise to fatigue in humans impairs skeletal muscle Na^+-K^+-ATPase activity, sarcoplasmic reticulum Ca^{2+} release, and Ca^{2+} uptake. *J Appl Physiol* **97,** 1414-1423.

Lescale-Matys L, Hensley CB, Crnkovic-Markovic R, Putnam DS & McDonough AA. (1990). Low K^+ increases Na,K-ATPase abundance in LLC-PK1/Cl4 cells by differentially increasing beta, and not alpha, subunit mRNA. *J Biol Chem* **265,** 17935-17940.

Lescale-Matys L, Putnam DS & McDonough AA. (1993a). Na^+-K^+-ATPase α_1- and β_1-subunit degradation: evidence for multiple subunit specific rates. *Am J Physiol* **264,** C583-590.

Lescale-Matys L, Putnam DS & McDonough AA. (1993b). Surplus Na^+ pumps: how low-K^+-incubated LLC-PK_1 cells respond to K^+ restoration. *Am J Physiol* **265,** C887-892.

Levenson R. (1994). Isoforms of the Na,K-ATPase: family members in search of function. *Rev Physiol Biochem Pharmacol* **123,** 1-45.

Lin SH, Lin YF & Halperin ML. (2001). Hypokalaemia and paralysis. *Qjm* **94,** 133-139.

Lindinger MI. (1995). Potassium regulation during exercise and recovery in humans: implications for skeletal and cardiac muscle. *J Mol Cell Cardiol* **27,** 1011-1022.

Lindinger MI, Hawke TJ, Lipskie SL, Schaefer HD & Vickery L. (2002). K^+ transport and volume regulatory response by NKCC in resting rat hindlimb skeletal muscle. *Cell Physiol Biochem* **12,** 279-292.

Lindinger MI & Heigenhauser GJ. (1988). Ion fluxes during tetanic stimulation in isolated perfused rat hindlimb. *Am J Physiol* **254,** R117-126.

Lindinger MI & Heigenhauser GJ. (1991). The roles of ion fluxes in skeletal muscle fatigue. *Can J Physiol Pharmacol* **69,** 246-253.

Lindinger MI, Willmets RG & Hawke TJ. (1996). Stimulation of Na^+, K^+-pump activity in skeletal muscle by methylxanthines: evidence and proposed mechanisms. *Acta Physiol Scand* **156,** 347-353.

Lindsay FH, Hawley JA, Myburgh KH, Schomer HH, Noakes TD & Dennis SC. (1996). Improved athletic performance in highly trained cyclists after interval training. Abstract. *Med Sci Sports Exerc* **28,** 1427-1434.

Lingrel JB, Van-Huysse J, O'Brien W, Jewell-Motz E & Schultheis P. (1992). Na,K-ATPase: structure-function studies. *Ren Physiol Biochem* **17,** 198-200.

Liu Y, Lormes W, Wang L, Reissnecker S & Steinacker JM. (2004). Different skeletal muscle HSP70 responses to high-intensity strength training and low-intensity endurance training. *Eur J Appl Physiol* **91,** 330-335.

Lunde PK, Dahlstedt AJ, Bruton JD, Lannergren J, Thoren P, Sejersted OM & Westerblad H. (2001). Contraction and intracellular Ca^{2+} handling in isolated skeletal muscle of rats with congestive heart failure. *Circ Res* **88,** 1299-1305.

Lytton J. (1985). Insulin affects the sodium affinity of the rat adipocyte Na^{+},K^{+}- ATPase. *J Biol Chem* **260,** 10075-10080.

Macdonald WA, Nielsen OB & Clausen T. (2005a). Na^{+}-K^{+} pump stimulation restores carbacholine-induced loss of excitability and contractility in rat skeletal muscle. *J Physiol* **563,** 459-469.

Macdonald WA, Nielsen OB & Clausen T. (2005b). Na^{+}-K^{+} pump stimulation restores carbacholine-induced loss of excitability and contrctility in rat skeletal muscle. *J Physiol*, jphysiol.2004.080390.

Madsen K, Franch J & Clausen T. (1994). Effects of intensified endurance training on the concentration of Na,K-ATPase and Ca-ATPase in human skeletal muscle. *Acta Physiol Scand* **150,** 251-258.

Malik N, Canfield V, Sanchez-Watts G, Watts AG, Scherer S, Beatty BG, Gros P & Levenson R. (1998). Structural organization and chromosomal localization of the human Na,K-ATPase β3 subunit gene and pseudogene. *Mamm Genome* **9,** 136-143.

Malik N, Canfield VA, Beckers M-C, Gros P & Levenson R. (1996). Identification of the mammalian Na,K-ATPase β3 subunit. *J Biol Chem* **271,** 22754-22758.

Marette A, Burdett E, Douen A, Vranic M & Klip A. (1992). Insulin induces the translocation of GLUT4 from a unique intracellular organelle to transverse tubules in rat skeletal muscle. *Diabetes* **41,** 1562-1569.

Marette A, Krischer J, Lavoie L, Ackerley C, Carpentier JL & Klip A. (1993). Insulin increases the Na^+-K^+-ATPase α_2-subunit in the surface of rat skeletal muscle: morphological evidence. *Am J Physiol* **265,** C1716-1722.

Martin-Vasallo P, Dackowski W, Emanuel J & Levenson R. (1989). Identification of a putative isoform of the Na,K-ATPase β subunit. Primary structure and tissue-specific expression. *J Biol Chem* **264,** 4613-4618.

Matsuda T, Murata Y, Kawamura N, Hayashi M, Tamada K, Takuma K, Maeda S & Baba A. (1993). Selective induction of α_1 isoform of (Na^+ + K^+)-ATPase by insulin/insulin-like growth factor-I in cultured rat astrocytes. *Arch Biochem Biophys* **307,** 175-182.

Matthews RP, Guthrie CR, Wailes LM, Zhao X, Means AR & McKnight GS. (1994). Calcium/calmodulin-dependent protein kinase types II and IV differentially regulate CREB-dependent gene expression. *Mol Cell Biol* **14,** 6107-6116.

McDonough AA, Azuma KK, Lescale-Matys L, Tang MJ, Nakhoul F, Hensley CB & Komatsu Y. (1992). Physiologic rationale for multiple sodium pump isoforms. Differential regulation of α1 vs α2 by ionic stimuli. *Ann N Y Acad Sci* **671,** 156-168; discussion 168-159.

McDonough AA, C.E. Magyar, C.B. Thompson & K.K. Azuma. (1994). Isoform specific Na,K-ATPase regulation in adaptation to potassium depletion in heart and muscle. In *International Society for Heart Research - XV European Section Meeting*, pp. 135-142. Monduzzi Editore, Copenhagen, Denmark.

McDonough AA, Tang MJ & Lescale-Matys L. (1990). Ionic regulation of the biosynthesis of NaK-ATPase subunits. *Seminars in Nephrology* **10,** 400-409.

McKenna MJ, Fraser SF, Li JL, Wang XN, Carey MF, Side EA, Morton J, Snell GI, Kjeldsen K & Williams TJ. (2003a). Impaired muscle Ca^{2+} and K^{+} regulation contribute to poor exercise performance post-lung transplantation. *J Appl Physiol* **95,** 1606-1616.

McKenna MJ, Gissel H & Clausen T. (2003b). Effects of electrical stimulation and insulin on Na^{+}-K^{+}-ATPase [^{3}H]ouabain binding in rat skeletal muscle. *J Physiol* **547,** 567-580.

McKenna MJ, Schmidt TA, Hargreaves M, Cameron L, Skinner SL & Kjeldsen K. (1993). Sprint training increases human skeletal muscle Na^+-K^+-ATPase concentration and improves K^+ regulation. *J Appl Physiol* **75,** 173-180.

Medbø JI, Jebens E, Vikne H, Refsnes PE & Gramvik P. (2001). Effect of strenuous strength training on the Na-K pump concentration in skeletal muscle of well-trained men. *Eur J Appl Physiol* **84,** 148-154.

Medved I, Brown MJ, Bjorksten AR, Leppik JA, Sostaric S & McKenna MJ. (2003). *N*-acetylcysteine infusion alters blood redox status but not time to fatigue during intense exercise in humans. *J Appl Physiol* **94,** 1572-1582.

Medved I, Brown MJ, Bjorksten AR & McKenna MJ. (2004a). Effects of intravenous *N*-acetylcysteine infusion on time to fatigue and potassium regulation during prolonged cycling exercise. *J Appl Physiol* **96,** 211-217.

Medved I, Brown MJ, Bjorksten AR, Murphy KT, Petersen AC, Sostaric S, Gong X & McKenna MJ. (2004b). *N*-acetylcysteine enhances muscle cysteine and glutathione availability and attenuates fatigue during prolonged exercise in endurance-trained individuals. *J Appl Physiol* **97,** 1477-1485.

Meissner G. (1994). Ryanodine receptor/Ca^{2+} release channels and their regulation by endogenous effectors. *Annu Rev Physiol* **56,** 485-508.

Mercer RW, Biemesderfer D, Bliss DP, Collins JH & Forbush B. (1993). Molecular cloning and immunological characterization of the γ polypeptide, a small protein associated with the Na,K-ATPase. *J Cell Biol* **121,** 579-586.

Milner-Brown HS & Miller RG. (1986). Muscle membrane excitation and impulse propagation velocity are reduced during muscle fatigue. *Muscle Nerve* **9,** 367-374.

Mircheff AK, Bowen JW, Yiu SC & McDonough AA. (1992). Synthesis and translocation of Na^+-K^+-ATPase α- and β-subunits to plasma membrane in MDCK cells. *Am J Physiol* **262,** C470-483.

Murphy R, McConell G, Cameron-Smith D, Watt K, Ackland L, Walzel B, Wallimann T & Snow R. (2001). Creatine transporter protein content, localization, and gene expression in rat skeletal muscle. *Am J Physiol* **280,** C415-422.

Murphy RM, Watt KK, Cameron-Smith D, Gibbons CJ & Snow RJ. (2003). Effects of creatine supplementation on housekeeping genes in human skeletal muscle using real-time RT-PCR. *Physiol Genomics* **12,** 163-174.

Musch TI, Wolfram S, Hageman KS & Pickar JG. (2002). Skeletal muscle ouabain binding sites are reduced in rats with chronic heart failure. *J Appl Physiol* **92,** 2326-2334.

Nakajima S, Nakajima Y & Bastian J. (1975). Effects of sudden changes in external sodium concentration on twitch tension in isolated muscle fibers. *J Gen Physiol* **65,** 459-482.

Nesher R, Karl IE, Kaiser KE & Kipnis DM. (1980). Epitrochlearis muscle. I. Mechanical performance, energetics, and fiber composition. *Am J Physiol* **239,** E454-460.

Ng YC, Nagarajan M, Jew KN, Mace LC & Moore RL. (2003). Exercise training differentially modifies age-associated alteration in expression of Na^+-K^+-ATPase subunit isoforms in rat skeletal muscles. *Am J Physiol* **285,** R733-740.

Nielsen JJ, Kristensen M, Hellsten Y, Bangsbo J & Juel C. (2003). Localization and function of ATP-sensitive potassium channels in human skeletal muscle. *Am J Physiol* **284,** R558-563.

Nielsen JJ, Mohr M, Klarskov C, Kristensen M, Krustrup P, Juel C & Bangsbo J. (2004a). Effects of high-intensity intermittent training on potassium kinetics and performance in human skeletal muscle. *J Physiol* **554,** 857-870.

Nielsen OB & Clausen T. (1996). The significance of active Na^+,K^+ transport in the maintenance of contractility in rat skeletal muscle. *Acta Physiol Scand* **157,** 199-209.

Nielsen OB & Clausen T. (1997). Regulation of Na^+-K^+ pump activity in contracting rat muscle. *J Physiol* **503,** 571-581.

Nielsen OB & Clausen T. (2000). The Na^+/K^+-pump protects muscle excitability and contractility during exercise. *Exerc Sport Sci Rev* **28,** 159-164.

Nielsen OB, de Paoli F & Overgaard K. (2001). Protective effects of lactic acid on force production in rat skeletal muscle. *J Physiol* **536,** 161-166.

Nielsen OB, Ørtenblad N, Lamb GD & Stephenson DG. (2004b). Excitability of the T-tubular system in rat skeletal muscle: roles of K^+ and Na^+ gradients and Na^+-K^+ pump activity. *J Physiol* **557,** 133-146.

Noguchi S, Higashi K & Kawamura M. (1990). A possible role of the β-subunit of (Na,K)-ATPase in facilitating correct assembly of the α-subunit into the membrane. *J Biol Chem* **265,** 15991-15995.

Noland RC, Hickner RC, Jimenez-Linan M, Vidal-Puig A, Zheng D, Dohm GL & Cortright RN. (2003). Acute endurance exercise increases skeletal muscle uncoupling protein-3 gene expression in untrained but not trained humans. *Metabolism* **52,** 152-158.

Nordsborg N, Bangsbo J & Pilegaard H. (2003a). Effect of high-intensity training on exercise-induced gene expression specific to ion homeostasis and metabolism. *J Appl Physiol* **95,** 1201-1206.

Nordsborg N, Mohr M, Pedersen LD, Nielsen JJ, Langberg H & Bangsbo J. (2003b). Muscle interstitial potassium kinetics during intense exhaustive exercise: effect of previous arm exercise. *Am J Physiol* **285,** R143-148.

Nygaard E. (1981). Skeletal muscle fibre characteristics in young women. *Acta Physiol Scand* **112,** 299-304.

Nørgaard A. (1986). Quantification of the Na,K-pumps in mammalian skeletal muscle. *Acta Pharmacol Toxicol (Copenh)* **58,** 1-34.

Nørgaard A, Kjeldsen K & Clausen T. (1984a). A method for the determination of the total number of ^{3}H-ouabain binding sites in biopsies of human skeletal muscle. *Scand J Clin Lab Invest* **44,** 509-518.

Nørgaard A, Kjeldsen K & Hansen O. (1984b). (Na^+ + K^+)-ATPase activity of crude homogenates of rat skeletal muscle as estimated from their K^+-dependent 3-O-methylfluorescein phosphatase activity. *Biochim Biophys Acta* **770,** 203-209.

O'Brien WJ, Lingrel JB & Wallick ET. (1994). Ouabain binding kinetics of the rat alpha two and alpha three isoforms of the sodium-potassium adenosine triphosphate. *Arch Biochem Biophys* **310,** 32-39.

O'Connell M, Robbins DC, Horton ES, Sims EA & Danforth E, Jr. (1979). Changes in serum concentrations of 3,3',5'-triiodothyronine and 3,5,3'-triiodothyronine during prolonged moderate exercise. *J Clin Endocrinol Metab* **49,** 242-246.

Ojuka EO, Jones TE, Nolte LA, Chen M, Wamhoff BR, Sturek M & Holloszy JO. (2002). Regulation of GLUT4 biogenesis in muscle: evidence for involvement of AMPK and Ca^{2+}. *Am J Physiol* **282,** E1008-1013.

Omatsu-Kanbe M & Kitasato H. (1990). Insulin stimulates the translocation of Na^+/K^+-dependent ATPase molecules from intracellular stores to the plasma membrane in frog skeletal muscle. *Biochem J* **272,** 727-733.

Orlowski J & Lingrel JB. (1988). Tissue-specific and developmental regulation of rat Na,K-ATPase catalytic α isoform and β subunit mRNAs. *J Biol Chem* **263,** 10436-10442.

Orphanides G, Lagrange T & Reinberg D. (1996). The general transcription factors of RNA polymerase II. *Genes Dev* **10,** 2657-2683.

Orphanides G & Reinberg D. (2002). A unified theory of gene expression. *Cell* **108,** 439-451.

Ortega A, Gonzalez-Serratos H & Lepock JR. (1997). Effect of the organic Ca^{2+} channel blocker D-600 on sarcoplasmic reticulum Ca^{2+} uptake in skeletal muscle. *Am J Physiol* **272,** C310-317.

Overgaard K, Fredsted A, Hyldal A, Ingemann-Hansen T, Gissel H & Clausen T. (2004). Effects of running distance and training on Ca^{2+} content and damage in human muscle. *Med Sci Sports Exerc* **36,** 821-829.

Overgaard K, Lindstrom T, Ingemann-Hansen T & Clausen T. (2002). Membrane leakage and increased content of Na^+ -K^+ pumps and Ca^{2+} in human muscle after a 100-km run. *J Appl Physiol* **92,** 1891-1898.

Overgaard K & Nielsen OB. (2001). Activity-induced recovery of excitability in K^+-depressed rat soleus muscle. *Am J Physiol* **280,** R48-55.

Overgaard K, Nielsen OB & Clausen T. (1997). Effects of reduced electrochemical Na^+ gradient on contractility in skeletal muscle: role of the Na^+-K^+ pump. *Pflugers Arch* **434,** 457-465.

Overgaard K, Nielsen OB, Flatman JA & Clausen T. (1999). Relations between excitability and contractility in rat soleus muscle: role of the Na^+-K^+ pump and Na^+/K^+ gradients. *J Physiol* **518,** 215-225.

Paterson DJ. (1996). Antiarrhythmic mechanisms during exercise. *J Appl Physiol* **80,** 1853-1862.

Pedersen TH, Clausen T & Nielsen OB. (2003). Loss of force induced by high extracellular $[K^+]$ in rat muscle: effect of temperature, lactic acid and β_2-agonist. *J Physiol* **551,** 277-286.

Pedersen TH, de Paoli F & Nielsen OB. (2005). Increased excitability of acidified skeletal muscle: role of chloride conductance. *J Gen Physiol* **125,** 237-246.

Pedersen TH, Nielsen OB, Lamb GD & Stephenson DG. (2004). Intracellular acidosis enhances the excitability of working muscle. *Science* **305,** 1144-1147.

Petersen AC, Murphy KT, Snow RJ, Leppik JA, Aughey RJ, Garnham AP, Cameron-Smith D & McKenna MJ. (2005). Depressed Na^+-K^+-ATPase activity in skeletal muscle at fatigue is correlated with increased Na^+-K^+-ATPase mRNA expression following intense exercise. *Am J Physiol* **289,** R266-274.

Pfeiffer R, Beron J & Verrey F. (1999). Regulation of Na^+ pump function by aldosterone is α-subunit isoform specific. *J Physiol* **516,** 647-655.

Pfliegler G, Szabo I & Kovacs T. (1983). The influence of catecholamines on Na, K transport in slow- and fast-twitch muscles of the rat. *Pflugers Arch* **398,** 236-240.

Pickar JG, Spier SJ, Harrold D & Carlsen RC. (1993). [^{3}H]ouabain binding in skeletal muscle from horses with hyperkalemic periodic paralysis. *Am J Vet Res* **54,** 783-787.

Pilegaard H, Ordway GA, Saltin B & Neufer PD. (2000). Transcriptional regulation of gene expression in human skeletal muscle during recovery from exercise. *Am J Physiol* **279,** E806-814.

Posterino GS, Lamb GD & Stephenson DG. (2000). Twitch and tetanic force responses and longitudinal propagation of action potentials in skinned skeletal muscle fibres of the rat. *J Physiol* **527,** 131-137.

Pressley TA, Ismail-Beigi F, Gick GG & Edelman IS. (1988). Increased abundance of Na^+-K^+-ATPase mRNAs in response to low external K^+. *Am J Physiol* **255,** C252-260.

Pressman BC & Fahim M. (1982). Pharmacology and toxicology of the monovalent carboxylic ionophores. *Annu Rev Pharmacol Toxicol* **22,** 465-490.

Proudfoot NJ, Furger A & Dye MJ. (2002). Integrating mRNA processing with transcription. *Cell* **108,** 501-512.

Psilander N, Damsgaard R & Pilegaard H. (2003). Resistance exercise alters MRF and IGF-I mRNA content in human skeletal muscle. *J Appl Physiol* **95,** 1038-1044.

Pu HX, Cluzeaud F, Goldshleger R, Karlish SJ, Farman N & Blostein R. (2001). Functional role and immunocytochemical localization of the gamma a and gamma b forms of the Na,K-ATPase gamma subunit. *J Biol Chem* **276,** 20370-20378.

Radzyukevich TL, Moseley AE, Shelly DA, Redden GA, Behbehani MM, Lingrel JB, Paul RJ & Heiny JA. (2004). The Na^+-K^+-ATPase α_2-subunit isoform modulates contractility in the perinatal mouse diaphragm. *Am J Physiol* **287,** C1300-1310.

Ramakrishnan V. (2002). Ribosome structure and the mechanism of translation. *Cell* **108,** 557-572.

Rayson B. (1993). Calcium: a mediator of the cellular response to chronic Na^+/K^+-ATPase inhibition. *J Biol Chem* **268,** 8851-8854.

Rayson BM. (1989). Rates of synthesis and degradation of Na^+-K^+-ATPase during chronic ouabain treatment. *Am J Physiol* **256,** C75-80.

Rayson BM. (1991). $[Ca^{2+}]_i$ regulates transcription rate of the Na^+/K^+-ATPase α_1 subunit. *J Biol Chem* **266,** 21335-21338.

Reed R & Hurt E. (2002). A conserved mRNA export machinery coupled to pre-mRNA splicing. *Cell* **108,** 523-531.

Reid MB, Haack KE, Franchek KM, Valberg PA, Kobzik L & West MS. (1992). Reactive oxygen in skeletal muscle. I. Intracellular oxidant kinetics and fatigue in vitro. *J Appl Physiol* **73,** 1797-1804.

Reid MB, Khawli FA & Moody MR. (1993). Reactive oxygen in skeletal muscle. III. Contractility of unfatigued muscle. *J Appl Physiol* **75,** 1081-1087.

Renaud JM & Light P. (1992). Effects of K^+ on the twitch and tetanic contraction in the sartorius muscle of the frog, Rana pipiens. Implication for fatigue in vivo. *Can J Physiol Pharmacol* **70,** 1236-1246.

Riis AL, Jorgensen JO, Moller N, Weeke J & Clausen T. (2005). Hyperthyroidism and cation pumps in human skeletal muscle. *Am J Physiol* **288,** E1265-1269.

Ritucci NA & Corbett AM. (1995). Effect of Mg^{2+} and ATP on depolarization-induced Ca^{2+} release in isolated triads. *Am J Physiol* **269,** C85-95.

Rousseau E, Ladine J, Liu QY & Meissner G. (1988). Activation of the Ca^{2+} release channel of skeletal muscle sarcoplasmic reticulum by caffeine and related compounds. *Arch Biochem Biophys* **267,** 75-86.

Sahlin K, Alvestrand A, Brandt R & Hultman E. (1978). Intracellular pH and bicarbonate concentration in human muscle during recovery from exercise. *J Appl Physiol* **45,** 474-480.

Sahlin K, Katz A & Broberg S. (1990). Tricarboxylic acid cycle intermediates in human muscle during prolonged exercise. *Am J Physiol* **259,** C834-841.

Sandiford SD, Green HJ, Duhamel TA, Perco JG, Schertzer JD & Ouyang J. (2004). Inactivation of human muscle Na^+-K^+-ATPase in vitro during prolonged exercise is increased with hypoxia. *J Appl Physiol* **96,** 1767-1775.

Sargeant RJ, Liu Z & Klip A. (1995). Action of insulin on Na^+-K^+-ATPase and the Na^+-K^+-$2Cl^-$ cotransporter in 3T3-L1 adipocytes. *Am J Physiol* **269,** C217-225.

Schleusing G & Noecker J. (1960). The effect of potassium deficiency and loading on extra-and intracellular mineral content of skeletal musculature in untrained and trained animals. *Med Welt* **31,** 1579-1583.

Schmidt TA, Hasselbalch S, Farrell PA, Vestergaard H & Kjeldsen K. (1994). Human and rodent muscle Na^+-K^+-ATPase in diabetes related to insulin, starvation, and training. *J Appl Physiol* **76,** 2140-2146.

Schmitt B, Fluck M, Decombaz J, Kreis R, Boesch C, Wittwer M, Graber F, Vogt M, Howald H & Hoppeler H. (2003). Transcriptional adaptations of lipid metabolism in tibialis anterior muscle of endurance-trained athletes. *Physiol Genomics* **15,** 148-157.

Schreck R, Rieber P & Baeuerle PA. (1991). Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-kappa B transcription factor and HIV-1. *EMBO J* **10,** 2247-2258.

Sejersted OM & Hallen J. (1987). Na, K homeostasis of skeletal muscle during activation. In *Muscular function in Exercise and Training (Med Sport Sci Vol 26)*, pp. 1-11. Karger, Basel.

Sejersted OM & Sjøgaard G. (2000). Dynamics and consequences of potassium shifts in skeletal muscle and heart during exercise. *Physiol Rev* **80,** 1411-1481.

Sen CK, Kolosova I, Hanninen O & Orlov SN. (1995). Inward potassium transport systems in skeletal muscle derived cells are highly sensitive to oxidant exposure. *Free Radic Biol Med* **18,** 795-800.

Sharabani-Yosef O, Nir U & Sampson SR. (2002). Thyroid hormone up-regulates Na^+/K^+ pump α2 mRNA but not α2 protein isoform in cultured skeletal muscle. *Biochim Biophys Acta* **1573,** 183-188.

Shindoh C, DiMarco A, Thomas A, Manubay P & Supinski G. (1990). Effect of *N*-acetylcysteine on diaphragm fatigue. *J Appl Physiol* **68,** 2107-2113.

Sjøgaard G. (1983). Electrolytes in slow and fast muscle fibers of humans at rest and with dynamic exercise. *Am J Physiol* **245,** R25-31.

Sjøgaard G, Adams RP & Saltin B. (1985). Water and ion shifts in skeletal muscle of humans with intense dynamic knee extension. *Am J Physiol* **248,** R190-196.

Sjøgaard G & Saltin B. (1982). Extra- and intracellular water spaces in muscles of man at rest and with dynamic exercise. *Am J Physiol* **243,** R271-280.

Sreter FA & Woo G. (1963). Cell water, sodium, and potassium in red and white mammalian muscles. *Am J Physiol* **205,** 1290-1294.

Stengelin MK & Hoffman JF. (1997). Na,K-ATPase subunit isoforms in human reticulocytes: evidence from reverse transcription-PCR for the presence of α1, α3, β2, β3, and γ. *Proc Natl Acad Sci U S A* **94,** 5943-5948.

Stephenson DG, Lamb GD & Stephenson GM. (1998). Events of the excitation-contraction-relaxation (E-C-R) cycle in fast- and slow-twitch mammalian muscle fibres relevant to muscle fatigue. *Acta Physiol Scand* **162,** 229-245.

Stepto NK, Hawley JA, Dennis SC & Hopkins WG. (1999). Effects of different interval-training programs on cycling time-trial performance. *Med Sci Sports Exerc* **31,** 736-741.

Stepto NK, Martin DT, Fallon KE & Hawley JA. (2001). Metabolic demands of intense aerobic interval training in competitive cyclists. *Med Sci Sports Exerc* **33,** 303 - 310.

Storz G, Tartaglia LA & Ames BN. (1990). Transcriptional regulator of oxidative stress-inducible genes: direct activation by oxidation. *Science* **248,** 189-194.

Strojnik V & Komi PV. (1998). Neuromuscular fatigue after maximal stretch-shortening cycle exercise. *J Appl Physiol* **84,** 344-350.

Sun P, Enslen H, Myung PS & Maurer RA. (1994). Differential activation of CREB by Ca^{2+}/calmodulin-dependent protein kinases type II and type IV involves phosphorylation of a site that negatively regulates activity. *Genes Dev* **8,** 2527-2539.

Sun X, Nagarajan M, Beesley PW & Ng YC. (1999). Age-associated differential expression of Na^{+}-K^{+}-ATPase subunit isoforms in skeletal muscles of F-344/BN rats. *J Appl Physiol* **87,** 1132-1140.

Sutro J. (1986). Kinetics of veratridine action on Na channels of skeletal muscle. *J Gen Physiol* **87,** 1-24.

Suwa M, Nakano H & Kumagai S. (2003). Effects of chronic AICAR treatment on fiber composition, enzyme activity, UCP3, and PGC-1 in rat muscles. *J Appl Physiol* **95,** 960-968.

Suwannachot P, Verkleij CB, Weijs WA, van Weeren PR & Everts ME. (1999). Effects of training on the concentration of Na^+, K^+-ATPase in foal muscle. *Equine Vet J Suppl*, 101-105.

Sweadner KJ & Rael E. (2000). The FXYD gene family of small ion transport regulators or channels: cDNA sequence, protein signature sequence, and expression. *Genomics* **68,** 41-56.

Sweeney G & Klip A. (1998). Regulation of the Na^+/K^+-ATPase by insulin: why and how? *Mol Cell Biochem* **182,** 121-133.

Tamkun M & Fambrough D. (1986). The (Na^+ + K^+)-ATPase of chick sensory neurons. Studies on biosynthesis and intracellular transport. *J Biol Chem* **261,** 1009-1019.

Taormino JP & Fambrough DM. (1990). Pre-translational regulation of the (Na^+ + K^+)-ATPase in response to demand for ion transport in cultured chicken skeletal muscle. *J Biol Chem* **265,** 4116-4123.

Terada S, Muraoka I & Tabata I. (2003). Changes in $[Ca^{2+}]_i$ induced by several glucose transport-enhancing stimuli in rat epitrochlearis muscle. *J Appl Physiol* **94,** 1813-1820.

Thompson CB, Choi C, Youn JH & McDonough AA. (1999). Temporal responses of oxidative vs. glycolytic skeletal muscles to K^+ deprivation: Na^+ pumps and cell cations. *Am J Physiol* **276,** C1411-1419.

Thompson CB, Dorup I, Ahn J, Leong PK & McDonough AA. (2001). Glucocorticoids increase sodium pump α_2- and β_1-subunit abundance and mRNA in rat skeletal muscle. *Am J Physiol* **280,** C509-516.

Thompson CB & McDonough AA. (1996). Skeletal muscle Na,K-ATPase α and β subunit protein levels respond to hypokalemic challenge with isoform and muscle type specificity. *J Biol Chem* **271,** 32653-32658.

Toledano MB & Leonard WJ. (1991). Modulation of transcription factor NF-kappa B binding activity by oxidation-reduction in vitro. *Proc Natl Acad Sci U S A* **88,** 4328-4332.

Tsakiridis T, Wong PP, Liu Z, Rodgers CD, Vranic M & Klip A. (1996). Exercise increases the plasma membrane content of the Na^+ -K^+ pump and its mRNA in rat skeletal muscles. *J Appl Physiol* **80,** 699-705.

Tunstall RJ, Mehan KA, Wadley GD, Collier GR, Bonen A, Hargreaves M & Cameron-Smith D. (2002). Exercise training increases lipid metabolism gene expression in human skeletal muscle. *Am J Physiol* **283,** E66-72.

van der Poel C & Stephenson DG. (2002). Reversible changes in Ca^{2+}-activation properties of rat skeletal muscle exposed to elevated physiological temperatures. *J Physiol* **544,** 765-776.

Veeneklaas RJ, Harun MA, Backx A, Mamade M, Joosten BJ & Everts ME. (2004). Effects of training on Na^+, K^+-ATPase contents in skeletal muscle and K^+ homeostasis of African draught bulls and cows. *J Vet Med* **51,** 321-326.

Venosa RA & Horowicz P. (1981). Density and apparent location of the sodium pump in frog sartorius muscle. *J Membr Biol* **59,** 225-232.

Verburg E, Hallen J, Sejersted OM & Vollestad NK. (1999). Loss of potassium from muscle during moderate exercise in humans: a result of insufficient activation of the Na^+-K^+-pump? *Acta Physiol Scand* **165,** 357-367.

Vincent HK, Powers SK, Stewart DJ, Demirel HA, Shanely RA & Naito H. (2000). Short-term exercise training improves diaphragm antioxidant capacity and endurance. *Eur J Appl Physiol* **81,** 67-74.

Voldstedlund M, Tranum-Jensen J & Vinten J. (1993). Quantitation of Na^+/K^+-ATPase and glucose transporter isoforms in rat adipocyte plasma membrane by immunogold labeling. *J Membr Biol* **136,** 63-73.

Walke W, Staple J, Adams L, Gnegy M, Chahine K & Goldman D. (1994). Calcium-dependent regulation of rat and chick muscle nicotinic acetylcholine receptor (nAChR) gene expression. *J Biol Chem* **269,** 19447-19456.

Wang J, Velotta JB, McDonough AA & Farley RA. (2001). All human Na^+-K^+-ATPase α-subunit isoforms have a similar affinity for cardiac glycosides. *Am J Physiol* **281,** C1336-1343.

Ward DS, Hamilton MT & Watson PD. (1996). Measurement of tissue volume during non-steady state high-intensity muscle contraction. *Am J Physiol* **271,** R1682-1690.

Wardzala LJ & Jeanrenaud B. (1981). Potential mechanism of insulin action on glucose transport in the isolated rat diaphragm. Apparent translocation of intracellular transport units to the plasma membrane. *J Biol Chem* **256,** 7090-7093.

Weil E, Sasson S & Gutman Y. (1991). Mechanism of insulin-induced activation of Na^+-K^+-ATPase in isolated rat soleus muscle. *Am J Physiol* **261,** C224-230.

Wessels HP & Spiess M. (1988). Insertion of a multispanning membrane protein occurs sequentially and requires only one signal sequence. *Cell* **55,** 61-70.

Wessler I. (1996). Acetylcholine release at motor endplates and autonomic neuroeffector junctions: a comparison. *Pharmacol Res* **33,** 81-94.

Westerblad H, Duty S & Allen DG. (1993). Intracellular calcium concentration during low-frequency fatigue in isolated single fibers of mouse skeletal muscle. *J Appl Physiol* **75,** 382-388.

Westerblad H & Lannergren J. (1986). Force and membrane potential during and after fatiguing, intermittent tetanic stimulation of single Xenopus muscle fibres. *Acta Physiol Scand* **128,** 369-378.

Westerblad H, Lee JA, Lamb AG, Bolsover SR & Allen DG. (1990). Spatial gradients of intracellular calcium in skeletal muscle during fatigue. *Pflugers Arch* **415,** 734-740.

Westerblad H, Lee JA, Lannergren J & Allen DG. (1991). Cellular mechanisms of fatigue in skeletal muscle. *Am J Physiol* **261,** C195-209.

Westgarth-Taylor C, Hawley JA, Rickard S, Myburgh KH, Noakes TD & Dennis SC. (1997). Metabolic and performance adaptations to interval training in endurance-trained cyclists. *Eur J Appl Physiol Occup Physiol* **75,** 298-304.

Weston AR, Myburgh KH, Lindsay FH, Dennis SC, Noakes TD & Hawley JA. (1997). Skeletal muscle buffering capacity and endurance performance after high-intensity interval training by well-trained cyclists. *Eur J Appl Physiol Occup Physiol* **75,** 7-13.

Williams M, Resneck W, Kaysser T, Ursitti J, Birkenmeier C, Barker J & Bloch R. (2001). Na,K-ATPase in skeletal muscle: two populations of β-spectrin control localization in the sarcolemma but not partitioning between the sarcolemma and the transverse tubules. *J Cell Sci* **114,** 751-762.

Williams RS & Neufer PD. (1996). Regulation of gene expression in skeletal muscle by contractile activity. In *The handbook of physiology,* ed. Rowell LB & Shepherd JT, pp. 1124-1150. Oxford University Press, New York.

Wolitzky BA & Fambrough DM. (1986). Regulation of the (Na^+ + K^+)-ATPase in cultured chick skeletal muscle. Modulation of expression by the demand for ion transport. *J Biol Chem* **261,** 9990-9999.

Wong JA, Fu L, Schneider EG & Thomason DB. (1999). Molecular and functional evidence for Na^+-K^+-$2Cl^-$ cotransporter expression in rat skeletal muscle. *Am J Physiol* **277,** R154-161.

Xie Y, Langhans-Rajasekaran SA, Bellovino D & Morimoto T. (1996). Only the first and the last hydrophobic segments in the COOH-terminal third of Na,K-ATPase α subunit initiate and halt, respectively, membrane translocation of the newly synthesized polypeptide. *J Biol Chem* **271,** 2563-2573.

Xie Z, Kometiani P, Liu J, Li J, Shapiro JI & Askari A. (1999). Intracellular reactive oxygen species mediate the linkage of Na^+/K^+-ATPase to hypertrophy and its marker genes in cardiac myocytes. *J Biol Chem* **274,** 19323-19328.

Yeung EW, Ballard HJ, Bourreau JP & Allen DG. (2003). Intracellular sodium in mammalian muscle fibers after eccentric contractions. *J Appl Physiol* **94,** 2475-2482.

Yu M, Stepto NK, Chibalin AV, Fryer LG, Carling D, Krook A, Hawley JA & Zierath JR. (2003). Metabolic and mitogenic signal transduction in human skeletal muscle after intense cycling exercise. *J Physiol* **546,** 327-335.

Zamofing D, Rossier BC & Geering K. (1989). Inhibition of N-glycosylation affects transepithelial Na^+ but not Na^+-K^+-ATPase transport. *Am J Physiol* **256,** C958-966.

Zhao H, Hyde R & Hundal HS. (2004). Signalling mechanisms underlying the rapid and additive stimulation of NKCC activity by insulin and hypertonicity in rat L6 skeletal muscle cells. *J Physiol* **560,** 123-136.

APPENDICES

Appendix 1 Standard forms and participation information sheets

Appendix 2 Raw data Study 1

Appendix 3 Raw data Study 2

Appendix 4 Raw data Study 3

Appendix 5 Raw data Study 4

Appendix 6 Raw data Study 5

Appendix 7 Photos from Experimental days

Appendix 1. Standard forms and participant information sheets

Appendix 1.1 Standard cardiovascular risk factor questionnaire

Appendix 1.2 Standard muscle biopsy and arterial-venous cannulation questionnaire

Appendix 1.3 Standard consent form

Appendix 1.4 Participation information sheets

Victoria University of Technology
PO Box 14428
MELBOURNE CITY MC VIC 8001
Australia

Telephone:
(03) 9688 4432
Facsimile:
(03) 9688 4891

School of Human Movement, Recreation and Performance
Footscray Park Campus
Building L, Ballarat Road, Footscray

VICTORIA UNIVERSITY OF TECHNOLOGY

CARDIOVASCULAR RISK FACTOR QUESTIONNAIRE

In order to be eligible to participate in the experiment investigating:
Effects of acute prolonged exercise on ion regulatory gene and protein expression in human skeletal muscle
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 that you think may prevent you from participating in strenuous exercise? No Yes, please elaborate _____
10. Are you currently pregnant or expect to become pregnant during the time in which this experiment is conducted? Yes No

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

Signed: _____ Date: _____

Victoria University of Technology
PO Box 14428
MELBOURNE CITY MC VIC 8001
Australia

Telephone:
(03) 9688 4432
Facsimile:
(03) 9688 4891

School of Human Movement, Recreation and Performance
Footscray Park Campus
Building L, Ballarat Road, Footscray

VICTORIA UNIVERSITY OF TECHNOLOGY

MUSCLE BIOPSY & ARTERIAL - VENOUS CANNULATION QUESTIONNAIRE

Effects of acute prolonged exercise on ion regulatory gene and protein expression in human skeletal muscle

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? 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? Yes No
   If yes, please elaborate... _____
   _____

7. Have you ever fainted when you had an injection or blood sample taken?
   Yes No Don't Know
   If yes, please elaborate _____

8. Have you previously had heparin infused or injected?
   Yes No Don't know

If yes, please elaborate_____

9. Do you or other members of your family have Raynauds disease, or suffer from very poor circulation in the fingers, leading to painful fingers that turn white/blue?

Yes No Don't know

If yes, please elaborate_____

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

Signature: _____ **Date:** _____

Victoria University of Technology
PO Box 14428
MELBOURNE CITY MC VIC 8001
Australia

Telephone:
(03) 9688 4432
Facsimile:
(03) 9688 4891

VICTORIA UNIVERSITY OF TECHNOLOGY

School of Human Movement, Recreation and Performance
Footscray Park Campus
Building L, Ballarat Road, Footscray

CONSENT FORM FOR PARTICIPANTS INVOLVED IN RESEARCH

INFORMATION TO PARTICIPANTS:

We would like to invite you to be a part of a study, which will investigate the effects of exercise on key proteins that regulate sodium, potassium and calcium in your muscles.

CERTIFICATION BY PARTICIPANT

I,
of
certify that I am at least 18 years old* and that I am voluntarily giving my consent to participate in the experiment entitled:

Effects of acute prolonged exercise on ion regulatory gene and protein expression in human skeletal muscle

being conducted at Victoria University of Technology by:
Associate Professor Michael McKenna, Dr Rodney Snow, Dr David Cameron-Smith and Ms Kate Murphy

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:
Ms Kate Murphy, Associate Professor Michael McKenna, Dr Rodney Snow and Dr David Cameron-Smith
and that I freely consent to participation involving the use on me of these procedures.

1. **Pre-experiment participant screening**
2. **Maximal exercise test on a stationary cycle**
3. **Prolonged exercise test on a stationary cycle**
4. **Heating of the hand and venous blood sampling**
5. **Muscle biopsies**

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 of the risks involved and am freely participating in this study.

Signed: ..... }
Witness other than the experimenter: } **Date:** .....
.....}

Any queries about your participation in this project may be directed to the researcher (Name: Assoc. Prof. 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 MC, Melbourne, 8001 (telephone no: 03-9688 4710).

PARTICIPANT INFORMATION SHEET

"Effects of acute exercise on skeletal muscle Na^+,K^+-pumps in humans: content, activity and gene expression"

INVESTIGATORS:
Associate Professor Michael McKenna, Ms Kate Murphy and Aaron Petersen, School of Human Movement, Recreation and Performance, Victoria University of Technology, Footscray, Victoria and Dr Rodney Snow, David Cameron-Smith, School of Health Sciences, Deakin University, Burwood, Victoria.

Aim of the study:
This study will investigate the effects of exercise on a key enzyme located in your muscle cells, known as the sodium, potassium pump. This enzyme helps to prevent your muscles from becoming fatigued, but we know little about its role in exercise. The first aim of this study is to find out whether exercise causes an increased or decreased availability of these pumps in your muscle. The second aim is to see whether a single bout of exercise is sufficient to cause the genes in your muscle cells to start producing new sodium, potassium pumps, which might then allow improvement in subsequent exercise sessions.
Results from this project will yield considerable social benefits by enhancing our understanding of the acute responses of muscle to exercise, factors influencing muscle fatigue, and the initial steps underlying normal training responses. Possible future applications may include the use of even single exercise bouts to enhance muscle function in patients before and after surgery.

Subject participation:
Should you choose to participate, as a you will be free to withdraw from the study at any time, without any adverse effects, reactions or discrimination. Your total time involvement would be from 3-4 weeks.

Exercise Testing Procedures:
Should you decide to participate in this study, you will be asked to undertake each of the three tests described below. You will be required to attend the Exercise Physiology Laboratory (Room L305, Building L), at the Footscray Campus of Victoria University of Technology on five separate occasions for an exercise test. Whilst each test is tiring, you will recovery from this very quickly. You will be asked to perform three different types of exercise tests:
(i) maximal aerobic fitness (VO_2 peak): this test involves continuous exercise on a cycle ergometer with the exercise intensity (effort) being progressively increased. We will closely monitor you and your heart electrical activity (ECG) during exercise to ensure your safety. The test is completed when you become too tired to continue (wish to stop), or unless we stop the test due to you having an abnormal response to exercise, such as unusual heart rhythm, inappropriate heart rate or sweating responses, chest pain, or sever shortness of breath. The most common event associated with maximal exercise tests is fainting. This will be prevented using our standard laboratory procedures. In the unlikely event of emergency situations, two members of the research team have current CPR (cardio pulmonary resuscitation) qualifications and the Western Hospital is minutes away by ambulance.
(ii) maximal leg muscle strength: this test involves contractions of your thigh muscles on a Cybex dynamometer, with your leg kicking speed controlled at a moderate speed.

(iii) leg muscle fatigue: this test involves repeated kicking contractions at 75% of your maximal leg strength, performed every 2 seconds, until your thigh muscles fatigue.

Order of Exercise Tests:

Should you choose to participate, the tests will be conducted in the following order. On the first visit to the laboratory you will be required to perform the VO_2 peak test. After a 20 minute rest you will then be asked to do a practise trial of the leg strength and the leg fatigue tests, to familiarise you with these tests. You will be to repeat the leg strength and the leg fatigue test on three more visits to the laboratory, so that we can measure how much your leg strength and fatigue varies. On your fifth visit, we will ask you to repeat the leg fatigue test again. During this test, muscles biopsy samples will be taken from your thigh muscle, and blood samples will be taken from a hand vein, at rest, and during exercise, as described below.

Blood Samples:

Should you choose to participate, blood samples will be taken as described. At specific intervals throughout the exercise test a small blood sample (each 5 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 approximately fifty (50) 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. 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 pH.

Muscle Biopsies and Muscle Fatigue Testing:

Should you choose to participate, muscle biopsy samples will be taken as described. On your fifth visit to the Exercise Physiology Laboratory, a muscle biopsy will be taken from your thigh muscle, at rest, immediately after you stop the fatigue test, and then 3 hours later. You will be asked to return to the laboratory for the final time (sixth visit) the day after the test, to have the last muscle biopsy taken at 24 hours after the end of the test. Thus a total of four biopsies will be taken over a 24 hour period. 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 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 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. On very rare occasions, some people have reported altered sensation (numbness or tingling) in the skin near the site of the biopsy. This is due to very small nerve being cut, but this sensation disappears over a period of a few weeks-to-months. Although the possibility of infection, significant bruising and altered sensation is quite small, if by chance it does eventuate, please inform us immediately and we will immediately consult the doctor who performed the biopsy to review the reported problems and recommend appropriate action.

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:

Assoc.Prof. Michael McKenna	**W: (03) 9688-4499**	**H (03) 5422-6089**
Dr Rod Snow	**W: (03) 9251-7315**	
Dr David Cameron-Smith	**W: (03) 9244-6502**	

Any queries about your participation in this project may be directed to the researcher (Name: Assoc. Prof. M. McKenna Ph. (03) 54226089). 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 MC, Melbourne, 8001 (telephone no: 03-9688 4710).

PARTICIPANT INFORMATION SHEET

Effects of acute prolonged exercise on ion regulatory gene and protein expression in human skeletal muscle

Investigators:
Associate Professor Michael McKenna and **Ms Kate Murphy**,
School of Human Movement, Recreation and Performance,
Victoria University of Technology, Footscray, Victoria
Dr. Rodney Snow and **Dr. David Cameron-Smith**,
School of Health Sciences, Deakin University, Burwood, Victoria.

Aims of the study:
This study will investigate the effects of exercise on key proteins located in your muscle cells. These proteins control sodium, potassium and calcium and in doing so, help prevent your muscles from becoming fatigued. The aim of this study is to find out whether exercise alters availability and activity of these proteins, and the genes for these proteins, in your muscle. Results from this project will enhance our understanding of the acute responses of muscle to exercise, factors influencing muscle fatigue, and the initial steps underlying normal training responses.

Subject participation:
Should you decide to participate, you will be free to withdraw from the study at any time, without any adverse effects, reactions or penalty. Your total time involvement would be 3 weeks.

Exercise Testing Procedures:
Should you decide to participate in this study, you will be asked to undertake each of the three tests described below. You will be required to attend the Exercise Physiology Laboratory (Room L305, Building L), at the Footscray Campus of Victoria University of Technology on three separate occasions for an exercise test. Whilst each test is tiring, you will recover from this very quickly. You will be asked to perform three different types of exercise tests:

(i) **Maximal aerobic fitness (VO_2 peak):** this test involves continuous exercise on a stationary cycle with the exercise intensity (effort) being progressively increased. We will closely monitor you and your heart electrical activity (ECG) during exercise to ensure your safety. The test is completed when you become too tired to continue (wish to stop), or unless we stop the test due to you having an abnormal response to exercise, such as unusual heart rhythm, inappropriate heart rate or sweating responses, chest pain, or severe shortness of breath. The most common event associated with maximal exercise tests is fainting. This will be dealt with using our standard laboratory procedures. In the unlikely event of emergency situations, two members of the research team have current CPR (cardio pulmonary resuscitation) qualifications and the Western Hospital is minutes away by ambulance.

(ii) **Prolonged cycling test:** this test involves continuous exercise on a stationary cycle, at an intensity corresponding ~75% of the highest work rate you achieved in the VO_2 peak test. This test is continued until you become too tired to continue, or

unless we stop the test due to you having an abnormal response to exercise, such as that detailed above. Similarly, we will continuously monitor you and your heart activity throughout the test.

Order of Exercise Tests:

Should you decide to participate, the tests will be conducted in the following order. On the first visit to the laboratory you will be required to perform the VO_2 peak test. You will then be asked to perform the prolonged cycling test on two more visits to the laboratory. On your second visit you will be asked to perform the prolonged exercise test for your familiarisation. On your third visit to the laboratory, we will ask you to repeat the prolonged cycling test again. During this test, muscles biopsy samples will be taken from your thigh muscle, and blood samples will be taken from a hand vein, at rest, during, and following exercise, as described on the following page.

Blood Samples:

Should you choose to participate, blood samples will be taken as described. At specific intervals throughout the exercise test, a small blood sample (each 5 ml) will be taken via a catheter placed into a vein in the back of your hand. The catheter consists of a needle and plastic 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 plastic 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 approximately fifty (50) ml of blood will be taken during the test. This is equivalent to approximately 4 tablespoons. Normally 400 ml is taken when you donate at the Blood Bank. Each time a blood sample is taken, a small volume of sterile saline (1-2 ml) will be injected to clear the catheter. Catheterisation 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 pH.

Muscle Biopsies and Muscle Fatigue Testing:

Should you choose to participate, muscle biopsy samples will be taken as described. On your fourth visit to the Exercise Physiology Laboratory, a muscle biopsy will be taken from your thigh muscle, at rest, fatigue, and at 3 h and 24 h post-exercise. **Thus, a total of four biopsies will be taken**. 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. 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 only lasts for 1-2 seconds. The size of muscle removed by the biopsy needle is similar to a 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 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. To minimise the risk of infection, the biopsy will be conducted under sterile conditions. On rare occasions, some people experience fainting episodes, but a qualified medical practitioner will perform the whole procedure. On very rare occasions, some participants have reported altered sensation (numbness or tingling) in the skin near the site of the biopsy. This is due to very small nerve being cut, but this sensation disappears over a period of a few weeks-to-months.
Although the possibility of infection (for example, pus, tenderness and/or redness), significant bruising and altered sensation is quite small, if by chance it does eventuate, please inform us immediately and we will immediately consult the doctor who performed the biopsy to review the reported problems and recommend appropriate action.

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 without penalty. Thank you for your co-operation.

Contact Numbers:

Assoc. Prof. Michael McKenna W: (03) 9688 4499 H (03) 5422 6089
Dr Rod Snow W: (03) 9251 7315

Dr David Cameron-Smith W: (03) 9244 6502

Any queries about your participation in this project may be directed to the researcher (Name: Assoc. Prof. M. McKenna Ph. (03) 54226089). 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 MC, Melbourne, 8001 (telephone no: 03-9688 4710).

PARTICIPANT INFORMATION SHEET

The effect of short term intensified interval training on skeletal muscle adaptation in well-trained cyclists

Investigators:
Professor John Hawley and Sally Clark (PhD student)
School of Medical Sciences, Division of Exercise Sciences, Exercise Metabolism Group, RMIT University, Bundoora Campus;
Associate Professor Michael McKenna, Robert Aughey (PhD student) and Kate Murphy (PhD student)
School of Human Movement, Recreation and Performance, Victoria University of Technology, Footscray Park Campus

Aims of the study:
Elite endurance athletes show an improved ability to sustain high absolute workrates for prolonged periods compared to less well-trained cyclists. This attenuation of muscle fatigue could be caused by many factors. In recent years, impairments in lactate, sodium, potassium, calcium and hydrogen regulation in skeletal muscle have been identified as important contributors to the fatigue process at several important *sites* these include:

(a) Lactate (lactic acid) produced in the muscle is transported into and out of the muscle membrane by two transporters. The abundance of lactate transporters in skeletal muscle is higher in highly trained athletes compared with untrained individuals. Furthermore, 8 weeks of high intensity training has been shown to increase lactate transporters and lactate/hydrogen transport capacity in untrained individuals. However it is not known whether a period of intensified training in already well-trained athletes can further increase the abundance of lactate transporters. Therefore the capacity of the skeletal muscle to take up lactate and utilise it may be a key determinant of endurance performance.

(b) Sodium and potassium are transported across the muscle membrane via an enzyme known as the sodium-potassium pump. This pump allows the electrical impulses that enable muscle contraction to spread into the muscle. The content of the sodium-potassium pump in skeletal muscle is changed after sprint and endurance training in healthy untrained participants. The effects of short-term intensified-interval training on the skeletal muscle sodium-potassium pump in well-trained subjects has not yet been investigated.

Accordingly, we aim to examine the effects of short-term intensified-training on skeletal muscle electrolyte regulation in already well-trained athletes, by measuring the effects on lactate and sodium-potassium regulation.

Subject participation:
Should you decide to participate in this study, you are free to withdraw from the study at any time, without any adverse effects, reactions or penalty.

Exercise Testing Procedures:

Should you decide to participate in this study, you will be required to attend the exercise physiology laboratory located in the Robert Magee building at the Bundoora West Campus of RMIT on 17 occasions over a 6-week period. Whilst each test is tiring, you will recover from this very quickly. You will undergo 2 weeks of initial exercise tests, 3 weeks of training and a week of re-testing. There will be a total of 8 exercise tests before, 7 interval training sessions and 5 post training exercise tests for the treatment group. Each exercise test is completed when you become too tired to continue (wish to stop), or unless we stop the test due to you having an abnormal response to exercise, such as an inappropriate heart rate or sweating responses, chest pain or severe shortness of breath. We will closely monitor you and your heart rate during exercise to ensure your safety. The most common event associated with maximal exercise testing is fainting. This will be prevented using our standard laboratory procedures. In the unlikely event of emergency situations, two members of the research team have current CPR (cardio pulmonary resuscitation) qualifications and an ambulance would be immediately called.

Preliminary tests:

1) Height, body mass, skinfolds and Maximal Oxygen Uptake ($V,\dot{O}_{2peak}$)

You will perform various baseline tests, including the measurement of your body mass (BM) and anthropometric measures including skinfolds and height. You will then perform a sub-maximal test on a cycle ergometer, to establish your lactate threshold. The initial intensity is very low but it will progressively increase until a lactate of 4 mmol/L is reached. After a short rest, you will perform an exercise test to fatigue (a max test) on a cycle ergometer. Fatigue is defined as the point at which you can no longer maintain your cadence above 60 – 70 rpm. From this test the desired work rate (% of peak sustained power output [PPO]) for defining the effort of the subsequent training sessions will be determined. During the test, expired air will be sampled as you breathe into a mouthpiece attached to an automated gas analyser. At the same time, we will also monitor your heart rate using the Polar Sports Tester. The results from these tests will provide you with a $V,\dot{O}_{2peak}$, peak sustained power output (PPO), lactate threshold and maximal heart rate (Hr_{max}). This data is then used to set your personalised training sessions.

2) 40 km Time Trial

Two days later will be required to report to the RMIT University Exercise Physiology Laboratory with your own bicycle, which will be mounted, on an air braked cycle ergometer. After a self-paced warm-up, you will then perform a simulated 40 km time. The only feedback you get is the distance covered.

3) Diet and Training Control

Within the next 48 hrs, you will report to the lab between 1700 and 1800 pm, where we will supervise a moderate training session lasting 60 min. You will then be provided with a special diet for the subsequent 36 hours. During this time, you will be requested to refrain from any further exercise until the next lab session. This diet/training control is undertaken in order to standardise your muscle and liver carbohydrate stores, which are important for the muscle tissue analysis.

4) Lactate Turnover test

36 hrs after the one-hour laboratory-training ride you will report to the lab between 0600 and 0800 after an overnight fast. A sterile catheter will be inserted into a forearm vein on one arm and another will be inserted into the back of the hand on the opposite side. The catheter consists of a needle and plastic 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 plastic 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). After each sample, the catheter will be flushed with 2-3 ml of a solution to prevent clotting. In order to get good blood flow to the hand, your hand will be placed in a waterproof bag and immersed in a warm water bath (~45°C) up to the wrist for a 10-minute period before each blood sample is collected. During this period, you can have only one hand on the handlebars whilst cycling. This will feel slightly awkward but will not impair your performance on the bike since it is a requirement that you remain seated during the test. Blood sampling and catheter insertion will be performed by staff who are trained in the correct sterile techniques of blood sampling and venepuncture.

The catheter in the forearm vein of the opposite arm will be used to infuse a radiolabeled fluid before and throughout exercise. The fluid will be infused continuously at a rate very slow from an auto syringe pump. Infusion will commence 90 minutes before the exercise and be maintained during 90 minutes of cycling. During the programme you will be exposed to a very small amount of radiation. Although the actual radiation dose you will receive is not known, it is estimated that it may be less than 1mSv. This amount of radiation is about the same as 6 months of natural background radiation. All people on earth are exposed to background radiation. Background radiation comes from the sun, the earth, the air, and all around us.

The ill-effects at very high doses of radiation have been well documented, for example, increased life-threatening cancer rates and sometimes death has been reported in populations exposed to nuclear explosions or in patients undergoing radio-therapy treatment. However, at very low doses of radiation, similar to those being received from being a participant in this research, the risks are not completely known and have to be estimated using theoretical models based on the very high dose data. The acknowledged theoretical model suggests that risk from 1mSv is about 1 in 20,000. This model is based on a conservative approach and the actual risk may be a lot smaller. Compared to other risks in everyday life, this risk is considered minor. For example, this theoretical risk is approximately the same as smoking 80 cigarettes, traveling 4000km by car, or traveling 40,000km by commercial aircraft.

The first 60 minutes of cycling will be at workload that represents 65% of your $\dot{V}O_{2peak}$ and the final 30 minutes will be at workload that represents 85% of $\dot{V}O_{2peak}$. Blood samples will be taken while you are resting at 15, 30, 60 and 90 min after the start of the infusion, at 5, 15, 30, 45, 59, 65, 75 and 90 min during exercise and at 1, 3 and 5 min post exercise. During the 5 min preceding each blood draw during exercise, you will breathe into a mouthpiece for gas analysis.

Training sessions:

In each of the 7 training sessions you will perform 8 x 5 min @ ~82% of your PPO (~85% $V,\dot{O}_{2peak}$), with a 60 s rest between each 5 min bout on the cycle ergometer. Two sessions will be performed in weeks 1 and 3, and 3 sessions in week 2 in the RMIT exercise physiology laboratory. On the days you are not training in the laboratory you are to maintain your normal training rides, but cover 15% less of the distance you were doing before the intensified training. You will be required to log all your training distances in a logbook provided, during the period of the study.

Post-training tests:

48 hours after the training has been completed, the maximal tests, 40 km time-trial and the submaximal ride will be repeated (as described above). These will be conducted on separate days, and will require the same preparatory day before the submaximal ride.

Blood Samples:
A total of 250ml of blood, equivalent to approximately 17 tablespoons, will be drawn over all visits, as described in **Section 4 "Lactate Turnover test"**

Muscle Biopsies
Should you decide to participate in this study, on one visit to the RMIT Exercise Physiology Laboratory, a resting biopsy will be taken from your thigh muscle. On two subsequent visits, a muscle biopsy will be taken from your thigh muscle, at rest, and immediately after you stop the test. **Thus a total of five biopsies will be taken during these three visits.** Muscle biopsies are routinely carried out in this 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 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 hours after biopsies and you should avoid heavy knocks. It is common for participants 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. A qualified medical practitioner will perform the whole procedure. To minimise infection, the biopsy will be conducted under sterile conditions. On very rare occasions, some people have reported altered sensation (numbness or tingling) in the skin near the site of the biopsy. This is due to a very small nerve being cut, but this sensation disappears over a period of a few weeks-to-months. Although the possibility of infection, significant bruising and altered sensation (for example puss, tenderness, numbness, tingling and/or redness) is quite small, if by chance it does eventuate, please inform us immediately and we will immediately consult the doctor who performed the biopsy.

By signing the informed consent form you are indicating that the tests and procedures have been explained to you and are understood by you. Also, it is accepted by the investigators and by yourself that you are participating voluntarily in the study and that you are free to withdraw from the investigation at any time without any penalty. Thank you for your co-operation.

Contact Numbers:

Professor John Hawley	**W:**	**99257353**		
Assoc. Prof. Michael McKenna	**W:**	**9688 4499**	**H:**	**(03) 5422-6089**

Any queries about your participation in this project may be directed to the researcher (Assoc. Prof. 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 MC, Melbourne, 8001 (telephone no: 03-9688 4710).

Appendix 2. Raw data Study 1

Effects of brief intense exercise on Na^+,K^+-ATPase isoform mRNA and protein expression in human skeletal muscle

Appendix 2.1 Study 1 Subject physical characteristics

Subject No.	Age (yr)	Height (cm)	Body mass (kg)
1	21	172.5	89.7
2	19	176.5	61.3
3	18	170.5	72.0
4	28	180.0	82.2
5	21	168.0	57.7
6	26	178.5	86.2
7	20	180.0	77.8
8	21	180.0	76.3
9	26	172.5	76.0
10	18	158.5	55.6
11	20	178.5	61.0
12	37	163.6	59.3
13	29	181.0	80.7
14	40	178.5	75.5
15	27	179.0	86.4
n	**15**	**15**	**15**
Mean	**25**	**174.5**	**73.2**
SD	**7**	**6.8**	**11.4**
SEM	**2**	**1.8**	**3.0**

Appendix 2.2 Peak O_2 consumption and exercise data from incremental exercise test

Subject No.	$\dot{V}O_{2peak}$ ($ml.kg^{-1}.min^{-1}$)	PPO (W)	Time to fatigue (s)
1	47.0	375	855
2	55.6	325	735
3	39.4	275	600
4	47.8	275	644
5	66.0	475	1105
6	45.2	350	816
7	61.5	425	970
8	58.7	400	945
9	28.1	250	558
10	43.0	250	572
11	52.7	300	675
12	43.1	275	628
13	56.3	375	886
14	70.0	500	1150
15	45.0	350	812
n	**15**	**15**	**15**
Mean	**50.5**	**346.7**	**796.7**
SD	**11.0**	**79.0**	**189.1**
SEM	**2.8**	**20.4**	**48.8**

$\dot{V}O_{2peak}$, peak O_2 consumption; PPO, peak power output; W, watts; s, seconds.

Appendix 2.3 Exercise data from muscle strength test

Subject No.	Max WO (J)	Target work (J)	Target work as % Max WO
1	246	99	40
2	147	59	40
3	133	53	40
4	109	41	38
5	215	82	38
6	202	72	36
7	197	78	40
8	155	63	41
9	144	46	32
10	83	39	47
11	99	44	44
12	156	54	34
13	349	94	27
14	165	60	36
15	88	62	70
n	**14**	**14**	**14**
Mean	**164**	**63**	**38**
SD	**69**	**19**	**10**
SEM	**21**	**5**	**1**

J, joules; WO, work output.

Appendix 2.4 Exercise data from invasive muscle fatigue test

Subject No.	Time to fatigue (s)	Total work (J)	Target work/ rep. (J)	Actual work/ rep. (J)	No. of repetitions	Av. repetition time (s)
1	192	12687	99	101	125	1.5
2	189	8802	59	59	148	1.3
3	159	5450	53	55	99	1.6
4	891	25552	41	42	603	1.5
5	788	44721	82	89	504	1.6
6	596	27850	72	72	385	1.6
7	420	22298	78	89	250	1.7
8	312	12245	63	66	186	1.7
9	119	4221	46	56	76	1.6
10	181	5042	39	39	128	1.4
11	615	18630	41	42	442	1.4
12	83	3040	61	52	59	1.4
13	98	6371	92	95	67	1.5
14	514	25142	68	74	342	1.5
15	118	5321	60	61	87	1.4
n	**15**	**15**	**15**	**15**	**15**	**15**
Mean	**352**	**15158**	**64**	**66**	**233**	**1.5**
SD	**268**	**11946**	**18**	**20**	**178**	**0.1**
SEM	**69**	**3084**	**5**	**5**	**46**	**0.0**

Av., average; rep., repetition.

Appendix 2.5 Reproducibility data for muscle fatigue test

Subject	Time to fatigue (s)				Total work (J)			
No.	Trial 1	Trial 2	Invasive trial	CV (%)	Trial 1	Trial 2	Invasive trial	CV (%)
1	209	257	192	15	13978	14398	12687	7
2	163	267	239	24	6714	10065	8802	20
3	117	85	159	31	4533	3244	5450	25
4	348	280	788	58	20800	21125	44721	47
5	1200	179	891	69	33995	4940	25552	70
6	175	140	596	84	8394	7264	27850	80
7	606	369	420	27	35037	21437	22298	29
8	170	180	259	24	8686	8962	12245	20
9	112	116	119	3	3848	3988	4221	5
10	241	475	181	52	5850	12984	5042	55
11	241	458	615	43	7181	14898	18630	43
12	61	76	83	15	2399	3415	3040	17
13	78	73	98	16	5437	5472	6371	9
15	76	84	118	24	3630	4480	5321	19
n	**14**	**14**	**14**	**14**	**14**	**14**	**14**	**14**
Mean	**271**	**217**	**340**	**35**	**11463**	**9762**	**14445**	**32**
SD	**302**	**139**	**273**	**23**	**10857**	**6302**	**12061**	**24**
SEM	**81**	**37**	**73**	**6**	**2902**	**1684**	**3223**	**6**

Appendix 2.6 Linearity of human Na^+,K^+-ATPase α_1-α_3 and β_1-β_3 and CYC primers to various inputs of cDNA

Gene	r^2 value	Regression equation	95% confidence intervals, slope	Mean efficiency ($10^{-1/slope}$-1)
α_1	0.9790	y = -3.1913x + 33.718	-4.049 to -2.351	-1.0
α_2	0.9936	y = -5.2653x + 28.985	-5.786 to -4.281	-1.0
α_3	0.9849	y = -6.5921x + 41.212	-7.938 to -5.155	-1.0
β_1	0.9828	y = -4.6501x + 28.123	-5.748 to -3.519	-1.0
β_2	0.9815	y = -2.6675x + 30.963	-3.360 to -1.974	-1.0
β_3	0.9195	y = -6.614x + 41.154	-10.162 to -3.105	-1.0
CYC	0.9904	y = -4.4244x + 28.649	-5.256 to -3.518	-1.0

Appendix 2.7 Brief intense exercise effects on Na^+,K^+-ATPase α_1, α_2 and α_3 mRNA expression in human skeletal muscle

Subject	α_1				α_2				α_3			
No.	Rest	Fat	+3 h	+24 h	Rest	Fat	+3 h	+24 h	Rest	Fat	+3 h	+24 h
1	1.00	5.62	9.75	3.24	1.00	3.48	0.53	0.38	1.00	3.35	0.10	1.59
2	1.00	0.53	1.25	4.93	1.00	0.20	0.78	1.76	1.00	3.35	5.46	1.20
3	1.00	0.09	0.16	0.37	1.00	0.06	0.08	1.04	1.00	3.35	1.51	0.26
4	1.00	4.39	1.28	6.96	1.00	10.56	1.47	0.71	1.00	0.88	1.08	0.09
5	1.00	3.24	1.63	7.39	1.00	5.24	6.02	7.76	1.00	1.47	0.46	1.38
6	1.00	0.15	0.12	0.04	1.00	0.04	0.02	0.15	1.00	0.23	0.11	0.59
7	1.00	2.27	1.07	0.65	1.00	4.48	8.94	4.48	1.00	10.27	2.29	1.46
8	1.00	2.86	0.56	1.38	1.00	0.39	0.93	0.20	1.00	1.05	4.79	2.33
9	1.00	2.46	1.13	3.56	1.00	3.48	0.80	2.27	1.00	3.35	1.11	2.57
10	1.00	4.75	1.04	5.69	1.00	6.16	13.41	2.07	1.00	2.01	0.49	0.75
11	1.00	0.47	0.42	1.15	1.00	1.85	8.78	0.73	1.00	7.39	6.19	0.93
12	1.00	4.72	2.27	6.75	1.00	9.02	7.04	0.74	1.00	6.87	8.94	1.67
13	1.00	0.66	1.62	0.19	1.00	1.22	4.48	0.19	1.00	0.01	2.86	0.37
14	1.00	2.24	0.37	3.11	1.00	2.57	9.42	2.19	1.00	3.35	2.86	1.62
n	**14**	**14**	**14**	**14**	**14**	**14**	**14**	**14**	**14**	**14**	**14**	**14**
Mean	**1.00**	**2.46**	**1.62**	**3.24**	**1.00**	**3.48**	**4.48**	**1.76**	**1.00**	**3.35**	**2.73**	**1.20**
SD	**0.00**	**1.90**	**2.42**	**2.70**	**0.00**	**3.33**	**4.43**	**2.10**	**0.00**	**2.96**	**2.68**	**0.75**
SEM	**0.00**	**0.51**	**0.65**	**0.72**	**0.00**	**0.89**	**1.18**	**0.56**	**0.00**	**0.79**	**0.72**	**0.20**

All data expressed relative to rest.

Appendix 2.8 Brief intense exercise effects on Na^+,K^+-ATPase β_1, β_2 and β_3 mRNA expression in human skeletal muscle

Subject	β_1				β_2				β_3			
No.	Rest	Fat	+3 h	+24 h	Rest	Fat	+3 h	+24 h	Rest	Fat	+3 h	+24 h
1	1.00	2.28	0.97	1.10	1.00	0.88	0.54	1.05	1.00	3.51	1.54	1.24
2	1.00	0.83	0.30	1.23	1.00	0.98	0.19	1.33	1.00	2.58	1.18	1.04
3	1.00	0.61	0.17	1.92	1.00	0.40	0.74	0.58	1.00	1.01	1.04	0.23
4	1.00	12.69	1.38	0.57	1.00	1.84	4.64	0.23	1.00	2.55	0.42	0.06
5	1.00	3.45	1.96	0.73	1.00	1.37	0.30	0.93	1.00	0.95	3.01	0.82
6	1.00	0.37	0.26	0.07	1.00	0.25	0.20	0.86	1.00	0.94	0.40	1.13
7	1.00	1.85	2.84	1.00	1.00	4.35	3.66	0.98	1.00	5.98	6.70	6.63
8	1.00	0.04	0.65	0.13	1.00	0.18	1.94	0.85	1.00	0.15	10.41	0.36
9	1.00	2.28	2.21	4.31	1.00	2.72	0.88	2.69	1.00	2.04	0.60	0.39
10	1.00	0.60	2.68	3.21	1.00	3.81	1.96	3.09	1.00	0.89	0.43	0.44
11	1.00	1.24	11.16	0.69	1.00	3.71	3.46	1.33	1.00	1.42	7.84	0.53
12	1.00	1.71	10.41	0.39	1.00	5.17	4.71	0.48	1.00	4.99	2.37	0.91
13	1.00	2.36	2.82	0.52	1.00	8.98	1.94	2.06	1.00	0.95	2.85	0.19
14	1.00	1.58	1.68	1.33	1.00	3.43	2.02	2.10	1.00	0.59	1.05	0.60
n	**14**	**14**	**14**	**14**	**14**	**14**	**14**	**14**	**14**	**14**	**14**	**14**
Mean	**1.00**	**2.28**	**2.82**	**1.23**	**1.00**	**2.72**	**1.94**	**1.33**	**1.00**	**2.04**	**2.85**	**1.04**
SD	**0.00**	**3.14**	**3.50**	**1.20**	**0.00**	**2.44**	**1.61**	**0.85**	**0.00**	**1.73**	**3.17**	**1.65**
SEM	**0.00**	**0.84**	**0.94**	**0.32**	**0.00**	**0.65**	**0.43**	**0.23**	**0.00**	**0.46**	**0.85**	**0.44**

Appendix 2.9 Brief intense exercise effects on Na^+,K^+-ATPase α_1, α_2 and α_3 protein expression in human skeletal muscle

Subject	α_1				α_2				α_3			
No.	Rest	Fat	+3 h	+24 h	Rest	Fat	+3 h	+24 h	Rest	Fat	+3 h	+24 h
1	1.00	0.93	0.37	2.33	1.00	0.92	0.60	1.38	1.00	0.95	4.26	2.30
2	1.00	0.70	0.58	1.06	1.00	0.69	1.32	0.99	1.00	1.09	2.74	2.13
3	1.00	0.64	1.15	0.39	1.00	0.92	0.20	1.38	1.00	0.88	1.08	0.86
4	1.00	0.35	0.50	0.66	1.00	0.41	0.81	0.41	1.00	0.76	1.24	1.39
5	1.00	0.19	0.80	0.63	1.00	1.69	1.44	1.34	1.00	1.12	1.13	1.13
6	1.00	2.56	1.50	3.27	1.00	1.36	0.51	0.61	1.00	0.87	1.37	0.87
7	1.00	1.79	3.54	1.09	1.00	1.03	1.40	1.59	1.00	1.63	0.54	1.06
8	1.00	0.97	0.87	0.15	1.00	0.75	0.85	1.27	1.00	0.95	0.78	0.31
9	1.00	1.12	0.60	0.62	1.00	0.47	0.61	0.99	1.00	0.95	6.68	14.08
10	1.00	1.08	0.77	1.89	1.00	0.96	1.01	1.10	1.00	0.22	0.31	0.31
11	1.00	0.27	0.31	0.58	1.00	0.28	0.44	0.48	1.00	2.91	5.00	6.32
12	1.00	1.04	0.51	0.86	1.00	1.12	0.28	0.49	1.00	0.35	0.21	0.27
13	1.00	1.54	0.61	1.97	1.00	0.79	1.11	1.06	1.00	1.37	1.08	0.78
14	1.00	0.35	0.78	0.28	1.00	1.36	1.31	1.46	1.00	0.69	3.54	2.13
15	1.00	0.38	0.15	0.07	1.00	1.00	0.29	0.26	1.00	0.41	0.66	0.20
n	**15**	**15**	**15**	**15**	**15**	**15**	**15**	**15**	**15**	**15**	**15**	**15**
Mean	**1.00**	**0.93**	**0.87**	**1.06**	**1.00**	**0.92**	**0.81**	**0.99**	**1.00**	**1.01**	**2.04**	**2.27**
SD	**0.00**	**0.65**	**0.81**	**0.91**	**0.00**	**0.38**	**0.43**	**0.43**	**0.00**	**0.64**	**1.96**	**3.60**
SEM	**0.00**	**0.17**	**0.21**	**0.24**	**0.00**	**0.10**	**0.11**	**0.11**	**0.00**	**0.17**	**0.51**	**0.93**

Appendix 2.10 Brief intense exercise effects on Na^+,K^+-ATPase β_1, β_2 and β_3 protein expression in human skeletal muscle

Subject	β_1				β_2				β_3			
No.	Rest	Fat	+3 h	+24 h	Rest	Fat	+3 h	+24 h	Rest	Fat	+3 h	+24 h
1	1.00	0.94	1.26	2.47	1.00	1.05	1.43	1.13	1.00	1.08	0.24	2.57
2	1.00	0.25	0.91	1.09	1.00	0.79	1.31	1.90	1.00	0.34	2.13	1.27
3	1.00	1.33	1.95	1.27	1.00	1.05	2.14	0.87	1.00	1.08	1.14	1.44
4	1.00	0.19	1.40	0.80	1.00	2.01	1.79	1.84	1.00	0.82	1.51	1.87
5	1.00	1.37	1.60	2.91	1.00	0.65	1.51	2.00	1.00	0.80	0.72	0.95
6	1.00	0.88	1.52	0.99	1.00	0.90	0.47	0.46	1.00	0.94	0.47	0.92
7	1.00	1.18	1.19	0.90	1.00	1.25	1.23	1.33	1.00	0.70	1.66	1.73
8	1.00	0.73	0.20	0.22	1.00	1.05	0.78	0.71	1.00	1.08	0.65	0.66
9	1.00	2.55	2.72	1.89	1.00	0.96	1.35	1.31	1.00	3.75	1.18	1.27
10	1.00	0.55	0.45	0.29	1.00	0.77	0.82	0.71	1.00	1.08	1.31	2.55
11	1.00	0.57	0.50	0.98	1.00	0.29	0.36	1.32	1.00	0.90	1.16	2.09
12	1.00	1.57	0.88	1.00	1.00	1.14	0.11	1.08	1.00	0.22	0.17	0.29
13	1.00	0.35	0.14	0.61	1.00	1.00	2.03	1.83	1.00	2.07	8.97	0.74
14	1.00	0.84	1.29	0.37	1.00	2.14	1.79	1.90	1.00	0.52	0.77	0.36
15	1.00	0.81	0.94	0.57	1.00	0.71	0.95	0.85	1.00	0.82	0.62	0.34
n	**15**	**15**	**15**	**15**	**15**	**15**	**15**	**15**	**15**	**15**	**15**	**15**
Mean	**1.00**	**0.94**	**1.13**	**1.09**	**1.00**	**1.05**	**1.20**	**1.28**	**1.00**	**1.08**	**1.51**	**1.27**
SD	**0.00**	**0.61**	**0.68**	**0.78**	**0.00**	**0.48**	**0.61**	**0.51**	**0.00**	**0.85**	**2.13**	**0.76**
SEM	**0.00**	**0.16**	**0.18**	**0.20**	**0.00**	**0.12**	**0.16**	**0.13**	**0.00**	**0.22**	**0.55**	**0.20**

Appendix 2.11 Brief intense exercise effects on the average post-exercise Na^+,K^+-ATPase α_1, α_2, α_3, β_1, β_2 and β_3 mRNA expression in human skeletal muscle

Subject	Average post-exercise mRNA expression (relative to rest; 1.00)					
No.	α_1	α_2	α_3	β_1	β_2	β_3
1	6.20	1.46	1.68	1.45	0.82	2.09
2	2.24	0.91	3.34	0.79	0.83	1.60
3	0.21	0.39	1.71	0.90	0.57	0.76
4	4.21	4.25	0.68	4.88	2.24	1.01
5	4.08	6.34	1.10	2.05	0.87	1.59
6	0.10	0.07	0.31	0.23	0.43	0.82
7	1.33	5.97	4.67	1.89	2.99	6.44
8	1.60	0.51	2.72	0.27	0.99	3.64
9	2.38	2.18	2.34	2.93	2.09	1.01
10	3.83	7.21	1.09	2.16	2.95	0.59
11	0.68	3.79	4.84	4.36	2.83	3.26
12	4.58	5.60	5.83	4.17	3.45	2.76
13	0.82	1.96	1.08	1.90	4.33	1.33
14	1.91	4.72	2.61	1.53	2.52	0.75
n	**14**	**14**	**14**	**14**	**14**	**14**
Mean	**2.44**	**3.24**	**2.43**	**2.11**	**2.00**	**1.97**
SD	**1.86**	**2.46**	**1.69**	**1.48**	**1.24**	**1.61**
SEM	**0.50**	**0.66**	**0.45**	**0.40**	**0.33**	**0.43**

Appendix 2.12 Brief intense exercise effects on [^{3}H]-ouabain binding site content in human skeletal muscle

Subject No.	Rest	Fat	+3 h	+24 h
1	164	239	212	207
2	229	256	205	260
3	356	445	405	377
4	354	361	328	343
5	316	240	224	278
6	341	359	347	329
7	297	291	288	263
8			263	182
9	239	239	376	280
10	327	328	317	366
11	285	304	275	232
12	346	342	415	332
13	281	322	285	278
14	251	195	266	232
15	283	302	319	276
n	**14**	**14**	**15**	**15**
Mean	**291**	**302**	**302**	**282**
SD	**56**	**65**	**65**	**57**
SEM	**15**	**17**	**17**	**15**

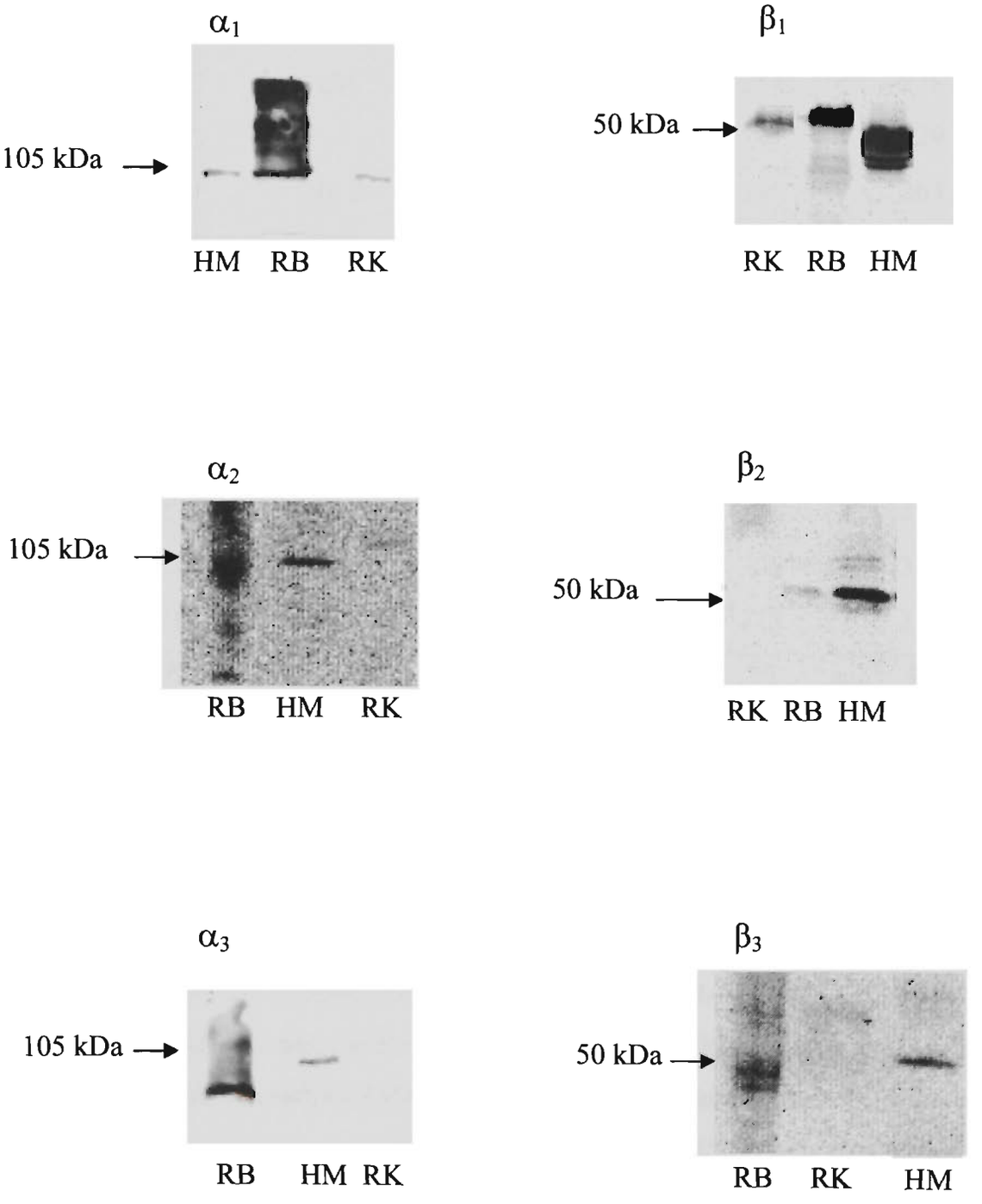
Blank, not measured. Muscle analyses completed by Aaron Petersen and presented in the following paper: Petersen, AC, Murphy, KT, Snow, RJ, Leppik, JA, Aughey, RJ, Garnham, AP, Cameron-Smith, D & McKenna, MJ. Depressed Na^+,K^+-ATPase activity in skeletal muscle at fatigue is correlated with increased Na^+,K^+-ATPase mRNA expression following intense exercise. *AJP Regul. Physiol,* In Review.

Appendix 2.13 Brief intense exercise effects on maximal 3-*O*-MFPase activity in human skeletal muscle

Subject No.	Rest	Fat	+3 h	+24 h
1	239	206	244	251
2	321	277	272	223
3	250	217	235	251
4	307	301	331	286
5	247	256	245	231
6		208		251
7			210	188
8	222	220	210	227
9	314	249	240	258
10	213	186	219	208
11	209	201	211	216
12	242	207	243	233
13		221		215
14	267	212	240	252
15	201	180	193	201
n	**12**	**14**	**13**	**15**
Mean	**253**	**224**	**238**	**233**
SD	**42**	**34**	**35**	**26**
SEM	**12**	**9**	**10**	**7**

Blank, not measured. Muscle analyses completed by Aaron Petersen and presented in the following paper: Petersen, AC, Murphy, KT, Snow, RJ, Leppik, JA, Aughey, RJ, Garnham, AP, Cameron-Smith, D & McKenna, MJ. Depressed Na^+,K^+-ATPase activity in skeletal muscle at fatigue is correlated with increased Na^+,K^+-ATPase mRNA expression following intense exercise. *AJP Regul. Physiol,* In Review.

Appendix 2.14 Representative immunoblots of Na^+,K^+-ATPase α_1, α_2, α_3, β_1, β_2 and β_3 isoforms in crude muscle homogenates of human vastus lateralis muscle (HM), rat brain (RB) and rat kidney (RK). Values at left indicate molecular weight of bands.



Appendix 2.15 Overview of muscle analyses performed and the method utilised, as well as the sample size used for each study in this thesis

Muscle analyses	Method used	Study 1	Study 2	Study 3	Study 4	Study 5
mRNA expression	Real-Time RT-PCR	✓	✓	✓	✓	✓
Protein abundance	Western blotting	✓	✓	✓		
Maximal Na^+,K^+-ATPase activity	Maximal 3-*O*-MFPase activity		✓		✓	
Muscle Na^+,K^+-ATPase content	[^{3}H]-ouabain binding site content		✓		✓	
n (number of humans used)		15	11	12	65*	
n (number of muscles used)						104

*n = 12 from Study 1, n = 8 from Study 2, n = 10 from Study 3, n = 35 from Aughey *et al.*, 2005.

Appendix 3. Raw data Study 2

Prolonged submaximal exercise induces isoform-specific transcriptional and translational responses of the Na^+,K^+-ATPase in human skeletal muscle

Appendix 3.1 Study 2 Subject physical characteristics

Subject No.	Age (yr)	Height (cm)	Body mass (kg)
1	31	180	80.1
2	23	167	64.6
3	21	156	58.5
4	23	167	54.7
5	32	155	45.8
6	23	165	65.4
7	20	173	64.8
8	22	181	72.3
9	24	188	83.8
10	21	179	62.7
11	23	172	62.0
n	**11**	**11**	**11**
Mean	**24**	**171**	**65**
SD	**4**	**10**	**11**
SEM	**1**	**4**	**4**

Appendix 3.2 Peak peak output and O_2 consumption from incremental exercise test

Subject No.	PPO (W)	$\dot{V}O_{2peak}$ ($ml.kg^{-1}.min^{-1}$)	$\dot{V}O_{2peak}$ ($l.min^{-1}$)
1	450	66.5	5.33
2	350	72.5	4.68
3	250	57.6	3.37
4	325	59.8	3.27
5	200	51.9	2.38
6	300	59.3	3.88
7	150	33.4	2.16
8	450	83.3	6.02
9	400	61.7	5.17
10	325	65.3	4.09
11	350	68.4	4.24
n	**11**	**11**	**11**
Mean	**323**	**61.8**	**4.05**
SD	**95**	**12.6**	**1.21**
SEM	**32**	**4.1**	**0.40**

PPO, peak power output

Appendix 3.3 Respiratory and exercise data from experimental prolonged exercise fatigue test

Subject No.	PO (W)	Av. $\dot{V}O_2$ (l.min-1)	Av. $\dot{V}O_2$ as (% $\dot{V}O_{2peak}$)	Av. $\dot{V}CO_2$ (l.min-1)	RER	Time to fatigue (s)
1	280	4.13	78.0	4.14	0.91	4853
2	250	3.75	80.0	3.62	0.97	1675
3	158	2.49	73.8	2.13	0.86	4073
4	177	2.38	72.9	2.19	0.91	3670
5	120	1.86	78.4	1.83	0.98	2423
6	190	2.77	71.3	2.94	1.08	2767
7	76	1.72	79.6	1.70	1.00	2352
8	316	4.46	74.1	4.12	0.95	2786
9	250	3.49	67.4	3.35	0.98	4620
10	200	2.77	67.7	2.63	0.98	1709
11	231	3.31	78.1	3.21	0.99	5021
n	**11**	**11**	**11**	**11**	**11**	**11**
Mean	**204**	**3.05**	**75.5**	**2.94**	**0.95**	**3268**
SD	**71**	**0.89**	**4.5**	**0.87**	**0.06**	**1234**
SEM	**23**	**0.31**	**1.5**	**0.30**	**0.02**	**363**

Av., average; $\dot{V}CO_2$, CO_2 output; RER, respiratory exchange ratio

Appendix 3.4 Prolonged submaximal exercise effects on arterialised venous plasma [K^+]

Subject No.	Rest	Ex 15	Ex 30	Ex 45	Ex 60	Ex 75	Fat	+1	+2	+5	+10
1	4.14	5.03	5.13	5.42	5.64	5.54	5.73	4.90	4.70	4.42	4.36
2	4.06	5.47					5.52	4.25	3.99	3.75	3.69
3	4.32	5.05	5.15	5.16	5.31		5.12		4.56	4.41	4.18
4	4.17	5.21	5.20	5.27	5.15		5.26	4.61	4.36	4.07	3.98
5	4.36	5.75	5.67	5.71			5.65	4.85	4.62	4.28	4.30
6	4.52	5.30	5.30				5.37		4.80	4.38	4.18
7	4.16	5.03	4.50				4.45		4.01	3.71	3.69
8	4.85	5.37	5.40	5.56			4.92	4.26	4.05	3.82	3.72
9	3.93	5.08	5.31	5.45	5.53	5.79	5.07	3.01	4.39	4.09	4.13
10	4.17	5.25	5.13	5.41			4.89	4.50	4.63	4.20	3.97
11	4.40	5.30	5.47	4.99	4.81	5.41	5.40	5.78	4.16	3.79	3.63
n	**11**	**11**	**10**	**8**	**5**	**3**	**11**	**8**	**11**	**11**	**11**
Mean	**4.28**	**5.26**	**5.23**	**5.37**	**5.29**	**5.58**	**5.22**	**4.52**	**4.39**	**4.08**	**3.98**
SD	**0.25**	**0.22**	**0.31**	**0.23**	**0.33**	**0.19**	**0.38**	**0.78**	**0.29**	**0.28**	**0.27**
SEM	**0.08**	**0.07**	**0.10**	**0.08**	**0.15**	**0.11**	**0.11**	**0.28**	**0.09**	**0.08**	**0.08**

Appendix 3.5 Prolonged submaximal exercise effects on Na^+,K^+-ATPase α_1, α_2 and α_3 mRNA expression in human skeletal muscle

	α_1				α_2				α_3			
Subject No.	Rest	Fat	+3 h	+24 h	Rest	Fat	+3 h	+24 h	Rest	Fat	+3 h	+24 h
1	1.00	1.67	1.47	2.26	1.00	2.23	1.24	0.77	1.00	0.46	0.89	0.18
2	1.00	0.61	0.58	0.29	1.00	0.82	1.13	0.37	1.00	1.49	0.94	2.66
3	1.00	1.18	2.34	0.78	1.00	3.15	2.38	2.28	1.00	4.01	0.31	1.47
4	1.00	0.59	0.59	3.29	1.00	1.67	2.69	0.61	1.00	0.74	0.26	0.05
5	1.00	3.26	1.15	4.75	1.00	2.05	0.70	0.12	1.00	0.89	0.69	0.02
6	1.00	1.05	1.27	0.84	1.00	0.79	1.16	0.07	1.00	2.20	0.84	0.00
7	1.00	1.09	1.55	3.38	1.00	0.78	0.75	0.91	1.00	4.81	2.85	3.12
8	1.00	0.78	1.12	1.68	1.00	0.80	1.21	1.39	1.00	4.78	0.64	0.89
9	1.00	0.96	1.49	1.75	1.00	2.24	1.66	0.86	1.00	0.42	0.20	0.24
10	1.00	1.30	0.26	0.52	1.00	1.50	0.88	0.63	1.00	2.20	1.26	0.89
11	1.00	2.24	2.17	2.78	1.00	0.77	1.65	3.28	1.00	2.20	0.63	0.28
n	**11**	**11**	**11**	**11**	**11**	**11**	**11**	**11**	**11**	**11**	**11**	**11**
Mean	**1.00**	**1.34**	**1.27**	**2.03**	**1.00**	**1.53**	**1.40**	**1.03**	**1.00**	**2.20**	**0.86**	**0.89**
SD	**0.00**	**0.84**	**0.64**	**1.41**	**0.00**	**0.86**	**0.64**	**0.97**	**0.00**	**1.65**	**0.73**	**1.09**
SEM	**0.00**	**0.27**	**0.19**	**0.43**	**0.00**	**0.27**	**0.19**	**0.29**	**0.00**	**0.50**	**0.22**	**0.33**

Appendix 3.6 Prolonged submaximal exercise effects on Na^+,K^+-ATPase β_1, β_2 and β_3 mRNA expression in human skeletal muscle

Subject	β_1				β_2				β_3			
No.	Rest	Fat	+3 h	+24 h	Rest	Fat	+3 h	+24 h	Rest	Fat	+3 h	+24 h
1	1.00	2.53	1.09	1.20	1.00	0.86	1.31	0.72	1.00	1.12	1.13	4.22
2	1.00	1.13	0.70	0.49	1.00	0.55	0.25	0.26	1.00	1.32	2.17	4.41
3	1.00	0.58	0.37	0.41	1.00	3.38	0.62	1.15	1.00	1.83	1.19	2.21
4	1.00	1.95	1.82	1.27	1.00	1.59	0.90	0.54	1.00	0.63	1.16	0.73
5	1.00	1.53	1.03	0.25	1.00	5.25	1.90	0.23	1.00	0.59	0.58	0.47
6	1.00	0.94	2.14	0.19	1.00	1.38	2.08	0.41	1.00	4.00	2.31	0.59
7	1.00	1.14	0.43	1.45	1.00	2.22	1.86	2.92	1.00	0.38	0.37	0.61
8	1.00	0.54	0.40	0.82	1.00	1.18	0.64	0.90	1.00	1.25	1.79	2.78
9	1.00	0.51	1.61	1.02	1.00	1.14	0.53	0.81	1.00	1.49	0.74	0.52
10	1.00	1.10	0.26	0.47	1.00	1.90	1.76	1.45	1.00	1.22	0.41	0.39
11	1.00	1.17	0.72	1.17	1.00	1.89	2.36	0.77	1.00	1.28	1.41	1.15
n	**11**	**11**	**11**	**11**	**11**	**11**	**11**	**11**	**11**	**11**	**11**	**11**
Mean	**1.00**	**1.20**	**0.96**	**0.80**	**1.00**	**1.94**	**1.29**	**0.92**	**1.00**	**1.39**	**1.21**	**1.64**
SD	**0.00**	**0.65**	**0.64**	**0.45**	**0.00**	**1.41**	**0.73**	**0.76**	**0.00**	**1.02**	**0.67**	**1.53**
SEM	**0.00**	**0.21**	**0.19**	**0.14**	**0.00**	**0.45**	**0.22**	**0.23**	**0.00**	**0.32**	**0.20**	**0.46**

Appendix 3.7 Prolonged submaximal exercise effects on Na^+,K^+-ATPase α_1, α_2 and α_3 protein expression in human skeletal muscle

Subject	α_1				α_2				α_3			
No.	Rest	Fat	+3 h	+24 h	Rest	Fat	+3 h	+24 h	Rest	Fat	+3 h	+24 h
1	1.00	0.63	1.35	0.98	1.00	0.65	2.40	1.87	1.00	0.91	2.43	1.39
2	1.00	0.87	2.03	1.94	1.00	0.66	2.93	1.07	1.00	1.21	1.31	3.13
3	1.00	0.68	0.48	0.87	1.00	0.71	0.15	0.49	1.00	1.18	0.35	2.27
4	1.00	1.10	0.38	1.02	1.00	0.83	1.02	3.44	1.00	1.51	0.54	0.85
5	1.00	0.78	0.28	0.88	1.00	1.27	0.78	0.93	1.00	1.70	0.91	1.52
6	1.00	1.20	0.77	0.55	1.00	0.68	0.28	0.18	1.00	1.48	0.87	2.40
7	1.00	1.41	0.79	1.00	1.00	0.64	0.37	0.66	1.00	0.82	0.73	0.90
8	1.00	0.55	0.87	0.74	1.00	0.29	0.38	0.34	1.00	0.65	0.73	0.86
9	1.00	0.40	0.26	0.28	1.00	0.69	0.14	0.52	1.00	1.14	0.53	0.88
10	1.00	1.43	1.39	1.32	1.00	0.40	0.73	0.25	1.00	1.06	2.50	1.22
11	1.00	0.47	1.01	1.59	1.00	0.38	1.36	0.72	1.00	1.68	2.17	5.39
n	**11**	**11**	**11**	**11**	**11**	**11**	**11**	**11**	**11**	**11**	**11**	**11**
Mean	**1.00**	**0.87**	**0.87**	**1.02**	**1.00**	**0.66**	**0.96**	**0.95**	**1.00**	**1.21**	**1.19**	**1.89**
SD	**0.00**	**0.37**	**0.55**	**0.46**	**0.00**	**0.26**	**0.93**	**0.95**	**0.00**	**0.35**	**0.80**	**1.38**
SEM	**0.00**	**0.11**	**0.17**	**0.14**	**0.00**	**0.08**	**0.28**	**0.29**	**0.00**	**0.10**	**0.24**	**0.42**

Appendix 3.8 Prolonged submaximal exercise effects on Na^+,K^+-ATPase β_1, β_2 and β_3 protein expression in human skeletal muscle

Subject	β_1				β_2				β_3			
No.	Rest	Fat	+3 h	+24 h	Rest	Fat	+3 h	+24 h	Rest	Fat	+3 h	+24 h
1	1.00	1.60	1.46	3.08	1.00	1.66	2.74	2.31	1.00	0.85	0.31	0.47
2	1.00	0.83	2.33	2.68	1.00	0.92	0.87	1.40	1.00	0.16	0.37	1.02
3	1.00	1.47	0.84	2.53	1.00	1.08	0.77	0.71	1.00	2.59	1.46	2.33
4	1.00	1.04	0.97	1.14	1.00	1.41	1.18	0.96	1.00	1.77	1.50	0.61
5	1.00	0.64	1.28	2.26	1.00	0.73	0.84	0.42	1.00	1.22	0.52	1.56
6	1.00	0.65	0.94	1.23	1.00	0.85	0.51	1.69	1.00	1.17	0.94	1.85
7	1.00	0.76	0.75	1.00	1.00	0.23	0.16	0.41	1.00	2.89	1.23	1.63
8	1.00	0.29	0.87	0.30	1.00	1.13	1.83	2.00	1.00	0.95	0.87	0.84
9	1.00	0.29	0.87	0.30	1.00	0.30	0.05	0.07	1.00	0.53	0.84	0.83
10	1.00	1.24	1.77	0.46	1.00	1.35	3.01	1.03	1.00	2.76	1.20	1.89
11	1.00	0.33	2.59	1.13	1.00	0.43	0.18	0.34	1.00	1.30	5.61	4.93
n	**11**	**11**	**11**	**11**	**11**	**11**	**11**	**11**	**11**	**11**	**11**	**11**
Mean	**1.00**	**0.83**	**1.33**	**1.47**	**1.00**	**0.92**	**1.10**	**1.03**	**1.00**	**1.47**	**1.35**	**1.63**
SD	**0.00**	**0.46**	**0.64**	**1.00**	**0.00**	**0.47**	**1.01**	**0.74**	**0.00**	**0.92**	**1.47**	**1.24**
SEM	**0.00**	**0.14**	**0.19**	**0.30**	**0.00**	**0.14**	**0.31**	**0.22**	**0.00**	**0.28**	**0.44**	**0.38**

Appendix 3.9 Prolonged submaximal exercise effects on the average post-exercise Na^+,K^+-ATPase α_1, α_2, α_3, β_1, β_2 and β_3 mRNA expression in human skeletal muscle

Subject No.	Average post-exercise mRNA expression (relative to rest; 1.00)					
	α_1	α_2	α_3	β_1	β_2	β_3
1	1.80	1.41	0.51	1.61	0.96	2.16
2	0.49	0.77	1.70	0.77	0.36	2.64
3	1.43	2.60	1.93	0.45	1.72	1.74
4	1.49	1.66	0.35	1.68	1.01	0.84
5	3.05	0.96	0.53	0.94	2.46	0.55
6	1.05	0.67	1.01	1.09	1.29	2.30
7	2.00	0.81	3.59	1.01	2.33	0.45
8	1.20	1.13	2.10	0.59	0.91	1.94
9	1.40	1.58	0.29	1.05	0.82	0.91
10	0.39	0.75	1.45	0.36	1.61	0.40
11	2.40	1.90	1.03	1.02	1.67	1.28
n	**11**	**11**	**11**	**11**	**11**	**11**
Mean	**1.52**	**1.30**	**1.32**	**0.96**	**1.38**	**1.38**
SD	**0.78**	**0.60**	**0.99**	**0.42**	**0.65**	**0.81**
SEM	**0.24**	**0.18**	**0.30**	**0.13**	**0.20**	**0.24**

Appendix 3.10 Prolonged submaximal exercise effects on muscle [^{3}H]-ouabain binding site content in human skeletal muscle

Subject No.	Rest	Fat	+3 h	+24 h
1	289	305	331	323
2	337	349	252	317
3	232	230	303	282
4	236	331	282	322
5	330	401	365	363
6	134	145	267	246
7	288	175	222	204
8	382	179	338	292
9	300	373	391	320
10	465	415	311	437
11	118	329	273	382
n	**11**	**11**	**11**	**11**
Mean	**283**	**294**	**303**	**317**
SD	**101**	**96**	**51**	**64**
SEM	**31**	**29**	**15**	**19**

Appendix 3.11 Prolonged submaximal exercise effects on maximal 3-*O*-MFPase activity in human skeletal muscle

Subject No.	Rest	Fat	+3 h	+24 h
1	142	122	191	194
2	173	145	190	212
3	274	155	155	166
4	274	253	272	238
5	222	194	167	202
6	264	175	148	157
7	297	322	269	301
8	232	184	220	203
9	226	220	279	312
10	121	86	117	204
11	252	163	215	135
n	**11**	**11**	**11**	**11**
Mean	**225**	**183**	**202**	**211**
SD	**57**	**65**	**55**	**55**
SEM	**17**	**19**	**16**	**17**

Appendix 3.12 Prolonged submaximal exercise effects on RNAP II, eIF-4E and 4E-BP1 mRNA expression in human skeletal muscle

Subject	RNAP II				eIF-4E				4E-BP1			
No.	Rest	Fat	+3 h	+24 h	Rest	Fat	+3 h	+24 h	Rest	Fat	+3 h	+24 h
1	1.00	1.14	0.97	1.67	1.00	2.02	1.64	1.39	1.00	0.93	4.85	1.74
2	1.00	2.60	0.49	1.14	1.00	0.76	0.53	0.79	1.00	0.21	0.77	0.66
3	1.00	4.12	4.47	2.58	1.00	1.01	1.05	0.47	1.00	1.56	1.85	0.67
4	1.00	0.70	2.14	0.78	1.00	0.40	0.67	0.76	1.00	0.84	1.71	1.11
5	1.00	1.10	0.44	1.14	1.00	1.33	0.30	0.27	1.00	1.23	0.45	0.15
6	1.00	4.25	1.27	0.16	1.00	1.08	1.27	0.19	1.00	0.49	1.27	0.13
7	1.00	5.74	1.69	0.55	1.00	0.59	0.36	0.78	1.00	1.15	0.83	0.93
8	1.00	1.21	3.48	1.14	1.00	0.54	0.80	2.84	1.00	0.53	1.16	1.61
9	1.00	4.97	1.79	0.91	1.00	1.23	0.77	0.59	1.00	1.37	1.71	1.06
10	1.00	2.39	0.86	0.89	1.00	1.01	0.17	0.17	1.00	0.93	0.32	0.96
11	1.00	0.36	0.36	1.53	1.00	1.12	0.55	1.40	1.00	0.98	1.29	2.67
n	**11**	**11**	**11**	**11**	**11**	**11**	**11**	**11**	**11**	**11**	**11**	**11**
Mean	**1.00**	**2.60**	**1.63**	**1.14**	**1.00**	**1.01**	**0.74**	**0.88**	**1.00**	**0.93**	**1.47**	**1.06**
SD	**0.00**	**1.88**	**1.31**	**0.64**	**0.00**	**0.45**	**0.44**	**0.77**	**0.00**	**0.40**	**1.23**	**0.74**
SEM	**0.00**	**0.57**	**0.40**	**0.19**	**0.00**	**0.14**	**0.13**	**0.23**	**0.00**	**0.12**	**0.37**	**0.22**

Appendix 3.13 Prolonged submaximal exercise effects on average post-exercise RNAP II, eIF-4E and 4E-BP1 mRNA expression in human skeletal muscle

Subject	Average post-exercise expression (relative to rest; 1.00)		
No.	RNAP II	eIF-4E	4E-BP1
1	1.26	1.68	2.51
2	1.41	0.69	0.55
3	3.72	0.84	1.36
4	1.21	0.61	1.22
5	0.89	0.64	0.61
6	1.90	0.85	0.63
7	2.66	0.57	0.97
8	1.94	1.39	1.10
9	2.56	0.87	1.38
10	1.38	0.45	0.74
11	0.75	1.02	1.65
n	**11**	**11**	**11**
Mean	**1.79**	**0.87**	**1.15**
SD	**0.89**	**0.37**	**0.58**
SEM	**0.27**	**0.11**	**0.17**

Appendix 4. Raw data Study 3

Effects of high-intensity intermittent exercise and training on skeletal muscle Na^+,K^+-ATPase mRNA and protein expression in already well-trained cyclists

Appendix 4.1 Study 3 Subject physical characteristics

Subject No.	Age (yr)	Height (cm)	Body mass (kg)		
			Baseline	Pre Train	Post Train
1	31	172	70.9	71.9	71.5
2	27	170	76.0	76.8	76.7
3	32	180	77.0	79.6	78.4
4	25	180	77.0	76.8	76.8
5	33	181		71.0	
6	31	173			
7	32	171	71.0	73.0	71.2
8	37	180	76.5	76.4	76.5
9	33	190	85.0		
10	32	185			
11	33	170	77.0	78.9	
12	31	180	71.0	74.5	
n	**12**	**12**	**9**	**9**	**6**
Mean	**31**	**178**	**75.7**	**75.4**	**75.2**
SD	**3**	**6**	**4.5**	**3.0**	**3.0**
SEM	**1**	**2**	**1.5**	**1.0**	**1.2**

Blank cells; missing data from RMIT laboratories where tests were performed.

Appendix 4.2 High-intensity intermittent training effects on peak power output and peak O_2 consumption during the incremental exercise test

Subject	PPO (W)				$\dot{V}O_{2peak}$ (ml.kg^{-1}.min^{-1})		
No.	Baseline	Pre-HIT	Mid-HIT	Post-HIT	Baseline	Pre-HIT	Post-HIT
1	359	359					
2	380	393			66.5	64.2	
3	430	422	455	438	73.4	69.4	73.7
4	355	355	370	380		64.9	64.5
5	365	365	370	370		60.7	62.5
6	315	335	335	343	61.9	59.5	59.0
7	350	353	360	352	61.8	60.9	66.4
8	390	385	393	377	64.0	67.0	64.6
9	415	415		435	66.3		66.4
10		355	355		62.6		
11	320	325	350	337	58.3		
n	**10**	**10**	**8**	**8**	**8**	**7**	**7**
Mean	**368**	**371**	**374**	**379**	**64.4**	**63.8**	**65.3**
SD	**37**	**32**	**37**	**39**	**4.5**	**3.6**	**4.5**
SEM	**12**	**10**	**13**	**14**	**1.6**	**1.4**	**1.7**

Blank, missing data from RMIT laboratories where tests were performed.

Appendix 4.3 High-intensity, intermittent exercise and training effects on Na^+,K^+-ATPase α_1, α_2 and α_3 mRNA expression

Subject	α_1				α_2				α_3			
No.	Pre Rest	Pre Ex	Post Rest	Post Ex	Pre Rest	Pre Ex	Post Rest	Post Ex	Pre Rest	Pre Ex	Post Rest	Post Ex
1	1.00	0.10	1.00	6.96	1.00	2.13	1.00	1.79	1.00	2.34	1.00	3.75
2	1.00	3.99	1.00	2.41	1.00	4.64	1.00	1.32	1.00	4.17	1.00	1.43
3	1.00	7.57	1.00	4.26	1.00	7.28	1.00	10.41	1.00	3.47	1.00	7.59
4	1.00	1.63	1.00	1.83	1.00	2.41	1.00	1.09	1.00	1.25	1.00	4.30
5	1.00	2.11	1.00	3.96	1.00	4.27	1.00	1.85	1.00	0.54	1.00	13.36
6	1.00	3.04	1.00	3.80	1.00	0.27	1.00	1.30	1.00	5.02	1.00	4.30
7	1.00	2.59	1.00	0.43	1.00	3.61	1.00	0.25	1.00	11.56	1.00	0.42
8	1.00	0.46	1.00	12.33	1.00	3.66	1.00	2.80	1.00	4.61	1.00	4.30
9	1.00	9.64	1.00	0.58	1.00	3.72	1.00	8.75	1.00	22.35	1.00	4.30
10	1.00	1.89	1.00	0.94	1.00	5.60	1.00	0.22	1.00	1.13	1.00	0.11
11	1.00	1.18	1.00	4.14	1.00	2.77	1.00	2.11	1.00	1.96	1.00	3.78
12	1.00	2.27	1.00	4.14	1.00	0.68	1.00	2.11	1.00	1.87	1.00	3.78
n	**12**	**12**	**12**	**12**	**12**	**12**	**12**	**12**	**12**	**12**	**12**	**12**
Mean	**1.00**	**3.04**	**1.00**	**3.82**	**1.00**	**3.42**	**1.00**	**2.83**	**1.00**	**5.02**	**1.00**	**4.28**
SD	**0.00**	**2.84**	**0.00**	**3.29**	**0.00**	**1.97**	**0.00**	**3.26**	**0.00**	**6.20**	**0.00**	**3.50**
SEM	**0.00**	**0.82**	**0.00**	**0.95**	**0.00**	**0.57**	**0.00**	**0.94**	**0.00**	**1.79**	**0.00**	**1.01**

Appendix 4.4 High-intensity, intermittent exercise and training effects on Na^+,K^+-ATPase β_1, β_2 and β_3 mRNA expression

Subject	β_1				β_2				β_3			
No.	Pre Rest	Pre Ex	Post Rest	Post Ex	Pre Rest	Pre Ex	Post Rest	Post Ex	Pre Rest	Pre Ex	Post Rest	Post Ex
1	1.00	0.85	1.00	9.76	1.00	0.43	1.00	3.70	1.00	0.52	1.00	5.29
2	1.00	1.39	1.00	2.52	1.00	1.66	1.00	7.34	1.00	9.80	1.00	4.86
3	1.00	3.08	1.00	4.38	1.00	3.69	1.00	7.75	1.00	0.70	1.00	1.73
4	1.00	0.67	1.00	1.53	1.00	0.06	1.00	0.12	1.00	0.18	1.00	0.27
5	1.00	1.01	1.00	1.42	1.00	0.98	1.00	1.18	1.00	1.58	1.00	1.61
6	1.00	1.13	1.00	0.72	1.00	2.30	1.00	12.20	1.00	1.82	1.00	1.50
7	1.00	1.70	1.00	0.09	1.00	0.43	1.00	2.30	1.00	2.64	1.00	0.27
8	1.00	2.09	1.00	9.24	1.00	2.30	1.00	1.71	1.00	2.19	1.00	0.88
9	1.00	0.98	1.00	1.06	1.00	8.88	1.00	3.70	1.00	0.40	1.00	0.68
10	1.00	1.12	1.00	0.59	1.00	1.16	1.00	1.54	1.00	4.42	1.00	0.60
11	1.00	1.86	1.00	2.21	1.00	3.85	1.00	1.60	1.00	1.62	1.00	0.51
12	1.00	0.50	1.00	2.21	1.00	1.87	1.00	1.60	1.00	0.99	1.00	0.51
n	**12**	**12**	**12**	**12**	**12**	**12**	**12**	**12**	**12**	**12**	**12**	**12**
Mean	**1.00**	**1.37**	**1.00**	**2.98**	**1.00**	**2.30**	**1.00**	**3.73**	**1.00**	**2.24**	**1.00**	**1.56**
SD	**0.00**	**0.72**	**0.00**	**3.24**	**0.00**	**2.40**	**0.00**	**3.57**	**0.00**	**2.66**	**0.00**	**1.72**
SEM	**0.00**	**0.21**	**0.00**	**0.94**	**0.00**	**0.69**	**0.00**	**1.03**	**0.00**	**0.77**	**0.00**	**0.50**

Appendix 4.5 High-intensity, intermittent exercise and training effects on Na^+,K^+-ATPase α_1, α_2 and α_3 protein expression

Subject	α_1				α_2				α_3			
No.	Pre Rest	Pre Ex	Post Rest	Post Ex	Pre Rest	Pre Ex	Post Rest	Post Ex	Pre Rest	Pre Ex	Post Rest	Post Ex
1	1.00	0.27	1.00	0.75	1.00	0.64	1.00	0.46	1.00	2.77	1.00	9.42
2	1.00				1.00				1.00			
3	1.00	0.53	1.00	1.07	1.00	0.88	1.00	0.35	1.00	1.25	1.00	1.12
4	1.00	0.33			1.00	1.52			1.00	1.22		
5	1.00	2.46			1.00	0.80			1.00	0.87		
6	1.00	0.85			1.00	2.67			1.00	2.80		
7	1.00	5.31			1.00	0.67			1.00	0.73		
8	1.00	1.02	1.00	7.99	1.00	2.24	1.00	8.96	1.00	1.06	1.00	3.07
9	1.00	2.67	1.00	0.60	1.00	0.24	1.00	0.67	1.00	0.83	1.00	0.48
10	1.00	3.30	1.00	1.25	1.00	0.99	1.00	0.30	1.00	1.88	1.00	1.51
11	1.00	1.22			1.00	0.77			1.00	0.74		
12	1.00	0.51			1.00	0.34			1.00	1.46		
n	**12**	**11**	**5**	**5**	**12**	**11**	**5**	**5**	**12**	**11**	**5**	**5**
Mean	**1.00**	**1.68**	**1.00**	**2.33**	**1.00**	**1.07**	**1.00**	**2.15**	**1.00**	**1.42**	**1.00**	**3.12**
SD	**0.00**	**1.59**	**0.00**	**3.17**	**0.00**	**0.77**	**0.00**	**3.81**	**0.00**	**0.76**	**0.00**	**3.65**
SEM	**0.00**	**0.48**	**0.00**	**1.42**	**0.00**	**0.23**	**0.00**	**1.70**	**0.00**	**0.23**	**0.00**	**1.63**

Blank, data not measured due to insufficient sample.

Appendix 4.6 High-intensity, intermittent exercise and training effects on Na^+,K^+-ATPase β_1, β_2 and β_3 protein expression

Subject	β_1				β_2				β_3			
No.	Pre Rest	Pre Ex	Post Rest	Post Ex	Pre Rest	Pre Ex	Post Rest	Post Ex	Pre Rest	Pre Ex	Post Rest	Post Ex
1	1.00	1.03	1.00	0.81	1.00	0.59	1.00	0.80	1.00	1.26	1.00	0.95
2	1.00				1.00				1.00			
3	1.00	0.69	1.00	1.13	1.00	2.39	1.00	0.80	1.00	1.83	1.00	0.29
4	1.00	1.33			1.00	1.14			1.00	0.13		
5	1.00	0.83			1.00	1.07			1.00	0.87		
6	1.00	0.37			1.00	0.36			1.00	0.68		
7	1.00	0.28			1.00	1.06			1.00	1.84		
8	1.00	0.90	1.00	9.98	1.00		1.00	0.97	1.00	1.38	1.00	0.75
9	1.00	0.65	1.00	2.30	1.00	4.02	1.00	0.85	1.00	1.25	1.00	0.52
10	1.00	2.29	1.00	1.35	1.00	1.44	1.00	0.15	1.00	1.07	1.00	1.63
11	1.00	0.82			1.00	2.74			1.00	0.76		
12	1.00	1.13			1.00	0.53			1.00	1.19		
n	**12**	**11**	**5**	**5**	**12**	**10**	**5**	**5**	**12**	**11**	5	**5**
Mean	**1.00**	**0.94**	**1.00**	**3.11**	**1.00**	**1.53**	**1.00**	**0.71**	**1.00**	**1.11**	**1.00**	**0.83**
SD	**0.00**	**0.54**	**0.00**	**3.88**	**0.00**	**1.17**	**0.00**	**0.32**	**0.00**	**0.50**	**0.00**	**0.51**
SEM	**0.00**	**0.16**	**0.00**	**1.74**	**0.00**	**0.37**	**0.00**	**0.14**	**0.00**	**0.15**	**0.00**	**0.23**

Blank cells, data not measured due to insufficient sample.

Appendix 4.7 High-intensity, intermittent exercise and training effects on [^{3}H]-ouabain binding site content

Subject No.	Pre-HIT	Post-HIT
1	235	132
2	351	
3	300	246
4	455	474
5	447	
6	424	487
7	264	326
8	325	
9		351
10	399	344
n	**9**	**7**
Mean	**356**	**337**
SD	**80**	**124**
SEM	**27**	**47**

Blank, data not measured due to insufficient sample. Muscle analyses completed by Rob Aughey and presented in the following paper: Aughey, RJ, Murphy, KT, Clark, SA, Garnham, AP, Snow, RJ, Cameron-Smith, D, Christie, JJ, Hawley, JA & McKenna, MJ. Muscle Na^+,K^+-ATPase isoform, content and activity responses to interval exercise and training in well-trained athletes. *J Physiol,* In Review.

Appendix 4.8 High-intensity, intermittent exercise and training effects on maximal 3-*O*-MFPase activity

Subject No.	Pre-HIT		Post-HIT	
	Rest	Ex	Rest	Ex
1	295	240	305	260
2	306	280	310	286
3	272	238	281	244
4	254	206	271	211
5	285	253	305	275
6	286	254	305	269
7	275	249	297	274
n	**7**	**7**	**7**	**7**
Mean	**282**	**246**	**297**	**260**
SD	**17**	**22**	**15**	**26**
SEM	**6**	**8**	**6**	**10**

Blank, data not measured due to insufficient sample. Muscle analyses completed by Rob Aughey and presented in the following paper: Aughey, RJ, Murphy, KT, Clark, SA, Garnham, AP, Snow, RJ, Cameron-Smith, D, Christie, JJ, Hawley, JA & McKenna, MJ. Muscle Na^+,K^+-ATPase isoform, content and activity responses to interval exercise and training in well-trained athletes. *J Physiol,* In Review.

Appendix 5. Raw data Study 4

Chronic endurance training but not gender modulate Na^+,K^+-ATPase mRNA expression, maximal activity and content in human skeletal muscle

Appendix 5.1 Study 4 Endurance trained males (ETM) physical characteristics

Subject No.	Age (yr)	Height (cm)	Body mass (kg)	$\dot{V}O_{2peak}$ ($ml.kg^{-1}.min^{-1}$)
1	28	176	71.5	68.5
2	24	190	78.8	70.2
3	25	185	77.9	68.9
4	36	190	78.3	68.6
5	28	179	70.9	70.9
6	26	180	68.1	68.7
7	20	173	64.8	67.4
8	24	178	68.4	68.5
9	21	187	80.0	69.1
10	30	189	68.9	70.2
11	21	171	65.0	74.5
12	25	176	75.7	61.2
13	33	185	74.0	72.2
14	26	180	80.0	59.6
15	20	176	64.0	69.9
16	27	173	68.9	78.4
17	24	182	66.0	72.7
18	36	179	67.0	66.8
19	33	183	92.7	56.8
20	19	174	61.0	67.0
21	27	174	60.9	70.6
22	21	192	80.5	65.0
23	25	173	72.0	66.5
24	25	182	73.2	64.3
25	29	162	59.0	73.2
26	32	181	75.0	68.8
27	29	168	64.0	67.4
28	34	176	67.5	67.4
29	29	173	70.3	68.1
30	21	186	75.0	63.3
31	30	175	70.0	63.7
32	23	181	68.5	72.7
33	32	177	78.9	61.1
34	19	185	82.0	66.4
35	29	169	66.5	66.9
36	31	172	70.9	64.0
37	27	170	76.0	66.5
38	32	180	77.0	73.4
39	25	180	77.0	64.9
40	33	181	74.3	60.7
41	31	173	74.3	61.9
42	33	190	85.0	66.3
43	32	185	74.3	62.6
44	31	180	71.0	64.0
45	22	181	72.3	83.3
n	**45**	**45**	**45**	**45**
Mean	**27**	**179**	**72.4**	**67.6**
SD	**5**	**7**	**6.7**	**4.9**
SEM	**1**	**1**	**1.0**	**0.7**

Appendix 5.2 Recreationally active males (RAM) physical characteristics

Subject No.	Age (yr)	Height (cm)	Body mass (kg)	$\dot{V}O_{2peak}$ ($ml.kg^{-1}.min^{-1}$)
1	21	173	89.7	47.0
2	19	177	61.3	55.6
3	21	168	57.7	56.0
4	20	180	77.8	51.5
5	21	180	76.3	58.7
6	29	181	80.7	56.3
7	27	179	86.4	45.0
8	31	180	80.1	66.5
9	23	167	64.6	56.5
10	24	188	83.8	61.7
11	23	172	62	58.4
n	**11**	**11**	**11**	**11**
Mean	**24**	**177**	**74.6**	**55.7**
SD	**4**	**6**	**11.2**	**6.2**
SEM	**1**	**2**	**3.4**	**1.9**

Appendix 5.3 Recreationally active females (RAF) physical characteristics

Subject No.	Age (yr)	Height (cm)	Body mass (kg)	$\dot{V}O_{2peak}$ ($ml.kg^{-1}.min^{-1}$)
1	21	167.0	55.0	47.8
2	26	178.5	86.2	45.2
3	18	158.5	55.6	43.0
4	20	178.5	61.0	52.7
5	37	163.6	59.3	43.1
6	21	156	58.5	57.6
7	23	167	54.7	59.8
8	23	165	65.4	59.3
9	32	155	45.8	51.9
n	**9**	**9**	**9**	**9**
Mean	**24.6**	**166**	**60.2**	**51.2**
SD	**6.2**	**9**	**11.2**	**6.7**
SEM	**2.1**	**3**	**3.7**	**2.2**

Appendix 5.4 Endurance trained males (ETM) Na^+,K^+-ATPase α_1, α_2, α_3, β_1, β_2 and β_3 mRNA expression

Subject No.	α_1	α_2	α_3	β_1	β_2	β_3
1	8.54	898.76	0.64	469.87	43.41	52.86
2	5.50	532.07	0.15	74.07	11.60	4.90
3	16.07	429.11	0.00	553.82	0.34	0.01
4	9.53	282.12	0.02	76.77	0.04	0.01
5	4.86	762.71	0.06	124.83	25.04	0.18
6	1.87	635.47	0.02	359.60	17.20	0.06
7	5.01	626.02	0.57	80.77	24.77	0.06
8	11.80	181.29	0.20	99.09	33.95	0.11
9	4.64	703.00	0.19	130.78	34.80	0.06
10	0.76	913.83	0.33	71.46	17.93	0.17
11	14.73	709.43	0.04	123.42	20.35	20.71
12	7.40	462.85	0.08	79.82	17.47	27.51
13	11.70	2195.42	0.01	98.19	15.07	61.22
14	4.00	1695.52	0.44	75.58	9.51	25.02
n	**14**	**14**	**14**	**14**	**14**	**14**
Mean	**7.60**	**787.69**	**0.20**	**172.72**	**19.39**	**13.78**
SD	**4.65**	**542.08**	**0.22**	**162.11**	**12.44**	**20.98**
SEM	**1.24**	**144.88**	**0.06**	**43.33**	**3.33**	**5.61**

Data relative to CYC mRNA expression.

Appendix 5.5 Recreationally active males (RAM) Na^+,K^+-ATPase α_1, α_2, α_3, β_1, β_2 and β_3 mRNA expression

Subject No.	α_1	α_2	α_3	β_1	β_2	β_3
1	35.99	1789.49	0.94	80.89	54.77	29.79
2	39.97	2891.96	0.79	593.15	136.72	100.87
3	33.82	1818.94	0.68	375.37	79.86	197.18
4	28.33	2885.02	0.18	194.40	45.57	40.74
5	7.75	1050.92	0.02	105.45	2.55	24.43
n	**5**	**5**	**5**	**5**	**5**	**5**
Mean	**29.17**	**2087.27**	**0.52**	**269.85**	**63.89**	**78.60**
SD	**12.69**	**793.51**	**0.40**	**214.53**	**49.35**	**72.98**
SEM	**5.68**	**354.87**	**0.18**	**95.94**	**22.07**	**32.64**

Data relative to CYC mRNA expression.

Appendix 5.6 Recreationally active females (RAF) Na^+,K^+-ATPase α_1, α_2, α_3, β_1, β_2 and β_3 mRNA expression

Subject No.	α_1	α_2	α_3	β_1	β_2	β_3
1	27.36	460.86	0.65	805.16	5.74	69.26
2	92.76	6779.13	0.06	550.80	160.53	71.17
3	8.61	282.07	0.36	72.44	33.91	20.87
4	9.21	318.99	0.11	62.10	6.02	11.24
5	7.79	441.63	0.03	64.54	8.17	20.62
6	0.71	509.92	0.00	140.47	8.60	0.19
7	1.21	1132.88	0.40	69.11	32.69	0.30
8	0.34	1464.09	0.38	134.75	9.38	0.41
9	0.33	1208.60	0.04	95.39	16.35	0.16
n	**9**	**9**	**9**	**9**	**9**	**9**
Mean	**16.48**	**1399.80**	**0.23**	**221.64**	**31.26**	**21.58**
SD	**29.85**	**2063.32**	**0.23**	**268.00**	**49.69**	**28.85**
SEM	**9.95**	**687.77**	**0.08**	**89.33**	**16.56**	**9.62**

Data relative to CYC mRNA expression.

Appendix 5.7 Endurance trained males (ETM) maximal 3-*O*-MFPase activity, muscle [^{3}H]-ouabain binding sites and endurance training duration

Subject No.	Maximal 3-*O*-MFPase activity (nmol.min^{-1}.(g wet wt)$^{-1}$	Muscle [^{3}H]-ouabain binding sites (pmol.(g wet wt)$^{-1}$	Yrs training
1	291	407	6
2	268	322	12
3	283	331	8
4	248	281	
5	312	307	10
6	280	407	
7	300	269	3
8	309	359	7
9	285	284	10
10	300	353	9
11	268	401	6
12	286	368	5
13	317	344	4
14	250	278	6
15	306	327	3
16	259	353	3
17	251	271	5
18	241	297	12
19	297	398	
20	295	336	
21	260	303	10
22	249		
23	270	280	9
24	253	301	3
25	283	332	3
26	289	332	12
27	282	359	
28	277	375	
29	291	265	
30	271		
31	278	302	4
32	280	296	5
33	262	300	7
34	264		
35	293	301	2
36	274	264	8
37		447	3
38	303	268	15
39	300	173	3
40	281	406	2.5
41	256	405	3.5
42	285	434	10
43	283		
44		400	8
45	232	382	5
n	**43**	**41**	**35**
Mean	**278**	**332**	**7**
SD	**20**	**58**	**5**
SEM	**3**	**9**	**1**

Blank cells, data not measured due to insufficient sample.

Appendix 5.8 Recreationally active males (RAM) maximal 3-*O*-MFPase activity and muscle [^{3}H]-ouabain binding sites

Subject No.	Maximal 3-*O*-MFPase activity (nmol.min^{-1}.(g wet wt)$^{-1}$	Muscle [^{3}H]-ouabain binding sites (pmol.(g wet wt)$^{-1}$
1	321	229
2	242	346
3	239	164
4	208	341
5	221	281
6	307	354
7	314	239
8	142	289
9	173	337
10	226	300
11	252	266
n	**11**	**11**
Mean	**240**	**286**
SD	**57**	**59**
SEM	**17**	**18**

Appendix 5.9 Recreationally active females (RAF) maximal 3-*O*-MFPase activity and muscle [^{3}H]-ouabain binding sites

Subject No.	Maximal 3-*O*-MFPase activity (nmol.min^{-1}.(g wet wt)$^{-1}$	Muscle [^{3}H]-ouabain binding sites (pmol.(g wet wt)$^{-1}$
1	209	298
2	210	297
3	201	283
4	250	356
5	267	251
6	274	232
7	274	236
8	222	330
9	264	134
n	**9**	**9**
Mean	**241**	**269**
SD	**30**	**65**
SEM	**10**	**22**

Appendix 6. Raw data Study 5

Muscle fibre-type, electrical stimulation and intracellular effects on Na^+,K^+-ATPase mRNA expression in rat skeletal muscle

Appendix 6.1 Study 5 Effect of 2, 5, 10 and 25 mM NAC on tetanic force production and tetanic characteristics in rat EDL muscle

	[NAC]	Muscle 1	Muscle 2
Force production	2 mM	104.0	117.2
	5 mM	102.1	116.2
	10 mM	86.9	97.8
	25 mM	90.7	108.4
$T_{rise, 10-90}$	2 mM	85.0	82.9
	5 mM	100.0	98.9
	10 mM	104.2	107.0
	25 mM	171.7	247.8
$T_{fall, 90-10}$	2 mM	92.6	96.8
	5 mM	96.0	93.3
	10 mM	108.3	103.6
	25 mM	92.3	100.0

Values are expressed as a percentage of control (100%). $T_{risc, 10-90}$, time of tetani to rise from 10-90% of peak height; $T_{fall, 90-10}$, time of tetani to fall from 90-10% of peak height.

Appendix 6.2 Linearity of rat Na^+,K^+-ATPase α_1-α_3 and β_1-β_3, and CYC primers to various inputs of cDNA

Gene	r^2 value	Regression equation	95% confidence intervals, slope	Mean efficiency ($10^{-1/slope}$-1)
α_1	0.9937	y = -1.5564x + 30.38	-1.758 to -1.329	-1.1
α_2	0.9223	y = -1.544x + 24.815	-2.368 to -0.713	-1.1
α_3	0.9945	y = -1.5714x + 31.203	-1.799 to -1.354	-1.1
β_1	0.9253	y = -1.6203x + 24.616	-1.758 to –1.329	-1.1
β_2	0.9758	y = -2.072x + 28.189	-2.744 to –1.413	-1.0
β_3	0.9766	y = -1.0354x + 28.522	-1.232 to –0.716	-1.1
CYC	0.9618	y = -1.183x + 25.258	-1.682 to -0.731	-1.1

Appendix 6.3 Effect of electrical stimulation on Na^+,K^+-ATPase α_1, α_2 and α_3 mRNA expression in rat EDL muscle

	α_1			α_2			α_3		
Animal No.	Con	Stim	Stim+3h	Con	Stim	Stim+3h	Con	Stim	Stim+3h
1	1.00	0.76	2.92	1.00	1.23	7.46	1.00	0.57	7.91
2	1.00	1.64	1.22	1.00	1.80	12.42	1.00	1.87	2.95
3	1.00	1.00	2.04	1.00	1.24	8.19	1.00	2.03	15.16
4	1.00	3.99	4.26	1.00	3.10	2.00	1.00	6.29	7.91
5	1.00	2.29	3.65	1.00	8.03	12.53	1.00	8.27	12.29
6	1.00	3.29	1.18	1.00	3.22	10.22	1.00	3.80	15.68
7	1.00		2.11	1.00		2.48	1.00		14.05
8	1.00		8.48	1.00		2.37	1.00		3.39
n	**8**	**6**	**8**	**8**	**6**	**8**	**8**	**6**	**8**
Mean	**1.00**	**2.16**	**3.23**	**1.00**	**3.10**	**7.21**	**1.00**	**3.80**	**9.92**
SD	**0.00**	**1.28**	**2.39**	**0.00**	**2.57**	**4.45**	**0.00**	**2.95**	**5.11**
SEM	**0.00**	**0.52**	**0.84**	**0.00**	**1.05**	**1.57**	**0.00**	**1.20**	**1.81**

Appendix 6.4 Effect of electrical stimulation on Na^+,K^+-ATPase β_1, β_2 and β_3 mRNA expression in rat EDL muscle

	β_1			β_2			β_3		
Animal No.	Con	Stim	Stim+3h	Con	Stim	Stim+3h	Con	Stim	Stim+3h
1	1.00	0.49	2.88	1.00	0.93	5.27	1.00	1.29	9.21
2	1.00	0.65	0.72	1.00	4.10	0.90	1.00	4.17	0.34
3	1.00	0.65	1.82	1.00	0.62	1.37	1.00	2.59	2.00
4	1.00	2.30	1.95	1.00	2.84	2.30	1.00	0.13	3.58
5	1.00	4.49	2.90	1.00	0.83	5.09	1.00	0.27	0.15
6	1.00	5.58	6.80	1.00	1.42	1.05	1.00	0.18	0.22
7	1.00		3.98	1.00		0.47	1.00		0.05
8	1.00		2.16	1.00		2.02	1.00		0.53
n	**8**	**6**	**8**	**8**	**6**	**8**	**8**	**6**	**8**
Mean	**1.00**	**2.36**	**2.90**	**1.00**	**1.79**	**2.31**	**1.00**	**1.44**	**2.01**
SD	**0.00**	**2.20**	**1.84**	**0.00**	**1.39**	**1.87**	**0.00**	**1.64**	**3.16**
SEM	**0.00**	**0.90**	**0.65**	**0.00**	**0.57**	**0.66**	**0.00**	**0.67**	**1.12**

Appendix 6.5 Effect of electrical stimulation on Na^+,K^+-ATPase α_1, α_2 and α_3 mRNA expression in rat soleus muscle

	α_1			α_2			α_3		
Animal No.	Con	Stim	Stim+3h	Con	Stim	Stim+3h	Con	Stim	Stim+3h
1	1.00	0.69	0.31	1.00	0.68	0.76	1.00	0.89	1.17
2	1.00	1.11	0.27	1.00	0.72	1.06	1.00	0.98	0.19
3	1.00	1.72	0.37	1.00	1.49	1.45	1.00	0.70	0.77
4	1.00	0.35	0.63	1.00	1.19	1.77	1.00	2.00	1.13
5	1.00	1.46	1.30	1.00	3.30	3.04	1.00	0.40	0.52
6	1.00	1.45	1.27	1.00	6.00	1.70	1.00	1.54	1.41
n	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**
Mean	**1.00**	**1.13**	**0.69**	**1.00**	**2.23**	**1.63**	**1.00**	**1.09**	**0.87**
SD	**0.00**	**0.52**	**0.48**	**0.00**	**2.08**	**0.79**	**0.00**	**0.58**	**0.45**
SEM	**0.00**	**0.21**	**0.20**	**0.00**	**0.85**	**0.32**	**0.00**	**0.24**	**0.19**

Appendix 6.6 Effect of electrical stimulation on Na^+,K^+-ATPase β_1, β_2 and β_3 mRNA expression in rat soleus muscle

	β_1			β_2			β_3		
Animal No.	Con	Stim	Stim+3h	Con	Stim	Stim+3h	Con	Stim	Stim+3h
1	1.00	0.47	0.65	1.00	1.01	1.32	1.00	1.56	0.65
2	1.00	1.07	0.25	1.00	1.47	0.16	1.00	3.38	0.42
3	1.00	0.51	0.77	1.00	3.14	0.95	1.00	0.21	1.55
4	1.00	1.35	1.31	1.00	8.55	2.19	1.00	3.22	0.57
5	1.00	0.32	1.19	1.00	1.02	0.38	1.00	0.49	3.18
6	1.00	0.51	1.19	1.00	2.61	1.47	1.00	6.27	6.05
n	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**
Mean	**1.00**	**0.70**	**0.90**	**1.00**	**2.97**	**1.08**	**1.00**	**2.52**	**2.07**
SD	**0.00**	**0.41**	**0.41**	**0.00**	**2.87**	**0.75**	**0.00**	**2.26**	**2.21**
SEM	**0.00**	**0.17**	**0.17**	**0.00**	**1.17**	**0.31**	**0.00**	**0.92**	**0.90**

Appendix 6.7 Effects of ouabain (Ouab), veratridine (Verat) and monensin (Monen) on Na^+ content in rat EDL muscle

Animal No.	Con	Ouab	Verat	Monen
1	53.2	75.0	75.9	54.3
2	43.0	90.0	46.7	50.3
3	45.3	92.5	44.3	55.0
4	40.6	100.3	47.5	
5	43.4	83.4	48.1	
6	54.4	81.3	55.2	
n	**6**	**6**	**6**	**3**
Mean	**46.6**	**87.1**	**53.0**	**53.2**
SD	**5.7**	**9.0**	**11.8**	**2.5**
SEM	**2.3**	**3.7**	**4.8**	**1.5**

Values are expressed in $\mu mol.(g\ wet\ wt)^{-1}$.

Appendix 6.8 Effects of ouabain (Ouab), veratridine (Verat) and monensin (Monen) on Na^+,K^+-ATPase α_1, α_2 and α_3 mRNA expression in rat EDL muscle

	α_1				α_2				α_3			
Animal No.	Con	Ouab	Verat	Monen	Con	Ouab	Verat	Monen	Con	Ouab	Verat	Monen
1	3.9	4.3	4.3	8.4	368.0	227.0	121.3	163.9	0.9	5.4	2.9	1.0
2	8.5	9.7	5.3	3.4	547.4	108.3	190.0	238.4	8.2	1.6	2.9	16.1
3	5.1	2.5	9.4	2.8	275.5	96.2	87.0	170.9	4.0	0.8	2.7	1.6
4	5.0	5.2	4.0	7.3	443.2	306.7	99.2	522.5	8.0	1.6	1.9	4.1
5	5.6	6.5	1.7	2.2	314.3	114.4	60.1	201.8	3.4	1.5	4.3	4.1
6	6.0	9.3	7.5	6.8	260.6	320.0	128.9	554.4	4.6	1.8	2.3	2.9
n	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**
Mean	**5.7**	**6.2**	**5.4**	**5.2**	**368.2**	**195.4**	**114.4**	**308.6**	**4.8**	**2.1**	**2.8**	**5.0**
SD	**1.6**	**2.8**	**2.7**	**2.7**	**110.3**	**102.9**	**44.5**	**180.2**	**2.8**	**1.7**	**0.8**	**5.6**
SEM	**0.6**	**1.2**	**1.1**	**1.1**	**45.0**	**42.0**	**18.2**	**73.6**	**1.1**	**0.7**	**0.3**	**2.3**

Appendix 6.9 Effects of ouabain (Ouab), veratridine (Verat) and monensin (Monen) on Na^+,K^+-ATPase β_1, β_2 and β_3 mRNA expression in rat EDL muscle

	β_1				β_2				β_3			
Animal No.	Con	Ouab	Verat	Monen	Con	Ouab	Verat	Monen	Con	Ouab	Verat	Monen
1	69.5	49.3	220.5	419.5	3.1	5.6	3.6	3.7	15.1	1.4	1.1	0.5
2	301.2	187.9	192.2	375.7	32.1	3.0	6.8	14.3	9.7	0.8	1.3	1.7
3	247.9	73.0	351.1	259.6	31.8	8.0	6.4	7.2	13.9	0.1	2.5	0.8
4	203.7	162.1	290.8	606.5	5.1	6.9	3.2	10.9	8.9	0.3	1.3	0.7
5	86.9	299.9	109.8	346.7	12.6	1.9	11.6	21.0	5.9	1.2	0.8	1.6
6	214.0	701.2	340.6	435.2	28.9	1.3	12.5	11.9	9.7	1.1	1.1	1.3
n	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**
Mean	**187.2**	**245.6**	**250.8**	**407.2**	**18.9**	**4.5**	**7.3**	**11.5**	**10.5**	**0.8**	**1.4**	**1.1**
SD	**91.2**	**240.5**	**93.8**	**115.8**	**13.6**	**2.8**	**3.9**	**6.0**	**3.4**	**0.5**	**0.6**	**0.5**
SEM	**37.2**	**98.2**	**38.3**	**47.3**	**5.5**	**1.1**	**1.6**	**2.4**	**1.4**	**0.2**	**0.2**	**0.2**

Appendix 6.10 Effect of caffeine on Na^+,K^+-ATPase α_1, α_2, α_3, β_1, β_2 and β_3 mRNA expression in rat EDL muscle

	α_1		α_2		α_3		β_1		β_2		β_3	
Animal No.	Con	Caffeine	Con	Caffeine	Con	Caffeine	Con	Caffeine	Con	Caffeine	Con	Caffeine
1	3.2	5.7	95.7	233.4	3.1	2.5	138.8	144.8	7.0	18.6	0.62	0.83
2	2.3	6.1	102.6	175.5	1.3	3.2	68.5	133.5	2.9	3.5	0.60	0.91
3	1.5	3.8	54.9	147.7	2.5	1.1	34.7	273.6	3.3	5.6	0.67	0.80
4	2.4	9.2	61.2	329.9	1.1	4.6	33.9	183.0	1.0	10.2	0.49	1.99
n	**4**	**4**	**4**	**4**	**4**	**4**	**4**	**4**	**4**	**4**	**4**	**4**
Mean	**2.4**	**6.2**	**78.6**	**221.6**	**2.0**	**2.9**	**69.0**	**183.7**	**3.6**	**9.5**	**0.59**	**1.13**
SD	**0.7**	**2.3**	**24.0**	**80.5**	**1.0**	**1.5**	**49.3**	**63.5**	**2.5**	**6.7**	**0.08**	**0.57**
SEM	**0.3**	**1.1**	**12.0**	**40.2**	**0.5**	**0.7**	**24.6**	**31.8**	**1.2**	**3.3**	**0.04**	**0.29**

Appendix 6.11 Effect of 13 mM K^+ on Na^+,K^+-ATPase α_1, α_2, α_3, β_1, β_2 and β_3 mRNA expression in rat EDL muscle

	α_1		α_2		α_3		β_1		β_2		β_3	
Animal No.	Con	13 mM K^+	Con	13 mM K^+	Con	13 mM K^+	Con	13 mM K^+	Con	13 mM K^+	Con	13 mM K^+
1	1.00	6.18	1.00	0.59	1.00	0.59	1.00	0.33	1.00	0.60	1.00	1.22
2	1.00	3.80	1.00	0.53	1.00	1.84	1.00	2.63	1.00	3.31	1.00	2.34
3	1.00	1.09	1.00	2.69	1.00	1.59	1.00	0.74	1.00	0.22	1.00	0.98
4	1.00	3.36	1.00	0.45	1.00	0.91	1.00	1.03	1.00	1.45	1.00	1.40
5	1.00	2.27	1.00	1.29	1.00	0.34	1.00	1.84	1.00	1.04	1.00	2.99
6	1.00	0.85	1.00	1.72	1.00	0.84	1.00	1.22	1.00	0.82	1.00	0.85
7	1.00	0.72	1.00	1.17	1.00	2.79	1.00	3.91	1.00	1.27	1.00	1.31
8	1.00	0.68	1.00	0.36	1.00	1.13	1.00	0.74	1.00	0.44	1.00	8.51
9	1.00	0.17	1.00	0.43	1.00	0.66	1.00	0.16	1.00	1.75	1.00	0.73
n	**9**	**9**	**9**	**9**	**9**	**9**	**9**	**9**	**9**	**9**	**9**	**9**
Mean	**1.00**	**2.12**	**1.00**	**1.03**	**1.00**	**1.19**	**1.00**	**1.40**	**1.00**	**1.21**	**1.00**	**2.26**
SD	**0.00**	**1.98**	**0.00**	**0.78**	**0.00**	**0.77**	**0.00**	**1.21**	**0.00**	**0.93**	**0.00**	**2.46**
SEM	**0.00**	**0.66**	**0.00**	**0.26**	**0.00**	**0.26**	**0.00**	**0.40**	**0.00**	**0.31**	**0.00**	**0.82**

Appendix 6.12 Effect of NAC on Na^+,K^+-ATPase α_1, α_2 and α_3 mRNA expression in rat EDL muscle

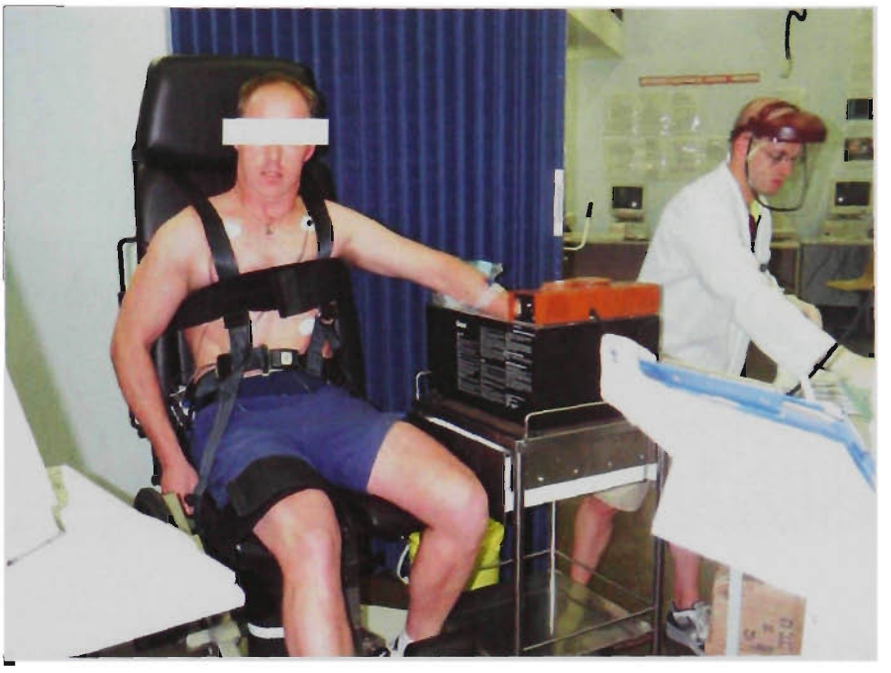
	α_1				α_2				α_3			
Animal No.	Con	Stim	NAC	Stim+NAC	Con	Stim	NAC	Stim+NAC	Con	Stim	NAC	Stim+NAC
1	1.6	4.0	2.6	3.0	26.2	206.3	132.7	40.6	0.3	8.23	1.17	0.75
2	4.5	14.6	2.5	1.7	23.9	276.2	97.8	53.6	0.5	4.25	0.75	1.40
3	2.3	5.6	6.4	3.6	28.7	75.6	131.5	47.1	0.1	7.71	3.97	2.47
4	0.9	10.4	7.7	4.5	56.4	158.7	170.5	34.5	0.3	10.60	3.15	2.39
5			5.9				292.4				5.99	
6			4.0				115.1				6.34	
n	**4**	**4**	**6**	**4**	**4**	**4**	**6**	**4**	**4**	**4**	**6**	**4**
Mean	**2.33**	**8.65**	**4.83**	**3.20**	**33.8**	**179.2**	**156.7**	**44.0**	**0.32**	**7.70**	**3.56**	**1.75**
SD	**1.56**	**4.85**	**2.14**	**1.17**	**15.2**	**84.3**	**70.7**	**8.3**	**0.15**	**2.62**	**2.35**	**0.83**
SEM	**0.78**	**2.43**	**0.87**	**0.58**	**7.6**	**42.1**	**28.9**	**4.1**	**0.08**	**1.31**	**0.96**	**0.41**

Appendix 6.13 Effect of NAC on Na^+,K^+-ATPase β_1, β_2 and β_3 mRNA expression in rat EDL muscle

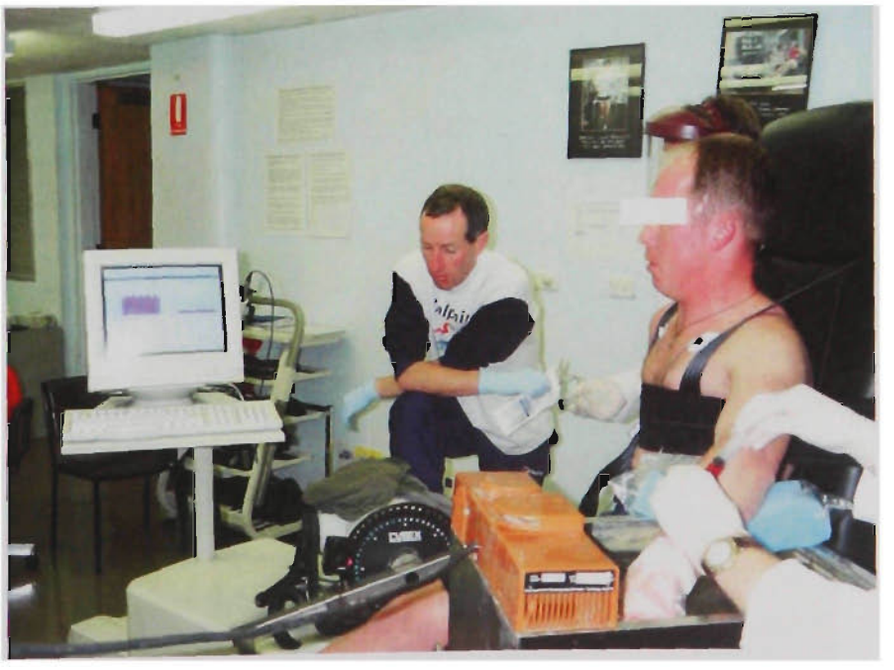
	β_1				β_2				β_3			
Animal No.	Con	Stim	NAC	Stim+NAC	Con	Stim	NAC	Stim+NAC	Con	Stim	NAC	Stim+NAC
1	21.4	255.4	534.4	3.3	1.6	4.90	3.98	0.34	1.8	0.87	0.13	0.35
2	86.0	189.8	198.1	4.9	4.5	2.04	1.59	0.68	4.5	1.27	0.42	0.33
3	79.0	141.6	269.8	1.2	4.2	6.30	7.58	1.76	2.1	0.77	0.70	1.20
4	158.3	105.7	302.0	1.1	0.8	6.48	9.99	1.58	1.0	0.56	0.60	0.77
5			429.2				13.18				1.22	
6			398.9				7.20				0.54	
n	**4**	**4**	**6**	**4**	**4**	**4**	**6**	**4**	**4**	**4**	**6**	**4**
Mean	**86.2**	**173.1**	**355.4**	**2.6**	**2.79**	**4.93**	**7.25**	**1.09**	**2.36**	**0.87**	**0.60**	**0.66**
SD	**56.1**	**64.8**	**121.9**	**1.8**	**1.83**	**2.05**	**4.13**	**0.69**	**1.50**	**0.30**	**0.36**	**0.41**
SEM	**28.1**	**32.4**	**49.8**	**0.9**	**0.91**	**1.03**	**1.69**	**0.34**	**0.75**	**0.15**	**0.15**	**0.21**

Appendix 7. Photos from Experimental days

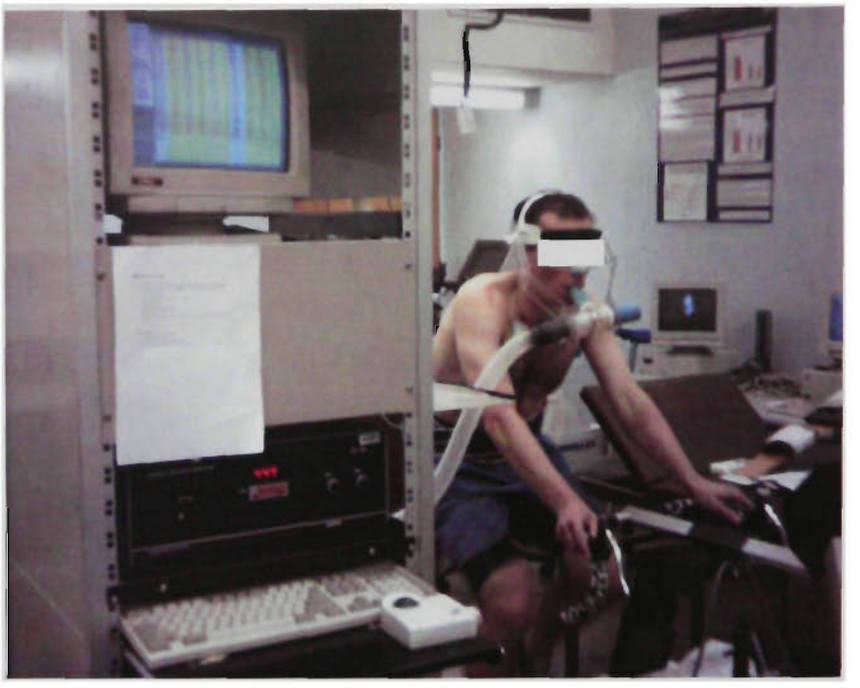
Study 1 Invasive muscle fatigue trial



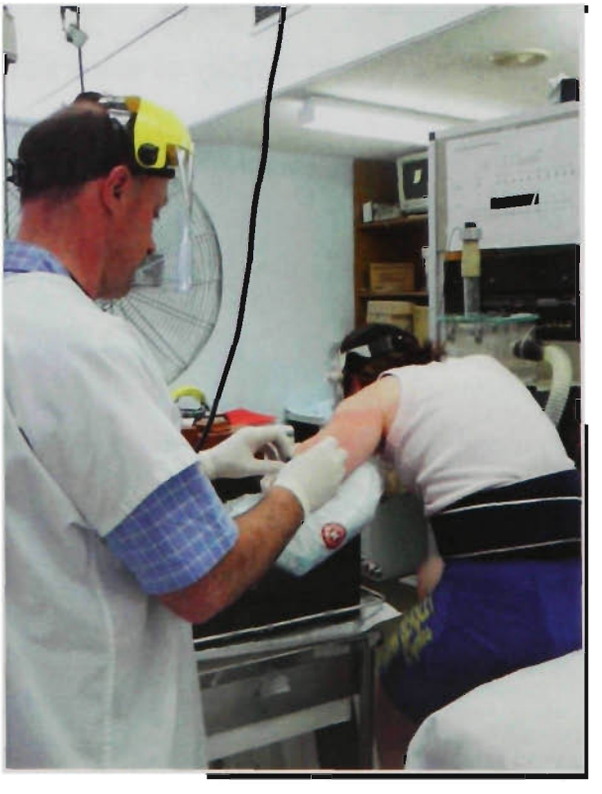
Study 1 Visual real-time display of work output



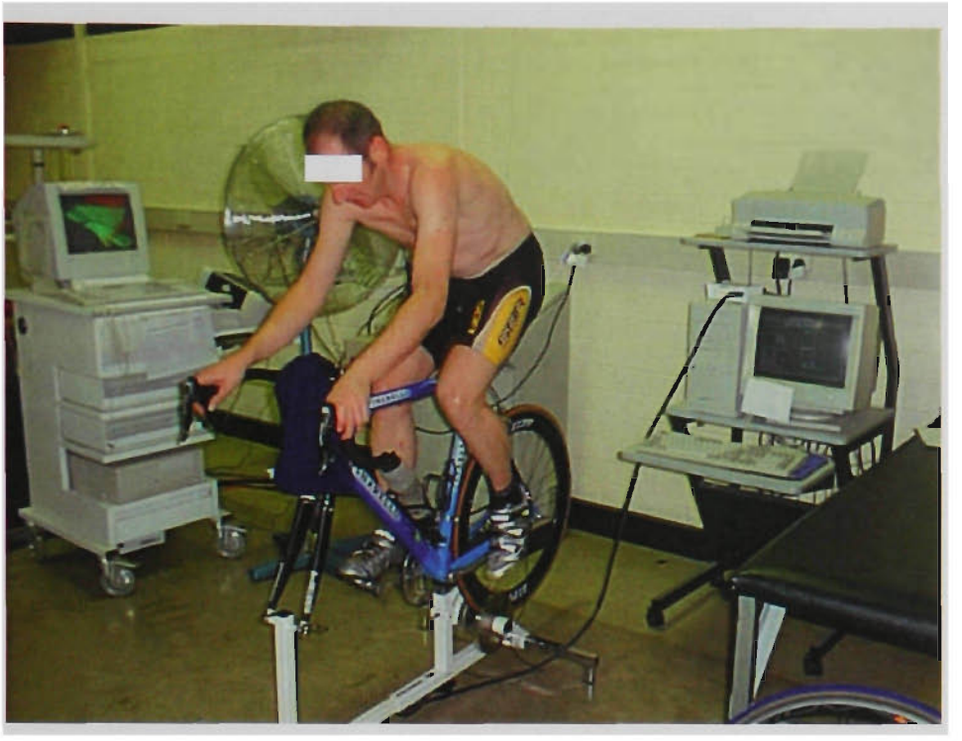
Study 2 Incremental exercise test



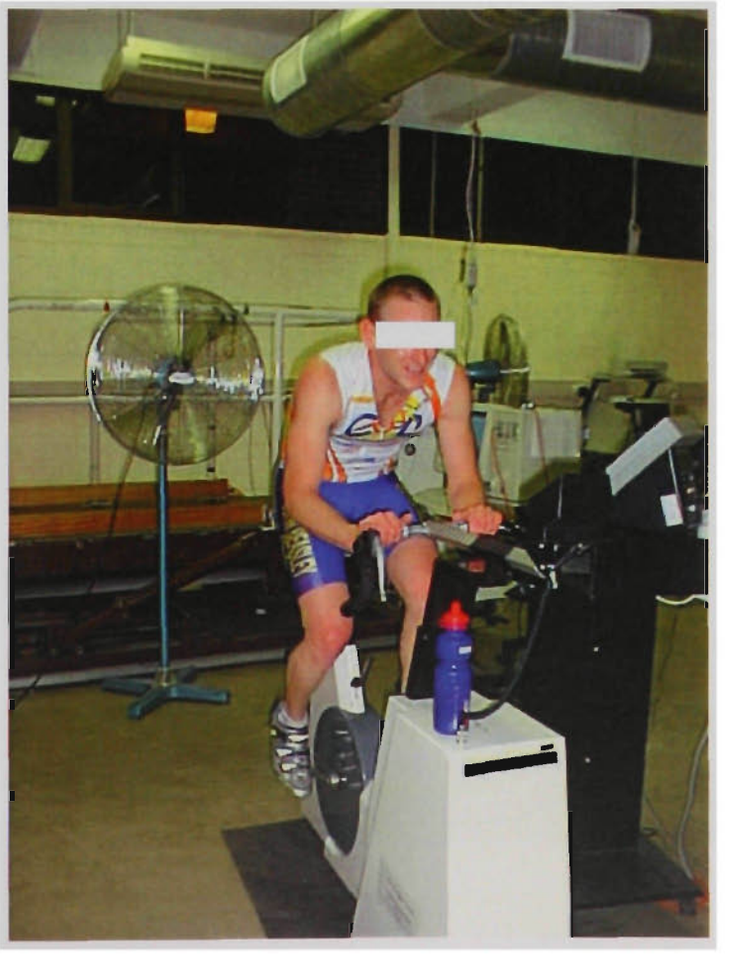
Study 2 Blood sampling during the prolonged exercise fatigue test



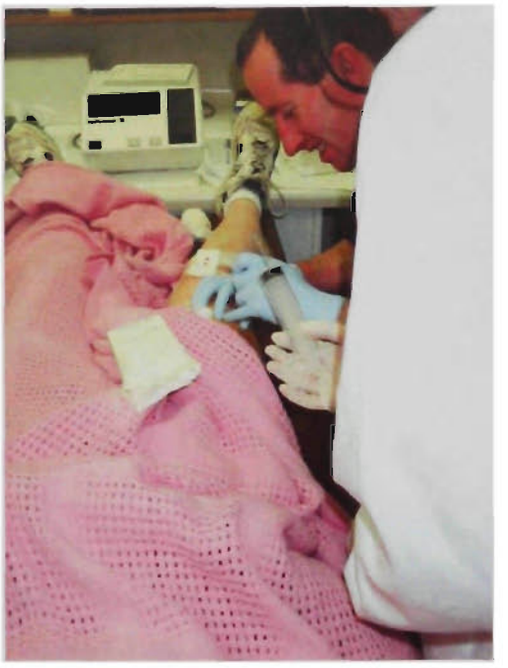
Study 3 Simulated 40 km time trial



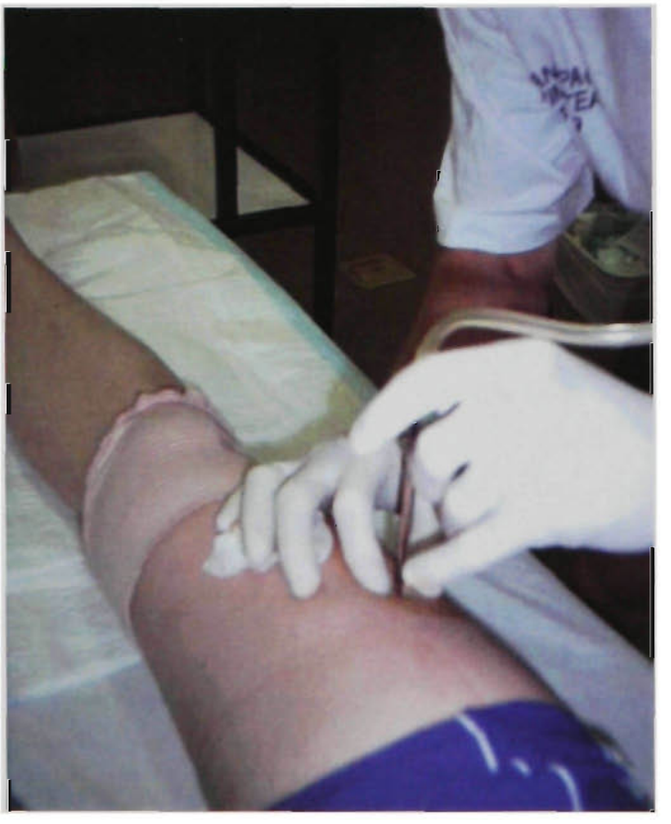
Study 3 High-intensity, intermittent training session



Study 4 Resting muscle biopsy



Study 4 Resting muscle biopsy



Study 5 Dissection of rat muscles

Study 5 Electrical stimulation of isolated muscles