Characterization of the skeletal muscle Na^+-K^+-ATPase, and the acute effects of exercise on Na^+-K^+-ATPase activity

by

Jonathon Richard Fowles

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ABSTRACT

The purpose of this thesis was to characterize the Na^+-K^+-ATPase (Na,K-pump) in skeletal muscle and to measure pump activity in response to exercise. To address these objectives, Na^+-K^+-ATPase activity, content and isoform distribution were measured in muscles of various fibre types. Subsequently, Na^+-K^+-ATPase was measured following acute exercise in both rat and human. The hypothesis was that muscles of varying fibre type composition would exhibit differences in Na^+-K^+-ATPase content and isoform distribution, and these factors would indicate muscle specific differences in Na^+-K^+-ATPase activity when measured *in vitro*. An additional hypothesis was that the Na^+-K^+-ATPase would be altered by exercise; to result in reduced Na^+-K^+-ATPase activity measured *in vivo* and *in vitro*.

In the first study, a comparative approach assessed pump characteristics between soleus (SOL), red gastrocnemius (RG), white gastrocnemius (WG), and extensor digitorum longus (EDL) skeletal muscles of the rat. These muscles are representative of primarily slow-oxidative type I fibres (SOL), fast oxidative-glycolytic type IIA fibres (RG), fast glycolytic type IIB fibres (WG), and muscle having a mixed complement of fast fibre types (EDL). Tissue was sampled at rest under anaesthetic, from a total of 38 male, 16 week old Wistar rats weighing 413 ± 6.0 g (mean ± SE). Na^+-K^+-ATPase activity was determined in homogenates (HOM) and isolated crude membranes (CM) for the regenerating ouabain-inhibitable hydrolytic activity assay (ATPase) and the 3-O-methylfluorescein K^+-stimulated phosphatase (3-O-MFPase) assays *in vitro*. In addition, pump content was determined by [^{3}H]ouabain binding, and the distribution of α1, α 2, α3, and β1, and β2 isoforms was determined by Western blot. Differences ($P<0.05$) in enzyme activity between muscles were observed in HOM (EDL>WG) and in CM (SOL>EDL=RG=WG) for the ATPase assay. For the 3-O-MFPase assay, differences ($P<0.05$) were also found for HOM (SOL>EDL=RG>WG) and CM (SOL=WG>RG). To determine if activity was related to pump content, differences in [^{3}H]ouabain maximum binding (Bmax) were observed in the order of EDL = RG > SOL = WG ($P<0.05$). Western blot results were similar between HOM and CM. In CM, a there was greater distribution of α1 in SOL than WG and EDL ($P<0.05$) with a similar relation observed for α2. The β1 was greater ($P<0.05$) in EDL and WG, and the β2 was greater in SOL and

RG ($P<0.05$). The presence of the α3 isoform, not previously observed in skeletal muscle, was identified in greater proportion in WG and EDL than SOL and RG ($P<0.05$). The α1 distribution correlated to HOM 3-O-MFPase ($r=0.79$, $P<0.05$) CM ATPase ($r=0.59$, $P<0.005$) and CM 3-O-MFPase activity ($r=0.33$, $P<0.05$). β1 distribution was related to HOM 3-O-MFPase ($r=0.61$, $P<0.005$) and CM ATPase ($r=0.42$, $P<0.05$) activity. HOM α2 distribution correlated to ouabain binding Bmax ($r=0.64$, $P<0.005$).

The purpose of the second study was to determine if Na^+-K^+-ATPase activity is altered following a single bout of aerobic exercise in rats. Female Sprague Dawley rats (age 14.7 ± 0.4 weeks, wt 275 ± 6.4g; mean ± SE) were run (RUN) on a treadmill at 21m/s and 8% grade (~65% VO_2max) until fatigue, or to a maximum of 2h. A second group of rats were kept on the treadmill for an additional 45 min of low-intensity exercise (10m/min and 8% grade) (RUN+). Directly following exercise, rats were anaesthetized, and soleus (SOL), red vastus lateralis (RV), white vastus lateralis (WV), and extensor digitorum longus (EDL) tissue was extracted and frozen for later analysis. K^+-stimulated 3-O-methylfluorescein phosphatase activity (3-O-MFPase) was determined as an indicator of Na^+-K^+-ATPase activity and glycogen depletion was used to identify use of each muscle during exercise. 3-O-MFPase was significantly decreased at RUN+ by 12% from CON when averaged over all muscles ($P<0.05$). No difference was found between CON and RUN. Glycogen was lower ($P<0.05$) by 65%, 57%, 44%, and 33% (SOL, EDL, RV, WV, respectively) at RUN, and there was no further depletion during continued exercise.

The purpose of the third study was to determine if exercise-induced depressions in Na^+-K^+-ATPase activity are associated with neuromuscular fatigue following isometric exercise in humans. To examine this hypothesis, force and EMG were measured in 14 volunteers (age, 23.4 ± 0.7 yrs, mean ± SE) in control (CON) and exercised (EX) legs, prior to (PRE) and immediately following (PST0), 1 hour post (PST1), and 4 hours post (PST4) isometric single leg extension exercise at ~60% of maximum voluntary contraction for 30 min using a 0.5 duty cycle (5s contraction, 5s rest). Tissue was obtained from vastus lateralis muscle prior to exercise (PRE) in CON, and following exercise in both the CON (PST0) and EX legs (PST0, PST1, PST4), for the measurement of Na^+-K^+-ATPase activity as determined by the 3-O-methylfluorescein phosphatase

assay (3-O-MFPase). Voluntary (MVC) and elicited (10, 20, 50, 100 Hz) muscle force were reduced 30-55% ($P<0.05$) at PST0, and did not recover ($P<0.05$) by PST4 in EX. Muscle action potential (M-wave) amplitude and area, and 3-O-MFPase activity at PST0-EX were less than PST0-CON ($P<0.05$) by 37%, 25%, and 38% respectively. M-wave area at PST1-EX was also less than PST1-CON ($P<0.05$). Changes in 3-O-MFPase activity correlated to changes in M-wave area across all timepoints ($r = 0.38$; $P<0.05$, $n=45$).

It is concluded from the results presented in this thesis that a number of factors can influence the measured activity of the Na^+-K^+-ATPase in skeletal muscle. Different activities may be observed between techniques, which highlights that the assay, isolation procedure, and muscle used, are important factors when interpreting measures of Na^+-K^+-ATPase activity. Na^+-K^+-ATPase activity is different between muscles of varying fibre composition, and a higher distribution of $\alpha 1$ and $\beta 1$ subunits, as found in SOL, confers the greatest Na^+-K^+-ATPase activity when measured on skeletal muscle preparations *in vitro*. This thesis also indicates that the *in vitro* activity of the skeletal muscle Na^+-K^+-ATPase is affected by a single session of exercise. This was demonstrated following prolonged aerobic exercise in rats, and following sustained isometric exercise in humans. The reduction in Na^+-K^+-ATPase activity was related to a loss of excitability and was associated with neuromuscular fatigue early in the recovery period from exercise. There are a number of possible mechanisms which may reduce intrinsic activity of the skeletal muscle Na^+-K^+-ATPase with exercise, and these possibilities remain a source of future investigation.

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Dedication

This thesis is dedicated to three people who inspired me to do what needed to be done, when it needed to be done.

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List of Abbreviations

ADP – adenosine diphosphate

AMP – adenosine monophosphate

ATP – adenosine triphosphate

ATPase- hydrolytic adenosine triphosphatase assay

Bmax-maximum binding

CM-crude membrane

CON- control

Ca^{2+} -Calcium

Cr- creatine

EDL-extensor digitorum longus muscle

EMG- electromyography

EX-Exercise leg

IMP-inosine monophosphate

IT-interpolated twitch tension

K^+-potassium

Kd-dissociation constant

kDa-kilodalton

KnPP- K^+-stimulated p-nitrophenol phophatase

M-wave- muscle compound action potential

MUA-motor unit activation

MVC-maximum voluntary contraction

Na^+-sodium

Na^+-K^+-ATPase sodium,potassium ATPase

PCr-phosphocreatine

Pt-peak twitch tension

PRE- pre-exercise

PST- post-exercise

RG-red gastrocnemius muscle

RUN-treadmill running

RV-red vastus muscle

SOL – soleus muscle

SR-sarcoplasmic reticulum

WG-white gastrocnemius muscle

WV-white vastus muscle

[^{3}H]-tritiated hydrogen

3-O-MF- 3-O-methylfluorescein

3-O-MFP-3-O-methylfluorescein phosphate

3-O-MFPase-3-O-methylfluorescein phosphatase

CHAPTER I

Introduction, Literature Review, and Statement of the Problem

INTRODUCTION

The Na^+-K^+-ATPase, is an integral membrane protein located in highest concentration at the sarcolemma and to a lesser extent in the t-tubules of skeletal muscle (Clausen, 1986). The Na^+-K^+-ATPase, also referred to as the Na,K-pump, couples the chemical hydrolysis of ATP to the vectoral transport of Na^+ out of, and K^+ into the muscle cell. This active transport mechanism restores ionic gradients primarily for the maintenance of membrane potential and 'excitability' of the cell at rest (Clausen *et al.*, 1998). Maintenance of ion gradients also contribute to the control of cell volume and facilitated transport of other ions and nutrients (Lang *et al.*, 1998). With heavy contractile demands that require a high frequency neuromuscular activation, the capacity of the Na,K-pump to restore ion gradients can be challenged by the rapid flux of Na^+ entering, and K^+ leaving the cell, during repetitive action potentials. Additionally, the build-up of metabolic by-products can alter the local environment surrounding the enzyme and further challenge pump function. Therefore, appropriate regulation of Na^+-K^+-ATPase activity is essential for maintaining ion gradients and muscle excitability. Despite a number of reviews published recently on the role of the Na,K-pump in muscle excitability and fatigue (Balog and Fitts, 1996; Clausen, 1996a; Clausen, 1996b; Clausen *et al.*, 1998; Green, 1998; Green, 2000; McComas *et al.*, 1994; Nielsen and Clausen, 2000; Nielsen and Harrison, 1998; Nielsen and Overgaard, 1996; Sejersted and Sjøgaard, 2000; Semb and Sejersted, 1996) the factors determining specific Na^+-K^+-ATPase protein expression and consequently, enzyme capacity in vivo are not defined. The enzymatic capability of the pump during exercise is also undetermined. The purpose for this thesis is to characterize the Na^+-K^+-ATPase in skeletal muscle and measure Na^+-K^+-ATPase activity in response to exercise.

Na,K-pump structure and function

The Na^+,K^+-ATPase has one catalytic (α) subunit with four known isoforms, one structural glycoprotein (β) subunit with three isoforms, and an additional regulatory γ subunit (Blanco and Mercer, 1998). The small γ subunit is not essential for enzyme activity and its role in skeletal muscle is minor (Therien and Blostein, 2000). The α catalytic subunit of the Na,K-pump traverses the membrane and has binding sites for Na^+, K^+, Mg^{2+}, ATP, P_i, and for the specific glycoside inhibitor, ouabain (Hundal *et al.*, 1993). The β-subunit is responsible for the correct insertion and stabilization of an active α-β complex in the plasma membrane (DeTomaso *et al.*, 1994; Fambrough *et al.*, 1994; Skou, 1992).

The isoforms of the α subunit have varying affinities for each substrate which confer kinetic specificity in a tissue specific manner. The α1 isoform is thought to be responsible for 'basal' activity in skeletal muscle because of its primary location at the membrane (Hundal *et al.*, 1994) and lower molecular activity when expressed in cultured cells (Jewell and Lingrel, 1991; Zahler *et al.*, 1997). The α2 is thought to be the major catalytic isoform (Hundal *et al.*, 1994) because of its greater specific activity than the α1 isoform (Zahler *et al.* 1997), greater content in both surface and internal membranes (Lavoie *et al.*, 1997), and greater translocation response to hormones (Hundal *et al.*, 1992; Lavoie *et al.*, 1996; Marette *et al.*, 1993) and exercise (Juel *et al.*, 2000; Tsakiridis *et al.*, 1996). The expression of α2 is also dynamically altered in response to chronic external stress (Azuma *et al.*, 1991; Azuma *et al.*, 1993; Thompson *et al.*, 1999; Thompson *et al.*, 2001; Thompson and McDonough, 1996).

Similar to the α2 subunits, β1 subunits also translocate from internal to external membranes in the acute response to insulin (Hundal *et al.*, 1992; Marette *et al.*, 1993) and exercise (Juel *et al.*, 2000). Chronic changes in the expression of β subunits are tissue and isoform specific (Azuma *et al.*, 1993; Gao *et al.*, 1999; Thompson *et al.*, 2001; Thompson and McDonough, 1996). β subunits influence extracellular K^+ activation and interaction with Na^+, and may take part in forming the phosphoenzyme complex from ATP (Blanco *et al.*, 1995). This function contributes to overall enzymatic activity with possibly a greater influence on K^+-stimulated phosphatase activity (Gick *et al.*, 1993; Lavoie *et al.*, 1997; Ng *et al.*, 1993). β1 subunits confer greater kinetic activity than β2 subunits (Blanco *et al.*, 1995; Crambert *et al.*, 2000; Hundal *et al.*, 1993), although activity of the enzyme *in vivo*, as inferred by measurement *in vitro*, is varied more by the interaction of α and β protein composition (Blanco and Mercer, 1998). Therefore, the Na^+-K^+-ATPase isoform complement particular to a given muscle fibre-type may regulate ion pumping capacity and consequently, muscle excitability, in a task-specific manner.

Different Na,K-pump subunit complexes are thought to have evolved distinct properties to respond to cellular requirements (Blanco and Mercer, 1998). In mammalian skeletal muscle, the distribution is such that slow oxidative (type I) fibres contain α1β1 and α2β1 complexes, fast glycolytic (type IIB) fibres contain α1β2 and α2β2 heterodimers (Hundal *et al.*, 1993; Hundal *et al.*, 1994), while fast oxidative-glycolytic (type IIA) have all four combinations (Thompson and McDonough, 1996). The α3 and β3 subunits are found in negligible quantities in skeletal muscle (Arystarkhova and Sweadner, 1997; Thompson *et al.*, 2001). Differences in isoform-specific enzymatic activity are observed when subunit complements are expressed in bacterial cultured cells (Blanco *et al.*, 1995; Crambert *et al.*,

2000) or when obtained by isolation in skeletal muscle (Hundal *et al.*, 1994; Lavoie *et al.*, 1997); however, fibre type-specific enzymatic activity has not been adequately demonstrated. A number of interventions from ionic to hormonal manipulations induce isoform specific adaptations in Na^+-K^+-ATPase expression in skeletal muscle (Azuma *et al.*, 1991; Azuma *et al.*, 1993; Bundgaard *et al.*, 1997; Thompson *et al.*, 1999; Thompson *et al.*, 2001; Thompson and McDonough, 1996), yet measures of enzyme activity do not consistently reflect these changes. Typically, the changes in isoform distribution measured by Western blot are not reflected in measures of pump content assessed by the standard ouabain-binding technique (Sun *et al.*, 1999). The results from both of these measures are often at odds with an *in vitro* measure of pump activity (Azuma *et al.*, 1993; Thompson *et al.*, 1999; Thompson *et al.*, 2001). The problem in determining fibre- and isoform-specific differences in pump activity is complex because of inherent difficulties in assessing Na^+-K^+-ATPase activity in mammalian skeletal muscle (Hansen and Clausen, 1988). A better understanding of the factors that influence Na^+-K^+-ATPase activity *in vivo* and the assessment of Na^+-K^+-ATPase activity *in vitro* may help to make sense of the available information on pump content, activity and isoform distribution. This information can provide insight into the factors that regulate pump expression in skeletal muscle.

It was previously postulated that the Na^+-K^+-ATPase content (Chin and Green, 1993) and activity (Ianuzzo and Dabrowski, 1987) may be associated to oxidative capacity of a fibre, although pump expression may be regulated in association with a number of characteristics of a particular fibre type. For example, Na^+-K^+-ATPase expression may be associated with the myosin ATPase that dictates the histochemical designation of fibre type. However, limited evidence suggests that the pump isoform complement may not be

regulated with the myosin heavy chain (MHC) (Sun *et al.*, 1999; Sweadner *et al.*, 1992). Changes in Na^+-K^+-ATPase during chronic low frequency stimulation have been observed to precede adaptations of oxidative enzymes (Green *et al.*, 1992; Hicks *et al.*, 1997), which highlights the importance of this enzyme in skeletal muscle's adaptive response to stress. Differences in steady state activity particular to an isoform complement and potential for activity modification of the various protein complexes by extracellular signals (Skou, 1992) imply that the activity of the pump is under continuous regulation in the living cell (Ewart and Klip, 1995).

Regulation of Na,K-pump capacity

Basal activity of the Na,K-pump primarily depends on the distribution of Na^+ and K^+ on either side of the plasma membrane and utilizes only ~2-8% of maximum pumping capacity *in vivo* (Clausen, 1986). In response to a single excitation impulse (action potential), voltage-gated Na^+ influx and K^+ efflux can stimulate up to twenty fold increase in Na,K pump activity (Clausen, 1996a). Epinephrine, norepinephrine, and insulin are also responsible for short-term activation of the pump after the original triggering event, in the form of acute hormonal control (Ewart and Klip, 1995). Extracellular signaling mechanisms commonly share phosphorylation/de-phosphorylation reactions to activate or inhibit protein kinases and phosphatases. The second, third and even fourth messenger cascades involving the Na,K-pump may be mediated through G-proteins to affect cell adenosine and guanosine 3',5'-cyclic monophosphate (cAMP, cGMP) concentration, activity of cAMP-dependent protein kinase A, C & G (PKA, PKC, PKG), and concentrations of eicosanoids, inositol phosphates (IP_3), cell calcium, or adenosine, cytokines, and endothelin (Bertorello and Katz, 1995; Blanco and Mercer, 1998). These signals may derive from catecholamines, calcitonin

gene-related peptide (CGRP; from nerve endings), free fatty acids, and cytoskeletal links (Therien and Blostein, 2000). Recent evidence suggests that these signaling mechanisms may also be targeted to specific isoforms of the separate Na^+-K^+-ATPase subunits (Gao *et al.*, 1999), through phosphorylation dependent mechanisms (Ragolia *et al.*, 1997), in a tissue-specific manner (Blanco and Mercer, 1998; Therien and Blostein, 2000). This may facilitate functional modification based on the expression of particular isozymes, and confer isoform-specific regulation (Blanco and Mercer, 1998).

Influencing the activity of the available pumps at the cell surface is just one way that the Na^+-K^+-ATPase pumping capacity can be regulated. The muscle cell also has mechanisms that acutely regulate the number of pumps at the membrane. For example, the hormone insulin has direct effects on translocating a 'hormone-responsive' pool of $\alpha 2\beta 1$ subunits to the muscle membrane (Hundal *et al.*, 1992; Marette *et al.*, 1993). This process is believed to be a mechanism that increases ion pumping capacity at the membrane (Hundal *et al.*, 1992; Marette *et al.*, 1993; Sampson *et al.*, 1994), particularly for the clearance of K^+ from the blood (Moore, 1983), Na^+-dependent amino acid clearance, and Na^+-H^+ exchange (Marette *et al.*, 1993). Since increased Na^+-K^+-ATPase activity is also observed *in vitro* following acute insulin administration (Lavoie *et al.*, 1996; Ragolia *et al.*, 1997), this explanation of increased transport capacity is plausible. This is consistent with the isoform and tissue specific regulation observed with the thyroid hormone (Azuma *et al.*, 1993; Horowitz *et al.*, 1990) and α & β adrenergic agonists (Gao *et al.*, 1999).

A recent report suggests that muscle contraction also mediates translocation of $\alpha 2\beta 1$ subunits to the membrane in humans (Juel *et al.*, 2000). This is in contrast to the original work done in rats where treadmill running induced an increase in both α subunits at the

membrane and β2 mRNA following the exercise (Tsakiridis *et al.*, 1996). In both studies, a greater extent of α than β translocation was observed, because β subunits are already in high concentration in the membrane (Lavoie *et al.*, 1997). Aggregation of α subunits to existing structural β subunits may obviate the need to translocate complete α-β complexes and may be a novel mechanism to regulate activity, as observed in buculo-virus-infected Sf-9 cells (DeTomaso *et al.*, 1994).

It is interesting to note that there have been no documented acute reductions in Na,K-pump activity in skeletal muscle by hormonal regulation (Clausen *et al.*, 1998). Phosphatase reactions associated with hormone action can reduce pump activity in other tissues, but not skeletal muscle (Therien and Blostein, 2000). Consequently, most of the secondary-signal mediated regulation of pump activity in skeletal muscle is assumed to be only stimulatory in nature. Inadequacies in pumping capacity under stress can therefore result from an inadequate original pump complement, or from an 'insufficient activation' of available pumps (Clausen *et al.*, 1998; Verburg *et al.*, 1999). Indirect evidence available from effects on other cellular ATPases implies that Na,K-pump activity can be acutely compromised by exercise (Green, 1998). Studies on mouse diaphragm muscle indicate that excessive intracellular Ca^{2+} can inhibit pump activity (Sulova *et al.*, 1998). Elevated intracellular Ca^{2+} is a common byproduct of intensive contractile activity (Westerblad and Allen, 1991) and is believed to have widespread effects on altering contractile function (Bruton *et al.*, 1998). A number of observations in the literature indicate that acute reductions of Na,K-pump activity measured *in vitro* result from ischemia, ischemia-repurfusion injury and possible damage by reactive oxygen species in cardiac muscle *in vivo* (Bersohn *et al.*, 1992; Bersohn, 1995; Kato

et al., 1998; Kim and Akera, 1987; Kramer *et al.*, 1984; Kukreja *et al.*, 1990; Xie *et al.*, 1990).

Only recently has there been evidence that the skeletal muscle Na,K-pump capacity may be compromised during exercise (Verburg *et al.*, 1999). These investigators provide indirect evidence that during sustained submaximal isometric contractions an increased loss of K^+ observed later in exercise, was due to an "insufficient activation of pumps". When this is considered in combination with the low K^+ reuptake observed after exercise, one might postulate that a time-dependent inactivation of Na,K-pumps may have occurred. A change in activity measured *in vitro* would likely indicate structural alterations of the enzyme, since measures under optimal conditions *in vitro* are not influenced by an altered metabolic environment *in vivo*. No published study presently exists examining the acute effects of exercise on Na^+-K^+-ATPase activity measured *in vitro*. However, the ability to answer whether pump activity is altered by exercise, and address the previously identified questions of muscle-specific pump expression, are dependent upon suitable measures of pump content and activity.

Measures of Na^+-K^+-ATPase in skeletal muscle

A complete enzymatic profile typically requires assessment of a number of variables, most notably the concentration of the enzyme, the ATP splitting rate (ATPase activity), and the transport capacity (use of energy to perform cellular work such as moving ions or other molecules). From these measures, assessment of the molecular activity (ATP splitting rate per enzyme molecule) and coupling ratio (transport capacity per ATP split) can be made to fully characterize the enzymes' ability to move substrate in a given task environment. These measurements are presented in the context of a functional enzyme in a membrane, so the

integrity of both the membrane and enzyme should also be considered (i.e. passive ion permeabilities, membrane competency, and membrane surface:cellular volume ratio). With regards to the Na,K-pump and determining its activity in skeletal muscle, a number of these issues have specific application.

The content of the Na^+-K^+-ATPase enzyme has classically been assessed by the [^{3}H]ouabain binding technique for skeletal muscle samples described by Norgaard *et al.* (1983; 1984). The total number of functional Na,K-pumps is determined by the binding of the specific cardiac glycoside inhibitor, ouabain, to the α-subunit of the enzyme in the presence of vanadate (Kjeldsen, 1986). This measure can infer a theoretical maximum pumping capacity when pump content is multiplied by the Na^+-K^+-ATPase molecular activity (i.e. maximal enzyme activity of a Na^+-K^+-ATPase molecule measured in isolation), assumed to be 8000 cycles·min^{-1}·pump^{-1} (Clausen, 1986; Horgan and Kuypers, 1987).

An additional measure obtained in some variations of the ouabain binding technique is the 'dissociation constant' or "Kd". The Kd is the negative reciprocal of the association constant, more appropriately describing the affinity of the enzyme (i.e. Na^+-K^+-ATPase) with its substrate (ouabain). In this procedure, muscle samples are incubated in concentrations of labeled and unlabeled ouabain from 5-5000nM (Kjeldsen, 1986; Norgaard *et al.*, 1984; Pickar *et al.*, 1997). Changes in affinity of the enzyme to ouabain should theoretically represent either isoform shifts (as may occur chronically) or modification of enzyme structure (which may occur acutely). It is interesting to note that many Kd assessments have indicated a single population of ouabain binding sites in skeletal muscle (Bundgaard *et al.*, 1997; Harrison *et al.*, 1994; Kjeldsen, 1986; Ng *et al.*, 1993; Norgaard *et al.*, 1984). This may be due to the fact that the α1 isoform of Na,K-pump in rat skeletal muscle is 250 times

more resistant to ouabain than is the α2 (Blanco *et al.*, 1995), and therefore the ouabain binding technique may only quantify the binding characteristics of the α2 subunits (Thompson *et al.*, 1999).

Hydrolytic activity of an ATPase is usually represented as specific activity, i.e., μmoles ATP split per milligram protein per minute or per hour. Estimation of hydrolytic activity involves measurement of specific activity (ATP split) as well as the protein content obtained in the preparation procedure. ATPase activity is normally assessed by the liberation of P_i or the coupling of ATP hydrolysis to a light sensitive product such as NADH through a series of enzymatic steps. Na^+-K^+-ATPase activity is low compared to other ATPases of the cell, making assessment in homogenates difficult. Isolation procedures typically have low yields and greater tissue requirements (Hansen and Clausen, 1996; Hundal and Aledo, 1996), which creates a problem for muscle specific determinations. Given that the primary step in ATP hydrolysis is a Na^+-dependent phosphorylation (Skou, 1992), hydrolytic activity is thought to be regulated by the α catalytic isoform and its varying affinities for Na^+ as a substrate (Jewell and Lingrel, 1991; Zahler *et al.*, 1997).

Because of the low proportion of Na^+-K^+-ATPase activity relative to other ATPases in the muscle cell, artificial substrates such as 3-O-methylflueroscein phosphate (3-O-MFPase) and p-nitrophenol phosphate (pNPP), are used in skeletal muscle as more sensitive and specific indicators of activity. K^+-dependent hydrolysis of chromogenic substrates, substitute for the aspartylphosphate intermediate of the ATPase (Horgan and Kuypers, 1987) to represent the terminal step in ATP hydrolysis (Huang and Askari, 1975). These phosphatase assays relate to actual ATPase activity (Horgan and Kuypers, 1987; Huang and Askari, 1975), but yield results that are less than the activity assessed by direct methods,

possibly because of reduced affinity for the artificial substrates. Phosphatase assays do not consistently represent changes at the isoform level, and therefore, may indicate something other than maximum hydrolytic activity of the enzyme. The β isoform stabilizes the K^+-bound E2 phosphoenzyme intermediate (Blanco *et al.*, 1995; Skou, 1992), which may explain why kinetic specificity in phosphatase assays may be conferred by the β subunits in rat tissue (Gick *et al.*, 1993; Lavoie *et al.*, 1997; Ng *et al.*, 1993).

It is interesting to note that the pump's hydrolytic, transport, and phosphatase activities can be modified independently of each other (Ball, 1986; Linnertz *et al.*, 1995; Zolotarjova *et al.*, 1995). This implies that different components of Na^+-K^+-ATPase function can be partitioned in their contributions to overall enzyme activity. These differences may be elicited by contrasting assays that optimize the separate functions, such as the ATPase for hydrolytic activity, and the 3-O-MFPase for phosphatase activity. Combining activity measures that assess different aspects of pump function in combination with identification of other pump characteristics, may help our understanding of factors that control pump activity.

With the number of difficulties associated with measuring skeletal muscle Na^+-K^+-ATPase *in vitro*, much of the work on muscle excitability and how it may relate to fatigue is done using another indirect measure, that of electromyography (EMG), and specifically, the muscle compound action potential (M-wave). Na^+-K^+-ATPase activity is electrogenic in nature, and therefore, contributes to membrane potential and the maintenance of sarcolemmal excitability (Clausen, 1996a). Changes in M-wave result primarily from a loss in muscle excitability, secondary to a loss in Na^+ and K^+ gradients (Cairns *et al.*, 1995; Overgaard *et al.*, 1997). Consequently, the *in vivo* activity of the Na,K-pump that maintains the ion gradients, is believed to directly influence the magnitude of the M-wave (Hicks and

McComas, 1989; Overgaard and Nielsen, 2001). A close relation has been observed between ion imbalance, changes in electromyogram and force loss in isolated rat muscle (Cairns *et al.*, 1995; Fitts, 1994; Hicks *et al.*, 1989; Overgaard *et al.*, 1997). A comparison of this relationship to enzymatic activity measured *in vitro* is not available. No relationship has similarly been demonstrated for human muscle between Na^+-K^+-ATPase, M-wave, and force, because of even greater difficulties in measuring Na^+-K^+-ATPase activity on human tissue compounded by the neural mechanisms in place to preserve muscle excitability in humans during voluntary exercise (Enoka and Stuart, 1992).

Summary

Collectively, the assessments of Na^+-K^+-ATPase activity in skeletal muscle have lacked integration and consistency, possibly because of a lack of information on the factors influencing skeletal muscle Na^+-K^+-ATPase activity assessed by different methods. It is possible that differences exist in the enzyme activity Na^+-K^+-ATPase subunits expressed between whole rat muscles of varying fibre composition. Enzyme activity should be directly influenced by the specific isoform complement expressed in the muscle fibres, although previous studies have not combined isoform characterization with appropriate enzymatic analysis. Different activity assays may be assessing different qualities of the enzyme's catalytic process, and this may explain the discordant results between chronic pump isoform adaptations and pump activity. The major objective in assessing fibre-specific differences in Na^+,K^+-ATPase characteristics, and Na^+,K^+-ATPase in response to exercise, is to lend some insight into factors that dictate Na^+,K^+-ATPase expression. The Na^+,K^+-ATPase is highly regulated in the acute and chronic response to stress (Green, 2000; Nielsen and Clausen, 2000), and yet the signals and mechanisms eliciting these changes are largely unknown.

STATEMENT OF THE PROBLEM

The purpose of the work presented in this thesis is to characterize the Na^+-K^+-ATPase (or Na,K-pump) in skeletal muscle and measure Na^+-K^+-ATPase activity in response to exercise. To address this, Na^+-K^+-ATPase activity, content and isoform distribution was measured in muscles of varying fibre types. Subsequently, Na^+-K^+-ATPase was measured following acute exercise in both rat and human muscle.

Specific study objectives include:

1. Comparing the validity, specificity, and variability of two assays used to measure Na^+-K^+-ATPase activity in skeletal muscle, and determine which technique is preferred for analysis of homogenate and crude membrane samples.
2. Determining the correlation between Na^+-K^+-ATPase content and isoform distribution to measurement of Na^+-K^+-ATPase activity in skeletal muscles of varying fibre composition.
3. Determining the fibre-specific effect of an isolation procedure used to obtain a crude membrane fraction, on Na^+-K^+-ATPase characteristics of activity, content and isoform distribution.
4. Determining the acute fibre-specific effect of exercise on Na^+-K^+-ATPase activity in rats, as measured by the 3-O-MFPase assay.
5. Determining the acute effect of sustained voluntary exercise on Na^+-K^+-ATPase activity in humans, as measured by the 3-O-MFPase assay

6. Establishing a relationship between the *in vitro* measure of Na^+-K^+-ATPase activity and the indirect measure of Na^+-K^+-ATPase by electromyography, during voluntary exercise in humans.

The specific hypotheses are:

1. The K^+-stimulated 3-O-methylfluorescein phosphatase (3-O-MFPase) assay will be the most specific and reliable, and the ouabain-inhibitable hydrolytic activity (ATPase) assay the most valid measure of Na^+-K^+-ATPase activity in skeletal muscle, in both homogenates and crude membrane fractions.
2. The 3-O-MFPase assay will be the preferred technique for use with homogenates, and the ATPase assay will be the preferred technique for use with crude membranes, to measure Na^+-K^+-ATPase activity in skeletal muscle.
3. Na^+-K^+-ATPase content, as measured by ouabain binding, will be greatest in muscles with the highest oxidative capacity (i.e. SOL, RG) and Na^+-K^+-ATPase content will correlate to the relative distribution of $\alpha 2$ subunits measured in homogenates of different skeletal muscles.
4. The relative abundance of $\alpha 2$ subunits between different skeletal muscles will best predict, by multiple regression, Na^+-K^+-ATPase activity measured by ATPase, in both homogenate and crude membranes.
5. The relative abundance of $\beta 1$ subunits between different skeletal muscles will best predict, by multiple regression, Na^+-K^+-ATPase activity measured by 3-O-MFPase, in both homogenate and crude membranes.

6. There will be no fibre-specific effect of the isolation procedure on measuring Na^+-K^+-ATPase characteristics in crude membrane fractions.

7. Na^+-K^+-ATPase activity will be reduced following a single bout of prolonged submaximal aerobic exercise in rats, when measured *in vitro* on muscle homogenates using the 3-O-MFPase assay.

8. Changes in Na^+-K^+-ATPase activity due to a single bout of exercise in rats will not be different between skeletal muscles of varying fibre type.

9. Na^+-K^+-ATPase activity and muscle excitability will be reduced during sustained, repetitive isometric exercise in humans, and will remain depressed for at least 60 minutes of passive recovery following the bout.

10. The EMG measure of M-wave area will correlate to the *in vitro* measure of Na^+-K^+-ATPase activity prior to and following voluntary exercise in humans.

Summary

To accomplish these objectives, three studies were performed.

1. In study one, I assessed Na^+-K^+-ATPase activity, pump content, and isoform distribution, in homogenates and crude membranes of skeletal muscles of varying fibre types. This study is presented in five parts, and forms the majority of the thesis work.
2. In study two, I investigated changes in Na^+-K^+-ATPase activity following a single bout of prolonged submaximal aerobic exercise in rat skeletal muscles of varying fibre types.
3. In study three, I measured Na^+-K^+-ATPase activity by 3-O-MFPase, and muscle excitability by EMG, in human skeletal muscle prior to and for a period of four hours of passive recovery following a single bout of sustained, moderate intensity voluntary isometric exercise in humans.

CHAPTER II

Characterization of Na^+-K^+-ATPase in rat skeletal muscles of varying fibre composition

ABSTRACT

The purpose of this study was to use a comparative approach assessing Na^+-K^+-ATPase characteristics in the soleus (SOL), red gastrocnemius (RG), white gastrocnemius (WG), and extensor digitorum longus (EDL) skeletal muscles of the rat. These muscles are representative of primarily slow-oxidative type I fibres (SOL), type I and fast oxidative-glycolytic type IIa fibres (RG), fast glycolytic type IIb fibres (WG), and muscle having a mixed complement of fast fibre types (EDL). Tissue was sampled at rest under anaesthetic, from a total of 38 male, 16 week-old Wistar rats weighing 413 ± 6.0 g (mean ± SE). Na^+-K^+-ATPase activity was determined in homogenates (HOM) and isolated crude membranes (CM) for the regenerating ouabain-inhibitable hydrolytic activity assay (ATPase) and the 3-O-methylfluorescein K^+-stimulated phosphatase (3-O-MFPase) assays *in vitro*. In addition, pump content was determined by [^{3}H]-ouabain binding, and the distribution of α1, α 2, α3, and β1, and β2 isoforms was determined by Western blot. Differences ($P<0.05$) in enzyme activity between muscles were observed for HOM (EDL>WG) and CM (SOL>EDL=WG) for the ATPase assay. For the 3-O-MFPase assay, differences ($P<0.05$) were also found for HOM (SOL>RG=EDL>WG) and CM (SOL=WG>RG). To determine if activity was related to pump content, differences in [^{3}H]-ouabain maximum binding (Bmax) were observed in the order of RG = EDL > SOL =WG ($P<0.05$), following the same trend as HOM ATPase activity. Western blot in CM indicated a greater distribution of α1 in SOL than WG and EDL ($P<0.05$) and a similar relation was observed for α2. The β1 isoform was greater ($P<0.05$) in EDL and WG, and the β2 was greater in SOL and RG ($P<0.05$). The presence of the α3 isoform, not previously observed in skeletal muscle, was identified in greater proportion in WG and EDL than SOL and RG ($P<0.05$). The α1 distribution was correlated

with HOM 3-O-MFPase ($r=0.79$, $P<0.05$) CM ATPase ($r=0.59$, $P<0.005$) and CM 3OMF activity ($r=0.33$, $P<0.05$). β1 distribution was correlated to HOM 3-O-MFPase ($r=0.61$, $P<0.005$) and CM ATPase ($r=0.42$, $P<0.05$) activity. HOM α2 distribution was correlated with ouabain Bmax ($r=0.64$, $P<0.005$). The results of this experiment indicate that a number of factors dictate the activity of the Na^+-K^+-ATPase in skeletal muscle, including the assay, isolation procedure, and the muscle studied. Na^+-K^+-ATPase activity is different between muscles of varying fibre composition, and a higher distribution of α1 and β1 subunits, as found in SOL, confers the greatest Na^+-K^+-ATPase activity when measured on skeletal muscle preparations *in vitro*.

INTRODUCTION

The Na^+-K^+-ATPase, or Na,K-pump, is an integral membrane protein that couples the chemical hydrolysis of ATP to the vectoral transport of Na^+ out of, and K^+ into, the cell. In skeletal muscle cells, this active transport mechanism restores ionic gradients for the control of cell volume and the facilitated transport of ions and nutrients (Lang *et al.*, 1998), and the maintenance of membrane potential and 'excitability' in response to neuromuscular activation (Clausen *et al.*, 1998). The effectiveness with which the Na^+-K^+-ATPase is able to perform this function is dependent upon the content and distribution of protein subunits and the precise control of regulatory signals that allow appropriate activation of the enzyme.

The contractile characteristics of muscle (i.e. force and speed of contraction) are typically linked to the proteins responsible for the excitation and contraction processes. In rat muscle, histochemically typed 'fast' fibres have IIA, IIX and IIB myosin heavy chains (MHC) (with high myosin ATPase activity), fast myosin light chains, and sarcoplasmic reticulum (SR) Ca^{2+} regulatory proteins specific to a rapid Ca^{2+} release and Ca^{2+} sequestration. A high glycolytic potential supports energy demands, and oxidative potential is variable between the fast fibre types. In addition, these muscle fibres require a high frequency of action potentials to develop and sustain maximum force. In contrast, 'slow' fibres with type I MHC (and low myosin ATPase activity) contain nearly the opposite functional complement of proteins, achieve a slower rate of force generation and relaxation, and do not require a high frequency of activation to attain optimal levels of force. These fibre type differences might suggest that the Na^+-K^+-ATPase content and isoform composition are dictated by the speed of the fibre and activation demand. However, it has been postulated that Na^+-K^+-ATPase content (Chin and Green, 1993) and activity (Ianuzzo

and Dabrowski, 1987) are associated with the oxidative capacity of the fibre, and limited evidence suggests that the pump isoform complement may not be regulated with myosin heavy chain (MHC) expression (Sun *et al.*, 1999; Sweadner *et al.*, 1992). Changes in Na^+-K^+-ATPase during chronic low frequency stimulation have been observed to generally precede adaptations of other proteins involved in excitation and contraction (Green *et al.*, 1992; Hicks *et al.*, 1997). These observations emphasize the importance of this enzyme in skeletal muscles' adaptive response to stress, although the factors that dictate the quantity and pattern of Na^+-K^+-ATPase subunit expression are largely unknown. A comprehensive investigation of pump activity, content, and isoform distribution in muscles with different fibre type composition may help to elucidate factors controlling expression of the skeletal muscle Na^+-K^+-ATPase.

The Na^+-K^+-ATPase is characterized as having one catalytic (α) subunit with four known isoforms, one glycoprotein (β) subunit with three isoforms, and an additional regulatory γ subunit (Blanco and Mercer, 1998). In mammalian skeletal muscle, the distribution is such that slow oxidative (type I) fibres contain $\alpha1\beta1$ and $\alpha2\beta1$ complexes, fast glycolytic (type IIB) fibres contain $\alpha1\beta2$ and $\alpha2\beta2$ heterodimers (Hundal *et al.*, 1993; Hundal *et al.*, 1994), and fast oxidative-glycolytic (type IIA) have all four combinations (Thompson and McDonough, 1996). The $\alpha3$ and $\beta3$ subunits are in negligible quantities in skeletal muscle (Arystarkhova and Sweadner, 1997; Thompson *et al.*, 2001) and characterization of the γ subunit in skeletal muscle is incomplete. These results suggest that in terms of isoform distribution, it is the β isoform that distinguishes slow from fast fibres. The $\beta1$ subunits are believed to confer greater enzymatic activity to the pump than the $\beta2$ subunits (Blanco *et al.*, 1995; Crambert *et al.*, 2000; Hundal *et al.*, 1993), so this may have

particular importance for characterizing fibre-specific Na^+-K^+-ATPase activity in skeletal muscle. The $\alpha 2$ is thought to be the major catalytic isoform (Hundal *et al.*, 1994), potentially conferring greater enzyme activity if preferentially expressed in a particular fibre.

Previous studies have identified Na,K-pump content and isoform distribution in a range of skeletal muscles of different fibre type composition (Hundal *et al.*, 1993; Sun *et al.*, 1999; Thompson *et al.*, 1999; Thompson *et al.*, 2001; Thompson and McDonough, 1996). However, the way in which these characteristics relate to muscle-specific Na^+-K^+-ATPase activity is unclear. For example, Thompson *et al.* (1999) reported no difference in Na^+-K^+-ATPase activity between soleus (SOL) and white gastrocnemius (WG) muscles despite a substantially different isoform composition. These authors also observed 94% and 70% decreases in $\alpha 2$ and $\beta 2$ subunits with K^+ deprivation, but only a 19% decrease in K^+-stimulated p-nitrophenol phosphatase (K-pNPP) activity. A number of other interventions from ionic to hormonal manipulations have induced isoform specific adaptations in Na^+-K^+-ATPase expression in skeletal muscle (Azuma *et al.*, 1991; Azuma *et al.*, 1993; Bundgaard *et al.*, 1997; Thompson *et al.*, 1999; Thompson *et al.*, 2001; Thompson and McDonough, 1996), although measures of enzyme activity do not consistently reflect these changes. Typically, the changes in isoform distribution measured by Western blot, are not reflected in measures of pump content assessed by the standard ouabain-binding technique (Sun *et al.*, 1999). Although Bundgaard *et al.* (1997) observed a relationship between changes in pump content and pump activity, the results of Western blot (isoform distribution) and ouabain binding (content) are often at odds with an *in vitro* measure of pump activity (Azuma *et al.*, 1993; Thompson *et al.*, 1999; Thompson *et al.*, 2001).

Distinct isoform combinations of the Na^+-K^+-ATPase are believed to confer kinetic specificity for varying cellular requirements (Blanco and Mercer, 1998). Differences in activity are observed when specific isoform complements are expressed in bacterial cultured cells (Blanco *et al.*, 1995; Crambert *et al.*, 2000) or when obtained by isolation of a group of red or white skeletal muscles (Hundal *et al.*, 1994; Lavoie *et al.*, 1997). Given the contradictory findings identified for specific skeletal muscles between pump content, subunit distribution, and activity, our understanding of the fibre-specific relationship between these variables is limited.

The problem in examining the relationship between fibre-type composition and pump activity is primarily due to inherent difficulties in assessing Na^+-K^+-ATPase activity in skeletal muscle (Hansen and Clausen, 1988). Sarcolemmal and t-tubular Na^+-K^+-ATPase content and activity are low compared to the content and activity of other ATPases of the cell, including other membrane bound enzymes. This fact makes valid assessment Na^+-K^+-ATPase activity difficult, whether in whole-muscle homogenates or enriched membrane fractions. Isolation procedures typically have low yields and greater tissue requirements (Hansen and Clausen, 1996; Hundal and Aledo, 1996) which also create problems for muscle specific determinations.

Artificial substrates, such as 3-O-methylfluorescein phosphate (3-O-MFP) and p-nitrophenol phosphate (pNPP), are used in phosphatase assays to analyze activity in skeletal muscle homogenates and enriched fractions, because these assays are sensitive and specific indicators of activity. These assays are purported to relate well to actual ATPase activity (Hansen and Clausen, 1988). This relationship was established in rat brain, cat heart, and human red cells (Huang and Askari, 1975), and a purified t-tubular membrane preparation

(Horgan and Kuypers, 1987), and therefore requires further confirmation in skeletal muscle of varying fibre types. These assays yield results that are less than the activity assessed by direct methods, possibly because of reduced affinity for the artificial substrates (Hansen and Clausen, 1988). Phosphatase assays do not consistently represent changes at the isoform level, so they may indicate something other than maximum hydrolytic activity of the enzyme.

The β isoform stabilizes the K^+-bound E2 phosphoenzyme intermediate (Blanco *et al.*, 1995; Skou, 1992), and therefore phosphatase assays may specifically identify enzyme activity between muscles of varying β isoform composition. Given that the primary step in ATP hydrolysis is a Na^+-dependent phosphorylation (Skou, 1992), hydrolytic activity assessed in a direct ATPase assay is thought to be regulated by the α catalytic isoform and its varying affinities for Na^+ as a substrate (Jewell and Lingrel, 1991; Zahler *et al.*, 1997). This property may help to specifically identify enzyme activity between muscle fibres of varying α isoform composition. Therefore, using both the hydrolytic and phosphatase assays to assess Na^+-K^+-ATPase activity between muscles of varying fibre type may help to relate muscle specific-differences in both α and β isoform distiribution.

Collectively, assessments of Na^+-K^+-ATPase activity in skeletal muscle have lacked integration and consistency, possibly because of a lack of information on the factors influencing skeletal muscle Na^+-K^+-ATPase activity assessed by different methods. There is some evidence to suggest that pump content may be related to oxidative potential of the fibre, but this requires confirmation in context of other variables used to characterize the Na^+-K^+-ATPase in skeletal muscle. The purpose of the present study is to assess activity, content, and isoform distribution characteristics of the Na^+-K^+-ATPase in skeletal muscles

of varying fibre composition. To achieve this, Na^+-K^+-ATPase characteristics were compared between soleus (SOL), red gastrocnemius (RG), extensor digitorum longus (EDL), and white gastrocnemius (WG) skeletal muscles of the rat. These muscles are representative of primarily slow-oxidative type I fibres (SOL), fast oxidative-glycolytic type IIa fibres (RG), fast glycolytic type IIb fibres (WG), and muscle having a mixed complement of fast fibre types (EDL) (Delp and Duan, 1996).

We hypothesize that in rat skeletal muscle, Na^+-K^+-ATPase hydrolytic activity (measured directly by the ATPase assay) should relate to the content of the $\alpha 2$ catalytic isoform, Na^+-K^+-ATPase phosphatase activity (measured by the 3-O-MFPase assay) should relate to the content of the $\beta 1$ isoform, and the standard ouabain binding assay should quantify the content of $\alpha 2$ subunits. We hypothesize that oxidative muscles will express greater relative amounts of $\alpha 2$ and $\beta 1$ subunits, and therefore, will have a higher pump content when measured by ouabain binding, and greater Na^+-K^+-ATPase activity, when measured *in vitro* by either the ATPase or the 3-O-MFPase assay. We will contrast these relationships to the current understanding that there is a direct relationship between and Na^+-K^+-ATPase content and activity, by measuring these characteristics in muscles of varying fibre type composition.

METHODS

Animals. Experiments were carried out using 38 male Wistar rats (age 16 weeks; weight 413 ± 6 grams , mean ± SE). Rats were housed in a room where the light-cycle was reversed (12h/12h) and rat chow and water were provided ad libitum. Care and treatment of the animals was in accordance with procedures outlined by the Canadian Council on Animal Care. All procedures were approved by the University of Waterloo Office for Ethics in Research.

Experimental Design. A comparative model was used to assess Na^+-K^+-ATPase characteristics between soleus (SOL), red gastrocnemius (RG), extensor digitorum longus (EDL), and white gastrocnemius (WG) skeletal muscles of the rat. Experimental methods were divided into five parts according to the following subdivisions: *Part 1*, Na^+-K^+-ATPase activity; *Part 2*, Validity, reliability, and specificity of Na^+-K^+-ATPase assays in HOM and CM preparations; *Part 3*, [^{3}H]ouabain binding and Na^+-K^+-ATPase content; *Part 4*, Isoform distribution quantified by Western Blot; *Part 5*, Relationships between Na^+-K^+-ATPase activity, content, and isoform distribution.

Na^+-K^+-ATPase activity (Part 1) was measured in whole-muscle homogenates (HOM) and homogenates prepared from isolated crude membranes (CM) using the direct ouabain-inhibitable hydrolytic activity assay (ATPase) and the 3-O-methylfluorescein K^+-stimulated phosphatase (3-O-MFPase) assays *in vitro*. From the measures in Part 1 maximum activity, specific activity, intra-assay variability and inter-assay variability were calculated between assays, for both HOM and CM, and the protein yield, purification, and recovery of activity were determined for CM (Part 2). [^{3}H]ouabain binding was used to quantify pump content (Bmax, maximum binding), as well as ouabain binding affinity (Kd, dissociation constant) on whole muscle tissue pieces (Part 3). Isoform distribution of α1, α2, α3, β1, and β2 isoforms was determined in both HOM and CM, by Western blot and relative

densitometry (Part 4). Linear, and multiple linear regression was used to identify correlations between Na^+-K^+-ATPase activity, content, and isoform distribution (Part 5).

Muscle preparation and isolation procedures. SOL, RG, EDL, and WG muscle samples were obtained from anaesthetized animals. Muscles were rapidly excised, quickly frozen in liquid nitrogen, and stored at –80°C until further preparation and/or analysis. Time between anaesthetization and extraction averaged ~5min. Individual muscles from 10 separate rats were obtained for preparation of HOM in Na^+-K^+-ATPase activity assays and Western blotting. For HOM, tissue from frozen muscle samples was homogenized (5% w/v) at 0°C for 2 x 20s @ 25,000 rpm (Polytron), in a buffer containing 250mM sucrose, 2 mM EDTA, 1.25 mM EGTA, 5mM NaN_3 and 10mM Tris (pH 7.40). The homgenate was aliquotted, and quickly frozen in liquid nitrogen until further analysis.

Muscles from both limbs of 2-3 rats (depending on muscle size) were pooled to prepare 'crude membranes' (CM) for Na^+-K^+-ATPase activity assays and Western blotting. The CM was prepared using a modification of procedures employed by Dombrowski *et al.* (1996), a technique developed to isolate plasma membrane, t-tubular, and internal membrane components of skeletal muscle. This technique uses various centrifugation and sucrose gradient separation steps to purify muscle membrane components. However, as the intent of this study was to use small tissue amounts for individual muscle Na^+-K^+-ATPase activity determinations, only the first few steps of this procedure were employed to produce a 'crude membrane' (CM) (identified by Dombrowski *et al.* as "pellet F2"). This fraction contains primarily sarcolemmal membranes and some internal membranes that contain Na^+-K^+-ATPase subunits. This fraction does have contamination from other internal membranes such as sarcoplasmic reticulum (SR) (Dombrowski *et al.*, 1996). The SR Ca^{2+}-ATPase was identified in this experiment to contribute to background activity in the ATPase assay. However, the extent of background relative to Na^+-K^+-ATPase activity was very stable between repeated analysis of CM samples (see Part 2 - Results). Therefore, the difference in

Na^+-K^+-ATPase activity in CM for the direct assay, was stable for an individual muscle. Na^+-K^+-ATPase activity in the direct assay was calculated as the difference between the presence and absence of the specific inhibitor, ouabain, and this accounts for background activity providing that the background is stable between separate runs, as indicated. It should be emphasized that the primary goal of the isolation procedure was *not* to obtain a 'pure' sarcolemmal preparation, but to obtain a sample adequately purified (~7-10 fold) to assess muscle specific Na^+-K^+-ATPase activity using a direct assay, without extensive tissue requirements (i.e. ~5 grams as per Dombrowski *et al.* 1996). The activity recovery obtained for CM in this experiment (see Part 2 – Results) was at least 4 fold higher than the activity recovery commonly obtained in complete isolation procedures (Hansen and Clausen, 1988).

Briefly, for preparation of CM, ~1g of frozen muscle (combined from 2-3 rats) was thawed, cleaned of connective tissue, and minced for 5 min in ice-cold isolation Buffer A (10mM $NaHCO_3$, 250mM sucrose, 5mM NaN_3, and 100μM polymethyl-sulphonyl fluoride [PMSF]; pH 7.0). Tissue was blotted and weighed (500-800mg wet weight), and homogenized for 2 x 5s @ 17,500 rpm with a polytron 3100 homogenizer in Buffer A (200mg in 3ml). Homogenate was centrifuged at 1300g for 10 min, the supernatant saved, the pellet resuspended in Buffer A (500mg in 3.75ml) and the homogenization and 1300g centrifugation was repeated. The supernatants were combined and centrifuged at 9000g for 10min. The supernatant was then centrifuged at 190,000g for 1h. The pellet was resuspended in 400 μl of Buffer A, aliquotted, and frozen in liquid nitrogen until further analysis.

Protein content of both the HOM and CM was determined by the method of Lowry as modified by Schacterle and Pollock (1973).

Part 1: Na^+-K^+-ATPase activity.

Samples were prepared according to the procedures outlined above for HOM and CM, and analyzed in two different Na^+-K^+-ATPase assays. Two assays were employed because of evidence that the pump's hydrolytic and phosphatase activities can be modified independently of each other (Ball, 1986; Linnertz *et al.*, 1995; Zolotarjova *et al.*, 1995), implying that different components of Na^+-K^+-ATPase may be selective in their contribution to overall enzyme activity. Therefore, each assay may distinguish functionally different aspects of Na^+-K^+-ATPase activity and possibly confer isoform-specific differences in enzyme capacity. Preparation of both HOM and CM samples, for both the ATPase and 3-O-MFPase assays, included four freeze thaw cycles to permeabilize membranes and expose binding sites for Na^+, K^+, ATP, and ouabain. Pilot testing indicated that this procedure produced similar results to other permeabilizing agents such as saponin, TritonX-100, and deoxycholate, with less intra-assay variability. Na^+-K^+-ATPase activity is expressed for all measures as $\mu mol \cdot mg\ protein^{-1} \cdot hr^{-1}$.

ATPase activity. The procedures for the direct ATPase assay were modified from that published by our laboratory for the Ca^{2+}ATPase (Chin and Green, 1996; Green *et al.*, 1997). Spectrophotometric (Shimadzu UV 160U) measurement of Na^+-K^+-ATPase activity was performed using a regenerating assay. The regenerating assay limits potential accumulation of inorganic phosphate, which in an end-point measure, can be inhibitory to maximal activity. Samples were freeze-thawed four times and incubated at 37°C for 15 min with or without 2mM ouabain in the buffer containing (in mM) 120 NaCl, 15 KCl, 50 TrisBase, 5 NaN_3, 1 EGTA, 5 $MgCl_2$, 10 PEP (pH 7.4), in the presence of PK and LDH. The assay was started with the addition of 0.3mM NADH and 5mM ATP. The linear portion of the slope (~6-8min) was compared between successive trials for each of ouabain and no-ouabain conditions to give a calculation of ouabain-inhibitable Na^+-K^+-ATPase activity. The average of three repeated determinations (no ouabain - ouabain) was used as the Na^+-K^+-

ATPase activity for a given sample. Analysis was identical between HOM and CM with the exception that 15-20 µl of HOM (~150 µg protein) and 10-15 µl of CM (~75 µg protein) were used.

3-O-MFPase activity. The K^+-stimulated 3-O-methylfluorescein phosphatase assay (3-O-MFPase) was assessed fluorometrically using a modified procedure from that of Huang and Askari (1975) and Horgan and Kuypers (1987), by using a higher substrate concentration (Fraser and McKenna, 1998; Huang and Askari, 1975). We have confirmed in a separate set of experiments (results not shown), that maximal activity was achieved at ~160 µM 3-O-methylfluorescein phosphate (3-O-MFP) in rat tissue, in both HOM and CM. The use of 1.25 mM EGTA, 5 mM NaN_3, was confirmed in a separate set of experiments to decrease non-specific activity in the assay, and optimize assessment of K^+-stimulated activity in rat muscle samples. Samples were freeze-thawed four times, and diluted 1/5 in cold homogenate buffer before being incubated for four min in medium containing (in mM) 5 $MgCl_2$, 1.25 EDTA, 1.25 EGTA, 5 NaN_3, and 100 Tris (pH 7.40). The K^+-stimulated activity of the Na^+-K^+-ATPase was determined by the increase in activity after the addition of 10 mM KCl. 3-O-MFPase activity was determined by the difference in slope before and after the addition of KCl. This method has greater reliability than can be determined by separate ouabain and no-ouabain assays. Incubation with 2mM ouabain inhibited >90% of K^+-stimulated 3-O-MFPase activity and confirmed the specificity of the assay. The 3-O-MFPase activity was also based on the average of three successive trials. Analysis was identical between HOM and CM samples, with the exception that ~30 µl of HOM (~30 µg protein) and ~15 µl of CM (~15 µg protein) were used.

Part 2: Validity, reliability, and specificity of Na^+-K^+-ATPase assays in HOM and CM preparations

The two assays used to assess Na^+-K^+-ATPase activity were compared for their validity, specificity and reliability between HOM and CM samples for each muscle.

Homogenate (HOM). The aforementioned variables were calculated for each assay. Validity was calculated by the % of activity measured by the assay relative to the theoretical maximum pumping capacity of the Na^+-K^+-ATPase for the muscle, as predicted by [^{3}H]ouabain binding (i.e. pump content in pmol/g wet weight) multiplied by the pump ATP turnover (assumed to be 8000/min (Clausen *et al.*, 1987)]. Specificity was indicated by the % of specific activity (i.e. K^+-stimulated activity for the 3-O-MFPase assay, or ouabain-inhibitable activity for the ATPase assay) relative to background activity (i.e. non-specific activity for the 3-O-MFPase assay, or Mg^{2+}-ATPase activity for the ATPase assay). Intra-assay reliability was indicated by the average coefficient of variation (C.V. = standard deviation/mean, %) for three repeated measures on a single sample (intra-assay C.V.). The inter-assay reliability was defined as the C.V. calculated on measures of different samples within a group of muscles (inter-assay C.V.).

Crude membrane (CM). The protein yield was calculated as the amount of protein available at the end of the isolation, relative to the known amount of wet weight tissue at the beginning of the isolation (mg protein/g wet weight). The protein yield is generic to the isolation procedure itself, whereas the following variables were calculated for each of the ATPase and 3-O-MFPase assays.

The validity was indicated in CM by the % of theoretical maximum activity, defined as the theoretical maximum calculated for HOM (as above) accounting for the recovery of HOM activity (per g wet weight tissue). The purification factor was defined as the amplification in Na^+-K^+-ATPase activity (per mg protein), relative to the HOM activity (per mg protein). Specificity and reliability were calculated identically to that of HOM.

Part 3: [^{3}H]Ouabain binding and Na^+-K^+-ATPase content.

[^{3}H]Ouabain binding was used to quantify pump content (Bmax, maximum binding), as well as ouabain binding affinity (Kd, dissociation constant) on whole muscle tissue samples. Attempts were made to determine Bmax and Kd in HOM and CM, although these data were not reliable. Whole muscle values would most relate to HOM characteristics. The procedures employed were modified from those used by Kjeldsen *et al.* (1986) and Pickar *et al.* (Pickar *et al.*, 1997). It is generally accepted that the standard ouabain binding assay (using 1000 nM [ouabain]) assesses only the content of α2 isoforms in rat skeletal muscle (Kjeldsen, 1988) because the α1 isoform in rat skeletal muscle is highly ouabain-resistant (Blanco and Mercer, 1998). Therefore, saturation binding in the range of 12.5-1000 nM ouabain was compared with binding curves using ouabain concentrations up to 5000nM, in combination with a modified washout period. A shorter washout period was used to minimize wash off of ouabain resistant α1 subunit binding, in order to quantify the two populations of subunits in skeletal muscle.

Bmax and Kd. In the modified procedure, muscle samples are incubated in concentrations of labeled and unlabeled ouabain from 12.5-5000 nM. Two muscle samples weighing between 2 and 8 mg were prewashed twice for 10 min periods in a Tris-sucrose buffer (10 mM Tris-HCl, 3 mM MgSO4, 1mM Tris-vanadate and 250 mM sucrose) containing sodium metavanadate ($NaVO_3$) at 0°C. Samples were incubated in the Tris-sucrose buffer with 12.5-50 nM of [^{3}H]-ouabain (0.9 μCi/ml) and unlabeled ouabain (50 nM – 4950 nM final concentration) for 180 min @ 37°C. After the unbound ouabain was removed by washing for three times for 15 min in ice-cold buffer, the samples were blotted, weighed, placed in 1.5-ml Eppendorf tubes, soaked in 1ml 5% trichloroacetic acid for 16 hr at room temperature, and then 0.5 ml of sample was counted for [^{3}H] radioactivity in a scintillation mixture. [^{3}H]Ouabain binding capacity was corrected for loss of specifically bound [^{3}H]ouabain during washout [1.05 as per Norgaard *et al.* (1984)], and unspecific

uptake and retention. Washout and retention of [^{3}H]ouabain was determined for each muscle for each assay relative to each ouabain concentration. This correction was less than 5% at 1000 nM, and less than 22% at 5000 nM total ouabain concentration. The isotopic purity of the [^{3}H]ouabain was 99% as determined by the supplier (New England Nuclear-Du Pont Canada).

The maximum binding capacity (Bmax) is calculated by non-linear regression (i.e. fit) of the saturation binding curve, using either the one- or two-component binding model. The affinity constants (Kd) are also obtained by linear regression of a binding curve and represented by linear transform on a Scatchard plot.

Part 4: Isoform distribution determined by Western blot.

Immunoblotting was performed on both HOM and CM, using the primary polyclonal antibodies specific to the α1, α2 and β1, and β2 isoforms (Upstate Biotechnologies, NY). In our hands, the polyclonal antibodies identified clear bands and had little background reactivity. An additional monoclonal antibody specific for the rat α3 subunit was also used (Affinity Bioreagents, NY), that showed high specificity and minimal cross-reactivity.

For any particular isoform, an equal amount of either HOM or CM sample was added to each lane, for each muscle. A total of 13 samples (4 muscles x 3/muscle + brain standard) were applied on an individual gel, and each gel was run in duplicate. Therefore, to complete an n of 6 for each isoform, two sets of two gels were run in parallel, each gel with a known content (2.5 or 5 μg) of brain standard for relative control. Rat brain has an α-β molar ratio of 1:1 and approximately equal subunit distribution (except α3, which is in high amounts) (Lavoie *et al.*, 1997) making it suitable as a relative control between muscle types in this experiment. Data in the results are presented relative to the density of the brain standard for a specific isoform only. For comparison of relative binding between isoforms

(and inferred molar distribution between subunits) please refer to the calculations in Appendix A.3.

Western blotting. Samples of HOM and CM (1.0 mg/ml) were suspended in the $NaHCO_3$ isolation buffer (described under isolation methods) and loaded into gels in equal amounts. Exactly 30 μg of HOM sample and 20 μg of CM sample from each muscle was electrophoresed in duplicate on separate 7.5% sodium dodecyl sulfate polyacrylamide (BIO-RAD Mini-PROTEAN II), with 3.75% stacking gels. After SDS-PAGE and a 15 min equilibration in cold transfer buffer (25mM Tris, 192mM glycine and 20% v/v methanol), the proteins were transferred to a polyvinylidene difluoride membrane (PVDF membrane, Bio-Rad) by placing the gel in transfer buffer and applying a high voltage (100V) for 45 min (Trans-Blot Cell, Bio-Rad). For the polyclonal antibodies, non-specific binding sites were blocked with 5 or 7.5% BSA in Tris-buffered saline (TBS, pH 7.5) at room temperature for 2h prior to incubation for 1h at room temperature with primary antibodies diluted in 5% non-fat milk (α1, 1:1000; α2, 1:500; β1, 1:1000; β2, 1:500). Following washing (3 x 3 min) in 0.1% TBS Tween 20 (TBS-T). A secondary antibody (goat anti-rabbit IgG1) was applied for 60 min diluted at 1:3000 (α1, α2, β1) or 1:4000 (β2) in TBS-T. For the monoclonal antibody specific to α3, blots were blocked with 5% milk overnight at room temperature, then prior to incubation with primary antibody for 1 h (1:500 dilution) and with secondary antibody goat ant-mouse for 1h (1:3000 dilution). An enhanced chemiluminescence procedure was used to identify antibody content (Amersham-ECL-RPN210691). After exposure to photographic film (Kodak Hyperfilm-ECL) blots were developed for 60-90 sec in Kodak GBX developing solution and fixed in Kodak GBX fixer. Relative isoform protein levels were determined by scanning densitometry (Scion Image© software) and values were determined relative to a known concentration of rat brain standard for each blot.

Part 5: Relationships between Na^+-K^+-ATPase activity, content, and isoform distribution

Linear regression was used to identify correlations (rectangular matrix) between Na^+-K^+-ATPase activity for the two assays, and between pump content and individual isoform distribution. Multiple linear regression (Pearson r) was used to identify the extent that Na^+-K^+-ATPase activity was predicted by isoform distribution.

Data Analysis

Statistical analysis was performed on Statistica for Windows R.4.5 software (Statsoft Inc., Tulsa, OK, 1993). Descriptive statistics included means and standard error (SE). One-way analysis of variance (ANOVA) with repeated measures was used to analyze pump characteristics by muscle. Two-way analysis of variance (ANOVA) with repeated measures was used to analyze differences between assay characteristics and measurements by muscle. Correlational analysis by linear and multiple linear regression was used to relate Na^+-K^+-ATPase characteristics. Post hoc analysis of mean values was performed using the Tukey test. Statistical significance was set at $P<0.05$.

RESULTS

Part 1: Na^+-K^+-ATPase activity

Na^+-K^+-ATPase activity in HOM and CM measured by ATPase and 3-O-MFPase, is summarized in Table 2.1.

HOM. EDL ATPase activity was 4 fold greater than WG ($P<0.05$). EDL ATPase activity was ~2 fold greater than both RG and SOL although this difference was not significant. For 3-O-MFPase, SOL had ~1.5 fold greater activity than both RG and EDL ($P<0.05$) which had nearly 2 fold greater activity than WG ($P<0.05$). In HOM, 3-O-MFPase activity was on average 18% of ATPase activity in the four muscles (range 9-29%).

CM. ATPase activity in SOL was ~1.5 fold greater than EDL and WG ($P<0.05$). RG ATPase activity was not significantly different than any other muscle, although its activity was intermediary between SOL and EDL. For 3-O-MFPase, RG was less than both SOL and WG ($P<0.05$) and EDL activity was not different from any muscle. In CM, 3-O-MFPase activity averaged 23% of ATPase activity in the four muscles studied (range 18-29%).

Table 2.1. Na^+-K^+-ATPase activity measured by ATPase and 3-O-MFPase assays in homogenates and crude membranes of different skeletal muscles.

		SOL	RG	EDL	WG
HOM	ATPase	0.97 ± 0.24	0.84 ± 0.29	1.87 ± 0.31	0.57 ± 0.34‡
	3-O-MFPase	0.27 ± 0.01	0.18 ± 0.01*	0.17 ± 0.01*	0.10 ± 0.01*†‡
CM	ATPase	8.12 ± 0.43	6.75 ± 0.66	5.77 ± 0.40*	5.10 ± 0.37*
	3-O-MFPase	1.50 ± 0.10	1.24 ± 0.06*	1.39 ± 0.06	1.47 ± 0.07†

Values are mean ± SE; HOM, n=10; CM, n=6. All values in μmol·mg protein^{-1}·hr^{-1}. HOM, homogenate; CM, crude membrane, ATPase, ouabain inhibitable hydrolytic activity; 3-O-MFPase, K^+-stimulated 3-O-methylfluorescein phosphatase activity, SOL, soleus, RG, red gastrocnemius; EDL, extensor digitorum longus; WG, white gastrocnemius.

* *Different from SOL ($P<0.05$);* † *Different from RG ($P<0.05$);* ‡ *Different from EDL ($P<0.05$). Significant main effects ($P<0.05$) for this analysis were: HOM < CM; ATPase > 3-O-MFPase.*

Part 2: Validity, reliability, and specificity of Na^+-K^+-ATPase assays in HOM and CM preparations

The two assays used to assess Na^+-K^+-ATPase activity were compared for their validity, specificity, and reliability (see Methods), between HOM (Table 2.2) and CM (Table 2.3) for different muscles.

HOM. The ATPase assay assessed 92%, 69%, 151%, and 77% of theoretical maximum capacity in SOL, RG, EDL, and WG, respectively. On average, the 3-O-MFPase assay resulted in less than 1/5 of the activity of the ATPase assay, but exhibited 2-3 fold greater specificity ($P<0.05$) and lower intra-assay C.V ($P<0.05$). Inter-assay C.V. was substantially lower for the 3-O-MFPase (range 11-24%) than the ATPase (79-186%) ($P<0.05$) because some samples for the ATPase had undetectable ouabain-inhibitable activity (SOL, n=2; RG, n=4; EDL, n=1; WG, n=6).

CM. The protein yield in the CM preparations was greater in RG than WG ($P<0.05$) with no differences between other muscles (overall average 7.4 ± 0.6 mg sarcolemmal protein per g wet weight tissue). The CM isolation resulted in purified Na^+-K^+-ATPase activity by an average of 8.3 fold across both assays for all muscles. The ATPase assay assessed a higher % of theoretical maximum than the 3-O-MFPase assay (33 % vs. 7 %, respectively) with SOL recovery greater in ATPase than all other muscles ($P<0.05$). The average recovery of HOM activity in the isolation was greater ($P<0.05$) for the 3-O-MFPase than ATPase assay (average recoveries were 44% and 31%, respectively). For the specific muscles, WG had greater ($P<0.05$) activity recovery for the 3-O-MFPase assay than all other muscles, and EDL had less ($P<0.05$) activity recovery than all other muscles in the ATPase assay. The isolation procedure translated to improved specificity ($P<0.05$) and reliabilities ($P<0.05$) over HOM for both assays, with WG exhibiting greater ($P<0.05$) improvements than the other muscles.

Table 2.2. Characteristics of ATPase and 3-O-MFPase assays in homogenates of different skeletal muscles.

	SOL	RG	EDL	WG
ATPase				
% of theoretical max	92 ± 21	69 ± 24	151 ± 26	77 ± 44[‡]
specific/background	24 ± 7	18 ± 7	34 ± 6	16 ± 12[*‡]
intra-assay C.V.	12.7	10.3	12.5	16.4
inter-assay C.V.	78.7	107.1	52.8	186.3
3-O-MFPase				
% of theoretical max	26 ± 1	15 ± 1[*]	14 ± 6[*]	13 ± 6[*]
specific/background	64 ± 11	52 ± 5	55 ± 3	36 ± 3
intra-assay C.V.	8.2	13.2	7.6	14.6
inter-assay C.V.	11.3	17.4	17.9	30.7

Values are mean ± SE; n=10. ATPase, ouabain inhibitable hydrolytic activity; 3-O-MFPase, K^+-stimulated 3-O-methylfluorescein phosphatase activity, SOL, soleus; RG, red gastrocnemius; EDL, extensor digitorum longus; WG, white gastrocnemius. Theoretical max indicates validity which is determined by the % activity achieved in the assay relative to the theoretical maximum pumping capability for the muscle, as predicted by [^{3}H]ouabain binding (i.e. pump content in pmol/g wet weight) multiplied by the pump ATP turnover (8000/min), corrected for protein content of homogenate (~150 mg protein/g wet weight). Specific/background indicates specificity, which is the % of specific activity (i.e. K^+-stimulated activity for the 3-O-MFPase assay, or ouabain-inhibitable activity for the ATPase assay) relative to background activity (i.e. non-specific activity for the 3-O-MFPase assay, or Mg^{2+}-ATPase activity for the ATPase assay). Intra-assay C.V. and inter-assay C.V. indicate reliability, which was calculated by the average intra-assay coefficient of variation for three repeated measures on a single sample (intra-assay C.V.), and by the variability of measures for different samples within a muscle (inter-assay C.V.).

[*] *Different from SOL (P<0.05);* [†] *Different from RG (P<0.05);* [‡] *Different from EDL (P<0.05). Significant (P<0.05) main effects from this analysis were: ATPase theoretical max > 3-O-MFPase theoretical max; 3-O-MFPase specific/background > ATPase specific/background.*

Table 2.3. Characteristics of ATPase and 3-O-MFPase assays in crude membranes of different skeletal muscles.

	SOL	RG	EDL	WG
CM protein yield				
mg protein/g wet weight	7.1 ± 0.3	8.7 ± 0.6*	8.0 ± 0.4	5.9 ± 0.3†
ATPase				
% of theoretical max	47 ± 3	33 ± 2*	26 ± 2*	26 ± 2*
recovery of HOM activity	49 ± 3	46 ± 3	16 ± 1*†	36 ± 3*‡
purification factor	8.3 ± 0.4	8.0 ± 0.8	3.3 ± 0.2*†	9.0 ± 0.6‡
specific/background	58 ± 2	65 ± 6	68 ± 6	70 ± 6
intra-assay C.V.	4.4	3.3	4.6	5.3
inter-assay C.V.	13.0	23.8	17.0	17.5
3-O-MFPase				
% of theoretical max	9 ± 1	6 ± <1	6 ± <1	7 ± <1
recovery of HOM activity	33 ± 3	39 ± 1	45 ± 1*	59 ± 2*†‡
purification factor	5.4 ± 0.3	7.4 ± 0.9	7.9 ± 0.6	16.5 ± 2.2*†‡
specific/background	170 ± 11	175 ± 5	199 ± 4	181 ± 5
intra-assay C.V.	4.2	3.5	6.2	8.6
inter-assay C.V.	13.7	12.3	10.1	12.3

Values are mean ± SE; n=6. CM, crude membrane. ATPase, ouabain inhibitable hydrolytic activity; 3-O-MFPase, K^{+}-stimulated 3-O-methylfluorescein phosphatase activity, SOL, soleus; RG, red gastrocnemius; EDL, extensor digitorum longus; WG, white gastrocnemius. Theoretical max indicates validity which is determined by the % activity achieved in the assay relative to the theoretical maximum pumping capability for the muscle, as predicted by ^{3}H-ouabain binding (i.e. pump content in pmol/g wet weight) multiplied by the pump ATP turnover (8000/min), corrected for protein content of homogenate (~150 mg protein/g wet weight). Recovery of HOM activity is the % recovery of activity per g wet weight of original tissue. Purification factor is the amplification of activity (per mg protein) relative to homogenate activity. Specific/background indicates specificity, which is the % of specific activity (i.e. K^{+}-stimulated activity for the 3-O-MFPase assay, or ouabain-inhibitable activity for the ATPase assay) relative to background activity (i.e. non-specific activity for the 3-O-MFPase assay, or Mg^{2+}-ATPase activity for the ATPase assay). Intra-assay C.V. and inter-assay C.V. indicate reliability, which was calculated by the average intra-assay coefficient of variation for three repeated measures on a single sample (intra-assay C.V.), and by the variability of measures for different samples within a muscle (inter-assay C.V. *Different from SOL (P<0.05); † Different from RG (P<0.05); ‡ Different from EDL (P<0.05). Significant (P<0.05) main effects from this analysis were: 3-O-MFPase purification >ATPase purification: 3-O-MFPase recovery of HOM activity >ATPase. recovery of HOM activity; 3-O-MFPase specific/background > ATPase specific/background.

Part 3: [^{3}H]Ouabain binding and Na^+-K^+-ATPase content

One-component model. Differences in [^{3}H]ouabain maximum binding (Bmax), determined on whole muscle samples, were observed in the order of RG = EDL > SOL = WG ($P<0.05$) for the total ouabain binding range of 12.5-1000 nM (Table 2.4). A difference in binding affinity (Kd) was only observed between RG and WG ($P<0.05$). The average saturation curve and Scatchard plot for SOL is presented in Figure 2.1. The Bmax determined using the one-component model, was used in subsequent analysis for relationships to other pump characteristics.

Two-component model. When plotted over the range of 12.5-5000nM, there was some indication of a 2-site binding model for RG and EDL muscle (r^2 = 0.83, 0.79 for RG and EDL, respectively). SOL and WG were not accurately predicted with a two component model (i.e. $r^2 < 0.68$). An average saturation curve and Scatchard plot is presented for EDL in Figure 2.2. Maximum binding was equal between the two binding sites (Bmax1 vs. Bmax2) for both RG (244 ± 71 vs. 240 ± 51) and EDL (291 ± 48 vs 283 ± 131). The Kd values for each binding isotherm (Kd1 vs. Kd2) differed greatly for both RG (45 ± 22 vs. 1143 ± 941) and EDL (50 ± 18 vs. 3734 ± 5195).

Table 2.4. Ouabain binding characteristics in different skeletal muscles

	SOL	RG	EDL	WG
Bmax	261 ± 17	371 ± 16*	368 ± 35*	244 ± 16†‡
Kd [nM]	61 ± 5	84 ± 7	76 ± 16	60 ± 8†
r^2	.938	.958	.955	.918

Values are mean ± SE; n=6 for each muscle. Bmax: maximum binding (pmol/g). Kd: dissociation constant [nM], r^2, the regression of the prediction equation used to depict the saturation binding plot. These Bmax, Kd, and r2 values were determined on binding isotherms containing 12.5-50 nM [^{3}H]ouabain to a total concentration of 12.5-1000nM ouabain. * *Different from SOL (P<0.05);* † *Different from RG (P<0.05);* ‡ *Different from EDL (P<0.05).*

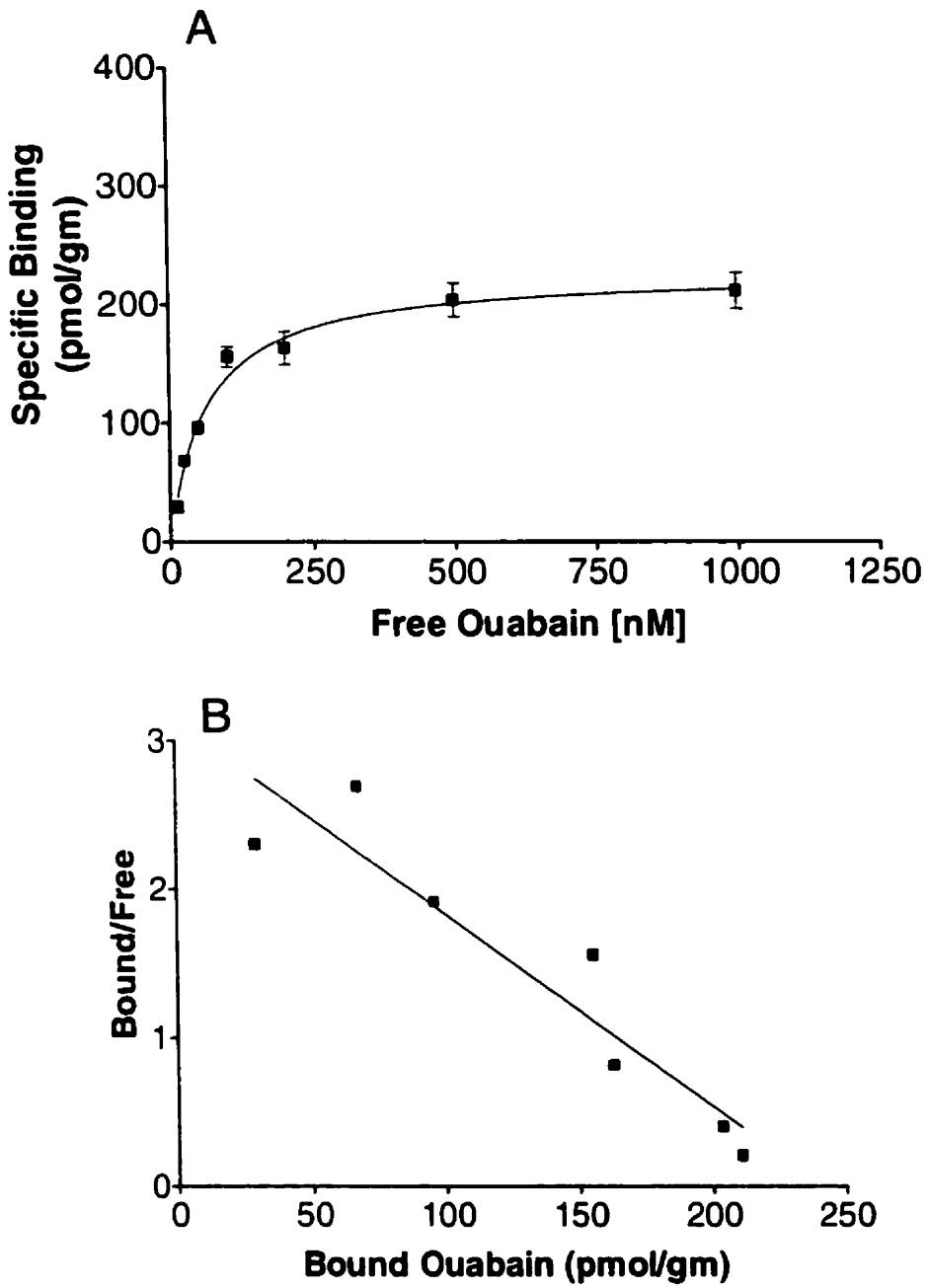

Figure 2.1. *Binding isotherm (A) and Scatchard Plot (B) for SOL muscle over the binding range of 12.5 to 1000 nM. The saturation curve clearly represent a plateau in maximum binding and the Scatchard plot demonstrates a linearity, both indicating a single binding site with affinity at low ouabain concentration.*

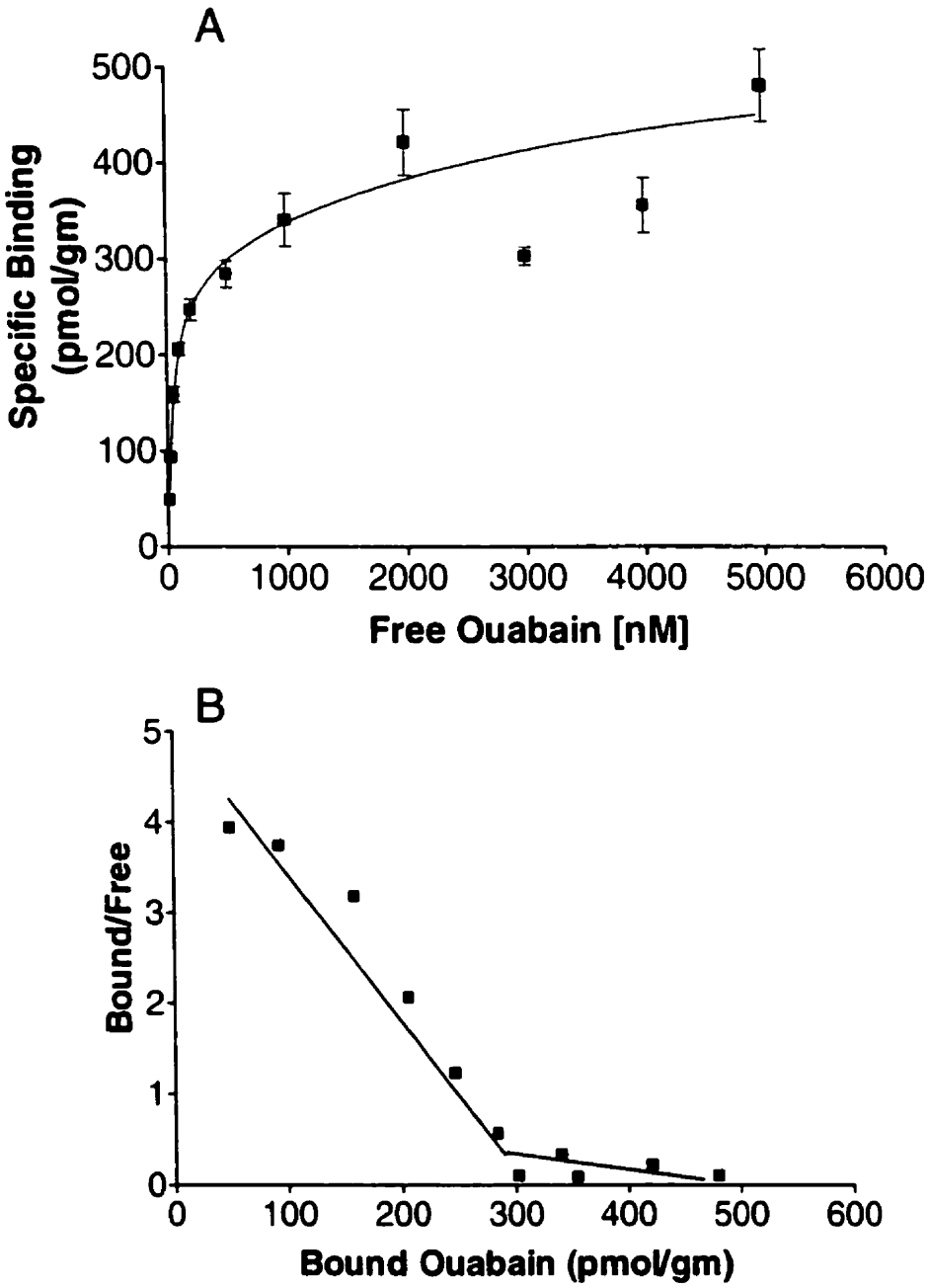

Figure 2.2. *Binding isotherm (A) and Scatchard Plot (B) for EDL muscle over the binding range of 12.5 to 5000 nM. The saturation curve plot clearly represent a biphasic binding curve and the Scatchard Plot shows non-linearity, indicating a two-component ouabain binding isotherm, one at low (~50 nM), and one at high (~3000 nM) ouabain concentration.*

Part 4: Isoform distribution quantified by Western blot

HOM. The relative distributions of isoforms for HOM, as determined using isoform-specific antibodies against α1, α2, β1, and β2 are presented in Figure 2.3. Representative blots for each antibody in HOM, are presented in Figure 2.4. In HOM, α1 was ~1.5 fold greater in SOL than RG ($P<0.05$), which was 1.5-2 fold greater than WG and EDL ($P<0.05$). Distribution of α2 showed a different pattern, with RG and EDL ~1.4 fold greater than WG ($P<0.05$), but not different than SOL. The β1 distribution was 1.5-2 fold greater ($P<0.05$) in SOL than in RG, EDL, WG. The β2 distribution was opposite to that of the β1, being 2-3 fold greater in RG, EDL, and WG, than SOL ($P<0.05$).

CM. Isoform distributions in CM are presented in Figure 2.5, and representative blots are presented in Figure 2.6. SOL content of α1 was ~2 fold greater than RG ($P<0.05$), and RG was 1.5 fold greater than WG and EDL ($P<0.05$). The distribution for α2 was ~1.3 fold greater in SOL and RG than WG and EDL ($P<0.05$). Distributions for β subunits exhibited dramatic differences between muscles. The β1 was ~1.5 fold higher in SOL than RG ($P<0.05$). RG was 2 fold higher than EDL ($P<0.05$) and WG was barely detectable. β2 was ~1.5 fold higher in WG than EDL ($P<0.05$), and EDL was ~2 fold higher than RG ($P<0.05$). In the CM, β2 presence was barely detectable in SOL.

Although the α3 is not considered to be abundant in Na^+-K^+-ATPase of skeletal muscle, clear bands were observed using a monoclonal antibody at a higher molecular weight (~135 kDa) than anticipated (~110 Kda) (Figure 2.7). The α3 was detected in HOM, in higher proportion in WG than RG and EDL, which were greater than SOL ($P<0.05$). Western blots on CM also identified the presence of the α3 isoform in greater proportion in WG and EDL than SOL and RG ($P<0.05$).

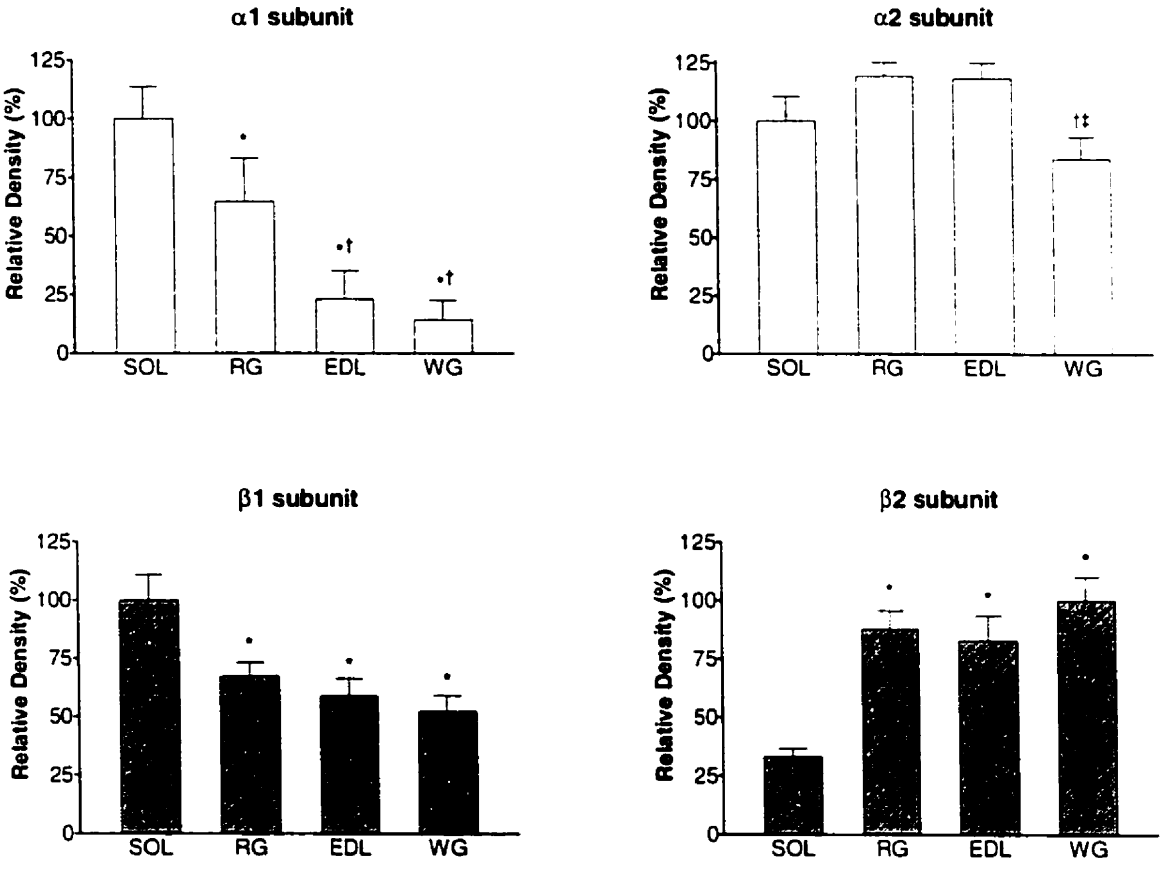

Figure 2.3. *Distribution of isoforms in homogenates (HOM) between soleus (SOL), red gastrocnemius (RG), extensor digitorum longus (EDL) and white gastrocnemius (WG) muscles. Density is calculated relative to a known standard of brain tissue for comparison between blots. The relative density was then adjusted relative to 100% for SOL in α1, α2, and β1, and against 100% for WG in β2 for comparison between muscles. These plots indicate relative differences between muscles for each isoform only, not between isoforms. For calculated molar ratios between isoforms, see App A.3.*

$^{\bullet}$ *Different from SOL (P<0.05);* † *Different from RG (P<0.05);* ‡ *Different from EDL (P<0.05).*

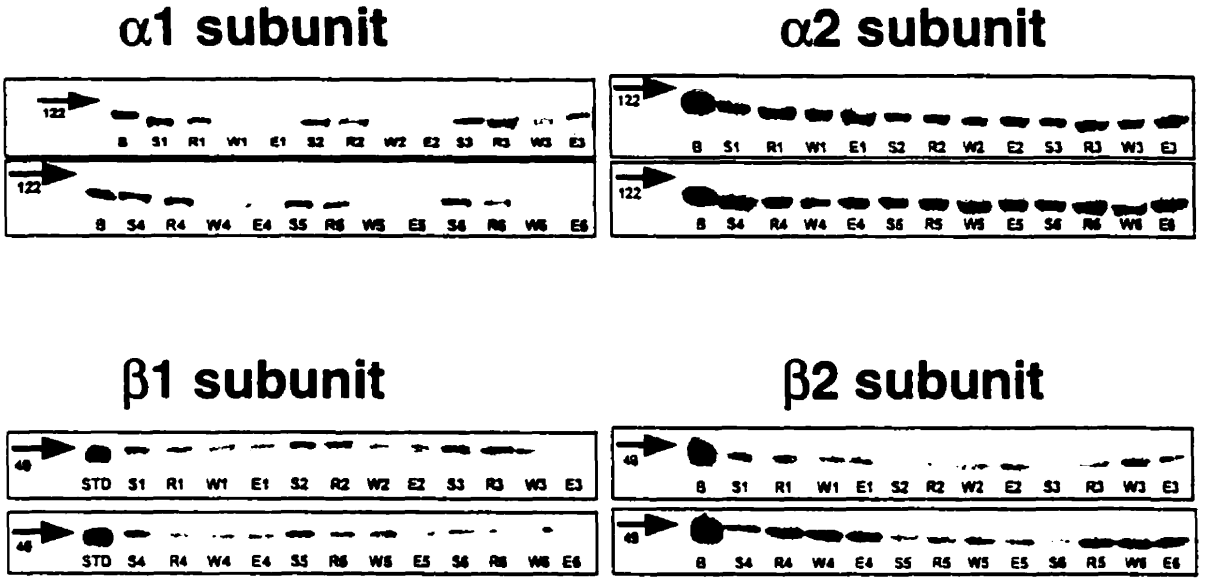

Figure 2.4. *Representative Western blots for homogenate (HOM), between soleus (S), red gastrocnemius (R), extensor digitorum longus (E) and white gastrocnemius (W) muscles. Scanning density is calculated relative to a known standard of brain tissue (B) for each plot. Arrow indicates the location of the molecular weight marker, in kDa.*

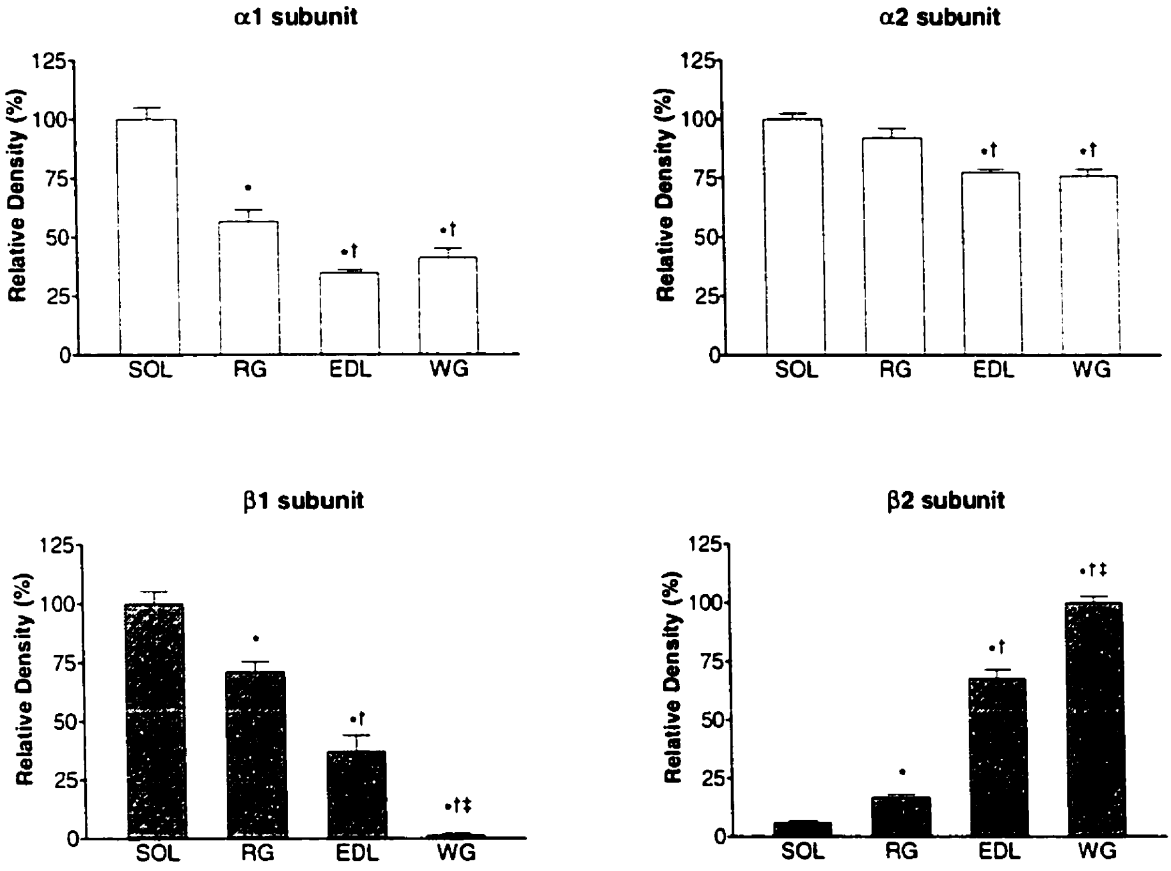

Figure 2.5. *Distribution of isoforms in Crude Membranes (CM,) between soleus (SOL), red gastrocnemius (RG), extensor digitorum longus (EDL) and white gastrocnemius (WG) muscles. Density is calculated relative to a known standard of brain tissue for comparison between blots. The relative density was adjusted relative to 100% for SOL in α1, α2, and β1, and against 100% for WG in β2 for comparison between muscles. These plots indicate relative differences between muscles for each isoform only, not between isoforms. For calculated molar ratios between isoforms, see App A.3.*

• Different from SOL (P<0.05); † Different from RG (P<0.05); ‡ Different from EDL (P<0.05).

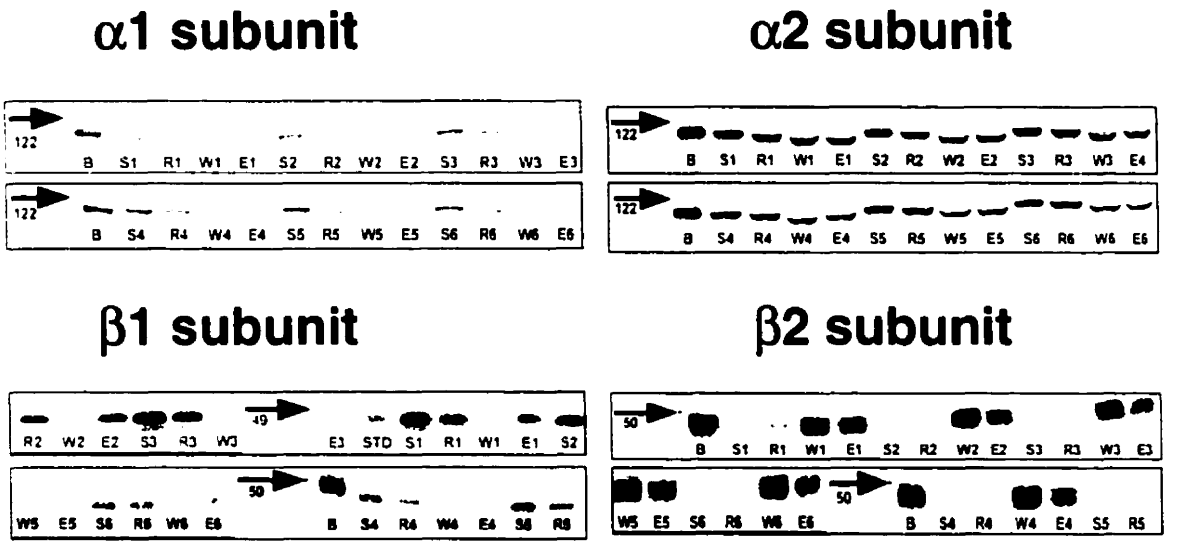

Figure 2.6. *Representative Western blots for crude membranes (CM) between soleus (S), red gastrocnemius (R), extensor digitorum longus (E) and white gastrocnemius (W) muscles. Scanning density is calculated relative to a known standard of brain tissue (B) for each plot. Arrow indicates the location of the molecular weight marker, in kDa.*

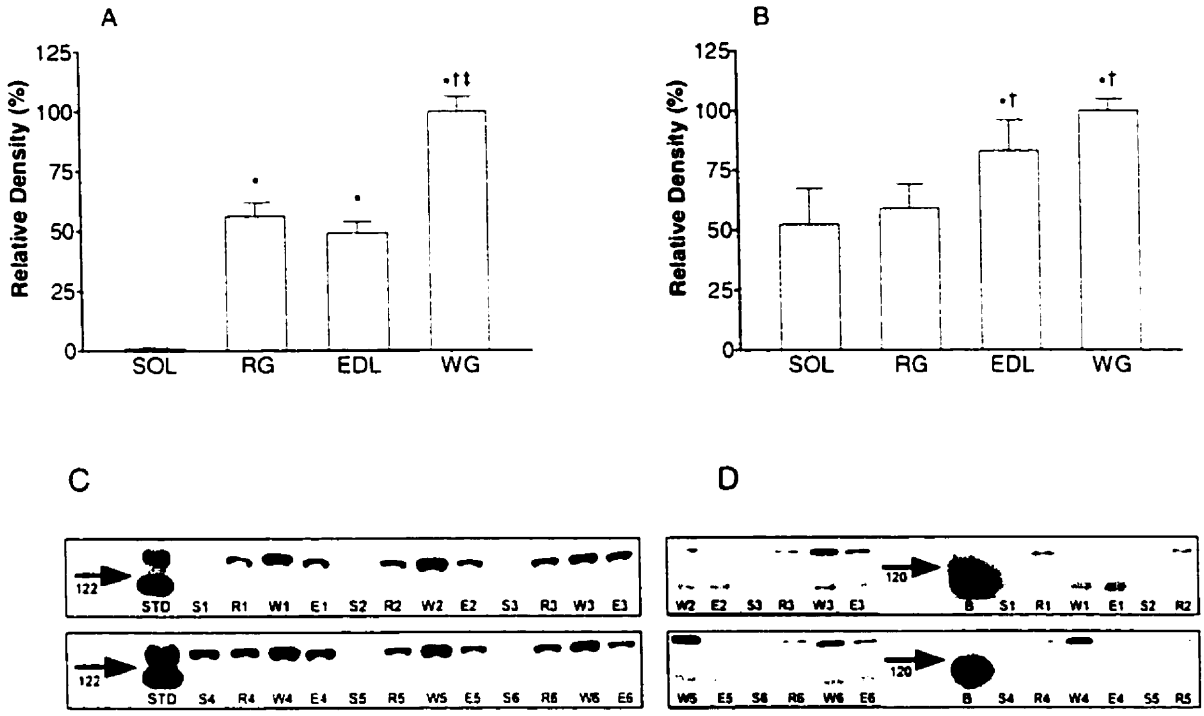

Figure 2.7. *Relative distribution between muscles for the α3 isoform in homogenates (A) and crude membranes (B) in soleus (SOL), red gastrocnemius (RG), extensor digitorum longus (EDL), and white gastrocnemius (WG) muscles. Representative blots for homogenates (C) and crude membranes (D) are also presented for soleus (S), red gastrocnemius (R), extensor digitorum longus (E) and white gastrocnemius (W) muscles. Relative density is calculated against a known standard of brain tissue (B) for each blot and the density for WG was then set at 100% for relative comparison between muscles for all blots. Note that the a3 appears at a higher (~135 kDa) molecular weight than the brain standard (~110 kDa), which is not normally expected for this isoform. When the brain standard is heat denatured as in (C), it also appears at the higher molecular weight, indicating possible unfolding of tertiary structure.* • *Different from SOL (P<0.05);* † *Different from RG (P<0.05);* ‡ *Different from EDL (P<0.05).*

Part 5: Relationships between Na^+-K^+-ATPase activity, content, and isoform distribution

A summary of multiple regression correlations between activity and isoform distribution is included in Table 2.5.

Correlations between activity assays. No correlation was found between ATPase and 3-O-MFPase activity for either HOM or CM ($P>0.05$).

Correlations between content and isoform distribution. The single site ouabain binding on whole muscle tissue pieces (12.5-1000 nM) was predicted by HOM $\alpha 2$ distribution ($r=0.64$, $P<0.005$).

Correlations between activity and isoform distribution. HOM 3-O-MFPase activity was highly predicted by multiple regression (multiple $r^2=0.76$, $P<0.001$), correlating to $\alpha 1$ distribution ($r=0.79$, $P<0.05$), and to $\beta 1$ distribution ($r=0.60$, $P<0.005$). CM ATPase activity was moderately-well predicted (multiple $r^2=0.41$, $P<0.05$) with the $\alpha 1$ most prominent ($r=0.69$, $P<0.05$) from the other predictors $\alpha 2$ ($r=0.53$) and $\beta 1$ ($r=0.42$). CM 3-O-MFPase activity was also predicted (multiple $r^2=0.39$, $P<0.05$), primarily by $\alpha 1$ distribution ($r=0.33$, $P<0.005$), although $\beta 2$ contribution nearly reached significance ($P=0.07$). HOM ATPase was not predicted by multiple regression (multiple $r^2= -0.01$, $P>0.5$), and was not correlated to any single isoform or measure of content.

Table 2.5. Summary of multiple regression Pearson r correlations between Na^+-K^+-ATPase activity and ouabain binding, and Na^+-K^+-ATPase isoform distribution in homogenates and crude membranes of different skeletal muscles.

		α1	α2	α3	β1	β2
Content:						
[^{3}H]ouabain	Bmax	-0.12	0.59*	-0.10	-0.29	-0.09
Activity:						
HOM	ATPase	-0.14	-0.19	0.21	0.03	0.37
	3-O-MFPase	0.79*	0.24	-0.86	0.63	-0.65
CM	ATPase	0.69*	0.53	-0.44	0.43	-0.64
	3-O-MFPase	0.32*	-0.03	0.06	-0.02	0.15

*Values are mean ± SE; Bmax, n=6; HOM, n=10; CM, n=6. HOM, homogenate; CM, crude membrane; Bmax, maximum binding, ATPase, ouabain-inhibitable hydrolytic activity; 3-O-MFPase, K^+-stimulated 3-O-methylfluorescein phosphatase activity. * significant correlation in the multiple regression equation for the particular variable; (P<0.05). Note that Bmax determined on whole muscle tissue, and isoform comparison is that determined in homogenates.*

DISCUSSION

The results of this experiment indicate that a number of factors dictate the activity of the Na^+-K^+-ATPase in skeletal muscle. Different activities are observed between techniques, which highlights that the assay, isolation procedure, and muscle used are important factors when interpreting measures of Na^+-K^+-ATPase activity. Contrary to our original hypothesis that ATPase activity would be predicted by α subunit distribution, and the 3-O-MFPase activity would be predicted by the β subunit distribution, both the HOM 3-O-MFPase and CM ATPase assays were predicted primarily by the α, and secondarily by the β subunit distributions. Given these considerations, SOL exhibited the highest Na^+-K^+-ATPase activity in these two measures based on its greater proportion of $\alpha 1$ and $\beta 1$ subunits relative to the other muscles. This is also contrary to our hypothesis that $\alpha 2$ and $\beta 1$ subunits would confer higher enzyme activity. Although the standard ouabain binding technique correlated highly with the content of $\alpha 2$ subunits in HOM, this relationship did not correlate to any measure of activity, which is also opposite to the classical understanding. The following discussion addresses many of the complex issues associated with measuring Na^+-K^+-ATPase activity, content, and isoform distribution in skeletal muscle. This detailed explanation will help in our understanding of the possible factors that control the expression of the Na^+-K^+-ATPase in skeletal muscle.

Part 1: Na^+-K^+-ATPase activity

When assessing an ATPase enzyme's catalytic capacity, the method that most accurately represents enzyme activity or turnover, is an assay of ATP hydrolysis. Direct assays of ATP hydrolysis, measure either the liberation of inorganic phosphate, or the absorbance change of a light sensitive product such as NADH, coupled directly to enzyme activity. This can be achieved in either a regenerating assay enzymatically linked to

maintain the by-products of reactions in equilibrium, or in an end-point assay where ATP hydrolysis creates a build up of inorganic phosphate. For the Na^+-K^+-ATPase, hydrolytic activity is composed of Na^+-dependent phosphorylation, and K^+-dependent phosphatase steps to complete a pump cycle in the presence of ATP (Skou, 1992). With all of these substrates in the assay medium, in the concentrations required for optimal activation, the direct, regenerating, ATPase assay could be considered as the most appropriate measure of Na^+-K^+-ATPase activity.

The literature rarely contains measures of Na^+-K^+-ATPase activity in crude muscle homogenates. This is because of the low proportion of Na^+-K^+-ATPase activity relative to the activity of other ATPases in the cell, unreliability due to inhomogeneity of homogenate suspensions, and problems in determining ouabain-inhibitable activity between separate runs of a variable assay (Horgan and Kuypers, 1987). Given these difficulties, many researchers use artificial substrates in K^+-stimulated phosphatase assays that are more specific to the Na^+-K^+-ATPase (Horgan and Kuypers, 1987), possibly in combination with an isolation procedure, to improve the measure of Na^+-K^+-ATPase relative to the high background activity in skeletal muscle. Although used extensively, the K^+-stimulated phosphatase assay can be criticized as not approximating the maximum hydrolytic capacity of the enzyme (Horgan and Kuypers, 1987).

In contrast to whole muscle homogenates, an isolation procedure requires greater amounts of tissue than can be supplied by a single muscle, and therefore a mixture of muscles are often used. Isolation procedures can also be criticized as not representing the entire population of pumps in a sample of muscle because of low tissue yields and low recovery of original enzyme activity (Hansen and Clausen, 1988) which may not represent the pump capability of the entire muscle pool (Hansen and Clausen, 1996). For these reasons, the information available on muscle-specific Na^+-K^+-ATPase activity is sparse.

Detailed in Part 2 of this discussion is the explanation of validity, specificity, and reliability of each assay, which must be considered when interpreting Na^+-K^+-ATPase activity data. The major objective of this portion of the experiment was to measure Na^+-K^+-ATPase activity between skeletal muscles of varying fibre type composition because there is limited data on this topic in the literature. Ianuzzo and Dabrowski (1987) studied changes in that Na^+-K^+-ATPase activity with training, and concluded that Na^+-K^+-ATPase activity was related to the oxidative capacity of the muscle. A potential criticism of their conclusion is that they failed to observe significant differences between muscles in the control state. Therefore, a difference in Na^+-K^+-ATPase activity between skeletal muscles of varying fibre type composition has not previously been identified and is consequently, a primary focus of this paper.

ATPase activity in HOM. For the ATPase assay, the determinations of activity in HOM were largely unreliable (Part 2). There was a significant difference between EDL and WG muscle, as might be expected from differences in pump content between these muscles (Part 3). The validity of the technique is questionable though, when 1 (EDL) to 6 (WG) samples out of 10 do not exhibit ouabain inhibitable activity. Na^+-K^+-ATPase subunits are present in the HOM (Part 4), although assessment of their activity is constrained by limitations with the technique. Therefore, the ATPase activities assessed in HOM are not suitable for comparison between muscles.

ATPase activity in CM. As a result of the difficulties in assessing ATPase activity in HOM, the comparison between muscles for ATPase is limited to the data on CM. The SOL CM ATPase activity was ~1.5 fold greater than the glycolytic EDL and WG muscles, respectively ($P<0.05$). RG exhibited activity between SOL and EDL activity, however RG was not significantly different from the three other muscles. This result contrasts that of Ianuzzo and Dabrowski (1987), who reported no significant differences between SOL, RG, WG, and plantaris muscles in their comparative assessment of fibre-specific Na^+-K^+-ATPase

activity in isolated muscle. As part of the methodology for the ATPase assay in the current experiment, four freeze thaw-cycles were used prior to measuring a sample to permeabilize membranes and expose latent enzymes. The freeze-thaw may explain the higher values in this experiment over those obtained by Ianuzzo and Dabrowski (1987), who used detergent only in the first homogenization prior to their isolation. The formation of vesicles during isolation can conceal a substantial portion of the catalytic structure involved in ATP hydrolysis (Hansen and Clausen, 1988), and/or limit access of ouabain to the lumenal portion of the enzyme. It is possible that this may have hampered Ianuzzo and Dabrowski (1987) in their ability to distinguish differences between muscles, and may explain the contradiction with the present experiment. This is the only comparative evaluation reported in the literature using a direct ATPase assay in skeletal muscle, so the present result of a fibre-type specific difference in Na^+-K^+-ATPase activity is a novel finding.

An interesting comparison between muscles of the partial isolation procedure used to obtain the CM, indicated that WG was purified to a greater extent than other muscles in this experiment (Part 2). This effect was evident even with a lower yield of protein per gram of wet weight original tissue. It is important to consider this difference when we compare our results to those of Ianuzzo and Dabrowski (1987), who used a more extensive isolation. The lack of difference that they observed may be due to varying purifications between muscles. The effect may be explained by the surface:volume area difference existing between fibre types (Delp and Duan, 1996), since removal of intracellular protein would be greater for a larger fibre, and therefore, would purify the sarcolemmal proteins to a greater extent. This aspect is also important when we compare other Na^+-K^+-ATPase characteristics for the CM between muscles. Nevertheless, WG still exhibited significantly lower CM ATPase activity compared to SOL, lending support to the hypothesis that differences in enzyme activity exist between skeletal muscles of varying composition.

3-O-MFPase activity in HOM. We found, as have others (Horgan and Kuypers, 1987; Huang and Askari, 1975) that the use of the artificial substrate in a phosphatase assay quantifies only a portion of directly assessed Na^+-K^+-ATPase activity. For both HOM and CM, the 3-O-MFPase assay exhibited less than 1/5 of the activity of the ATPase assay. In our assay, improved conditions were also employed. Previous reports of 3-O-MFPase activity in skeletal muscle homogenates range from 0.018 μmoles·mg protein^{-1}·hr^{-1} in mixed gastrocnemius (Sun *et al.*, 1999), to 0.07 μmoles·mg protein^{-1}·hr^{-1} in mixed hindlimb (Norgaard *et al.*, 1984). These activities were obtained with 3-O-MFP concentrations of 10 and 20 μM respectively, which is only 1/8 of the optimal substrate concentration originally reported by Horgan and Kuypers (1988). Horgan and Kuypers identified the optimal concentration around 160 μM for the assay, but used 20 μM for a number of comparisons in their experiment because of the cumbersome dilutions required for use with their measuring equipment. A number of researchers have subsequently employed the lower concentration in their work.

It is possible that this methodological issue influences identification of tissue specific differences. In this experiment, the differences in HOM 3-O-MFPase activity were highly significant between muscles, assessed at 160 μM 3-O-MFP. SOL was ~1.5 fold greater than RG and EDL, and 3 fold greater than that for WG. This is in contrast to a report that K^+-stimulated phosphatase activity is not different between SOL and WG (Thompson *et al.*, 1999). Thompson *et al.* used the K^+-pNPP assay for their evaluation, which also is limited by the use of a low concentration of the artificial substrate. The activities reported by Thompson *et al.* were 0.034 and 0.033 μmoles·mg protein^{-1}·hr^{-1} for SOL and WG, respectively, which is less than 3% of the theoretical maximum Na^+-K^+-ATPase activity for these muscles. It is again, uncertain if methodology can explain the different outcomes

between experiments. The present results indicate that distinct tissue differences exist from SOL to WG in HOM 3-O-MFPase activity.

3-O-MFPase in CM. In our assay, differences in activity were observed for SOL>RG ($P<0.05$) but surprisingly, RG<WG as well. This is not the same hierarchical relationship observed for HOM 3-O-MFPase and CM ATPase in this experiment, of SOL greater than WG, nor is it consistent with the position of Hundal *et al.* (1993) that K^+-stimulated phosphatase is higher in red than white tissue. The different results obtained in this experiment might be explained however, by the ~2 fold greater recovery of HOM 3-O-MFPase activity in WG (and greater purification) vs SOL muscle. This greater recovery for WG was specific to the 3-O-MFPase assay, which may explain the discrepant observation.

Summary. The Na^+-K^+-ATPase activity results suggest greater activity in soleus, to lesser activity for WG, given the considerations for each technique. Referring to literature values would suggest that Na^+-K^+-ATPase activity is greater in muscles with greater oxidative potential, and is not related to fibres with high speeds of contraction.

Part 2: Validity, reliability, and specificity of Na^+-K^+-ATPase assays and the CM isolation

The information gleaned from the results of this experiment emphasizes the limitations in measuring Na^+-K^+-ATPase in skeletal muscle. We compared Na^+-K^+-ATPase activity measured by ATPase and 3-O-MFPase assays *in vitro*. There were some distinct differences for validity, specificity, and reliability indicated between the two assays, for both HOM and CM.

ATPase assay in HOM. We observed similar problems with the ATPase assay in HOM, as described in the literature. Some samples for the ATPase had undetectable ouabain-inhibitable activity because of a high variability between ouabain-inhibited and no-ouabain assays on the same homogenate sample. This produced a marked variability in

activity between samples of the same muscles. EDL exhibited the lowest group variation at 53%, likely because of its high proportion of pumps quantified by ouabain binding, and because of the significantly greater proportion of ouabain-inhibitable activity relative to background activity (i.e. specificity). WG exhibited the greatest group variation at 186% since 6 out of 10 samples had undetectable ouabain-inhibitable activity. This was expected when one considers the 35% lower concentration of pumps, and 50% lower specificity in WG than EDL. These differences also raise the important consideration of fibre morphology in assessing Na^+-K^+-ATPase characteristics.

WG muscle contains a very high % of type IIB fibres which have ~2 fold greater cross-sectional area than the type I, IIA, and IIX fibres that make up 75% of the composition of EDL muscle (Delp and Duan, 1996), (refer to Appendix 1 for detailed summary of fibre characteristics of muscles in this experiment). For a muscle fibre that is 2 fold larger in muscle cross-sectional area, there is 1/2 the surface area relative to volume. Assuming that the density of muscle is a constant, the volume represents weight. When represented per milligram of protein in an assay, a lower proportion of sarcolemmal protein contributes to the total protein assayed in a sample. This is in light of the fact that maximal Ca^{2+}-ATPase and myosin ATPase activities can be 10-fold higher than that for the Na^+-K^+-ATPase represented per milligram protein (Williams *et al.*, 1998). Given that the Na^+-K^+-ATPase prevails in the surface membrane and the Ca^{2+}-ATPase and myosin ATPase compose the majority of internal ATPases, the difficulty in comparing Na^+-K^+-ATPase activity across muscle fibres of varying composition, and morphology, is understandable.

One of the positive features of the ATPase assay in HOM was that it approximated the theoretical maximum enzyme capacity for SOL, where reliability was sufficient to realize a high validity. This result suggests that the assay can in fact assess the maximum activity of the enzyme *in vitro*, given certain limitations. The theoretical maximum capacity was predicted from the number of pumps quantified by ouabain binding multiplied by the

molecular activity of the Na^+-K^+-ATPase (Clausen, 1986; Hansen and Clausen, 1988). The SOL best predicted its theoretical maximum value at 92%, whereas activity for EDL was higher (151%) and RG and WG were lower (69% and 77%, respectively) than their theoretically predicted values. The activity for SOL of 0.97 μmoles·mg protein^{-1}·hr^{-1} is higher than that reported by Gick *et al.* (1993) for mixed muscle homogenate (0.55 μmoles·mg protein^{-1}·hr^{-1}), and similar to that reported by Thompson *et al.* (2001) for a mixed muscle post-nuclear (i.e. one purification step to amplify signal) homogenate (1.03 μmoles·mg protein^{-1}·hr^{-1}). The variation between the theoretical maximum values and actual values can be explained by the variability and low specificity of the assay as previously described, and by the fact that the predicted value, as calculated by the equation of Clausen (1986), estimates ATP molecular activity at 8000/min. This value is still used by this group (Nielsen and Clausen, 2000). One of the primary objectives of this experiment was to determine if the molecular activity was different between muscles, based on the varying isoforms exhibited in different muscles (Thompson and McDonough, 1996). The Na^+-K^+-ATPase activity results already presented imply that there may be a muscle-specific difference in molecular activity.

3-O-MFPase assay in HOM. The positive feature of using the 3-O-MFPase to measure Na^+-K^+-ATPase activity compared with the ATPase assay, is a markedly higher specificity. This feature allowed reliable determination of activities for all muscles in HOM. The higher activity measured in oxidative SOL than the primarily glycolytic WG is consistent to the relationship demonstrated for CM ATPase activity that may also support the assay's validity.

Specifics of the CM isolation. The partial isolation employed in this experiment was done to amplify Na^+-K^+-ATPase activity, while concurrently attempting to maintain as much of the original homogenate activity as possible. A 'crude membrane' was prepared, using the first few steps of a membrane isolation procedure developed by Dombrowski *et al.* (1996),

that yielded a preparation with ~7.5 fold greater Na^+-K^+-ATPase activity (per milligram protein) than HOM, while recovering ~35% of the original HOM activity. This is significant when we consider that a single purification step, as employed by Thompson *et al.* (2001) does not amplify Na^+-K^+-ATPase activity (as compared to our HOM values), and most full isolations recover only 2-8% (Hansen and Clausen, 1988; Nielsen and Overgaard, 1996) or possibly as high as 15% (Hundal and Aledo, 1996) of the original HOM activity.

ATPase in CM. The partial isolation procedure employed markedly improved specificity and reduced variability, as evidenced by the fact that ouabain-inhibitable ATPase activity was reliably obtained for every CM sample assayed, even WG. The activity values ranging from 5.7-8.12 μmoles·mg protein^{-1}·hr^{-1} exceed values reported by Ianuzzo and Dabrowski (1987) for purified skeletal muscle (2.77 - 3.49 μmoles·mg protein^{-1}·hr^{-1}) and approximate the value reported for a highly purified t-tubular membrane preparation (10.26 μmoles·mg protein^{-1}·hr^{-1}) assessed by Horgan and Kuypers (1987). The values obtained in this experiment are also impressive when compared to the ~18-26 μmoles·mg protein^{-1}·hr^{-1} obtained for isolated rat heart muscle (Dixon *et al.*, 1992; Pierce and Dhalla, 1983), which has ~4x greater Na^+-K^+-ATPase activity than skeletal muscle (Gick *et al.*, 1993). Even with these positive features, the CM ATPase activities only obtain ~33% of maximum theoretical activity. The validity of the assay, although improved over previous reports in the literature, can still be criticised for not representing the entire portion of pumps available and because of the differential recovery of HOM activity observed between muscles.

3-O-MFPase in CM. The specificity for the 3-O-MFPase assay was even greater after purification, and the improved conditions of the assay also produced activities comparable to the maximal activity reported by Horgan and Kuypers (1987) for purified t-tubular membrane. This activity is however, still only ~7%, of the theoretical maximum activity of the enzyme. This calls into question the 'validity' of the CM 3-O-MFPase results, especially given that the purification can selectively yield activity for a particular muscle

such as the WG. As is identified in this experiment, this can be due to a selective purification of certain Na^+-K^+-ATPase subunits (Part 1,4).

Summary. The evaluation of methods to assess Na^+-K^+-ATPase activity between different skeletal muscles indicated that the muscle, the assay, and the purification procedure all interact. The ATPase assay is a more direct measure of catalytic capacity although its use for HOM is constrained by variability. For CM, the ATPase assay is limited by the amount of tissue required for isolation and therefore, the number of animals that can be sampled for a given experiment. The 3-O-MFPase assay is more specific and reliable which allows determinations in HOM, but is affected by its low activity relative to theoretical maximum values, and possibly by muscle-specific preferences in isolation with CM.

Part 3: [^{3}H]Ouabain binding and Na^+-K^+-ATPase content

[^{3}H]Ouabain binding is the most commonly used assay for characterizing Na^+-K^+-ATPase in skeletal muscle. There is extensive literature for changes in maximum ouabain binding (Bmax), indicating a change in the content of Na,K-pumps, under a variety of interventions for the standard maximum binding assay on whole muscle tissue pieces. There is less information available when Bmax is combined with an assessment of binding affinity (Kd).

Bmax. In comparison to published data, the values of SOL=WG<RG=EDL are consistent to the relationship reported by Bundgaard *et al.* (1997) and Thompson *et al.* (2001) for SOL, EDL, and WG. In earlier work from our laboratory, comparison across muscles indicated SOL values were more similar to that of EDL (Chin and Green, 1993). Given that oxidative enzyme activity was similar between these muscles, and muscle fibre composition fundamentally different, Chin and Green (1993) concluded that pump content might be related to muscle oxidative capacity. With the discrepant results to the present experiment it would be difficult to support this conclusion.

An explanation for the discrepancy in results may be provided by the work of Harrison *et al.* (1994) who identified that Na,K-pump content is related to fibre morphology. Harrison *et al.* (1994) examined guinea pig muscles' response to warm and cold. These researchers indicated that smaller fibres had significantly greater membrane area per unit volume, which significantly related to ouabain binding. The authors calculated that in two treatments of cold and warm environments for each muscle, the difference in membrane area accounted for 55, 81, and 85% of the differences in Bmax for latissimus, sartorius, and soleus muscles, respectively. This assertion is consistent with the fact that fibre morphology may explain differences in specific versus non-specific enzyme activity assessed on different muscles (Part 1, 2). Fibre morphology can be used to explain to differences in ouabain-binding between muscles used in this experiment.

Average fibre cross-sectional areas for SOL, RG, EDL and WG muscles are 4054, 3004, 2922, and 3923 μm^2, respectively, as calculated from published values (Delp and Duan, 1996) for male, 16-18 wk old Wistar rats, (see Appendix 1 for detailed breakdown). The corresponding comparison to pump content in this experiment (261, 371, 368, and 244 pmol/g, respectively) exhibits a striking inverse relationship. The rats examined by Delp and Duan (1996) were the same breed, age, and weight (409 g vs 413 g) as the rats examined in this experiment, so these published are suitable for comparison. This is contrasted to the younger (~10 weeks), lighter (259 g), Wistar rats examined by Chin and Green (1993). It is interesting to note that if we refer to another experiment from this laboratory by Smith *et al.* (1989) on muscle morphology of rats during development, an appropriate average cross-sectional area for SOL muscle for a 10 week old, male, Wistar rat is ~3000 μm^2. Using the previous inverse relation, this would correspond to a ouabain binding value of ~370 pmol/g, which is very similar to what Chin and Green report (359 pmol/g).

According to the inverse-relationship paradigm, for two fibres to have equal pump concentrations, a fibre twice the size of another would actually require twice the pumps on

its surface relative to its volume. Since muscle cross-sectional areas were not determined on the same fibres used for ouabain binding in this experiment, no correlation can be done to confirm these associations. However, the results presented do support the theory that the measurement of content of membrane Na,K-pumps, may be influenced by fibre morphology. The associations identified in this experiment warrant further investigation.

Kd. Determination of binding affinity, in combination with Bmax, requires much more extensive measurements than does the standard assay. Previous reports have also failed to indicate changes in Kd, relative to chronic changes in pump content (Bundgaard *et al.*, 1997; Pickar *et al.*, 1997). In these studies, and others (Kjeldsen, 1986; Norgaard *et al.*, 1983), only a single population of binding sites is quantified in the saturation binding isotherm for whole skeletal muscle pieces, even though two distinctly different binding affinities are present for the two α subunits (Blanco and Mercer, 1998). This has led others (Lavoie *et al.*, 1997; Thompson *et al.*, 1999; Thompson *et al.*, 2001) to use the binding affinity technique as a sole quantification of $\alpha 2$ subunit composition in skeletal muscle. Published Kd values for SOL have ranged from 60 nM (Kjeldsen, 1986) to 75 nM (Pickar *et al.*, 1997), which is similar to the 61nM determined for SOL in this experiment. The only significant difference in Kd in this experiment, was observed between RG (84 nM) and WG (60nM). This is contrasted to a report by Bundgaard *et al.* (1997), who reported a Kd of 92 nM for WG. Fibre morphology may play a role in this result as previously described, although the higher Kd may result from a dilution from a second binding site.

A two-site binding model. Using a modification of procedures for the saturation binding assay over a range of ouabain concentrations from 12.5-5000 nM, saturation binding isotherms gave some indication of a 2-site binding model for RG and EDL muscle (r^2 = 0.83, 0.79, respectively). Prediction equations were not accurate in a two-site binding model for SOL and WG, possibly due to the lower total binding capacity in these muscles or the low n in this sample. Figure 2.3 clearly indicates two components on the saturation curve,

and a non-linear scatchard plot for the binding isotherm, in EDL. This has not previously been reported for skeletal muscle. The standard assay by Kjeldsen (1987) employs a long washout period, which may remove bound ouabain from low affinity sites. The present experiment used a shorter washout and multiple determinations in the higher binding range to indicate the possible presence of a two-component model for RG and EDL.

When calculated with a two-component model, the Kd values for the high affinity site actually become evident at a lower concentration, 45 and 50 nM for RG and EDL respectively. The low affinity sites exhibited affinities higher than 1100 nM, and therefore, may artificially 'dilute' a Kd value to a higher value in a single component model. It is uncertain what the experimental impact of these data may be. It is possible that the full capacity Na^+-K^+-ATPase by ouabain-binding may be underestimated with the standard techniques, which would explain why the EDL HOM ATPase activity was higher (but not RG HOM ATPase lower) than expected from the calculation from binding capacity (see Table 2.2.). It is also unclear why a two-component model could not be identified for SOL and WG given that these muscles express both α isoforms. Further research is warranted to explore these questions.

Part 4: Isoform distribution quantified by Western blot

The major objective of this thesis project was to determine muscle specific, and therefore, isoform-specific characteristics of the Na^+-K^+-ATPase. It is clearly evident from the activity and binding data that characteristic differences do occur between muscles. We used Western blot to assess the relative abundance of each of the known Na^+-K^+-ATPase isoforms in skeletal muscle. The relative abundance was calculated in reference to a known standard of brain tissue which expresses all Na^+-K^+-ATPase isoforms and has been used previously for subunit comparisons to skeletal muscle (Lavoie *et al.*, 1997; Thompson and McDonough, 1996). The relative isoform distributions displayed for both HOM (Figures

2.3), and CM (Figure 2.5) clearly demonstrate fibre-specific Na^+-K^+-ATPase isoform complements. Some interesting aspects of the analysis between HOM and CM were identified in this experiment so these data are discussed collectively. Previous studies have not assessed the α3 subunit skeletal muscle so this will also be addressed as a separate topic in this section.

α1 and α2 subunit distribution. Similar to the results of Thompson and McDonough, the distribution of α subunits was such that α1 was greater in oxidative muscles (SOL and RG) than glycolytic muscles (WG and EDL), and the α1 distribution was similar between HOM and CM. Abundance of α2 was greater in EDL and RG than WG, and the distribution was somewhat modified by the isolation. The latter point confirms the previous suggestion that α2 may have greater abundance in intracellular compartments (Lavoie *et al.*, 1997). The α1 subunit is thought to have a 'housekeeping' function (Hundal *et al.*, 1993) and a more stable position in the surface membrane, whereas the α2 subunit is known to translocate to the membrane in response to stress (Hundal *et al.*, 1992; Juel *et al.*, 2000). This is similar to the relationships of GLUT1 and GLUT4 proteins involved in glucose transport at the sarcolemma (Hayashi *et al.*, 1997; Lund *et al.*, 1995; Marette *et al.*, 1992). The isolation of 'crude membranes' in this experiment does not fully capture internal membranes (Dombrowski *et al.*, 1996) and therefore, surface membranes may be retained to a greater extent. This observation may have further relevance when discussed in relation to the Na^+-K^+-ATPase activity measures presented in Part 5.

β1 and β2 subunit distribution. For the 'structural' β subunits, similar distributions were observed for HOM and CM. β1 was greater ($P<0.05$) in oxidative SOL than in glycolytic RG, EDL, and WG. β2 distribution was opposite to that of the β1, being greater in RG, EDL, and WG, than SOL. This is consistent with previous reports (Hundal *et al.*, 1994; Thompson *et al.*, 1999; Thompson and McDonough, 1996). An observation from this experiment was that the CM preparations were 'cleaner' than HOM (i.e. there was less

content of β2 in SOL and β1 in WG, in CM vs. HOM). It is possible that this effect may result from cross-reactivity with the polyclonal antibodies used in this experiment. Additionally, some researchers employ de-glycosylation to increase antibody binding to β subunits (Thompson *et al.*, 2001; Thompson and McDonough, 1996). Our blots were clear and provided adequate determination of relative density between muscles (Figure 2.6), so it is doubtful that this affected the comparative results for this experiment. Nevertheless, the use of polyclonal antibodies is a limitation that can be addressed with use of monoclonal antibodies in future experiments. An evaluation of the results of Thompson and McDonough (1996) might also indicate that the contrast between HOM and CM may be biased by the isolation procedure, as has been identified to affect assessment of other Na^+-K^+-ATPase characteristics.

Thompson and McDonough evaluated isoform distributions in SOL, RG, EDL and WG. Their results for the α1 and α2 isoforms are similar to that obtained in HOM in this experiment. As well, their results for the β isoforms are similar to that obtained for CM in this experiment. Thompson and McDonough used homogenates for their α Western analysis, but employed a single isolation step to purify their samples for analysis of β subunits. Although the β subunits are thought to more evenly distributed between surface and intracellular compartments, they do have intracellular pools (Lavoie *et al.*, 1997), susceptible to mobilization with insulin (Hundal *et al.*, 1992; Marette *et al.*, 1993) and contraction (Juel *et al.*, 2000; Tsakiridis *et al.*, 1996). These intracellular pools may be preferentially discarded during an isolation, and lead to 'cleaner' preparations. This possibly has been documented by Lavoie *et al.* (1997). These researchers identified that each step of their isolation resulted in a loss of β1 subunits from preparation of 'red' skeletal muscle. If this phenomenon is real, and can be compared to the results of this experiment, it implies that there may also be a fibre- and isoform-specific distribution of surface:intracellular pools of Na^+-K^+-ATPase subunits which are sensitive to the isolation procedure. Again, further

clarification of this issue is required, since the isolation procedure employed in this experiment is the first report to the author's knowledge, to compare muscle-specific differences in purification for the Na^+-K^+-ATPase.

Identification of the α3 subunit. Another interesting finding from the Western blot results in this experiment was the presence of the α3 subunit in both HOM and CM, in a muscle specific distribution pattern. Although the α3 subunit is not considered to be abundant in Na^+-K^+-ATPase of skeletal muscle (Blanco and Mercer, 1998), clear bands were observed for this subunit using a monoclonal antibody. This antibody was highly selective and produced a single band for both the brain standard and the muscle samples. The brain standard has been previously quantified by Western blot to have a high content of α3 subunits compared to skeletal muscle (Lavoie *et al.*, 1997). The interesting aspect of the skeletal muscle bands was that these bands were observed at a higher molecular weight (~135 kDa) than anticipated. The normal brain standard produced a band at the expected molecular weight of ~110 kDa in CM (Figure 2.7). When the brain standard was denatured with high heat as shown in the Western blot for HOM in Figure 2.7, a band was observed at both the expected, and at the higher molecular weight observed for skeletal muscle. Banding near the higher molecular weight marker due to denaturation indicates that the protein likely unfolded, and therefore, had slower transport on the gel. What this also indicates is that the α3 isoform in skeletal muscle may have a different tertiary structure from that expected from a monoclonal antibody produced in a cell line. Additionally, what is detected on the gel may not be the α3 at all. Nevertheless, the band identified here as α3 was detected in highest proportion in more glycolytic WG than RG and EDL, greater than SOL ($P<0.05$), in both HOM and CM.

Summary. The Western blot data clearly identifies that muscle specific distributions of Na^+-K^+-ATPase subunits exist. The findings from this study are consistent to the results identified by other researchers. The exception of the novel identification of the α3 subunit in

skeletal muscle invites confirmation. An equally novel observation to this experiment is the relationship of isoform distribution to Na^+-K^+-ATPase activity and content. This topic is addressed as the final component of this discussion.

Part 5: Relationships between Na^+-K^+-ATPase activity, content, and isoform distribution

Relationship between Na^+-K^+-ATPase activity and ouabain binding and ouabain binding to isoform distribution. The data presented in this study clearly indicate differences in Na^+-K^+-ATPase activity, content and isoform distribution between skeletal muscles of varying fibre type composition. Historically, the relationship between these variables has been dictated by the classical assumption that Na^+-K^+-ATPase activity is linearly related to the number of pumps quantified by ouabain binding (Clausen, 1986; Clausen *et al.*, 1998; Hansen and Clausen, 1988). Given the fibre-specific differences in the Na^+-K^+-ATPase characteristics, this assumption may be incorrect.

EDL exhibited high ouabain-binding content in this experiment, and displayed the highest Na^+-K^+-ATPase activity measured by direct ATPase in HOM (i.e. the measure probably most representative of ouabain binding in muscle tissue pieces). This relationship would support the classical assumption. Contrary to the classical assumption is the observation that SOL had lower ouabain binding than EDL but more activity in HOM 3-O-MFPase, another measure believed to quantify pump content (Hansen and Clausen, 1988; Horgan and Kuypers, 1987). Ouabain binding on whole muscle tissue samples (12.5-1000 nM) was strongly predicted by only the $\alpha 2$ distribution in HOM for all muscles ($r=0.64$, $P<0.005$), which confirms that ouabain binding only relates to the quantity of one of the two (and tentatively, three) major catalytic isoforms in rat skeletal muscle. The other catalytic isoforms may also contribute to fibre-specific Na^+-K^+-ATPase activity and this would not be accounted for in the one-component oubain binding assay.

The novel results from the modified binding affinity technique also provides evidence, at least in two of the four muscles, that the classic oubain assay does not accurately quantify the total number of pumps. An inaccurate account of pump content can not accurately predict activity by the classical relation. More work is required to develop both the two-component binding assay and the direct ATPase assay in HOM, to further evaluate this hypothesis. These data suggest that the classic assumption of linearity between ouabain binding and Na^+-K^+-ATPase activity is invalid for rat skeletal muscle, where major differences in ouabain affinity exist between isoforms. Applying this assumption to quantify ion transport capacity (Clausen, 1986; Clausen *et al.*, 1987; Clausen, 1996) and K^+ balance in rat muscle (Nielsen and Clausen, 2000) as it relates to muscle fibre specific fatigue (Clausen *et al.*, 1987; Overgaard *et al.*, 1997) may be inappropriate as a consequence.

Additional evaluation of the Bmax measure indicates that fibre morphology may also play a greater role to pump 'content' than the actual 'concentration' of pumps in a surface membrane. There is a striking inverse relationship between Bmax values and average fibre cross-sectional area (Part 3), which also indicates that quantifying pump activity by ouabain binding is more complex than indicated by a simple linear relationship between content and activity. It is possible that the $\alpha 2$ isoform distribution may confer greater activity to HOM ATPase activity, as was the hypothesis for this experiment, however, the variability in the HOM ATPase data prohibits making any conclusions in support of this hypothesis.

Relationship between Na^+-K^+-ATPase activity and isoform distribution. Probably the most provocative issue is why the activity measured by 3-O-MFPase and ATPase did not correlate by muscle for HOM or CM. This may be because of the variability in the HOM ATPase and the preferential purification in CM 3-O-MFPase as previously described, although an evaluation of the relationships to isoform distribution may also help to answer this question. From the multiple regression equations between isoform distribution and

activity for each of 3-O-MFPase and ATPase and each of HOM and CM, some consistent associations are evident.

The distribution of α1 isoform was the principal predictor for HOM 3-OMFPase ($r=0.79$, $P<0.05$), CM ATPase ($r=0.69$, $P<0.05$), and CM 3-OMFPase ($r=0.325$, $P<0.05$) in this experiment. The distribution of β1 also related to activity in HOM 3-O-MFPase ($r=0.63$) and CM ATPase ($r=0.42$), although α1 assumed most of the predictive value and therefore, the β1 contributions were not significant in the multiple regression. The α2 isoform also failed to significantly predict activity in any assay, although it contributed ($r=0.53$) to the multiple regression for CM ATPase. Similarly, the β2 failed to predict activity in any assay, although its relationship in CM 3-O-MFPase nearly reached significance ($r=0.16$, $P=0.07$). The α3 isoform did not factor in any assay, other than a negative relationship (N.S.) in HOM 3-O-MFPase, supporting the current understanding that the α3 isoform has little catalytic contribution in skeletal muscle (Blanco and Mercer, 1998; Therien and Blostein, 2000; Thompson *et al.*, 1999; Thompson *et al.*, 2001).

Two interesting features arise from the multiple regression results between Na^+-K^+-ATPase and isoform distribution in this experiment. First, was the fact that the α2 isoform, believed to be the 'major catalytic isoform' in skeletal muscle (Hundal *et al.*, 1994), failed to predict activity in any assay. The rat skeletal muscle α2 isoform has greater specific activity than the α1 isoform (Jewell and Lingrel, 1991; Zahler *et al.*, 1997), is present in both surface and internal membranes, and exhibits a translocation response to hormones (Hundal *et al.*, 1992; Lavoie *et al.*, 1996; Marette *et al.*, 1993) and exercise (Juel *et al.*, 2000; Tsakiridis *et al.*, 1996). The α2 also has a more dynamic response to chronic external stress (Azuma *et al.*, 1991; Azuma *et al.*, 1993; Thompson *et al.*, 1999; Thompson *et al.*, 2001; Thompson and McDonough, 1996). The findings from this experiment may mean that the α2 isoform's

influence on Na^+-K^+-ATPase activity measured in resting muscle is minor, and therefore, its role may be restricted to acute and chronic modifications of pump activity.

Additionally, the results may also support the previous assertion that the isolation procedure may preferentially maintain surface membranes, and therefore, over-emphasize the α1 contribution to activity. This might explain results in the CM assays, but not the HOM 3-O-MFPase assay, where α1 was also the significant predictor. Crambert *et al.* (2000) have recently observed that the α1 isoform from human muscle has greater activity than α2. This fact introduces the possibility of species-specific molecular activity differences between isoforms from the rats sampled in this experiment, and those used in the previous transfection experiments.

The second feature from the Western blot results is that the β2 isoform contribution nearly reached significance in the CM 3-O-MFPase assay only. This finding may help to explain the peculiar hierarchy established between muscles for the CM 3-O-MFPase, whereby activity in WG exceeded RG, and was not different from SOL or EDL. It was previously identified that WG muscle exhibited greater purification than the other muscles in the CM 3-O-MFPase assay, likely due to preferential purification of surface membranes. The β2 isoform may be the more structural of the two isoforms, existing primarily in the membrane, since β1 exhibits a translocation response to hormones (Hundal *et al.*, 1992; Marette *et al.*, 1993) and exercise (Juel *et al.*, 2000). Therefore, a higher proportion of β2 could be maintained in the 'crude membrane' than β1 during the isolation. This is also supported by the calculations of relative molar abundance between muscles (see Appendix A.2. for the rationale and calculations for this comparison). The calculations suggests that for the CM 3-O-MFPase assay only, β2 is likely contributing to activity more than β1 because of relative purification differences, not because of a higher specific activity contribution to activity. Given the justification, the β1 isoform likely confers higher kinetic

specificity than the β2 isoform in all situations, and the muscle specific activity hierarchy from oxidative muscle (highest) to glycolytic muscle (lowest) is conserved.

Summary. The relationships between activity and isoform distributions provide insight into which assay is the 'best' representation of kinetic activity at the molecular level. The activity of HOM 3-O-MFPase was the best predicted by multiple regression (multiple r^2=0.76, P<0.001). CM ATPase (multiple r^2=0.41, P<0.05) and CM 3-OMFPase (multiple r^2=0.39, P<0.05) were also predicted, and the low r^2 values are likely a function of the low sample size (n=6), relative to the number of predictor variables (n=5). HOM ATPase was not predicted at all (multiple r^2= -0.01, P>0.5) because of the substantial variation in this measure. Therefore, the 'best' assay representing relative molecular measures, is likely the HOM 3-O-MFPase assay, although, this requires qualification. The 3-O-MFPase assay only approximates 13-26% of theoretical maximum capacity of the enzyme. This is thought to relate to the lower affinity of the enzyme for the artificial substrate (Clausen, 1986), although the low activity may be related to phosphatase activity being the rate-limiting step in ATP hydrolysis. The fact that β1 contributed less than α1 to the multiple regression of HOM 3-O-MFPase activity may refute this possibility.

The paradox in choosing the 'best overall measure of Na^+-K^+-ATPase activity' is that the CM ATPase assay measures hydrolytic activity directly, but does not relate as well (given the small n, in this experiment) to molecular findings. Details of the isolation procedure likely influence the relationships established in this experiment and should remain an important consideration for any measure of Na^+-K^+-ATPase activity on purified membrane (Hansen and Clausen, 1988; Hansen and Clausen, 1996). In summary, it appears that the 3-O-MFPase assay is the best measure of Na^+-K^+-ATPase activity in homogenates, and the ATPase assay is the best measure of Na^+-K^+-ATPase activity in CM, given that the CM retains a substantial portion of the original HOM activity, as was the case in this experiment.

Overall Summary and Conclusions

It is concluded from the results of this experiment that the assay, isolation procedure, and muscle used are important factors when interpreting measures of Na^+-K^+-ATPase activity. The HOM 3-O-MFPase assay was the assay best predicted by molecular assessment of subunit distribution, and second was the CM ATPase assay. The HOM 3-O-MFPase and CM ATPase assays were both predicted primarily by the α and secondarily by the β subunit distributions. The muscle that expressed the highest activity in both of these assays was SOL, which was based on its greater proportion of $\alpha 1$ and $\beta 1$ subunits relative to the other muscles. Although the standard ouabain binding technique correlated highly with the content of $\alpha 2$ subunits in HOM, this relationship did not correlate to a measure of activity. The muscle characteristics of morphology, oxidative capacity and protein complement all interact to designate the quantity of ouabain binding as well as muscle- and isoform specific Na^+-K^+-ATPase activity in skeletal muscle, and therefore, are not adequately explained by a simple linear relation between pump content measured by ouabain binding, and activity. Given the many novel findings in this experiment, future investigation needs to re-examine the basic principles involved in our understanding of the Na^+-K^+-ATPase, and the many factors that contribute to its measurement in skeletal muscle.

CHAPTER III

Reduced activity of muscle Na^+-K^+-ATPase following prolonged running in rats

ABSTRACT

The purpose of this study was to determine if Na^+-K^+-ATPase activity is altered in muscles of different fiber composition following a single session of aerobic exercise in rats. Female Sprague Dawley rats (weight 275 ± 21g; mean ± SE) were run (RUN) on a treadmill at 21m/min and 8% grade until fatigue, or to a maximum of 2h. A second group of rats were kept on the treadmill for an additional 45 min of low-intensity exercise recovery (at 10m/min and 8% grade) (RUN+). Directly following exercise, rats were anaesthetized, and tissue extracted from Soleus (SOL), red vastus lateralis (RV), white vastus lateralis (WV), and extensor digitorum longus (EDL) and frozen for later analysis. 3-O-Methylfluorescein phosphatase activity (3-O-MFPase) was determined as an indicator of Na^+-K^+-ATPase activity and glycogen depletion identified recruitment of each muscle during exercise. 3-O-MFPase was decreased at ($P<0.05$) RUN+ by an average of 12% from CON in all muscles. No difference was found between CON and RUN. Glycogen was lower ($P<0.05$) by 65%, 57%, 44%, and 33% (SOL, EDL, RV, WV, respectively) at RUN, and there was no further depletion during the continued low-intensity exercise period. The results of this study indicate that inactivation of Na^+-K^+-ATPase occurs following prolonged aerobic exercise and that the inactivation that occurs is not specific to muscles of different fibre type composition. The inactivation of the Na^+-K^+-ATPase suggests intrinsic structural modifications by mechanisms that are unclear.

INTRODUCTION

The Na^+-K^+-ATPase, or Na,K-pump, catalyzes coupling of the chemical hydrolysis of ATP to the vectoral transport of Na^+ out of, and K^+ into the cell. In skeletal muscle, basal activity of the Na,K-pump depends primarily on the distribution of Na^+ and K^+ on either side of the plasma membrane and utilizes only ~2-8% of maximum pumping capacity *in vivo* (Clausen, 1986). During contractile work, transport of the Na^+ and K^+ by the Na,K-pump rapidly restores ionic gradients following an excitatory electrical potential or action potential (Nielsen and Clausen, 2000). The capacity of the Na,K-pump can be challenged by heavy contractile demands and by changes in the local environment (i.e. ionic and metabolite build-up, substrate depletion), and therefore, appropriate regulation of Na^+-K^+-ATPase activity is essential for maintaining transport capacity and muscle excitability. Despite a number of reviews published recently on the role of the Na,K-pump in muscle excitability and fatigue (Balog and Fitts, 1996; Clausen, 1996a; Clausen, 1996b; Clausen *et al.*, 1998; Clausen and Nielsen, 1994; Green *et al.*, 2000; Green, 1998; McComas *et al.*, 1994; Nielsen and Clausen, 2000; Nielsen and Harrison, 1998; Nielsen and Overgaard, 1996; Sejersted and Sjøgaard, 2000; Semb and Sejersted, 1996) the factors controlling both the acute and long-term regulation of the pump with exercise are still largely undetermined.

Acute regulation of Na,K-pump can occur by influencing the activity of the pumps and by modulating the number of pumps at the cell surface (Blanco and Mercer, 1998). With contraction, the passive Na^+ influx and K^+ efflux can stimulate up to a 20 fold increase in Na,K-pump activity (Clausen, 1996b; Nielsen and Clausen, 2000). Substrates, cytoskeletal components, catecholamines, and hormones can also provide additional short-term activation (Bertorello and Katz, 1995; Ewart and Klip, 1995; Therien and Blostein, 2000). In addition, an increase in number of pumps at the sarcolemma can occur acutely by translocating a specific pool of Na,K-pump subunits from intracellular sites to the muscle membrane (DeTomaso *et al.*,

1994; Hundal *et al.*, 1992), potentially increasing the number of functional Na^+-K^+-pumps. Collectively, these cellular processes increase the transport capacity of the Na^+-K^+-ATPase during exercise, and therefore, help to maintain ion gradients, excitability, and contractility. Despite the extensive research into mechanisms that may increase pump transport capacity during activity, it is unclear if the intrinsic Na^+-K^+-ATPase activity can be altered by exercise, as has been observed for the other ATPases in the cell. As an example, Williams *et al.* (1998) observed that maximal activity of the actomyosin ATPase was reduced with a single exercise bout. Several investigators have also observed decreased Ca^{2+}-ATPase activity after fatiguing exercise in rats (Belcastro *et al.*, 1993; Byrd, 1992; Williams *et al.*, 1998; Williams and Klug, 1995). Although the exact mechanisms involved in the inactivation of these ATPases remains unclear, structural damage induced by the generation of free radicals is strongly suspected (Kourie, 1998). The evidence from cardiac muscle Na^+-K^+-ATPase indicates that damage by circulating free radicals can occur (Kim and Akera, 1987). Collectively, the results suggest that catabolic processes associated with exercise may alter the structure of the skeletal Na^+-K^+-ATPase, reduce membrane excitability, impair conduction of action potentials and contribute to fatigue. No study presently exists examining the intrinsic activity of the skeletal muscle Na^+-K^+-ATPase in response to exercise.

The goal of this study was to determine if the Na^+-K^+-ATPase activity is altered in muscles of different fiber composition following prolonged endurance running in rats. Our hypothesis was that the Na^+-K^+-pump is intrinsically modified during exercise resulting in a reduced Na^+-K^+-ATPase activity and that the effects of exercise are not specific to fibre type composition of the muscle. This hypothesis was tested in muscle homogenates using a 3-O-methylfluorescein K^+-stimulated phosphatase assay (3-O-MFPase), as an indicator of Na^+-K^+-ATPase activity.

METHODS

Animals. Female Sprague Dawley rats (age 12.1 ± 0.7 weeks; weight 275 ± 21 grams, mean ± SE) were utilized for the study. Rats were housed in a room where the light cycle was controlled (12h/12h) and rat chow and water were provided *ad libitum*. Care and treatment of the animals was in accordance with procedures outline by the Canadian Council on Animal Care. All procedures were approved by the University of Waterloo Office for Ethics in Research.

Experimental Design. To investigate the effect of a single session of aerobic exercise on Na^+-K^+ pump function, rats were randomly assigned to one of three groups (n=10 per group). In one group, rats were run (RUN) on a treadmill at 21m/min and 8% grade (~65% peak aerobic power) until fatigue, or to a maximum of 2h. A second group of rats were run on the treadmill as for the RUN protocol, then were kept on the treadmill for an additional 45 min of low-intensity exercise (i.e. continued fast walking @ 10m/min and 8% grade) (RUN+). By reducing the speed of the treadmill we were able to increase exercise duration. A third group of rats served as control (CON) for the anaesthetic and surgical procedures. This design, including the exercise protocol, is similar to that previously used to investigate Ca^{2+}-ATPase activity following running and active recovery (Ferrington *et al.*, 1996). Directly following exercise (or at rest in CON), rats were anaesthetized, and a muscle sample was immediately obtained from soleus (SOL), extensor digitorum longus (EDL), red vastus lateralis (RV) and white vastus lateralis (WV) and plunged into liquid nitrogen for later analysis of muscle metabolites, glycogen content and Na^+-K^+ pump characteristics. Time for anaesthetization and surgery following exercise averaged ~5min.

Muscle metabolites and glycogen depletion. Muscle glycogen (Glyc) and metabolites including ATP, PCr, creatine (Cr) and lactate were analyzed fluorometrically after extraction from freeze dried tissue according to procedures previously published (Green *et al.*, 1989). In

addition, we have measured the contents of the adenine nucleotides (ATP, ADP, AMP) and inosine monophosphate (IMP) using ion-pair reversed-phase high-performance liquid chromatography (HPLC) (Ingebretson *et al.*, 1982) as modified by our group (Green *et al.*, 1989). All samples were analyzed in duplicate. On a given analytical day, equal numbers of tissue samples from each muscle and group were measured.

Na^+-K^+-ATPase activity. Activity of the Na^+-K^+-ATPase was assessed fluorometrically using the K^+- stimulated 3-O-methylfluorescein phosphatase assay (3-O-MFPase) modified from the procedures of Huang and Askari (1975) and Horgan and Kuypers (1987) but using higher substrate concentration (Fraser and McKenna, 1998; Huang and Askari, 1975). We have confirmed in a separate set of experiments (results not shown) that maximal activity was achieved at ~160 μM substrate concentration in rat tissue. Additionally, the use of 1.25 mM EGTA, 5mM NaN_3, was also employed to optimize enzyme activity in rat muscle samples. Briefly, tissue from frozen muscle samples was homogenized (5% w/v) at 0°C for 2 x 20s @ 25,000 rpm (Polytron), in a buffer containing (in mM) 250 sucrose, 2 EDTA, 1.25 EGTA, 5 NaN_3 and 10 Tris (pH 7.40). Homogenates were freeze-thawed four times, and diluted 1/5 in cold homogenate buffer. Approximately 30 μg of protein (~30 μl homogenate) was incubated for four minutes in medium containing (in mM) 5 $MgCl_2$, 1.25 EDTA, 1.25 EGTA, 5 NaN_3, and 100 Tris (pH 7.40). The K^+-stimulated activity of the Na^+-K^+-ATPase was determined by the increase in activity after the addition of 10 mM KCl, at a substrate concentration of 160 μM 3-O-MFP. The activity of 3-O-MFPase was determined by the difference in slope before and after the addition of KCl. It was confirmed in a separate set of experiments that the change in slope with the addition of KCl is completely eliminated with ouabain. The Na^+-K^+-ATPase activity, which was based on the average of three trials, is expressed in nmol·mg $protein^{-1}$·hr^{-1}. Protein content of the homogenate was determined by the method of Lowry as modified by Schacterle and Pollock (Schacterle and Pollock, 1973).

Data analysis. Statistical analysis was performed using Statistica for Windows V.4.5 software (Statsoft. Inc., Tulsa, OK, 1993). Descriptive statistics included means and standard error (SE). Two-way analysis of variance (ANOVA) with repeated measures was used to analyze difference in Na^+,K-pump activity between the three conditions (CON, RUN, RUN+) and within muscle groups. Identical procedures were employed for analyses of the metabolite data. Paired analysis was used to assess the activity response to exercise between muscles. Post hoc analysis of mean values was performed using the Tukey test. Statistical significance was set at $P<0.05$.

RESULTS

Exercise protocol, metabolites and glycogen. The average running duration at 21m/min (and 8% grade) for the exercise was 102.4 ± 5.2 min for both RUN and RUN+ groups. The RUN+ group exercised for an additional 45 min at 10m/min and 8% grade. The glycogen depletion pattern indicated that each muscle was used during the prolonged, low intensity protocol (Fig. 3.1.). Muscle glycogen was reduced ($P<0.05$) between 33 and 66% at RUN in the muscles examined. No differences were observed between muscles in the RUN group for glycogen content. No further depletion in glycogen content was observed in RUN+.

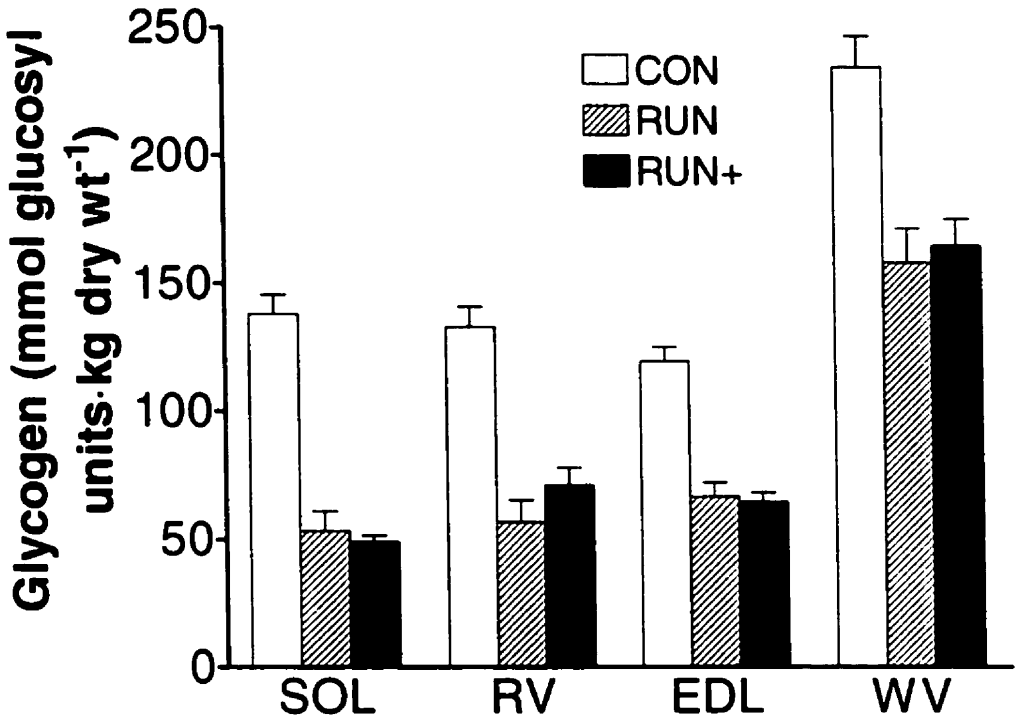

Figure 3.1. *Glycogen content in different locomotor muscles of rats. Values are mean ± SE; n=10 per group except EDL where n=7. CON, non-exercise control; RUN, running at 21m/min @ 8% grade for 2 hrs; RUN+, RUN protocol + 45 min of fast walking at 10m/min; SOL, soleus; EDL, extensor digitorum longus; RV, red vastus; WV, white vastus. There was a main effect for group whereby RUN and RUN+ were different from CON (P<0.05). Glycogen depletion ranged between 66-33% from SOL to WV muscle although muscle specific differences were not significant.*

Exercise had little effect in altering the adenine nucleotides (Table 3.1.). The only difference observed was an increased ADP content at RUN relative to CON ($P<0.05$) in SOL. Exercise also had minimal effects on high energy phosphates and metabolites namely ATP, PCr, Cr and lactate (Table 3.2.). Increases in Cr at RUN+ relative to CON ($P<0.05$), were observed but only for EDL ($P<0.05$). Main effects were observed for adenine nucleotides and for the high energy phosphates and metabolites between muscles, typical to what we have previously published (Dossett-Mercer *et al.*, 1994).

Table 3.1. Effects of exercise on adenine nucleotide and IMP concentrations in the different muscles.

		SOL	RV	EDL	WV
ATP	CON	21.0 ± 0.7	26.2 ± 1.0	30.0 ± 0.3	29.5 ± 0.8
	RUN	22.7 ± 0.8	28.2 ± 0.6	29.4 ± 0.9	30.7 ± 0.4
	RUN+	22.0 ± 0.7	26.8 ± 1.1	31.3 ± 0.5	30.9 ± 0.5
ADP	CON	4.18 ± 0.13	3.77 ± 0.11	4.57 ± 0.14	4.04 ± 0.08
	RUN	4.80 ± 0.20*	3.75 ± 0.09	4.69 ± 0.08	4.19 ± 0.07
	RUN+	4.15 ± 0.20	3.90 ± 0.20	4.86 ± 0.05	4.31 ± 0.05
AMP	CON	0.32 ± 0.03	0.21 ± 0.02	0.15 ± 0.02	0.13 ± 0.02
	RUN	0.36 ± 0.03	0.20 ± 0.01	0.15 ± 0.01	0.15 ± 0.03
	RUN+	n.d.	0.19 ± 0.04	0.17 ± 0.02	0.15 ± 0.02
TAN	CON	25.6 ± 0.8	30.2 ± 1.0	34.8 ± 0.3	33.7 ± 0.8
	RUN	27.8 ± 1.0	32.1 ± 0.7	34.2 ± 0.9	35.0 ± 0.5
	RUN+	25.1 ± 0.8	30.9 ± 1.1	36.3 ± 0.5	35.3 ± 0.5
IMP	CON	n.d.	0.24 ± 0.03	0.27 ± 0.04	0.12 ± 0.03
	RUN	n.d.	0.30 ± 0.04	0.32 ± 0.03	0.12 ± 0.04
	RUN+	n.d	0.29 ± 0.04	0.27 ± 0.03	0.09 ± 0.03

*Values are means ± SE in mmol kg dry weight^{-1}; N=10 all muscles, except EDL (n=7). SOL, soleus; EDL, extensor digitorum longus; RV, red vastus lateralis; WV, white vastus lateralis; ADP, adenosine diphosphate; AMP, adenosine monophosphate; TAN, total adenine nucleotides; IMP, inosine monophosphate; CON, control; RUN, treadmill run; RUN+, run + 45min fast walk. n.d. designates that the particular measure for the specified muscles was not detectable. *Significantly different (P<0.05) vs CON.*
Main effects (P<0.05) for muscle type were found. For ATP, SOL<RV<EDL=WG. For ADP, RV<WV=SOL<EDL. For AMP, EDL=WV<RV<SOL. For TAN, SOL<RV<EDL=WV.

Table 3.2. Effects of exercise on high energy phosphates and metabolites in the different muscles.

		SOL	RV	EDL	WV
PCr	CON	45.7 ± 2.3	58.8 ± 4.7	85.4 ± 5.9	74.7 ± 4.5
	RUN	41.2 ± 1.8	69.9 ± 3.0	82.8 ± 6.8	79.3 ± 5.6
	RUN+	44.0 ± 1.6	57.9 ± 5.9	75.0 ± 7.9	77.1 ± 4.2
Cr	CON	44.3 ± 2.8	78.9 ± 3.8	51.5 ± 4.4	72.7 ± 5.6
	RUN	57.5 ± 2.3	71.2 ± 3.3	61.5 ± 7.2	75.4 ± 3.2
	RUN+	60.1 ± 3.0	75.5 ± 4.7	76.0 ± 8.4*	74.2 ± 6.1
Lactate	CON	8.2 ± 0.9	12.6 ± 1.8	12.9 ± 2.0	16.9 ± 2.5
	RUN	9.1 ± 0.9	7.4 ± 2.6	11.5 ± 2.2	15.1 ± 2.2
	RUN+	5.7 ± 0.8	7.8 ± 1.6	14.9 ± 4.1	15.6 ± 4.0

Values are means ± SE in mmol kg · dry weight^{-1}; N=10 all muscles, except EDL (n=7).
SOL, soleus; EDL, extensor digitorum longus; RV, red vastus lateralis; WV, white vastus lateralis; PCr, creatine phosphate; Cr, creatine; CON, control; RUN, treadmill run; RUN+, run + 45 min fast walk.
Main effects (P<0.05) for muscle type were found.
For PCr, SOL<RV<EDL=WV. For Cr, SOL<EDL<RV=WV. For Lac, SOL=RV<EDL=WV.
**Significantly different (P<0.05) vs CON.*

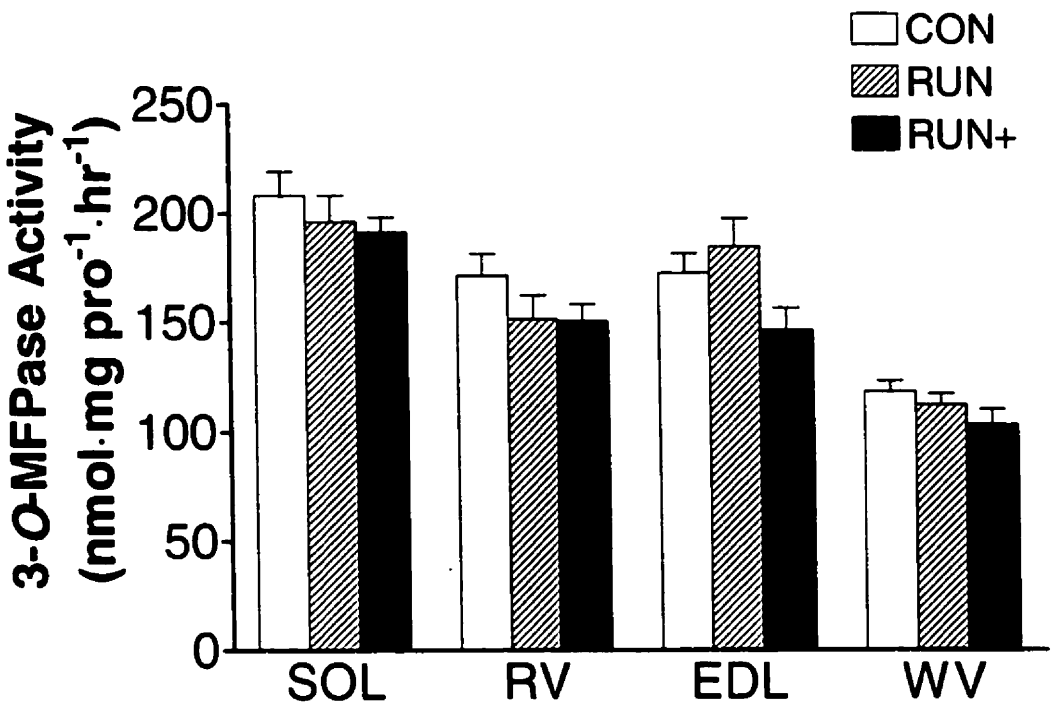

Figure 3.2. *Na^+-K^+-ATPase activity, measured by 3-O-methylfluorescein phosphatase activity (3-O-MFPase) in different locomotor muscles of rats. Values are mean ± SE; n=10 per group except EDL where n=7. CON, non-exercise control; RUN, running at 21m/min @ 8% grade for 2 hrs; RUN+, RUN protocol + 45 min of fast walking at 10m/min; SOL, soleus; RV, red vastus; EDL, extensor digitorum longus; WV, white vastus. There was a main effect for group whereby RUN+ was different from CON (P<0.05).*

Na^+-K^+-ATPase activity. Na^+-K^+-ATPase activity, measured by 3-O-MFPase, decreased ($P<0.05$) by approximately 12% from CON to RUN+ when averaged over all muscles (168 ± 7 vs 149 ± 7) nmol·mg protein^{-1}·h^{-1}. The decrease was not specific to muscle. No differences were observed between CON and RUN and between RUN and RUN+ for any muscle. No differences were found in non-specific, or background, activity between CON, RUN and RUN+ for any muscle.

DISCUSSION

As hypothesized, we have found that the exercise protocol that we employed induced a reduction in Na^+-K^+-ATPase activity when measured in muscle homogenates *in vitro*.

Although there is a strong indication for Na^+-K^+-ATPase activity to decrease in all muscles except the EDL at RUN ($p=0.12$), an additional 45 min at reduced exercise intensity was needed to obtain a significant reduction. Our results for RUN are similar to what has been found for the Ca^{2+}-ATPase activity in response to a similar bout of exercise (Byrd *et al.*, 1989). However, unlike Ca^{2+}-activity, where an overshoot was observed to occur during the additional exercise (Ferrington *et al.*, 1996), our results for the Na^+-K^+-ATPase activity indicate that further exercise depresses activity. The effect of the additional exercise on Na^+-K^+-ATPase activity did not depend on the muscle examined.

The basis of our hypothesis, namely that a reduction in Na^+-K^+-ATPase activity would occur with prolonged exercise, was derived from previous reports of declines in SR Ca^{2+}-ATPase that occurred in response to similar types of exercise (Belcastro *et al.*, 1993; Byrd *et al.*, 1989; Williams *et al.*, 1998). However, not all studies report a reduction in Ca^{2+}-ATPase activity with exercise (Chin and Green, 1996; Dossett-Mercer *et al.*, 1994; Ferrington *et al.*, 1996). Although the reasons for the discrepancies remain unclear, differences in exercise protocols, muscles examined, assay procedures and species appear important (Green, 1998). Our study suggests that at least some of these factors may be important with Na^+-K^+-ATPase activity given the additional exercise that was needed to induce changes.

The inactivation in Na^+-K^+-ATPase activity that we have observed with exercise is likely to represent structural modifications to the enzyme since our measurements were performed under optimal conditions *in vitro*. There are a number of cellular mechanisms that may explain

the intrinsic changes to the enzyme, the inactivation process and the specific effect on the Na^+-K^+-ATPase activity. The most notable possibilities for acute inactivation may result from free-radical damage (Kourie, 1998), Ca^{2+}-activated proteolysis (Belcastro *et al.*, 1998) and heat denaturation (Febbraio, 2000), all of which can increase with exercise (Armstrong *et al.*, 1991; Sjodin *et al.*, 1990). Free radical damage has been demonstrated to reduce Na^+-K^+-ATPase activity in cardiac tissue (Kim and Akera, 1987), in a time and concentration-dependent manner, following exposure to reactive compounds (Kato *et al.*, 1998). Evidence from studies on mouse diaphragm also indicate that excessive intracellular Ca^{2+} can inhibit Na,K-pump activity (Sulova *et al.*, 1998). Prolonged exposure to heat stress has been demonstrated to affect a number of metabolic processes in skeletal muscles, including Ca^{2+}-ATPase activity (Febbraio, 2000). Future study is required to determine which of these possible mechanisms may predominate, as well as identifying the site on the enzyme that is altered.

One possible mechanism that was investigated as a source of inactivation in this experiment was that of substrate depletion. The skeletal muscle Na^+-K^+-ATPase appears to preferentially utilize aerobic glycolysis for metabolism (James *et al.*, 1996). Furthermore, James et al. (1999) have reported that activity of the pump is dependent upon glycogen as a substrate, and therefore, glycogen depletion may induce fatigue. It is unlikely that the 33-65% range of glycogen depletion observed had a significant effect on Na^+-K^+-ATPase activity in this experiment. However, recent evidence suggests that Na^+-K^+-ATPase activity is regulated through ankyrin-spectrin links to the cytoskeleton (Therien and Blostein, 2000), so it is possible that even subtle changes in glycogen may change the structural balance necessary for optimal enzymatic function. The analytical procedure for measuring 3-O-MFPase activity involves four freeze-thaw cycles to permeabilize membranes for the optimal activation by K^+, so it is unlikely

that altered membrane effects *in vivo* persist to alter activity beyond the optimal conditions *in vitro*.

This is the first study to identify that an acute reduction in skeletal muscle Na^+-K^+-ATPase can occur with exercise. These effects were observed following a period of additional activity, after the initial treadmill running exercise. This suggests that the volume of activity is a critical factor. One possible explanation for this might be that the mechanisms previously identified, in isolation or in combination, may take time to manifest. During continued exercise, the processing of free radicals to produce hydrogen peroxide for example, may be what reduces the skeletal muscle Na^+-K^+-ATPase in the same manner as observed for the cardiac Na^+-K^+-ATPase (Kato *et al.*, 1998). Similarly, the effects of elevated calcium may have delayed time-course, since the causes of damage with this process can be attributed to the Ca^{2+}-activated mobilization of calpain (Belcastro *et al.*, 1998). Increased free calcium has been presumed to activate a number degradative cascades (Belcastro *et al.*, 1998), and this effect may be related to total amount of work done during an exercise bout (Chin and Allen, 1996). Similarly, heat denaturation due to elevated muscle temperature may also be cumulative.

In this experiment we have also examined whether a muscle-specific response in Na^+-K^+-ATPase activity occurred with exercise. As indicated by a planned comparison, the response of EDL muscle was significantly different from that of the other muscles. EDL muscle exhibits considerably faster fatigue than SOL (Everts *et al.*, 1988). This greater fatigue is attributed, at least in terms of muscle excitability, to a greater number of Na^+-channels relative to Na,K-pumps (Harrison *et al.*, 1997). This increased ion leak relative to ion pump capacity, can result in a rundown of ion gradients, and result in fatigue (Nielsen and Clausen, 2000). This fact may explain why EDL Na^+-K^+-ATPase activity was unaffected at RUN compared to other muscles,

possibly because of early fatigue, and a lack of involvement in the treadmill running. Glycogen content measurements showed that EDL was similarly depleted to other muscles, so EDL must have been recruited at some point, either early or late in RUN. Involvement must also have continued once the lower-intensity phase began, since at EDL had similarly depressed activity to the other muscles at RUN+.

A final issue when interpreting the results of this experiment is that Na^+-K^+-ATPase activity was measured in homogenates using the 3-O-MFPase assay. K^+-dependent hydrolysis of the 3-O-methylfluorescein phosphate chromogenic substrate substitutes for the aspartylphosphate intermediate of the ATPase (Horgan and Kuypers, 1987) to represent the terminal step in ATP hydrolysis (Huang and Askari, 1975). These phosphatase assays relate to actual ATPase activity (Horgan and Kuypers, 1987; Huang and Askari, 1975), but yield results that are less than the activity assessed by direct methods, possible because of reduced affinity for the artificial substrates. Based on the studies cited, it is interesting to note that Kato et al. (1998) observed a reduction in β1 subunit density in combination with reduced Na^+-K^+-ATPase activity from hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCL) exposure, and that administration of DTT (reducing agent) prevented losses only in phosphatase activity (K^+ p-NPPase). According to current theory, the β subunit is responsible for the correct insertion and stability of an active α-β subunit complex in the plasma membrane (Fambrough *et al.*, 1994; Skou, 1992; Therien and Blostein, 2000). This structural role may have physiological significance, evidenced by a strong correlation between mRNA of the β1 unit and activity of the Na,K-pump in a variety of tissues including skeletal muscle (Gick *et al.*, 1993). Although the exact role the β subunit plays in the pump reaction cycle is still uncertain, the β isoform is believed to stabilize the K^+-bound E2 phosphoenzyme intermediate and therefore support

phosphatase activity of the enzyme (Skou, 1992). The presence of three disulfide bonds in the β subunit amino acid structure (Blanco and Mercer, 1998) makes it particularly susceptible to oxidation and damage (Beggah *et al.* 1997; Kourie 1998) and therefore may be responsible for the observations of Kato *et al.* (1998), and the results of the present experiment.

Summary. The present study is the first to directly measure, albeit through an indirect indicator (3-O-methylfluorescein), that skeletal muscle Na^+-K^+-ATPase activity is acutely modified by exercise. Many factors could affect Na^+-K^+-ATPase activity during the prolonged exercise session performed in this experiment, and these mechanisms are supported by plausible explanations from the literature. The observations from this experiment are consistent with previous findings for cardiac muscle Na^+-K^+-ATPase and confirm a preliminary report that activity is reduced following repeated submaximal leg extension exercise to fatigue in skeletal muscle (McKenna *et al.*, 1996). These findings reintroduce the postulation that Na^+-K^+-ATPase may contribute to acute neuromuscular fatigue in skeletal muscle, and highlights the role of the Na^+-K^+-ATPase as a key control site for regulating the electrical signal reaching the contractile apparatus. Confirmation of these findings, and elucidation of the mechanisms for the reduced activity with exercise, are avenues for future investigation.

Perspectives. Evidence from the rat chronic stimulation model suggests that the Na^+-K^+-ATPase may be one of the first proteins modified in an adaptive cascade, restoring muscle excitation and allowing functional alterations to continue in response to repeated exposure to a training stress (Hicks *et al.*, 1997). There are a number of intracellular signals that may facilitate this rapid upregulation, including translocation of subunits and increased mRNA following exercise (Tsakiridis *et al.*, 1996), changes in intracellular water and ionic content (Lang *et al.*, 1998; Therien and Blostein, 2000) and changes in local phosphorylation state and/or redox potential of the cell (Korge and Campbell, 1995). An additional stimulus may be alteration of the enzyme and the need to stimulate turnover and remodeling. It is reasonable to postulate, given the results of this experiment, that the Na,K-pump is acutely altered during exercise, then quickly compensates to facilitate the rapid changes in pump concentration observed with chronic exposure to stress (Green *et al.*, 2000; Nielsen and Clausen, 2000).

CHAPTER IV

Neuromuscular fatigue is associated with reduced Na^+-K^+-ATPase activity following isometric exercise in humans

ABSTRACT

Previously we have demonstrated that postcontractile force depression (PCD) in humans is associated with an impairment in SR Ca^{2+} pump function (Tupling et al., Am. J. Physiol. 278: R87-R94, 2000). The purpose of this study was to investigate the hypothesis that reductions in Na^+-K^+-ATPase activity are also associated with neuromuscular fatigue following a similar bout of exercise. In control (CON) and exercised (EX) legs, leg extyensor force and EMG from the vastus medialis were measured in 14 volunteers (age, 23.4 ± 0.7 yrs, mean ± SE) prior to (PRE) and immediately following (PST0), 1h post (PST1), and 4h post (PST4) isometric single leg extension exercise at ~60% of maximal voluntary contraction (MVC) for 30 min using a 0.5 duty cycle (5s contraction, 5s rest). Tissue was obtained from vastus lateralis muscle prior to exercise (PRE) in CON, and following exercise in both the CON (PSTO) and EX legs (PST0, PST1, PST4), for the measurement of Na^+-K^+-ATPase activity as determined by the 3-O-methylfluorescein phosphatase assay (3-O-MFPase). Voluntary (MVC) and elicited (10, 20, 50, 100 Hz) muscle force was reduced 30-55% ($P<0.05$) at PST0, and did not recover by PST4. Muscle action potential (M-wave) amplitude and area, and 3-O-MFPase activity at PST0-EX were less than PST0-CON ($P<0.05$) by 37%, 25%, and 38% respectively. M-wave area at PST1-EX was also less than PST1-CON ($P<0.05$). Changes in 3-O-MFPase activity correlated to changes in M-wave area across all timepoints ($r = 0.38$; $P<0.05$, $n=45$). These results demonstrate that Na^+-K^+-ATPase activity is reduced by sustained isometric exercise in humans from that in a matched control leg, and this reduction in Na^+-K^+-ATPase activity may contribute to a loss of excitability as indicated by M-wave alterations. In general, these effects are reversed early in the recovery period.

INTRODUCTION

A number of intracellular sites have been mechanistically linked to depressed force following activity. Accumulating evidence suggests that an impairment in excitation-contraction coupling can be a primary source of force failure with severe fatigue (Stephenson *et al.*, 1998; Westerblad *et al.*, 1998), although other sites may contribute to varying degrees (Green, 1997). More specifically, the role of the sarcolemma and t-tubular membranes in conducting repetitive action potentials has been identified as a possible contributor to fatigue in humans during voluntary exercise (Green, 1998; Sejersted and Sjøgaard, 2000). Failure at the level of the sarcolemma could occur because the reduction in the transmembrane gradients for Na^+ and K^+ during exercise reduces the ionic membrane potential, muscle excitability and force (Nielsen and Clausen, 2000).

Previous work studying the sarcolemma as a site in fatigue has relied primarily on properties of the electromyogram (EMG) and subsequently, the muscle compound action potential (M-wave), as an indirect measure of sarcolemmal excitability. Reduced M-wave amplitude and/or area has been directly related to reduced force in experiments employing sustained muscle stimulation in both animals (Badier *et al.*, 1999; Harrison and Flatman, 1999; Overgaard *et al.*, 1999) and humans (Bigland-Ritchie *et al.*, 1979; Hultman *et al.*, 1983). However, acute decreases in M-wave amplitude and area are not commonly observed during volitional exercise, especially if the fatigue is induced by high intensity, short duration (< 5 min) activity (Behm and St-Pierre, 1997; Bigland-Ritchie *et al.*, 1982). This may be due to M-wave potentiating effects that counteract or supersede the effects of fatigue directly following these types of contraction schedules (McComas *et al.*, 1994).

In contrast, contractions repeated for longer durations appear to induce greater M-

wave depressions than brief, high-force, fast-fatiguing contractions (Behm and St-Pierre, 1997; Fuglevand *et al.*, 1993). In studies by Arnaud et al. (1997) and Jammes et al. (1997), the depression in M-wave amplitude persisted for a minimum of 15 min following bouts of supramaximal cycling and progressive cycling to fatigue, respectively. Persistent reductions in M-wave properties have also been observed by others (McFadden and McComas, 1997) during recovery following sustained contractile activity. Collectively, these finding suggest that reduced excitability may have a more significant role in fatigue induced by sustained exercise than previously believed. It is possible that acute alterations of membrane proteins may be the basis for sustained M-wave changes, and consequently, contribute to the long-lasting fatigue of post-contractile depression (PCD) that is commonly observed after sustained isometric exercise exercise (Skurvydas and Zachovajevas, 1998; Tupling *et al.*, 2000).

The Na^+-K^+-ATPase is an integral membrane protein that maintains ionic gradients at the sarcolemma by pumping $3Na^+$ out and $2K^+$ ions into the cell per ATP cleaved. Its activity is increased in response to repetitive action potentials. This pump activity is also electrogenic in nature and, therefore, contributes to membrane potential and the maintenance of sarcolemmal excitability (Clausen, 1996a). Changes in M-wave result primarily from a loss in muscle excitability, secondary to a loss in Na^+ and K^+ gradients (Cairns *et al.*, 1995; Overgaard *et al.*, 1997). Consequently, the catalytic activity of the Na^+-K^+-pump to maintain ion gradients, is believed to directly influence the magnitude of the M-wave (Hicks and McComas, 1989; Overgaard and Nielsen, 2001). Although the Na^+-K^+-ATPase has a wide dynamic range of activity to maintain excitability *in vivo* (Nielsen and Clausen, 2000), no studies presently exist examining if exercise intrinsically alters the enzymatic activity of the

Na^+-K^+-ATPase in skeletal muscle during exercise.

A recent study by Verburg *et al.* (1999) supports this possibility. These investigators reported that during sustained submaximal exercise, an increased loss of K^+ from muscle was observed late in the exercise, which was attributed to an "insufficient activation of pumps". The "insufficient activation", could be due to a time-dependent inactivation of the Na^+-K^+-ATPase, resulting in a loss of enzymatic activity and an inability to maintain K^+ homeostasis, membrane excitability and consequently force. Evidence from cardiac muscle Na^+-K^+-ATPase indicates that the pump may be susceptible to damage by free radicals (Kato *et al.*, 1998; Kim and Akera, 1987; Xie *et al.*, 1990), which are produced during contractile activity (Sjodin *et al.*, 1990). Recently, our laboratory has demonstrated that moderate intensity isometric contractions for 30 min in humans was associated with prolonged reductions in sarcoplasmic reticulum (SR) Ca^{2+}-ATPase pump function *in vitro* which could have contributed to PCD (Tupling *et al.*, 2000). PCD is a type of fatigue characterized by a depression in force across low and high frequencies of stimulation following exercise (Skurvydas and Zachovajevas, 1998; Tupling *et al.*, 2000). It is possible, particularly with the high level of activation required to sustain this type of isometric exercise schedule, that the Na^+-K^+-ATPase may be similarly affected, and result in reduced enzyme activity measured *in vitro* following exercise.

The purpose of this study was to determine if Na^+-K^+-ATPase activity is affected by sustained, moderate intensity, isometric exercise, and to investigate the interrelationship between Na^+-K^+-ATPase activity, muscle excitability and neuromuscular fatigue. We have hypothesized that Na^+-K^+-ATPase activity and muscle excitability will be reduced during exercise, and will remain depressed during recovery as was observed for the Ca^{2+}-ATPase

using a similar protocol.

METHODS

Subjects. Fourteen healthy active volunteers [7 men, 7 women, age 23.4 ± 0.7 yrs, (0 ± SE)], participated in the study. Nine of these subjects volunteered for the biopsy component of the study. The study carried the approval of the Office of Human Research at the University of Waterloo and all participants were fully informed of all experimental procedures and associated risks prior to obtaining written consent.

Experimental Design. To investigate the relationship between Na^+-K^+ pump function and PCD, subjects completed a moderately intense isometric exercise protocol as previously described (Tupling *et al.*, 2000), with minor modifications. Subjects performed isometric single leg extension at a target force of ~60% of maximum voluntary contraction (MVC) for 30 min using a 0.5 duty cycle (5s contraction, 5s rest) to induce PCD.

Force and EMG were measured prior to (PRE) and immediately following (PST0), 1 hour post (PST1), and 4 hours post (PST4) exercise both in the control (CON) and exercise (EX) legs. Muscle tissue samples were obtained using the muscle biopsy technique under suction, prior to exercise (PRE) in a rested control leg (CON) and directly following exercise (PST0) in both the CON and EX legs, and at one hour post (PST1) and four hours post (PST4) in the EX leg only. The exercise leg was randomized between subjects. Tissue sampling sites were prepared prior to initial mechanical measurements, and a total of two biopsies were extracted from each site at each time-point. The initial biopsy was quickly plunged into liquid N_2 and stored for later analysis of Na^+-K^+ pump activity. The second

sample was used for analysis of Na^+-K^+ pump concentration. During the exercise protocol, tissue was extracted as rapidly as possible directly following (<4s) the last 5s contraction.

The subjects reported to the laboratory 2-4 days prior to the beginning of the experiment. This visit was used as an initial accommodation and testing session for measurement of MVC force and forces at different electrical stimulation frequencies. During the initial visit, the exercise task was also practiced. Prior to exercise on the experimental day, the force-frequency measures were repeated (to account for differences in stimulating electrode placement between days). Approximately 5 min after the initial mechanical measurements, the subjects began the fatigue protocol. An oscilloscope screen was clearly marked to indicate the target force (60% MVC) that each subject was required to achieve during repetitive activity. Periodically during activity, a brief force record was used to verify that the subjects were meeting target force. When both biopsy and mechanical measurements were made at a testing time-point, contractile measurements in the EX limb followed the biopsy (~ 30s), which were then followed by mechanical measurements in CON leg.

Muscle stimulation and force measurements. The experimental setup for measuring muscle contractile characteristics in isometric knee extension has been described previously (Green and Jones, 1989; Tupling *et al.*, 2000). Briefly, for all force measurements, both voluntary and involuntary, the participant sat upright in a straight backed chair with hips and leg firmly secured and the knee at 90° to the thigh and the arms folded across the chest. A 5cm wide plastic cuff, placed around the lower right leg just proximal to the ankle malleoli, was tightly attached to a linear variable differential transducer (LVDT). The LVDT was amplified by a Daytronic carrier preamplifier at 1kHz, converted to a digital signal, and fed

into a 12 bit A/D converter and then into an IBM computer for analysis. Positioning of the LVDT was such that an angle of pull at 180E was achieved for each participant. Calibration was performed prior to each test session with weights of known amounts.

Twitches and tetani were elicited by stimulation of the quadriceps muscle by a Grass Model S48 stimulator with isolation unit. Two aluminum chloride electrodes (8 x 13 cm) coated with warm electrode gel were used to deliver the electrical impulse to the quadriceps muscle. The ground electrode was placed centrally on the anterior aspect of the thigh just above the patella while the active electrode was toward the hip on the proximal portion of the belly of the vastus lateralis. Each electrode was secured firmly with tensor wrapped around the leg and over the top of the electrode to ensure good contact between the skin and the electrode. Twitches (Tw) were evoked using a single supramaximal (~ 150 volts) impulse of 50 μs duration, while tetani at low (10 and 20 Hz) and high (50 and 100 Hz) frequencies, were induced using a voltage that elicited approximately 55% of MVC at 100Hz with a pulse duration of 50 μs and train duration of 1s. The voltage was kept constant throughout the testing for each individual subject. Tetanic force, regardless of frequency of stimulation, was taken as the peak force recorded. Maximum voluntary contractions with twitch interpolation (IT) were obtained as the best of two trials. For each MVC, IT was used to calculate motor unit activation (MUA) by the formula of potentiated twitch-IT/potentiated twitch x 100 (Behm *et al.*, 1996). During a typical trial, subjects were first assessed for supramaximal twitch properties, followed by tetanic stimulations from low to high frequency, followed by the MVC's.

Electromyography. The area required for muscle biopsies and positioning of stimulation electrodes precluded the use of the vastus lateralis for EMG recordings.

Preliminary testing indicated that vastus medialis was stimulated through the surface electrode arrangement employed, and changes in vastus medialis EMG correlated with changes in vastus lateralis EMG at the specific joint angle used in this experiment (unpublished). EMG recordings were made with 10 mm diameter Ag/AgCl (Meditrace 60) surface electrodes. Electrodes were placed over the belly of the vastus medialis (interelectrode distance was ~2 cm), with one ground electrode positioned on the lateral epicondyle of the tibia. The skin was shaved, abraded and cleaned with alcohol. Electrode positioning was not altered during a single testing trial.

The EMG signal (20-500 Hz bandwidth) was passed through an AC amplifier (National Instruments, AT-MIO-16H multi-function board). The gain was calibrated to optimize signal amplitude for A/D conversion and collected at 2028 Hz. Custom modified NIAD software (National Instruments© 1999) was used to acquire EMG and force records and analyze raw data (Labview™ 5.1 software routine). Raw EMG signals from voluntary contractions were full wave rectified, and the resulting signal was integrated over the duration of the contractions. Integrated EMG was divided by time (for a 1s window) to achieve average integrated EMG (AEMG, mV) in either MVC or repetitive contractions. For electrically stimulated contractions, single twitch compound muscle action potential (M-wave) were analyzed for peak-peak amplitude (mV), duration (ms), and area (μVs). Amplitude was defined as the sum of absolute values for maximum and minimum points of the biphasic M-wave. Duration was defined as the time (ms) from positive deflection 2 standard deviations above baseline to return to the time when M-wave returned to baseline after minimum negative deflection. The area was calculated as the integral of the entire area enclosed by the first positive deflection to the return to baseline after completion of the

biphasic M-wave (i.e. as delineated by the parameters of amplitude and duration).

Na^+-K^+-ATPase activity. Activity of the Na^+-K^+-ATPase was assessed flurometrically using the K^+-stimulated 3-O-methylfluorescein phosphatase assay (3-O-MFPase) following the procedures of Fraser and McKenna (Fraser and McKenna, 1998), with minor modifications. K^+-dependent hydrolysis of the 3-O-methylfluorescein phosphate chromogenic substrate substitutes for the aspartylphosphate intermediate of the ATPase (Horgan and Kuypers, 1987) to represent the terminal step in ATP hydrolysis (Huang and Askari, 1975). Briefly, tissue from the frozen muscle biopsy was homogenized (5% w/v) at OEC for 2 x 20s @ 25,000 rpm (Polytron), in a buffer containing 250mM sucrose, 2 mM EDTA, and 10mM Tris (pH 7.40). Homogenates were freeze-thawed four times, and diluted 1:4 in cold homogenate buffer. Approximately 30 μg of protein (~25 μl homogenate) was incubated for 4 min in medium containing 5mM $MgCl_2$, 1.25 mM EDTA, and 100 mM Tris (pH 7.40). The K^+-stimulated activity of the Na^+-K^+-ATPase was determined by the increase in phosphatase activity (i.e. appearance of 3-OMF) after the addition of 10mM KCl, at a substrate concentration of 160 μM 3-O-MFP. The appearance of 3-O-MF was calibrated to known standard of 3-O-MF (40nmoles) prior to analysis each day. 3-O-MFPase activity was determined by the difference in slope before and after the addition of KCl. In a separate set of experiments, it was demonstrated that the difference in slope was completely eliminated by ouabain, which is used to inhibit Na^+-K^+ATPase activity. The Na^+-K^+-ATPase activity, which was based on an average of four trials for each sample, is expressed in nmol·mg $protein^{-1}$·hr^{-1}. Protein content of the homogenate was determined by the method of Lowry as modified by Schacterle and Pollock (Schacterle and Pollock, 1973).

To test the hypothesis that M-wave area is an indicator of membrane excitability

based on the electrogenic contribution of the Na^+-K^+-ATPase to membrane potential, we correlated M-wave area to Na^+-K^+-ATPase activity measured by 3-O-MFPase. M-wave area is generally interpreted as a measure of the magnitude of the total current passage at the recording electrode, which is a function of the size of the individual action potentials and the number of activated fibres (Overgaard and Nielsen, 2001). Only time-points where a matched set of both biopsy and M-wave measurements were available (i.e. PRE-CON, PST0-CON, PST0-EX, PST1-EX, PST4-EX), were used in the correlation of M-wave area with pump activity.

Na^+-K^+-ATPase content. The content of Na^+-K^+ pumps in muscle biopsy samples was determined using the assay developed by Norgaard et al. (Norgaard *et al.*, 1984a), and employed previously in our laboratory (Green *et al.*, 1993). Using this procedure, two samples from each biopsy weighing between 2 and 8 mg are prewashed twice for 10 min periods in a Tris-sucrose buffer (10 mM Tris-HCl, 3 mM MgSO4, 1mM Tris-vanadate and 250 mM sucrose) containing sodium metavanadate ($NaVO_3$) at 0EC. Samples were incubated in the Tris-sucrose buffer with [^{3}H]-ouabain (1.8 μCi/ml) and unlabeled ouabain (1 μM final concentration) for 2 x 60 min at 37°C. After the unbound ouabain was removed by washing four times for 30 min in ice-cold buffer, the samples were blotted, weighed, placed in 1.5 ml Eppendorf tubes, soaked in 1ml 5% trichloroacetic acid for 16 h at room temperature, and then 0.5 ml of sample was counted for ^{3}H radioactivity in a scintillation mixture. [^{3}H]ouabain binding capacity was corrected (x1.05) for loss of specifically bound [^{3}H]ouabain during washout (Norgaard *et al.*, 1984a). No correction was made for non-specific uptake and retention of ^{3}H which was estimated at < 3% (H.Green, unpublished observations). The isotopic purity of the [^{3}H]ouabain was 99% as determined by the

supplier (New England Nuclear-Du Pont Canada).

Data analysis. Statistical analysis was performed on Statistica for Windows R.4.5 software (Statsoft A Inc., Tulsa, OK, 1993). Descriptive statistics included means and standard error (SE). One-way analysis of variance (ANOVA) with repeated measures was used to analyze Na^+-K^+-ATPase properties over time. Correlational analysis was used to relate M-wave area to Na^+-K^+-ATPase activity and twitch force. Two-factor ANOVA's were used to compare differences in force and EMG measures between the CON and EX condition for each time-point. Differences between EX and CON legs were determined by paired comparison. Post hoc analysis of main effects was performed using the Tukey test. Statistical significance was set at $P<0.05$.

RESULTS

Force Measurement. The target force was maintained throughout most of the protocol, although some subjects were unable to maintain a target ~60% of MVC in the last minutes of the exercise session (data not presented). All subjects completed 30 min of exercise despite the neuromuscular fatigue.

Evoked quadriceps force was depressed ($P<0.05$) across all electrical stimulation frequencies at PST0 in EX (Figure 4.1.A). The depression ranged from an average of ~50% at low frequencies (Tw, 10Hz, 20Hz) to ~33% at high frequencies (50Hz, 100Hz). Force remained depressed ($P<0.05$) below PRE across all frequencies by an average of ~25% after 4 hours of recovery. The only change in CON stimulated force was at PST0, where there was an ~10% increase ($P<0.05$) in 20, 50 and 100Hz force. There were no differences from PRE at any other time in the CON condition (Figure 4.1.B).

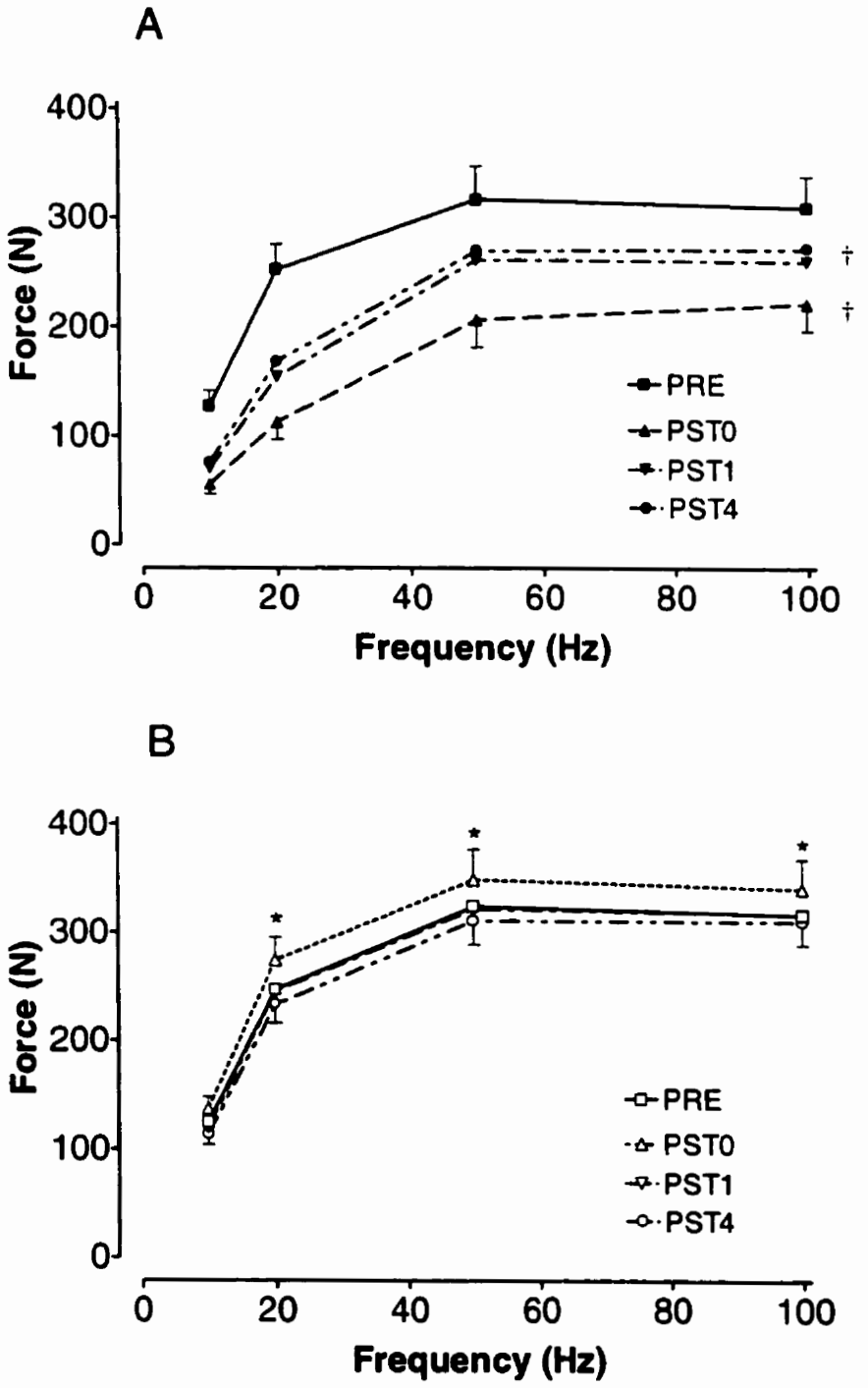

Figure 4.1. *Stimulated force in exercise (A) and control (B) limbs prior to (PRE), immediately following (PST0), and 1h (PST1) and 4h (PST4) following exercise.*
** Significantly different from PRE (P<0.05; n=14). † Symbols beside lines in A indicate reduced force across all frequencies.*

Table 4.1. Force characteristics following sustained voluntary isometric exercise

		PRE	PST0	PST1	PST4
CON	Tw	131.9 ± 11.8	151.2 ± 10.9	132.9 ± 11.3	130.2 ± 11.3
	MVC	501.7 ± 38.1	486.5 ± 39.8	468.9 ± 38.8	447.4 ± 38.4
	% Act	92.0 ± 1.4	90.9 ± 1.4	91.0 ± 2.6	91.6 ± 1.6
	% AEMG	100	94.5 ± 5.7	90.3 ± 4.7	89.1 ± 4.9
EX	Tw	138.5 ± 11.6	80.7 ± 8.7*	94.4 ± 6.5*	113.4 ± 8.4 *
	MVC	552.4 ± 46.9	323.1 ± 36.0 *	395.8 ± 39.1*	407.9 V 40.5 *
	% Act	96.4 ± 1.6	75.9 ± 4.2 *	83.5 ± 3.2*	87.4 ± 3.3*
	% AEMG	100	67.8 ± 3.7 *	79.9 ± 6.6*	89.6 ± 6.5

*Values are means ± SE (n=14). Tw, evoked peak twitch (N); MVC, maximum voluntary contraction (N); %Act; percentage motor-unit activation in an MVC (see text; %), %AEMG, average integrated electromyography in an MVC as a percentage of PRE MVC AEMG. CON, control leg; EX, exercise leg. *indicates significant difference from PRE (P<0.05).*

In the exercised leg, MVC was depressed at PST0 by 42% (P<0.05) and recovered by 16% of PRE (P<0.05) at PST4, although still remaining below PRE values (P<0.05) (Table 4.1). The reduction in MVC was associated with a 21% reduction in motor-unit activation at PST0, and activation remained below PRE by 13% and 9%, at PST1 and PST4, respectively (P<0.05). There was no significant changes in MVC force or motor-unit activation in the CON limb.

Electromyography. The AEMG measured from the vastus medialis at the beginning of the repetitive contractions was 52% of AEMG observed with the PRE MVC. During the exercise, AEMG during repetitive contractions increased by 20% (P<0.05) over the protocol (results not presented). The AEMG during MVC was reduced (P<0.05) 33% and 23% at PST0 and PST1, respectively. There was no change in AEMG at MVC in the control limb.

There was no difference in PRE values between CON and EX for the M-wave parameters investigated, namely amplitude, duration, and area. Both M-wave amplitude and area were altered ($P<0.05$) in response to the exercise (Figure 4.2.). M-wave amplitude at PST0-EX was 37% less than PST0-CON ($P<0.05$). No further differences were observed between EX and CON in recovery. M-wave area in EX was 25% less than CON at both PST0, and PST1 ($P<0.05$) (Figure 4.2.A, 4.2.C). M-wave area and amplitude in EX was not different from CON at PST4. There was no significant change in M-wave duration over time in either leg (Figure 4.2.B).

Na^+-K^+-ATPase function. Na^+-K^+-ATPase activity, measured by 3-O-MFPase, was 81.2 ±12.8 nmol·mg pro^{-1}·hr^{-1} at PRE for CON. At PST0 in EX, 3-O-MFPase was 38% less than PST0 for CON (58.8 ± 9.8 nmol·mg pro^{-1}·hr^{-1} vs 90.6 ± 13.2 nmol·mg pro^{-1}·hr^{-1}; $P<0.05$) due to the exercise (Figure 4.3.A). 3-O-MFPase activity was not different from PRE at any time-point. [^{3}H]Ouabain binding indicated that total number of Na^+-K^+-ATPase pumps quantified by this technique, did not change over time (Figure 4.3.B). Linear regression indicated a low correlation between 3-O-MFPase and M-wave area ($r=0.35$; $P=0.02$; $n=45$) (Figure 4.4.). In addition, 3-O-MFPase exhibited a correlation to Na^+-K^+-ATPase pump content ($r=0.48$; $P<0.001$; $n=42$).

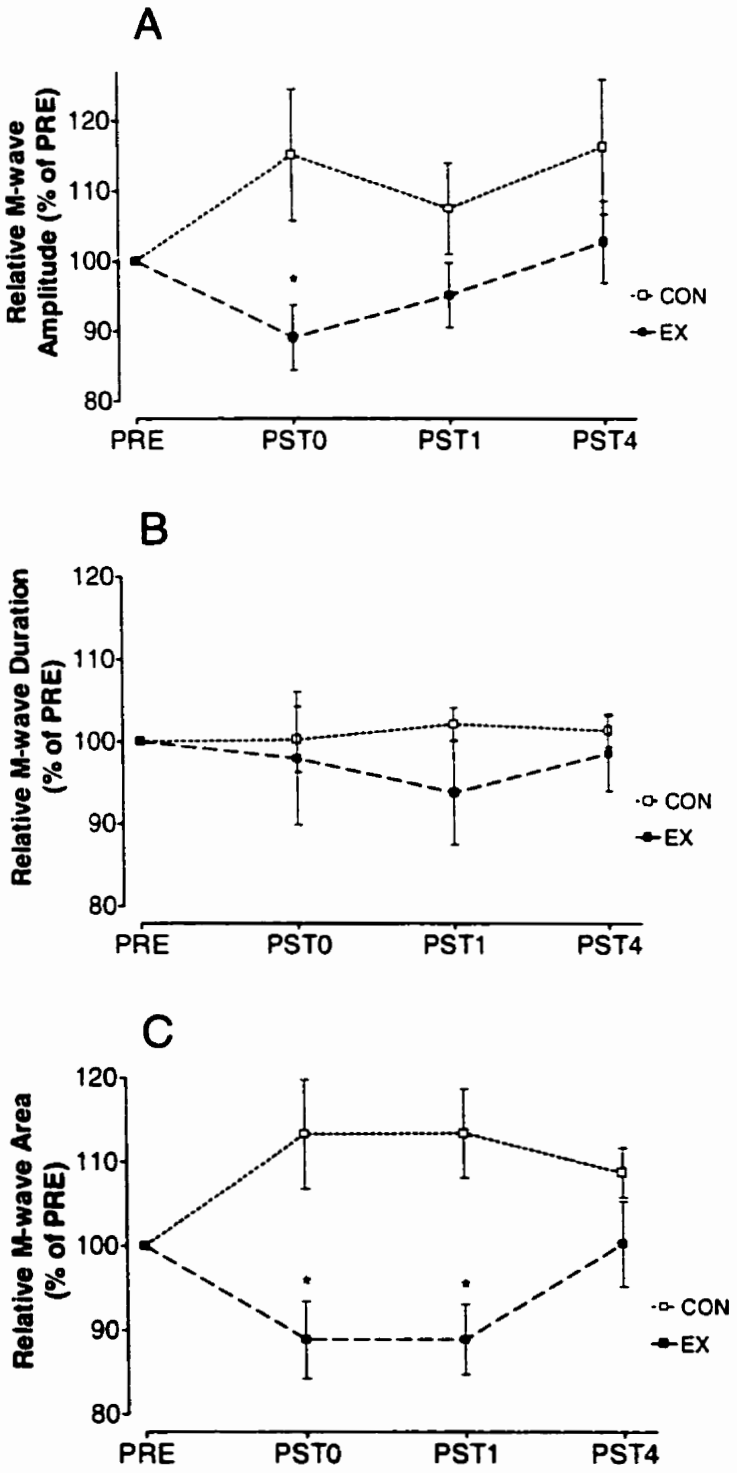

Figure 4.2. *M-wave amplitude (A), duration (B), and area (C) (see definitions in text), represented relative to pre-exercise (PRE) values, in exercise (EX) and control (CON) limbs, prior to (PRE), immediately following (PST0), and 1h (PST1) and 4h (PST4) following exercise. * Significantly different from PRE (P<0.05, n=14).*

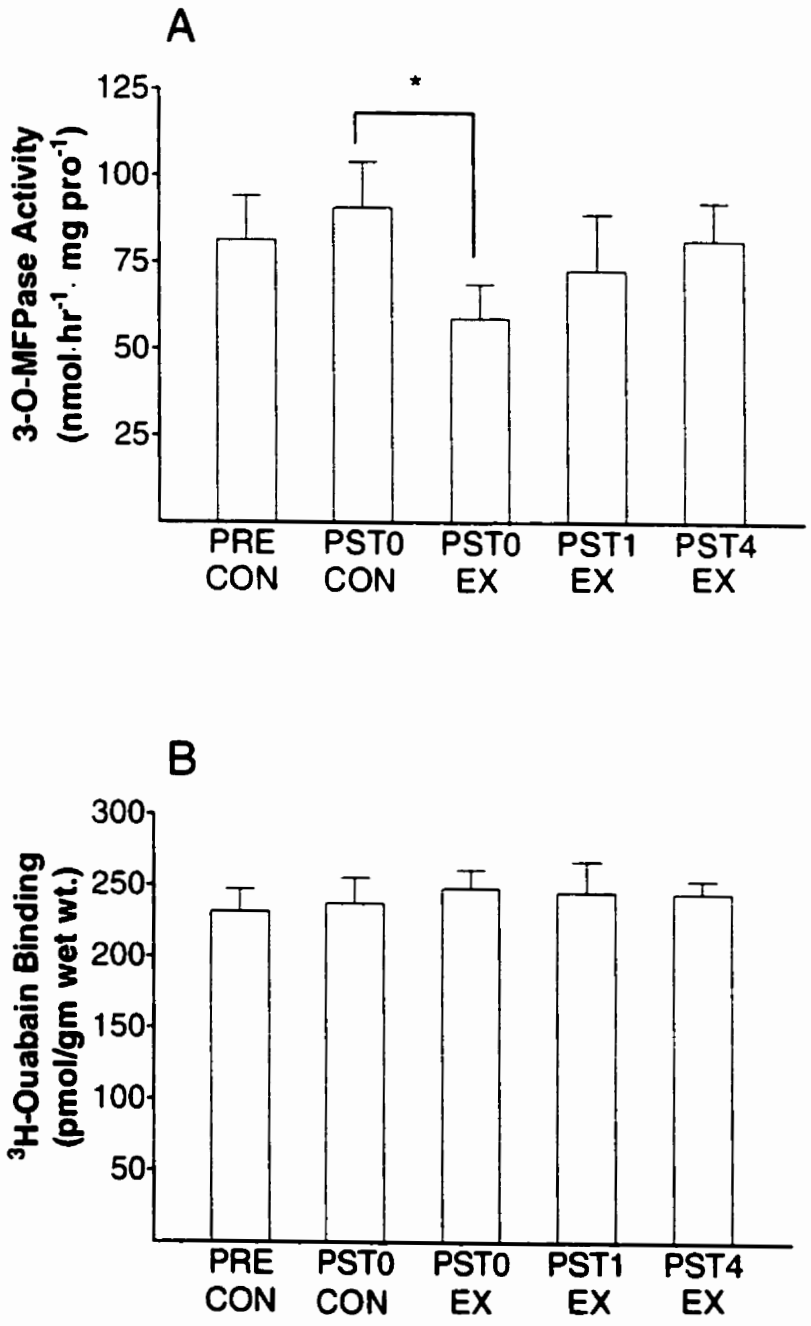

Figure 4.3. *Na^+-K^+-ATPase activity, indicated by 3-O-methylfluorescein phosphatase activity (3-O-MFPase), and Na^+-K^+-ATPase content indicated by [^{3}H]-Ouabain binding in response to exercise. Biopsies were taken before and immediately after exercise in the control (CON) leg (PRE, PST0) and immediately following, 1h, and 4h after exercise in the exercise (EX) leg (PST0, PST1, PST4, respectively). * Significantly different (P<0.05).*

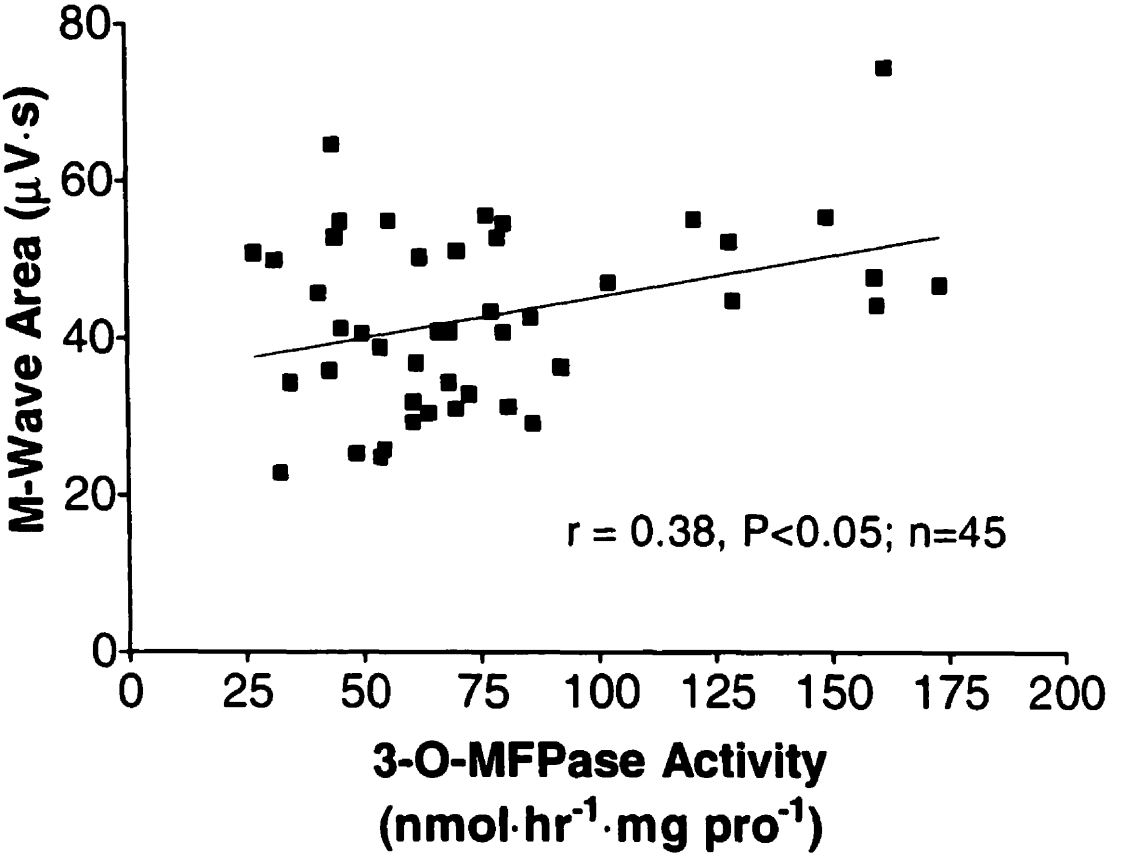

Figure 4.4. *Correlation between Na^+-K^+-ATPase activity, indicated by 3-O-methylfluorescein phosphatase activity (3-O-MFPase), and M-wave area in this experiment.*

DISCUSSION

In this study, we hypothesized that Na^+-K^+-ATPase activity and muscle excitability would be reduced during an isometric exercise protocol previously shown to be associated with a prolonged impairment in SR Ca^{2+} pump function (Tupling *et al.*, 2000). Using the 3-O-MFPase assay, we demonstrated a 35% difference in Na^+-K^+-ATPase activity between the exercised and control limb directly following the contractile protocol. This change was also accompanied by depressions in M-wave amplitude and area. The fact that M-wave and Na^+-K^+-ATPase activity recovered within 4 hours following exercise, demonstrates the rapidly adaptable nature of the Na^+-K^+ pump in skeletal muscle. These results also suggest that, at least for the isometric task studied, compromised Na^+-K^+-ATPase activity and reduced sarcolemmal excitability can contribute to muscle fatigue.

Exercise protocol and fatigue. PCD is a type of fatigue characterized by a depression in force across low and high frequencies of stimulation following exercise (Skurvydas and Zachovajevas, 1998; Tupling *et al.*, 2000). The repetitive isometric exercise protocol employed in this experiment depressed force output across all muscle stimulation frequencies and in maximal voluntary contractions. Although some recovery was evident, fatigue persisted for up to 4 hours following exercise. This protocol was previously observed to cause acute alterations of the Ca^{2+} pump, reducing maximal activity of the Ca^{2+}-ATPase for at least of one hour following exercise (Tupling *et al.*, 2000). The authors suggested that Ca^{2+} cycling could be one of several sites of fatigue with this protocol. Previous investigations on the cause(s) of PCD identified a failure of neuromuscular transmission (Bigland-Ritchie *et al.*, 1979), although it was later demonstrated that muscle fibers retained the ability to generate normal action potentials during PCD (Lannergren and

Westerblad, 1987). The results of this experiment indicate that, given the high level of activation required to sustain the voluntary isometric exercise schedule, impaired excitability of the sarcolemma appears to contribute to neuromuscular fatigue that was observed. However, definitive evidence that impaired sarcolemmal excitability is mechanistically linked to fatigue would depend on demonstrating impaired t-tubule excitability and coupling between the t-tubule and the SR Ca^{2+}-release channel. During recovery, excitability was restored at a point where force was still depressed. This observation supports other findings that indicate other sites of fatigue are responsible for more prolonged reductions in force during the period following exercise [as reviewed by Fitts (1994) and Lannergren et al. (1996).

Inactivation of the Na^+-K^+-ATPase. The capacity of the Na,K-pump to restore ionic gradients can be challenged by heavy contractile demands and resultant changes in the local environment (i.e. ionic and metabolite build-up, substrate depletion), and therefore, appropriate regulation of Na^+-K^+-ATPase activity is essential for maintaining transport capacity and muscle excitability (Nielsen and Clausen, 2000). Complex regulatory controls exist to ensure that Na,K-pump capacity is 'activated' during exercise to maintain excitability. Intrinsic activity of the pump is increased over a 20-fold range by mechanisms associated with the action potential (Everts and Clausen, 1994) and acute hormonal control (Bertorello and Katz, 1995), and pump capacity is increased by translocation of a specific pool of Na,K-pump subunits from intracellular sites to the muscle membrane (Juel *et al.*, 2000). Despite a wealth of information published on the role of the Na,K-pump in muscle excitability and fatigue, the actual enzymatic capacity of the pump in skeletal muscle to meet the demands of exercise remains unknown. Most experiments rely on indirect measures of

excitability and pump function in response to a short-term fatiguing event and only recently has there been evidence, albeit indirect, that Na,K-pump inactivation can occur during sustained exercise in humans (Verburg *et al.*, 1999). Verburg *et al.* (1999) determined that after a period of sustained submaximal exercise, increased loss of K^+ from muscle occurred which persisted in brief recovery. This observation suggests that a reduction in Na^+-K^+-ATPase activity occurred as the exercise progressed. Our results offer evidence that inactivation of the Na^+-K^+-ATPase can occur, at least with sustained isometric exercise.

There are a number of possible mechanisms to explain the intrinsic reduction in pump activity that was observed in this experiment when measured *in vitro* under supposedly optimal conditions. Changes in the metabolic environment were observed previously with this protocol (Tupling *et al.*, 2000). These effects are most likely to exact their greatest impact on Na^+-K^+-ATPase activity *in vivo*. Reductions in Na^+-K^+-ATPase activity measured under 'optimal' conditions *in vitro*, likely reflect structural alterations to the enzyme. The most notable causes for acute inactivation include temperature denaturation (Febbraio, 2000), Ca^{2+}-activated proteolysis (Belcastro *et al.*, 1998), and free-radical damage (Kourie, 1998), all of which can increase with exercise (Armstrong *et al.*, 1991; Sjodin *et al.*, 1990). Studies on mouse diaphragm indicate that excessive intracellular Ca^{2+} can inhibit Na^+-pump activity (Sulova *et al.*, 1998). A number of reports also indicate that free radical damage can reduce Na^+-K^+-ATPase activity in cardiac muscle (Kato *et al.*, 1998; Kim and Akera, 1987; Kramer *et al.*, 1984; Kukreja *et al.*, 1990). Free radical damage could also affect the Na^+-K^+-ATPase, since the beta subunit of the pump has a number of disulphide bonds that are susceptible to oxidation (Kourie, 1998). Interestingly, the SR Ca^{2+}-ATPase activity is also inhibited by repetitive contractions and structural modifications to the

nucleotide binding domain appear to be responsible for the impairment (Dux *et al.*, 1990). Regardless of the mechanisms, the fact that Na^+-K^+-ATPase activity had returned to PRE values within 4 hours of recovery indicates that the alterations that occur as a result of exercise are rapidly reversible.

Electromyography. M-wave amplitude and area assessed in the exercise limb was significantly lower than M-wave in an unperturbed control limb in this experiment immediately following exercise. Although the decrease in M-wave amplitude in the exercise leg of 12-14% was not significant on its own, similar but significant decreases have been shown with a long-duration voluntary fatigue protocol using the ankle plantarflexors and leg extensors (Behm and St-Pierre, 1997) or first dorsal interosseus muscle (Fuglevand *et al.*, 1993). It has been proposed that the rundown of gradients for Na^+ and K^+ during intense exercise reduce the ion potential at the membrane, and thereby reduce M-wave and force (Nielsen and Clausen, 2000). A reduction in ion gradients with exercise can result from Na^+ and K^+ fluxes during successive action potentials exceeding the ability of membrane transport to maintain the gradients. Although complex regulatory controls exist, including the concentration of Na^+ and specific ion channels in the sarcolemma, the Na^+-K^+-ATPase is the principal mechanism for maintaining excitability (Sejersted and Sjøgaard, 2000). The present results indicate that M-wave reduction following exercise may be due at least in part to intrinsic modifications of the Na^+-K^+-ATPase. Impaired pump function would indicate a compromised capability of the enzyme to maintain membrane excitability through its electrochemical contribution to membrane potential, resulting in depolarization, and reduced M-wave.

It should be noted that the reduced M-wave observed in this experiment immediately

following exercise was relative to the M-wave in an unperturbed control limb. The trend towards higher M-wave amplitude and area in CON following exercise was not significant. This effect has been observed previously (Kuiack and McComas, 1992), where non-contracting muscle fibers exhibit hyperpolarization, likely the result of catecholamine spillover associated with exercise of a single limb (Savard *et al.*, 1989). The fact that a trend towards increased M-wave amplitude was observed in the non-exercised limb would suggest that the exercised-induced effects on M-wave amplitude are best appreciated by comparing the exercise limb with the non-exercised limb.

Relationship of M-wave and Na^+-K^+-ATPase. The depression of M-wave following activity and subsequent return to PRE levels within 4 hours can be explained, at least in part, by alteration of structure and subsequent repair, or activation of, Na^+-K^+-ATPase subunits in recovery. This is a rapid time-course, but the Na^+-K^+-ATPase has very dynamic acute and chronic responses to exercise (Green, 2000; Nielsen and Clausen, 2000). These effects, combined with the possible effects on the M-wave and Na^+-K^+-ATPase in the CON limb, likely supported the weak correlation ($r=0.35$, $P<0.05$) observed between Na^+-K^+-ATPase activity and M-wave area in this experiment. M-wave area is interpreted as a measure of the magnitude of the total current passage at the recording electrode, which is a function of the size of the individual action potentials and the number of activated fibres (Overgaard and Nielsen, 2001). Either factor may be affected by fatigue, although the integrity of the Na^+-K^+-ATPase determines the ion gradients that these variables represent in a measure of M-wave area, making it suitable for correlation to Na^+-K^+-ATPase measured *in vitro*. A positive and highly significant correlation was observed between Na^+-K^+-ATPase activity and concentration in the control state ($r=0.48$, $P<0.001$, $n=18$), which supports the

hypothesis that the measure of activity is related to the number of available pumps. The capacity of these pumps is indicated by the measure of activity.

The relatively low correlation observed between Na^+-K^+-ATPase activity and M-wave highlights the more complicated nature of human experimentation as compared to the isolated nature of animal research, where studies support a direct relationship between recovery of membrane potential with activity of the Na^+-K^+-ATPase (Hicks *et al.*, 1997; Hicks and McComas, 1989; Overgaard *et al.*, 1997; Overgaard *et al.*, 1999; Overgaard and Nielsen, 2001). A similar relation in Na^+-K^+-ATPase activity with exercise has not been previously published for human muscle because of difficulties in measuring the Na^+-K^+-ATPase activity. We used a newly modified assay for Na^+-K^+-ATPase activity (Fraser and McKenna, 1998) to show that the combined effects of increased M-wave in CON, and decreased M-wave in EX, were related to the measured activity of the Na^+-K^+-ATPase. There are a number of assumptions and limitations associated with the use of M-wave as an indication of excitability and force in skeletal muscle (Dowling, 1997). A positive correlation of M-wave amplitude with force (twitch) was also identified in this experiment (unpublished), demonstrating a link between Na^+-K^+-ATPase, M-wave, and force in human muscle, as has been demonstrated previously for animal muscle (Overgaard *et al.*, 1999).

Conclusions. The results of this experiment indicate that the Na^+-K^+-ATPase can be altered by a single bout of heavy isometric exercise in humans. The association observed between Na^+-K^+-ATPase activity and measures of membrane excitability obtained by EMG, support the contention that indirect measures of neuromuscular activity can reflect cellular processes measured through biochemical procedures in human muscle. The alteration in activity and the short time course for recovery emphasize the rapidly adaptive nature of the

Na^+-K^+-ATPase, and imply that reduced sarcolemmal excitability may contribute to neuromuscular fatigue in humans during exercise. It is likely that other cellular processes contribute more to fatigue in the prolonged period of recovery. No doubt the role of Na^+-K^+-ATPase in a multi-factorial fatigue process, is dependent upon the intensity, duration and type of exercise performed.

Perspectives. Investigation of the skeletal muscle Na^+-K^+-ATPase indicates that it is highly adaptable to a wide variety of training protocols (Green, 2000; Nielsen and Clausen, 2000). The rapid concentration changes observed for the Na^+-K^+-ATPase in response to training imply that acute events likely lead to the chronic adaptation. There are a number of intracellular signals that may predispose the enzyme to rapid adaptations, one of them possibly being inactivation of the enzyme and the need to stimulate turnover and remodeling. It reasonable to postulate that the stress of exercise induces structural modifications that reduces the activity of the enzyme and results in a need for compensation and adaptation. The results obtained in this study would support such a hypothesis. Heavy contractile activity that induces both high and low frequency fatigue, challenges the capacity of the Na^+-K^+-ATPase to maintain transmembrane Na^+ and K^+ gradients and excitability. By mechanisms that are as yet uncertain, structural modifications occur to the Na^+-K^+-ATPase, resulting in reduced activity. The fact that the Na^+-K^+-ATPase activity and EMG were restored within four hours indicates that this modification has a rapid time-course for recovery. This rapid accommodation is also observed in response to repeated training bouts for this key sarcolemmal protein.

CHAPTER V

General Discussion, Conclusions, and Future Directions

GENERAL DISCUSSION

The primary objective of this thesis was to characterize the Na^+-K^+-ATPase in skeletal muscle of varying fibre type composition. This involved measuring Na^+-K^+-ATPase activity, content, and isoform distribution in a range of rat skeletal muscles, in order to determine possible differences in Na^+-K^+-ATPase protein expression between fibres of different contractile function. From this assessment, and the establishment of analytical procedures to accurately measure Na^+-K^+-ATPase activity in skeletal muscle, the effect of exercise on Na^+-K^+-ATPase activity was investigated. Na^+-K^+-ATPase activity was measured in rat skeletal muscles of varying fibre type, to determine if there is a fibre type- and therefore, Na^+-K^+-ATPase isoform-specific, response to exercise in rat skeletal muscle. Additionally, measurement of Na^+-K^+-ATPase activity was applied to the human exercise model to determine the acute response to exercise, and the relationship between biochemical (direct), and electrophysiological (indirect) measures of Na^+-K^+-ATPase activity in human skeletal muscle.

The overall hypothesis for the thesis was that there are differences in Na^+-K^+-ATPase characteristics between muscles of varying fibre composition, and these differences would be quantified by measures of activity, content and isoform distribution. Additionally, it was hypothesized that the Na^+-K^+-ATPase would be inactivated by exercise, and this inactivation would be identified by a reduction in Na^+-K^+-ATPase activity, either *in vitro*, or in conjunction with an indirect, *in vivo* measure of activity, as is commonly employed in the human model. There were a number of specific hypotheses formulated to address these primary questions, and these will be addressed individually.

The major focus of the thesis work was to measure fibre-specific differences in Na^+-K^+-ATPase activity. There are very few reports of skeletal muscle Na^+-K^+-ATPase activity in the literature, largely because of the difficulties inherent to its measurement. Consequently, another major objective was to identify suitable parameters of validity, specificity, and reliability for the assays presently available, so the Na^+-K^+-ATPase activity results could be interpreted appropriately. Two assays were employed (ATPase and 3-O-MFPase) because of the possibility that each may indicate the different aspects of the pump's hydrolytic and phosphatase activities (Ball, 1986; Linnertz *et al.*, 1995; Zolotarjova *et al.*, 1995). Two preparations were used (HOM and CM) because of the implications to validity, specificity and reliability, commonly overlooked in establishing the scientific authenticity of an assay.

A hypothesis was that the K^+-stimulated 3-O-methylfluorescein phosphatase (3-O-MFPase) assay would be the most specific and reliable, and the oubain-inhibitable hydrolytic activity (ATPase) assay the most valid measures of Na^+-K^+-ATPase activity in skeletal muscle, in both homogenates and crude membrane fractions. The direct ATPase assay exhibited higher activity than the K^+-stimulated phosphatase assay (Ch.II, Pt.1), and therefore, is a more valid assessment of Na^+-K^+-ATPase activity. However, the ATPase assay was more variable, particularly in HOM, and the greater reliability identified for the 3-O-MFPase (Ch.II, Pt.2), confirmed our next hypothesis, that the 3-O-MFPase assay would be the preferred technique for use with HOM. The HOM 3-O-MFPase was actually the assay best predicted by isoform distribution (Ch.II, Pt.4), so one could argue that it is the most

valid representation of Na^+-K^+-ATPase subunit complement for a given muscle. However, the 3-O-MFPase only quantified ~18% of theoretical maximum activity in HOM, and ~7% in CM, and therefore, is questionable as the 'best overall' measure of Na^+-K^+-ATPase activity. The CM ATPase quantifies a greater % of theoretical maximum activity (Ch.II, Pt.2) and was well predicted by multiple regression, given the small (n=6) sample size used in the analysis. However, the CM does require more tissue, and can produce a selective yield by muscle (Ch.II, Pt.2). Given all these considerations, the 3-O-MFPase assay is probably the 'best' assay for HOM and the ATPase assay the 'best' assay in CM, given that the CM has an adequate recovery (Hansen and Clausen, 1996). In any particular experimental situation, the choice of assay should be between HOM 3-O-MFPase, and CM ATPase, considering the limitations of equipment and tissue available for analyses.

Another major focus of Ch II, was to address the hypothesis that Na^+-K^+-ATPase content, as measured by oubain binding, would be greatest in muscles with the highest oxidative capacity, and this would relate to higher Na^+-K^+-ATPase activity. Although oxidative capacity was not measured but inferred, which is a limitation of this study, the results suggested that fibre morphology (also not measured, but inferred) plays an important role in ouabain-binding as a measure of pump content (Ch. II, Pt.3). What was identified was that the standard ouabain binding technique is highly correlated to the relative distribution of $\alpha 2$ subunits measured in homogenates of different skeletal muscles (Ch II, Pt.4,5). This is consistent with our hypothesis, which was based on the suggestions in the literature (Kjeldsen, 1988; Thompson *et al.*, 1999) that the standard technique only quantifies a single population of high-affinity sites in rat skeletal muscle. By modifying the standard technique,

evidence was provided that binding at two sites is possible (Ch.II, Pt.3). This result was only obtained in two out of four muscles, for reasons unknown. This novel finding requires further investigation, but also highlights the problems associated with the classical assumption (Clausen, 1996; Hansen and Clausen, 1988).

Given that the standard ouabain-binding technique only quantifies α2 subunits in rat skeletal muscle, and that ouabain- quantified pump content should relate to Na^+-K^+-ATPase activity, a hypothesis was that the relative abundance of α2 subunits between different skeletal muscles would best predict Na^+-K^+-ATPase activity measured by ATPase, in both homogenate and crude membranes. This hypothesis was also grounded by the assertions of Hundal (Hundal *et al.*, 1993) who indicated that the α2 is the 'major catalytic subunit' in skeletal muscle. From the results of our experiment, this hypothesis is rejected. There was no relationship established between any activity measure and α2 distribution in this experiment, for either HOM or CM (Ch.II, Pt.5). The interesting feature was that α1 distribution was the best predictor of in Na^+-K^+-ATPase activity in three out of four assays. The α1 isoform is described to have a 'basal' role in Na^+-K^+-ATPase ion transport (Hundal *et al.*, 1994), and this may be supported by these results, given they were obtained from resting muscle. The distribution of α1 was consistent with a relationship of higher activity in oxidative (SOL) muscle, to lower activity in glycolytic (WG) muscle.

One of the limitations in assigning importance to the conclusions of α1 and α2 Na^+-K^+-ATPase activity, is the fact that HOM ATPase activity was variable. Under optimal conditions, the HOM ATPase assay would likely be the most valid assay to assess muscle specific relationships to activity, because it would best represent whole muscle activity. If

one compares the α2 distribution to the relative activities of HOM ATPase between muscles, there are potential similarities, and it is possible that a relationship could be indicated between activity and α2 distribution, under improved conditions for the assay. However, for this experiment, extensive work was done to optimize conditions for the assay. The values reported are only the second to appear for a direct measure of Na^+-K^+-ATPase activity in HOM. Still, these conditions were not sufficient to give complete data out of ten samples for any particular muscle (range was 4-9 'values' out of 10). The SOL was the closest to the 'theoretical maximum value' (Clausen, 1996); however, using this value is also tenuous given the limitations and assumptions discussed in detail in Ch. II. It should be identified that the affinities for ouabain do not differ between isoforms in human skeletal muscle, and therefore, applying values of oubain binding to theoretical Na^+-K^+-ATPase in human skeletal muscle may be more appropriate.

An additional hypothesis in explaining relationships between Na^+-K^+-ATPase characteristics was the idea that the relative abundance of β1 subunits between different skeletal muscles would best predict Na^+-K^+-ATPase activity measured by 3-O-MFPase, in both homogenate and crude membranes. This hypothesis was based on molecular evidence that suggested the β isoform stabilizes the K^+-bound E2 phosphoenzyme intermediate (Blanco *et al.*, 1995; Skou, 1992), and therefore, may specifically assess enzyme activity between muscle of varying β isoform composition. Since β1 activity is higher than β2 (Blanco *et al.*, 1995; Crambert *et al.*, 2000; Hundal *et al.*, 1993), the expectation was to find a relationship of β1 to 3-O-MFPase activity. A relationship was identified for HOM, but not CM (Ch.II, Pt.5). Interleaved to this hypothesis, was the assumption that there would be no

fibre-specific effect of the isolation procedure on measuring Na^+-K^+-ATPase characteristics in crude membrane fractions. Given that there was a fibre-specific difference in isolation and purification (Ch.II, Pt.2) which influenced the CM 3-O-MFPase results, the conclusion that the β1 isoform likely confers higher kinetic specificity than the β2 isoform in all situations and the muscle specific activity hierarchy from oxidative muscle (highest) to glycolytic muscle (lowest), was conserved.

From the measures of Na^+-K^+-ATPase activity established in Ch II, an objective as to determine if Na^+-K^+-ATPase activity would be reduced following a single session of prolonged aerobic exercise in rats. Na^+-K^+-ATPase activity was measured *in vitro* on muscle homogenates using the 3-O-MFPase assay, as it was established that this assay was best predicted by molecular measures, is specific, reliable, and suited for use with small tissue amounts. Na^+-K^+-ATPase activity was reduced following exercise (Ch. III) and it is possible that one of several mechanisms, namely free-radical damage (Kourie, 1998), Ca^{2+}-activated proteolysis (Belcastro *et al.*, 1998) and heat denaturation (Febbraio, 2000), may act in isolation or in combination to inactivate the Na^+-K^+-ATPase and reduce its activity. Because it was the additional period of fast walking after the initial running period that elicited the significant changes in activity, it is possible that these factors are dependent upon the total amount of contractile work done during the exercise.

The fibre-specific response to exercise was also investigated. The hypothesis was that changes in Na^+-K^+-ATPase activity due to a single bout of exercise in rats would not be different between skeletal muscles of varying fibre type. The results indicated that the inactivation at the completion of the extended exercise session was consistent across

muscles; however, the effects at the end of the 2 hr of running exercise were not significantly different. By paired comparison, EDL muscle was the only muscle with Na^+-K^+-ATPase activity that was not lower than CON. There was only a trend in the other muscles for a reduction in activity (P=0.12), so it is uncertain if this effect has physiological relevance. EDL muscle exhibits considerably faster fatigue than SOL (Everts *et al.*, 1988), and this is attributed, at least in terms of muscle excitability, to a greater number of Na^+-channels relative to Na,K-pumps (Harrison *et al.*, 1997). This increased ion leak relative to ion pump capacity, can result in a rundown of ion gradients, and result in fatigue (Nielsen and Clausen, 2000), possibly explaining EDL's lack of 'inactivation' at the end of exercise. Since this suggestion is speculative, it invites further investigation. In light of the differences in Na^+-K^+-ATPase characteristics between muscles, it is possible that differences in acute 'inactivation' exist, especially given the isoform specific regulation of pump content and activity (Therien and Blostein, 2000).

A limitation of the exercise study may arise from the use of only the 3-O-MFPase as our indicator of Na^+-K^+-ATPase activity. Although proven to be the 'best' measure for homogenates, it is possible that assessment by the direct ATPase assay may produce different results. This is based on the evidence that the pump's hydrolytic and phosphatase activities can be modified independently of each other (Ball, 1986; Linnertz *et al.*, 1995; Zolotarjova *et al.*, 1995). The HOM 3-O-MFPase did relate to both $\alpha 1$ and $\beta 1$ isoform distributions, so identification of this problem may require more detailed micro-structural analysis, as opposed to comparison of activity assay results.

The final objective was to apply the techniques developed for Na^+-K^+-ATPase assessment to the human model. There are number of difficulties associated with assessing neuromuscular excitability and the relationships to fatigue (Enoka and Stuart, 1992). In spite of this, we were able to demonstrate a relationship between *in vitro* and *in vivo* measures of excitability in humans following fatiguing exercise. There are a number of assumptions associated with our conclusions, namely that 3-O-MFPase activity is representative of Na^+-K^+-ATPase activity in human skeletal muscle samples, EMG changes from the vastus medialis are representative of changes in the vastus lateralis where the biopsies were taken, and changes in EMG and 3-O-MFPase activity represented relative to the contra-lateral control limb are the most physiologically relevant comparison for the data. The low, albeit significant, correlation may be representative of these limitations. Nevertheless, this is the first study to show that Na^+-K^+-ATPase activity and muscle excitability are reduced during sustained, repetitive isometric exercise in humans, and the first study to show any relationship between these measures in humans. The original hypothesis was that the decline in Na^+-K^+-ATPase activity would remain depressed for an hour following the exercise, as was observed for the Ca^{2+}-ATPase (Tupling *et al.*, 2000). The observation that the Na^+-K^+-ATPase had a more rapid time-course for recovery, exhibited in both the EMG and Na^+-K^+-ATPase activity measures, leads to the conclusion that reduced Na^+-K^+-ATPase activity and excitability likely contributes only to short-term fatigue.

In reference to the prolonged running study in rats, where it was postulated that the volume of contractile activity may factor in the acute inactivation of the Na^+-K^+-ATPase, the type of exercise utilized in humans would also support this notion. The exercise was

designed to sustain as high a force possible, for as long as possible. Intensity might also factor into this equation, although the intensity of work done with the isometric exercise was far below the forces considered to be 'high-intensity' in a resistive model. This supports the conclusions of Sejersted and Sjogaard (Sejersted and Sjøgaard, 2000), that medium intensity and volume contractile work exhibits the greatest challenge to K^+ balance in muscle. Given the results of this thesis, the difficulty in maintaining K^+ balance may be due in part to alterations of the Na^+-K^+-ATPase by this type of exercise.

CONCLUSIONS

The results presented in this thesis indicate that a number of factors dictate the activity of the Na^+-K^+-ATPase in skeletal muscle. Different activities are observed between techniques, which highlights that the assay, isolation procedure, and muscle used are important factors when interpreting measures of Na^+-K^+-ATPase activity. Na^+-K^+-ATPase activity is different between muscles of varying fibre composition, and a higher distribution of $\alpha 1$ and $\beta 1$ subunits, as found in SOL, confers the greatest Na^+-K^+-ATPase activity when measured on skeletal muscle preparations *in vitro*. The exercise studies indicate that the Na^+-K^+-ATPase is susceptible to acute inactivation, and that this inactivation may contribute to impaired excitability during exercise.

Perspectives

The role of the Na^+-K^+-ATPase is to maintain ionic balance at the sarcolemma. In a fatigue resistant environment such as in SOL, high Na^+-K^+-ATPase activity is suited to this role. This is in contrast to the fact that ionic imbalance occurs at the sarcolemma in fatigue, largely because of the inability of the Na^+-K^+-ATPase to keep pace with ion transport. The expression of low Na^+-K^+-ATPase activity in fatigable WG also appears to facilitate this occurrence. This implies that the Na,K-pump can both resist and promote fatigue. When one considers that the Na^+-K^+-ATPase is upregulated immediately upon activation, is acutely inactivated by continued exercise and is dynamically regulated by the absence or presence of repeated contractile work, one concludes that the Na^+-K^+-ATPase plays a key role in the muscle's acute and chronic response to stress.

FUTURE DIRECTIONS

These are the first investigations to provide a comprehensive evaluation of Na^+-K^+-ATPase measures in skeletal muscle, both at rest and in response to exercise, in both rat and human skeletal muscle. A number of novel findings arise from these studies, and invite further investigation. Some of these future directions include:

1. Developing the ATPase assay to improve its specificity and reliability, in order to further investigate the relationship between isoform distribution and fibre specific Na^+-K^+-ATPase activity. This may include Na^+ and K^+ dependencies, to detect changes in ion sensitivity induced by acute or chronic stress.
2. Determining the relationship of fibre morphology to the measurement of Na^+-K^+-ATPase activity and pump content determined by ouabain binding, and relating the characteristics to fatigue resistance, or fatigue promotion, in skeletal muscles of varying fibre type composition.
3. Investigating the physiological relevance in skeletal muscle, if any, of the $\alpha 3$ and $\beta 3$ isoforms.
4. Investigating the mechanisms of Na^+-K^+-ATPase inactivation following a single bout of exercise in rats and/or humans and identify the molecular/structural basis and physiological impact as it relates to the stress-adaptation response.
5. Confirming the relationship between the *in vitro* measure of Na^+-K^+-ATPase activity and the indirect *in vivo* measure of Na^+-K^+-ATPase assessed by electromyography during voluntary exercise in humans using different contraction schedules, to ascertain what parameters identify limitations by the Na^+-K^+-ATPase to performance

This thesis work has facilitated further investigation into the mechanisms regulating the activity, content, and isoform distribution of the Na^+-K^+-ATPase. Given the novel findings of an acute inactivation of the enzyme with exercise, and the fact that the molecular mechanisms regulating adaptation to exercise have not been addressed, the exercise model provides a fruitful avenue of further research. Investigating the acute and chronic effects of exercise should help with our understanding of factors controlling Na^+-K^+-ATPase expression in skeletal muscle.

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Appendices

Appendix 1 - Fibre composition and oxidative capacity of different skeletal muscles

Appendix 2 – Muscle specific, individual values of Na^+-K^+-ATPase activity measured in different skeletal muscles

Appendix 3 – Relative density of α and β subunits obtained by Western Blot in different skeletal muscles

Appendix 1 – Fibre composition and oxidative capacity of selected skeletal muscle fibres.

Table A.1. Fibre type characteristics of different muscles in the rat, summarized from Delp and Duan (1996).

Characteristic		SOL	RG	EDL	WG
Oxidative capacity	CS activity	21.3 ± 2.3	36.2 ± 1.6	21.6 ± 2.1	8.1 ± 0.7
fibre population (%)	type I	84 ± 6	51 ± 4	4 ± 1	0
	type IIA	7 ± 0	35 ± 3	20 ± 3	0
	type IIX/D	9 ± 6	13 ± 7	38 ± 3	8 ± 4
	type IIB	0	1 ± 1	38 ± 3	92 ± 4
fibre area (μm^2)	type I	4140 ± 230	3235 ± 530	1460 ± 165	0
	type II	3740 ± 145	2653 ± 208	1605 ± 50	0
	type IIX/D	3500 ± 708	3003 ± 155	2215 ± 78	2545 ± 485
	type IIB	0	3565 ± 145	4478 ± 140	4043 ± 553
	average	4054	3004	2923	3923

Values are 0 ± SE; based on data from three male Sprague-Dawley rats aged 16-18 weeks. SOL, soleus; RG, red gastrocnemius; EDL, extensor digitorum longus; WG, white gastrocnemius; CS, citrate synthase ($\mu mol\ min^{-1}\ g^{-1}$); I, type I fibre; IIA, type IIA fibre; IIX/D, type IIX/D fibre; IIB, type IIB fibre. Average fibre area is calculated by multiplying the %type x area for each type, summing and dividing by 100%. Fibre types were histochemically determined.

Appendix 2 - Na^+-K^+-ATPase activity measured in homogenates and crude membranes of different muscle fibres on two activity assays.

Table A.2.1 Na^+-K^+-ATPase activity measured in homogenates on the ATPase assay for different skeletal muscles.

	SOL	RG	EDL	WG
rat 1	1.367	1.008	1.837	0.000
rat 2	0.676	0.000	2.766	0.000
rat 3	0.700	0.000	0.000	0.000
rat 4	0.000	1.064	1.189	0.000
rat 5	0.956	1.606	2.176	1.057
rat 6	0.495	1.176	1.708	0.538
rat 7	1.817	0.000	3.000	0.000
rat 8	0.000	2.776	2.242	3.384
rat 9	2.388	0.000	3.063	0.706
rat 10	1.340	0.760	0.730	0.000
mean	0.974	0.839	1.871	0.568
sd	0.767	0.904	1.001	1.061
se	0.242	0.286	0.317	0.335
n	10	10	10	10
cv	78.7%	107.7%	53.5%	186.6%

Values are means ± SE; n=10 (μmoles·mg protein^{-1}·hr^{-1}) for each muscle. SOL, soleus; RG, red gastrocnemius; EDL, extensor digitorum longus; WG, white gastrocnemius.

Table A.2.2 Na^+-K^+-ATPase activity measured in homogenates on the 3-O-MFPase assay for different skeletal muscles.

	SOL	RG	EDL	WG
rat 1	0.236	0.144	0.194	0.081
rat 2	0.274	0.140	0.222	0.087
rat 3	0.291	0.166	0.157	0.122
rat 4	0.333	0.154	0.173	0.065
rat 5	0.285	0.231	0.200	0.101
rat 6	0.265	0.220	0.140	0.114
rat 7	0.240	0.168	0.136	0.089
rat 8	0.239	0.188	0.160	0.050
rat 9	0.252	0.200	0.139	0.154
rat 10	0.292	0.166	0.145	0.113
mean	0.271	0.178	0.167	0.098
sd	0.031	0.031	0.030	0.030
se	0.010	0.010	0.009	0.009
n	10	10	10	10
cv	11.3%	17.4%	17.9%	30.7%

Values are means ± SE; n=10 (μmoles·mg protein^{-1}·hr^{-1}) for each muscle. SOL, soleus; RG, red gastrocnemius; EDL, extensor digitorum longus; WG, white gastrocnemius.

Table A.2.3 Na^+-K^+-ATPase activity measured in crude membranes on the ATPase assay for different skeletal muscles.

	SOL	RG	EDL	WG
rat 1	6.942	8.695	5.763	4.517
rat 2	7.374	8.181	6.829	4.360
rat 3	8.088	5.161	4.462	4.293
rat 4	9.184	4.981	6.809	6.359
rat 5	9.594	7.573	4.840	6.029
rat 6	7.555	5.917	5.925	5.103
mean	8.123	6.751	5.772	5.110
sd	1.056	1.603	0.980	0.893
se	0.431	0.655	0.400	0.365
n	6	6	6	6
cv	13.0%	23.8%	17.0%	17.5%

Values are means ± SE; n=6 (μmoles·mg protein^{-1}·hr^{-1}) for each muscle. SOL, soleus; RG, red gastrocnemius; EDL, extensor digitorum longus; WG, white gastrocnemius.

Table A.2.4 Na^+-K^+-ATPase activity measured in crude membranes on the 3-O-MFPase assay for different skeletal muscles.

	SOL	EDL	RG	WG
rat 1	1.312	1.401	1.272	1.589
rat 2	1.659	1.292	1.379	1.394
rat 3	1.428	1.171	1.079	1.217
rat 4	1.737	1.519	1.393	1.649
rat 5	1.764	1.536	1.298	1.647
rat 6	1.131	1.442	1.031	1.336
mean	1.505	1.393	1.242	1.472
sd	0.256	0.140	0.153	0.182
se	0.104	0.057	0.062	0.074
n	6	6	6	6
cv	17.0%	10.1%	12.3%	12.3%

Values are means ± SE; n=6 (μmoles·mg protein^{-1}·hr^{-1}) for each muscle. SOL, soleus; RG, red gastrocnemius; EDL, extensor digitorum longus; WG, white gastrocnemius.

Appendix 3 – Relative density of α and β subunits obtained by Western Blot in different skeletal muscles

Table A.3. Relative density of α and β subunits obtained by Western Blot in different skeletal muscles

		SOL	RG	EDL	WG
HOM	α subunits				
	α1	54 ± 10	35 ± 12*	21 ± 7*†	16 ± 6*†
	α2	62 ± 5	72 ± 3	73 ± 3	52 ± 4†‡
	α3	<1 ± <1	34 ± 4*	30 ± 3*	60 ± 4*†‡
	β subunits				
	β1	39 ± 3	26 ± 2*	23 ± 2*	21 ± 2*
	β2	8 ± 1	22 ± 2*	20 ± 3*	25 ± 3*
CM	α subunits				
	α1	64 ± 3	36 ± 3*	22 ± 2*†	26 ± 3*†
	α2	97 ± 2	90 ± 3	75 ± 1*†	74 ± 2*†
	α3	8 ± 3	8 ± 1	14 ± 1	16 ± 1*†
	β subunits				
	β1	50 ± 1	33 ± 1*	15 ± 1*†	<1 ± <1*†‡
	β2	6 ± 1	17 ± 1*	69 ± 4*†	103 ± 3*†‡

Values are means ± SE; n=6 for each subunit of each muscle. SOL, soleus; RG, red gastrocnemius; EDL, extensor digitorum longus; WG, white gastrocnemius; H, homogenate; CM, crude membrane. Relative density is % relative to a control standard of known concentration of rat brain, corrected for the molar ratio between subunits as determined Lavoie et al. (1997). * *different from SOL;* † *different from RG;* ‡ *different from EDL; (P<0.05).*

Lavoie *et al.* (1997) determined the relative molar ratios between rat brain, and red (oxidative) skeletal muscle. To relate the present data to those of Lavoie *et al.*, I first assumed that the red skeletal muscle examined by Lavoie *et al.*, is representative of the RG muscles examined in this experiment. Using RG as the control between these two experiments, the relative content between RG isoforms was corrected by the ratios identified by Lavoie *et al.* for brain, so that the brain standard in each of the blots in this experiment represents a relative molar ratio of 1. The values for α2 isoforms were corrected by 43/31

and the values for β1 were corrected by 227/151, representing the molar ratio established by Lavoie for rat brain for the α2/α1 isoforms, and the β1/β2 isoforms, respectively. The molar ratio of α:β subunits was approximately equal for rat brain. This calculation is done only for comparative purposes, and would require extensive analysis to confirm.

Given these assumptions, the relative abundance of WG β2 is 103 relative units in CM, and SOL is only 50 relative units in CM. SOL β1 purified 1.3 from HOM to CM, whereas WG β2 purified 4 fold. Previous reports indicated higher β1 supported phosphatase activity than β2 (Hundal *et al.* 1993, Lavoie *et al.* 1997). In fact, if the β1 contribution to activity is multiplied by a factor of 2.7 to represent the greater activity, the isoform contribution to total activity equals the activity results obtained for CM 3-O-MFPase. Furthermore, if one accounts for the specificity of purification [(4/1.3 = 3.06x) less purification of β1 from HOM to CM), the isoform contribution to activity in CM equals similar relative relationships between muscles observed for the HOM 3-O-MFPase activity. The synopsis of the mathematical model suggests that for the CM 3-O-MFPase assay only, β2 is likely contributing to activity more than β1 because of the relative purification differences, not because of specific activity contribution to activity.