Acute Regulation of Na\(^+\)-K\(^+\)-ATPase Activity in Skeletal Muscles of Different Fibre Type Composition in Response to Insulin Exposure

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A thesis presented to the University of Waterloo in fulfillment of the thesis requirement for the degree of Master of Science in Kinesiology

Waterloo, Ontario, Canada, 2007

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Author’s Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made available electronically to the public.
Abstract

The Na\(^+\)-K\(^+\)-ATPase (pump) is a transmembrane, multi-subunit (α and β) protein that is expressed in all cells, and particularly in skeletal muscle cells. In one cycle, it pumps 3 Na\(^+\) ions out of the cell and 2 K\(^+\) ions into the cell at the expense of 1 ATP molecule. This enzyme is responsible for maintaining muscle cell excitability. This is of particular importance during contractile activity, when the flux of Na\(^+\) and K\(^+\) across the cell membrane is high. The activity of the Na\(^+\)-K\(^+\)-ATPase is highly regulated and very responsive to hormonal stimuli. Previous research has shown that 20-30 min insulin exposure in vivo induces the translocation of pumps from intracellular stores to the plasma membrane. However, no study has examined the catalytic properties of this enzyme in response to short insulin exposures. The objective of this study was to investigate the response of the Na\(^+\)-K\(^+\)-ATPase to short insulin incubation in vitro in muscles of different fibre type. It was hypothesized that the short insulin treatment would result in an increase in pump activity, not only through translocation but also increased intrinsic activity. Using an in vitro model, rat soleus (Sol), red gastrocnemius (RG), and white gastrocnemius (WG) muscle homogenates were incubated at 37°C for 5 min with and without 75\(\mu\)M insulin (Ins). Next, in order to separate mechanisms of translocation and intrinsic activation, the plasma (SLP) and endosomal (EN) membranes were separated through a fractionation procedure. This allowed the investigation of insulin-induced increases in intrinsic activity in SLP and EN fractions of Na\(^+\)-K\(^+\)-ATPase; SLP and EN (non-treated) membranes were incubated at 37°C for 5 min with and without 75\(\mu\)M insulin. Lastly, muscle homogenates were insulin-treated for 5 min at 37°C with 625\(\mu\)M insulin prior to fractionation. These SLP and EN fractions (insulin-treated) were then incubated at 37°C for 5 min with and without 75\(\mu\)M...
insulin. Na⁺-K⁺-ATPase maximal activity ($V_{\text{max}}$, mmol·mg prot⁻¹·h⁻¹) and $k_m$ (substrate affinity), $\alpha_2$ content, and tyrosine phosphorylation (Tyr-P) were probed. It was found that insulin increased $V_{\text{max}}$ (P<0.05) in Sol and RG, but not WG, homogenates (Con vs Ins, Sol=221±17 vs 256±21; RG=190±14 vs 256±18; WG=104±4.6 vs 99±1.8). In non-treated fractions, insulin increased $V_{\text{max}}$ (P<0.05) in Sol and RG SLP fractions (Con vs Ins, Sol=1710±186 vs 1970±231; RG=1476±128 vs 1655±139). A main effect, Con<Ins (P<0.05) was observed in non-treated WG SLP. Insulin also increased $V_{\text{max}}$ in non-treated RG EN (Con vs Ins, 246±38 vs 304±43). In insulin-treated fractions, insulin increased $V_{\text{max}}$ in RG SLP only (Con vs Ins, 1145±119 vs 1426±150). Increased $V_{\text{max}}$ was not observed in insulin-treated fractions when compared to non-treated fractions. No evidence of translocation or increased Tyr-P was detected with insulin treatment via $\alpha_2$ Western blotting. Short insulin exposure induced increases in Na⁺-K⁺-ATPase activity, and these increases were due to stimulation of intrinsic activity and not due to translocation.
Acknowledgements

I’d like to start off by thanking my committee members, Drs. Howie Green, Russ Tupling, and Ken Stark, for their guidance throughout this process. I’d especially like to thank Dr. Green, who has provided me with mentorship throughout my career at the University of Waterloo. He has committed much time and energy in helping me attain the scientific skills I have today. He has been a great influence in the way I approach science.

I’d also like to thank my family: my parents Pat and Julie and my sister Kelly. They have provided me with infinite support in all of my endeavors. Their love and reassurance has given me the confidence to follow through on any goal I set forth.

I’d like to thank the members of my lab. I had a great deal of fun working with all of them. They definitely made coming to work enjoyable. Also, they provided me with motivation to keep trying when experiments were not working well, helpful advice when I was stuck on a problem, and modesty when I thought nothing could go wrong. I’d especially like to thank Jing Ouyang, whose technical support and assistance throughout my experiments made all of the difference. Without her, I would not have completed all of my experiments in the timeframe in which I did. I’d also like to thank Todd Duhamel, who has provided me with guidance and mentorship since the undergraduate years of my research career.

Lastly, I’d like to thank all of my friends I have made here in Waterloo. Without them the academic life would not have been so appealing. They made sure I didn’t forget to have fun once in a while and that there was more to life than being in the lab. I’d like to especially mention Mike Williams-Bell, Riley Stewart, Chris Vigna, Justin Chung, and Andrew Robertson.
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List of Abbreviations

ACh = acetylcholine

3-O-MFP = 3-O-methyl fluorescein phosphate

cAMP = adenosine 3’-5’-cyclic monophosphate

CaM = calmodulin

CHO = carbohydrate

CS = Citrate Synthase

DAG = diacylglycerol

DTT = dithiothreitol

EN = endosomal fraction

Epi = epinephrine

EDL = extensor digitorum longus

GLU = glucose

Ins = insulin

K⁺ = potassium ion

\(k_m\) = Michealis-Menton constant, represents substrate concentration at which 0.5 \(V_{max}\) is achieved.

MAPK = mitogen activated protein kinase

M-wave = muscle compound action potential

Na⁺ = sodium ion

Norepi = norepinephrine

PI3 kinase = phosphatidylinositol 3-kinase

PKA = Protein Kinase A

PKC = Protein Kinase C
PLA$_2$ = phospholipase A$_2$

PLC = phospholipase C

PLM = phospholemman

PPase = protein phosphatase

RG = red gastrocnemius

RV = red vastus lateralis

Ser = serine

SR = sarcoplasmic reticulum

SLP = sarcolemmal particulate fraction

Sol = soleus

Thr = threonine

Tyr = tyrosine

Tyr-P = phosphorylated tyrosine residue

$V_{\text{max}}$ = maximal Na$^+$-K$^+$-ATPase activity

WG = white gastrocnemius

WV = white vastus lateralis
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Introduction
Skeletal muscle is specialized for developing varying amounts of force, which is essential for performing the diverse tasks necessary for survival. A system must be in place that allows the muscle cell to respond to commands from neural input by conducting signals to the interior of the muscle cell. In a resting muscle cell, the membrane potential is ~70mV, with extracellular concentrations of Na\(^+\) and K\(^+\) of ~145 and 4.5mM, respectively, and intracellular concentrations of Na\(^+\) and K\(^+\) of ~4.5 and ~145mM, respectively (65). Neural input causes the release of acetylcholine (ACh) from nerve endings at the neuromuscular junction, which in turn binds to receptors on the muscle motor end plate and initiates an action potential (AP) (39). An AP is caused by the opening of voltage-gated Na\(^+\) channels in the sarcolemmal membrane, which allows a rapid influx of Na\(^+\) and depolarization of the sarcolemmal membrane (39). This depolarization signal is propagated across the sarcolemma and into the T-tubules, which initiates an intracellular release of Ca\(^{2+}\) from the sarcoplasmic reticulum (SR) and subsequent muscle contraction (39). An outward flux of K\(^+\), through voltage-sensitive K\(^+\) channels, is necessary to restore resting membrane potential (65).

This system relies on the concentration gradients for Na\(^+\) and K\(^+\) to conduct action potentials. However, with repetitive action potentials, the gradients of these ions become depleted. During contractile activity, AP frequency reaches up to 55Hz depending on the characteristics of activity and the fibre type of the muscle (57). Such a stress could deplete the Na\(^+\) and K\(^+\) concentration gradients in a relatively short time. Thus, for repetitive action potentials to occur, a structure is needed that can rapidly transport Na\(^+\) and K\(^+\) against their
respective concentration gradients in order to maintain muscle cell responsiveness to neural input. The Na\(^+\)-K\(^+\) ATPase (pump) is the trans-membrane enzyme responsible for maintaining and restoring muscle cell excitability through the active transport of Na\(^+\) and K\(^+\). The Na\(^+\)-K\(^+\) pump cycles 3 Na\(^+\) ions out of the cell and 2 K\(^+\) ions into the cell at the expense of a single molecule of ATP (37). This results in the net movement of a single positive charge out of the cell and repolarization of the membrane following action potential propagation. The resting membrane potential is re-established and voltage-gated Na\(^+\) channels re-activated. Restoration of the Na\(^+\) and K\(^+\) gradients to resting concentrations allows membrane excitability to be protected.

The large increases in Na\(^+\) and K\(^+\) flux that occur with contractile activity require a rapid increase in Na\(^+\)-K\(^+\)-ATPase activity. As such, intricate regulatory mechanisms exist to match pump activity to task demand. Na\(^+\)-K\(^+\) ATPase activity is low at rest but increases dramatically within seconds of contractile activity due to activation by increased intracellular Na\(^+\) concentrations (57). Hormonal stimulation of the Na\(^+\)-K\(^+\)-ATPase can further increase pump activity within minutes (57). Furthermore, there are multiple isoforms of the Na\(^+\)-K\(^+\)-ATPase, each exhibiting unique catalytic properties, which are differentially expressed in tissue and muscle fibre types to confer differential regulation and responses to contractile stimuli (10).

**Na\(^+\)-K\(^+\)-ATPase Structure and Cellular Localization**

The Na\(^+\)-K\(^+\)-ATPase (pump) is a transmembrane, multi-subunit protein that is expressed in all cells, and particularly in skeletal muscle cells. These pumps are localized in the plasma membrane, which is composed of the sarcolemma and the T-tubules, and in intracellular
sites. Although fractionation techniques have confirmed the existence of a pool of Na\(^+\)-K\(^+\) pumps distinct from the plasma membrane pool (27; 69), the exact localization of these sites has yet to be elucidated. Some evidence suggests that these intracellular sites include tubular and vesicular structures located in subsarcolemmal and triadic regions (53). The Na\(^+\)-K\(^+\) ATPase consists of a \(~110\) kDa \(\alpha\) subunit and a \(~40\) kDa heavily glycosylated \(\beta\) subunit (10). Both of these proteins are required for enzymatic function (10). More recently, two members of the FXYD protein family have been associated with the Na\(^+\)-K\(^+\) pump: FXYD1, also called phospholemman (PLM), and FXYD2, also called the \(\gamma\) subunit of the Na\(^+\)-K\(^+\) ATPase (29). These proteins, although not essential for catalytic activity, bind to and modify the behaviour of the Na\(^+\)-K\(^+\)-ATPase (29). Unlike PLM, the \(\gamma\) subunit has not yet been detected in skeletal muscle (73).

**Muscle isoforms of the Na\(^+\)-K\(^+\)-ATPase: Distribution and Properties**

Several isoforms exist for the \(\alpha\) and \(\beta\) subunits of the Na\(^+\)-K\(^+\) pump, each with distinct properties. There are 4 \(\alpha\) isoforms, although only three have been reported in skeletal muscle (\(\alpha_1\), \(\alpha_2\), and \(\alpha_3\)), and three \(\beta\) isoforms (\(\beta_1\), \(\beta_2\) and \(\beta_3\)), all of which have been detected in skeletal muscle (10). The \(\alpha\) subunit contains the catalytic site which binds Na\(^+\), K\(^+\), and ATP (10). The \(\beta\) subunit serves a regulatory function and is necessary for both the transport of \(\alpha\beta\) heterodimers from intracellular synthesis sites to the plasma membrane and for the catalytic activity of the enzyme (10). Na\(^+\)-K\(^+\)-ATPase catalytic properties, which include the maximal rate of ATPase activity (\(V_{\text{max}}\)) as a measure of pump capacity for ATP usage and the \(k_{50}\) as a measure of substrate (Na\(^+\), K\(^+\), or ATP) affinity, are influenced by both the \(\alpha\) and the \(\beta\) isoform (10).
These isoforms appear to be distributed in tissue and fibre type specific manners (22). The $\alpha_1\beta_1$ combination is found in nearly every tissue while the other $\alpha$ and $\beta$ isoforms are more restricted in their expression (10). Much work has been done to elucidate the specific expression patterns within skeletal muscles of different fibre types. Muscle fibres are generally classified according to their myosin heavy chain composition; these classes, in order of slowest to fastest velocity of contraction, are type I, type IIA, type IIX(D), and type IIB (63). These classes are consistent across species (63). However, it has been reported that the properties of the Na$^+$-K$^+$ ATPase correlate with the oxidative potential of muscle fibres more so than with contractile speed of the fibres (22). In rat skeletal muscle the oxidative potential of fibre types ranks in the order of type IIA>$\text{I}$$>$IID/X>$\text{IIB}$ fibres (18) whereas in humans the order is type I$>$IIA$>$IID/X fibres (21). Slow oxidative (type I) fibres contain $\alpha_1\beta_1$ and $\alpha_2\beta_1$ complexes (22), the $\alpha_1$ isoform representing 15-25% of the total pool of Na$^+$-K$^+$ ATPase in soleus (Sol) (35). The fast glycolytic fibres (type IIX, IIb), such as white gastrocnemius (WG), contain $\alpha_1\beta_2$ and $\alpha_2\beta_2$ complexes while fast oxidative-glycolytic fibres (type IIA), such as red gastrocnemius (RG), have all four combinations (22). The $\alpha_3$ and $\beta_3$ isoforms have been reported to be present in negligible quantities in skeletal muscle (22). However, a recent report detecting these two isoforms in human vastus lateralis muscle (56) illustrates the need for further investigation into their distribution and role in the different fibre types. Also, even though a single isoform may be found in multiple fibre types, the expression levels of the Na$^+$-K$^+$ pump isoforms are skewed. In a comparison between rat skeletal muscles of different fibre types [Sol, RG, extensor digitorum longus (EDL), and WG], it was reported that Sol contained the greatest amount of $\alpha_1$ and $\beta_1$ isoforms while having significantly less $\beta_2$ isoform than the other three muscles (22). All muscles had
comparable amounts of α2 isoform (22). Based upon this fibre type analyses, it seems as though the β isoform distinguishes slow from fast fibres (22). In terms of $V_{\text{max}}$, the rank order from highest to lowest was Sol > RG = EDL > WG (22). The fibre type distribution of Na+-K+ pumps in rat skeletal muscle is summarized in Table 1.

Table 1: Relative fibre type specific expression of rat Na+-K+ ATPase isoforms

<table>
<thead>
<tr>
<th>Type</th>
<th>Type I</th>
<th>Type IIA</th>
<th>Type IIX/IIB</th>
</tr>
</thead>
<tbody>
<tr>
<td>α1</td>
<td>100</td>
<td>75</td>
<td>25</td>
</tr>
<tr>
<td>β1</td>
<td>100</td>
<td>70</td>
<td>70</td>
</tr>
<tr>
<td>α2</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>β2</td>
<td>25</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>dominant isozymes</td>
<td>α1β1, α2β1</td>
<td>α1β1, α2β1, α1β2, α2β2</td>
<td>α1β2, α2β2</td>
</tr>
</tbody>
</table>

[ref (22)]

The catalytic properties of the Na+-K+ ATPase are primarily determined by the α isoform and modified by the β isoform (10). The use of heterologous expression systems has allowed the separate expression of each isoform; however, attempts to characterize the enzymatic properties of the Na+-K+ ATPase isoforms have resulted in contradictory results (10). The tissue under study and the specific αβ heterodimers both influence catalytic properties (10). For example, the renal α1β1 isozyme was reported to have lower ATP, similar K+, and higher Na+ affinities than the neuronal enzyme composed of α2 and α3 isoforms (10). It has also been reported that the α1 and α2 isoforms have similar affinities for Na+ and K+, but that these affinities are higher and lower than those of the α3 isoform, respectively (70). These reports are most likely influenced in part by the membrane environment in which the different experiments were conducted (i.e. different species, expression systems, or tissues). Consistent positive correlations between molecular activity (enzyme activity/enzyme number) and increasing levels of polyunsaturation and unsaturation index (avg. number of double bonds per fatty acid residue) have been reported.
Furthermore, ‘cross over’ experiments, in which membranes from tissues of species that display high molecular activity were exchanged with membrane from the same tissue of a species with lower molecular activity, have shown that the Na\(^+\)-K\(^+\) pumps display molecular activities shifting in the direction of the added membrane source (20).

Measures performed on isoforms synthesized in heterologous expression systems from rat cDNA clones show apparent affinity for Na\(^+\) ranks in the order of \(\alpha_2\beta_2 > \alpha_2\beta_1 = \alpha_1\beta_1 = \alpha_3\beta_2 > \alpha_3\beta_1\); apparent affinity for K\(^+\) ranks in the order of \(\alpha_1\beta_1 > \alpha_2\beta_1 = \alpha_2\beta_2 > \alpha_3\beta_1 = \alpha_3\beta_2\); and activation by ATP is equivalent for \(\alpha_2\) and \(\alpha_3\) isoforms, which is approximately four times lower than that of the \(\alpha_1\beta_1\) complex (10). These catalytic properties of the different rat Na\(^+\)-K\(^+\) ATPase \(\alpha\) isoforms are summarized in Table 2.

**Table 2: Catalytic properties of rat Na\(^+\)-K\(^+\) ATPase isoforms**

<table>
<thead>
<tr>
<th></th>
<th>(\alpha_1)</th>
<th>(\alpha_2)</th>
<th>(\alpha_3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na(^+) affinity</td>
<td>medium</td>
<td>highest</td>
<td>lowest</td>
</tr>
<tr>
<td>K(^+) affinity</td>
<td>highest</td>
<td>medium</td>
<td>lowest</td>
</tr>
<tr>
<td>ATP activation</td>
<td>highest</td>
<td>low</td>
<td>low</td>
</tr>
</tbody>
</table>

[ref (10)]

In *Xenopus* oocytes, the catalytic properties of all nine human isoform combinations have also been characterized (16). The \(\alpha_1\) isoform displayed the highest turnover rates and Na\(^+\) affinity while K\(^+\) activation varied depending on the \(\alpha\)-\(\beta\) combination (16). The difference in K\(^+\) activation was particularly pronounced in the comparison between the \(\alpha_2\beta_1\) and \(\alpha_2\beta_2\) isoymes, in which the \(\alpha_2\beta_1\) isozyme showed a more than 2 fold higher affinity for K\(^+\) than the \(\alpha_2\beta_2\) isozyme (16). Also, the \(\alpha_2\) isoform displayed higher Na\(^+\) affinity than the \(\alpha_3\) isoform (16). Voltage dependence was influenced by the \(\alpha\) isoform present, with \(\alpha_2\) showing a steeper voltage dependence than \(\alpha_1\) and \(\alpha_3\) being nearly voltage-independent (16). The
catalytic properties of the different human Na\(^+\)-K\(^+\) ATPase \(\alpha\) isoforms are summarized in Table 3.

<table>
<thead>
<tr>
<th></th>
<th>(\alpha_1)</th>
<th>(\alpha_2)</th>
<th>(\alpha_3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(V_{\text{max}})</td>
<td>highest</td>
<td>medium</td>
<td>lowest</td>
</tr>
<tr>
<td>Na(^+) affinity</td>
<td>highest</td>
<td>medium</td>
<td>lowest</td>
</tr>
<tr>
<td>K(^+) affinity</td>
<td></td>
<td></td>
<td>depends on (\alpha\beta) combination</td>
</tr>
<tr>
<td>voltage dependence</td>
<td>medium</td>
<td>highest</td>
<td>lowest</td>
</tr>
</tbody>
</table>

[ref (16)]

Specific cellular roles for the Na\(^+\)-K\(^+\) ATPase isoforms also appear to exist. In human soleus muscle, the \(\alpha_1\) isoform has been identified mainly in the sarcolemma while the \(\alpha_2\) isoform was distributed in both the sarcolemma and T-tubules (14). A similar distribution was observed in rat skeletal muscle (14). Furthermore, \(\alpha_2\), \(\beta_1\), and \(\beta_3\) subunits have been shown to populate intracellular membranes as well (48). The \(\alpha_2\) content always exceeds that of the \(\alpha_1\), ranging from \((\alpha_2: \alpha_1)\) 1.6 in surface membranes to 3-7 in internal membranes, and the \(\beta\) content always exceeds that of the \(\alpha\) (48). This suggests that the expression pattern of the Na\(^+\)-K\(^+\) ATPase isoforms is skewed in order to achieve optimal enzymatic activity in skeletal muscle.

Higher activity reported for the \(\alpha_2\) isoform when compared to the \(\alpha_1\) isoform (10) may indicate a greater role for the \(\alpha_2\) isoform during contractile activity. Given such observations, and the ubiquitous expression of the \(\alpha_1\beta_1\) complex, it has been hypothesized that the \(\alpha_2\) isoform is the major catalytic isoform (22). However, evidence from tissues other than skeletal muscle suggest that it is unlikely the \(\alpha_1\) isoform is strictly active during rest. In tissues such as the kidney and rat heart, where the \(\alpha_1\) isoform predominates, similar regulation of the Na\(^+\)-K\(^+\) ATPase is observed as in skeletal muscle (14). Thus, the \(\alpha_1\) isoform appears to respond to the same stimuli that have been attributed to increased \(\alpha_2\) pump
activity in skeletal muscle. Interactions with the surrounding membrane environment may explain some of the differences in catalytic activity reported between the $\alpha_1$ and $\alpha_2$ isoforms in skeletal muscle and discrepancies in the role for the $\alpha_1$ isoform in different tissues. A study using gene targeted mice lacking one copy of either the $\alpha_1$ or $\alpha_2$ isoform gene reported that reduction in the $\alpha_2$ isoform resulted in an increase in isometric force while reduction in the $\alpha_1$ isoform resulted in a decrease in isometric force (36). This illustrates that, although the exact roles of these two $\alpha$ isoforms are unclear, they do in fact play different roles in maintaining muscle cell excitability.

In summary, the differences in the catalytic properties of the different $\text{Na}^+\text{-K}^+$ pump isoforms suggests that the expression of multiple $\alpha$ and $\beta$ isoforms within a given cell is not redundant (70). Diversity in the isoform characteristics of the $\text{Na}^+\text{-K}^+$ ATPase allows for tissue and fibre type specific roles of the enzyme. Therefore, the ability of the $\text{Na}^+\text{-K}^+$ ATPase to maintain $\text{Na}^+$ and $\text{K}^+$ gradients, as determined by $V_{\text{max}}$ and protein abundance, will be influenced by the isoform combinations of the $\alpha$ and $\beta$ subunits. This allows for the possibility of differential regulation of these isoforms so that a given stimulus may independently influence the catalytic properties of the $\text{Na}^+\text{-K}^+$ pumps in different cellular compartments, fibres, or tissues.

**Measurement of $\text{Na}^+\text{-K}^+$ ATPase Properties**

The distinct properties associated with each isoform depend on differences in catalytic properties. As previously stated, the catalytic properties of interest are the $V_{\text{max}}$ and the $k_{50}$. The $k_{50}$ is the substrate concentration at which half $V_{\text{max}}$ is achieved. These properties can be measured *in vitro* using a kinetic assay of ATPase activity or through measurement of $\text{Na}^+$-
K⁺ pump current. The standard assay for measuring Na⁺-K⁺ ATPase activity in skeletal muscle homogenate is the K⁺ stimulated 3-O-methylfluorescein phosphatase (3-O-MFPase) assay. This is an indirect measure of activity, as the direct hydrolysis of ATP is not measured. Of all of the ATPases in skeletal muscle, the Na⁺-K⁺ ATPase accounts for <10% of the total ATP usage at rest and this percentage is not likely to increase during work (14). As such, it is difficult to measure direct hydrolytic activity in muscle homogenate without first purifying the sample. Purification of Na⁺-K⁺ ATPase fractions often leads to low recovery of the total Na⁺-K⁺ ATPase content and thus limits generalizations based on these findings (14); it is unknown if these yields constitute a representative sample of the total pump content. The 3-O-MFPase assay has been optimized as described by Fraser and McKenna (26) and later modified by Barr et al (4). This assay can provide measures of catalytic properties by measuring K⁺ stimulated 3-O-MFPase activity over increasing concentrations of K⁺ so as to develop a kinetic curve for Na⁺-K⁺ ATPase activity. However, the 3-O-MFPase assay is not without limitations. One major issue with this assay is the non-specific background activity. Variability in background activity can provide issues with obtaining reliable measures. This background is ~70% of the total measured activity. Thus, it would be preferred to measure Na⁺-K⁺ ATPase activity using a hydrolytic assay specific to the Na⁺-K⁺ ATPase in crude muscle homogenate.

**Na⁺-K⁺ ATPase Responses to Contractile Activity**

Based on the diversity of the Na⁺-K⁺ ATPase isoforms, it is not surprising that the Na⁺-K⁺ pumps from muscles composed of different fibre types respond differently to contractile stimuli. The demands introduced by contractile activity require the catalytic rate of the Na⁺-
K⁺ ATPase to be rapidly increased. The catalytic rate depends not only on protein-isoform abundance, but also on acute regulatory influences and translocation. Skeletal muscle contains a high concentration of Na⁺-K⁺ pumps that can be activated in response to contractile activity (57). In muscle, Na⁺-K⁺ ATPase activity is low at rest but increases dramatically within seconds of contractile activity due to activation by increased intracellular Na⁺ concentrations (57). This quick response is necessary to prevent rundown of Na⁺ and K⁺ gradients caused by the large influx of Na⁺ and efflux of K⁺ with membrane depolarization. A rundown of these gradients has been shown to cause reductions in tetanic force output in isolated rat Sol (60). Activation of the Na⁺-K⁺ pumps by means of salbutamol (β₂ agonist), insulin, or epinephrine resulted in recovery of force to 80-90% of the normal level (60). Using an in vitro method that stimulates the nicotinic ACh receptors at the motor end plate of an isolated muscle instead of methods that vary the Na⁺ and K⁺ concentrations in an incubation medium, a more physiological response of Na⁺ and K⁺ gradients can be mimicked (51). Under conditions mimicking intense exercise, the ability of the Na⁺-K⁺ ATPase to restore muscle excitability and contractility was confirmed through the use of salbutamol, epinephrine, and calcitonin gene-related peptide (51). These studies illustrate the importance of acute regulatory factor(s) activation of the Na⁺-K⁺ ATPase in restoring muscle excitability during contractile activity.

Repetitive Exercise and Na⁺-K⁺ ATPase Responses

A number of studies have investigated the acute response of Na⁺-K⁺ ATPase activity to various exercise protocols, in both rats and humans (23-25; 50). In general, these studies indicate a reduction of Vₘₐₓ with repetitive exercise. In rat, this pattern of reduced Vₘₐₓ has been observed across all muscle types [Sol, red vastus lateralis (RV), EDL, and white vastus
lateralis (WV)] (23). In humans, sustained, moderate-intensity, isometric knee extension exercise (24), heavy isokinetic exercise (25), and prolonged cycling exercise at 50% (68) and 75% (50) VO$_{\text{peak}}$ have resulted in reductions in $V_{\text{max}}$ of the enzyme at fatigue. Together, these studies show that fatiguing exercise reduces $V_{\text{max}}$ in muscle across a diverse range of exercise types, durations, and intensities (50). It has been suggested that this response to exercise is obligatory in the regulation of skeletal muscle homeostasis (25).

In contrast, repetitive contractile activity in rats, as performed by electrical stimulation (500 ms train at 30Hz every 1.5 seconds for 90 min) in vivo, has been shown to increase $V_{\text{max}}$ as measured on Sol muscle homogenates in vitro (69). Also, some studies performed in humans that have investigated M-wave (muscle compound action potential) properties with contractile activity have not reported depressions in M-wave properties as would be expected with decreased $V_{\text{max}}$. The M-wave is a measure of the excitability of the cell membrane and consequently an indirect measure of the ability of the Na$^{+}$-K$^{+}$ ATPase to maintain ion gradients (61). Increases in M-wave duration are associated with decreases in the conduction speed of an action potential across the sarcolemma and t-tubules (66). Decreases in M-wave amplitude and area are generally associated with reductions in the ability to restore Na$^{+}$ and K$^{+}$ gradients across the sarcolemma (66). Brief isometric maximum voluntary contractions (MVCs) in the adductor pollicis (8) and short duration fatigue in the plantar flexors through intermittent isometric contractions (5) found no decreases in M-wave properties. This suggests that not all contractile activity results in inactivation of pump activity; there appears to be a dependence on duration, intensity, and type of activity in the response of the Na$^{+}$-K$^{+}$ pumps. Muscle excitability is not commonly compromised during volitional exercise if fatigue is induced by high intensity, short duration
contractions; however, contractions repeated for longer durations appear to induce greater reductions in $V_{\text{max}}$ or membrane excitability (24).

Whereas measurements of $V_{\text{max}}$ in vitro suggest a pattern of pump inhibition with contractile activity, the physiological response of the Na$^+$-K$^+$-ATPase to contractile activity is activation. This discrepancy illustrates the difference between the measurement of enzyme capacity ($V_{\text{max}}$) and in vivo activity. Although Na$^+$-K$^+$-ATPase activity may remain higher at fatigue when compared to rest, its maximal capacity of transport has decreased. As mentioned previously, increased intracellular Na$^+$ concentration is a potent stimulus to increase Na$^+$-K$^+$ pump activity within seconds of membrane depolarization (57). Translocation may be one of the mechanisms for increasing Na$^+$-K$^+$ ATPase activity. Transient translocation of pump subunits from intracellular membranes to the plasma membrane can be induced by both contractile activity and insulin (38; 43; 44; 53; 72). These studies generally utilize fractionation techniques to identify increases in sarcolemmal Na$^+$-K$^+$ pump content and subsequent decreases in the Na$^+$-K$^+$ pump content of intracellular stores. In humans, short, high intensity exercise induces the translocation of $\alpha_2$ and $\beta_1$ subunits (44) while in rats both long-lasting low-intensity running and short-lasting high-intensity contractions induce translocation of all isoforms present, in both oxidative and glycolytic fibres (43). However, Sandiford et al (69) demonstrated translocation of $\alpha_1$ and $\alpha_2$ subunits but no translocation of any $\beta$ subunits in rat soleus using in vivo electrical stimulation. Translocation was associated with increased $\alpha_2$ Tyr phosphorylation (69). Interestingly, it has been reported that the translocation of $\alpha_2$ and $\beta_1$ subunits following insulin injection was restricted to muscles composed of mostly oxidative fibres (49), which
is contrary to observations during contractile activity. Thus, there may be differences between contractile and insulin-induced translocation.

Although strong evidence exists that translocation is a mechanism for Na\(^+-\)K\(^+\) ATPase activation during contractile activity, it is unclear as to what isoforms undergo translocation, the mechanism through which translocation occurs, and whether it is individual isoforms or functional heterodimers that translocate.

*Changes in Na\(^+-\)K\(^+\) pump Expression in Response to Contractile Activity*

The stimulation protocol employed by Sandiford et al (69) induced an increase in \(\alpha_1\) protein by 90 min of stimulation, providing evidence that just a single bout of exercise provides enough stimulus to increase the expression of selected \(\alpha\) subunits. This illustrates the highly malleable nature of the Na\(^+-\)K\(^+\) ATPase. Evidence for such rapid responses to stress exists in humans as well. It has been shown that just 6 min of intense knee extensor exercise upregulates the mRNA expression of all six Na\(^+-\)K\(^+\) ATPase isoforms in VL, but not protein expression (56). Thus, a very brief stimulus is sufficient to initiate a cellular response towards increasing Na\(^+-\)K\(^+\) pump content. A follow up study that used this same exercise protocol reported a transient depression in \(V_{\text{max}}\) from rest to fatigue that recovered by 3 hours post-exercise (62). Depressed \(V_{\text{max}}\) was inversely correlated to the increase in expression of \(\alpha_1\) and \(\alpha_2\) mRNA, which suggests that reversible depression in muscle \(V_{\text{max}}\) with fatiguing exercise may act as a stimulus to increase muscle Na\(^+-\)K\(^+\) ATPase gene expression (62).

More long term training studies, performed in humans, have reported increased protein turnover. Using a 6 day training model of cycle exercise at 60-65% \(VO_2\) \(_{\text{peak}}\) for 2 hours/day, Green et al (31) reported an increase in pump content by day 3, but no increase in
V\textsubscript{max} until day 6. Analysis of isoform content revealed that only \( \alpha_2 \) had increased by 3 days of training but \( \beta_1 \) increased after 6 days of training (31). Thus, an increase in the content of one subunit alone is not enough evidence to conclude increased catalytic function; measurements of activity are required to assess whether increases in protein turnover result in increases in functional heterodimers. Other training protocols also increase pump content. For example, resistance training for 30 min, 3 times per week for 16 weeks was sufficient to increase \( \alpha_1, \alpha_2, \) and \( \beta_1 \) protein content (17). Sprint training (17) and cycle (60-65% VO\textsubscript{2peak}) training (31) have also been shown to increase the content of Na\textsuperscript{+}-K\textsuperscript{+} pumps. Training infers protective effects on V\textsubscript{max}. It has been shown, in well-trained athletes, that chronic intermittent hypoxia does not depress V\textsubscript{max} as previously shown in untrained individuals (2). In theory, the increase in pump content that occurs with training infers upon the cell a greater potential to maintain Na\textsuperscript{+} and K\textsuperscript{+} gradients through an increase in pumping capacity. This, in turn, could delay time to fatigue with exercise in trained individuals.

**Regulation of the Na\textsuperscript{+}-K\textsuperscript{+} ATPase**

Given the requirement for rapid modulation of Na\textsuperscript{+}-K\textsuperscript{+} ATPase activity, it is not surprising that this enzyme appears to be under complex regulatory control, both acute and long-term. Acute regulation involves either direct effects on the kinetic behaviour of the enzyme or translocation of Na\textsuperscript{+}-K\textsuperscript{+} pumps from intracellular membranes to the plasma membrane (73). One key mechanism through which this regulation is carried out in the skeletal muscle cell is second messenger mediated kinase and phosphatase activity (70; 73). Residues specific to a given isoform can be covalently modified, which in turn can induce conformational changes that influence enzyme catalytic properties or availability. The major stimuli for such changes
are hormones, but other factors such as substrate availability, metabolic by-products, and redox state of the cell may also modify Na\(^+\)-K\(^+\) pump function. Long term regulation generally involves alterations in de novo synthesis or degradation (73), in which hormones may also play a role.

The major signaling cascades associated with Na\(^+\)-K\(^+\) pump modulation are:

1) adenosine 3’-5’-cyclic monophosphate (cAMP) activation of protein kinase A (PKA)

2) the diacylglycerol (DAG – formed by phospholipase C (PLC)) activation of protein kinase C (PKC)

3) free intracellular Ca\(^{2+}\) activation of calmodulin (CaM) kinase

4) guanosine 3’-5’-cyclic monophosphate (cGMP) activation of protein kinase G (PKG)

[ref (70; 73)]

Further downstream modulators of Na\(^+\)-K\(^+\) properties include tyrosine kinases, protein phosphatases 1, 2A and 2B (PP1, PP2A and PP2B respectively), and phospholipase A\(_2\) (PLA\(_2\)) (73). There is evidence to suggest that signaling cascades activating kinases correlate with inhibition of pump activity and signaling cascades activating phosphatases correlate with stimulation of pump activity (69). This does not mean that all protein kinases inhibit pump activity; for example, PKC has been reported to both activate and inhibit pump activity depending on tissue and isoform expression (69). Thus, different responses to signaling cascades are possible depending on tissue, isoform diversity (in both the signaling proteins and the target proteins), and terminal action of the cascade (i.e. phosphorylation or dephosphorylation).
Different phosphorylation sites on the α isoform have been identified for PKA and PKC (69), and this most likely also plays a role in whether pump activity is stimulated or inhibited. The main site of PKA stimulated phosphorylation is serine (Ser)-943 (10). PKA inhibits the pump through direct phosphorylation of the E1 state (73). However, it was recently shown in COS cells that the concentration of Ca^{2+} ions is important in determining whether PKA stimulates or inhibits Na^+-K^+ ATPase activity (73). In various systems, PKA has been shown to inhibit Na^+-K^+ ATPase activity by activating PLA_2, activating a protein phosphatase inhibitor, and phosphorylating monomeric actin (73). This same pathway has been shown to activate Na^+-K^+ ATPase activity by phosphorylating polymeric actin (73). There also appears to be isoform specific effects of PKA activation. PKA activation in insect cells expressing rat Na^+-K^+-ATPase isoforms stimulated pump activity of α_3β_1 but inhibited pump activity of α_1β_1 and α_2β_1 isozymes (10).

PKC phosphorylation sites have been identified at Ser-16, Ser-23, and threonine (Thr)-15, although Ser-16 is only present in the α_1 isoform and Thr-15 is only present in the α_2 isoform (10; 13). The effects of PKC are tissue specific and depend on the isoform of PKC involved (73). In the kidney proximal tubule PKC has been reported to inhibit Na^+-K^+ pump activity, both through activation of the PLA_2 pathway and through causing endocytosis of pumps via direct phosphorylation (73). There are multiple putative cytosolic PKC phosphorylation sites on the Na^+-K^+ ATPase α subunit, with the site of phosphorylation appearing to be important in whether PKC activates or inhibits pump activity (73). There is also evidence that phosphorylation occurs preferentially in the E2 conformation (73).

Tyrosine phosphorylation by non-receptor tyrosine kinase c-src appears to occur at tyrosine (Tyr)-10 on the α_1 isoform and on Tyr-543 on the α_2 isoform (13). PKG, activated
by increases in cGMP, inhibits Na\(^{+}\)-K\(^{+}\) pump activity in skeletal muscle (73). Regulation by these processes is not as well understood as those by PKA and PKC.

The FXYD1 protein phospholemman (PLM) also elicits a regulatory role on skeletal muscle Na\(^{+}\)-K\(^{+}\) ATPase function; however, this role is not well defined. In native skeletal muscle tissue, PLM has been shown to associate with the \(\alpha_1\beta\)-isozymes and less efficiently with \(\alpha_2\beta\)-isozymes (15). In Xenopus oocytes, interaction of PLM with Na\(^{+}\)-K\(^{+}\) pumps results in a small decrease in extracellular K\(^{+}\) affinity and a nearly 2 fold decrease in intracellular Na\(^{+}\) affinity (15). It has been suggested that reduced Na\(^{+}\) affinity of the Na\(^{+}\)-K\(^{+}\) pumps associated with PLM is necessary for efficient muscle contractility; maximal transport rates are not achieved before sufficient depolarization and the capacity to increase rates with further increases in Na\(^{+}\) exists (15). However, studies in PLM-deficient mice suggest that PLM stimulates rather than inhibits the Na\(^{+}\)-K\(^{+}\) ATPase, as the Na\(^{+}\) affinity was not different between wild type and PLM-deficient mice (29). PLM phosphorylation has been reported through PKA and PKC mediated pathways (15).

In mouse ventricular myocytes, PKA phosphorylates PLM at Ser-68 and PKC phosphorylates PLM at both Ser-63 and Ser-68 (33). PKC activation leads to phosphorylation of PLM at Ser-63 and Ser-68 and increased \(V_{\text{max}}\), without altering Na\(^{+}\) affinity (33). This effect is not observed in PLM knockout mice (33). Furthermore, this effect has been shown to be to \(\alpha_2\beta\) and not \(\alpha_1\beta\) isozymes (7). PKA activation increased PLM phosphorylation at Ser-68 and increased Na\(^{+}\) affinity (but not \(V_{\text{max}}\)), an effect that was additive with that of PKC (33). This effect is similar in both \(\alpha_2\beta\) and \(\alpha_1\beta\) isozymes (7). Thus, although the functional effects of PLM on Na\(^{+}\)-K\(^{+}\) ATPase activity in skeletal muscle remain
unclear, evidence from cardiac myocytes suggests that PLM may play a key role in regulating the effects of signaling cascades, in particular PKC.

Figure 1: Overall summary of messenger signaling influences on the Na\(^+\)-K\(^+\) ATPase

**(Hormonal Regulation of Na\(^+\)-K\(^+\)-ATPase Function)**

Two key hormones involved in the long term regulation of muscle Na\(^+\)-K\(^+\)-ATPase are aldosterone and thyroid hormone. Aldosterone is involved in long term regulation of Na\(^+\)-K\(^+\) pump biosynthesis (73). This hormone directly interacts with receptor complexes on nuclear DNA to sustain long-term increases in expression of \(\alpha_1\) and \(\beta_1\) isoforms of the Na\(^+\)-K\(^+\) ATPase (73). There is evidence that cAMP-inducible factors mediate this effect and that this effect can be abolished by calcineurin (PP2B) inhibition (73). Thyroid hormone is a long term regulator of steady state Na\(^+\)-K\(^+\) ATPase activity (70). Through effects on gene
transcription, mRNA stability, and transport of subunits from the nucleus to the cytosol, thyroid hormone stimulates pump activity by increasing the number of pumps without altering the catalytic properties of the enzyme (70). This regulation is isoform specific; for example, in rat, it is the $\alpha_2$ and $\beta_2$ isoforms that are upregulated with thyroid hormone treatment (70).

The catecholamines have been shown to have marked effects on the Na$^+$-K$^+$ ATPase. Epinephrine (Epi) has been shown to increase pump activity in rat skeletal muscle as measured through active Na$^+$-K$^+$ transport, presumably through increased cAMP and activation of the PKA pathway (70). This effect leads to membrane hyperpolarization, which is more pronounced in type I fibres when compared to type II fibres (14). Norepinephrine (Norepi) stimulates pump activity and hyperpolarization in liver and kidney tissue (70; 73), as well as in skeletal muscle (14). This mechanism involves a Norepi-induced increase in intracellular free calcium concentration ([Ca$^{2+}$]$_i$), which activates calcineurin, keeping the pumps in an active, dephosphorylated state (73).

Modulation of Na$^+$-K$^+$ ATPase activity by Epi and Norepi involves both $\alpha$ and $\beta$ adrenergic receptors. Generally, $\beta$-adrenergic stimulation is associated with cAMP and PKA activation while $\alpha$-adrenergic stimulation is associated with PKC activation (73). The significance of catecholamine induced increases in Na$^+$-K$^+$ pump activity has been well documented. During exercise, plasma catecholamine concentrations increase in an intensity-dependent manner (34). Recent evidence suggests that $\beta_2$ adrenergic stimulation causes a rapid increase in the affinity of Na$^+$-K$^+$ pumps for intracellular Na$^+$ through measurement of $^{86}$Rb$^+$ uptake (11). This increased affinity does not require Na$^+$ influx but does appear to require the generation of action potentials (11). Such a stimulus was shown to substantially
restore tetanic force in isolated Sol and EDL muscles during electrical stimulation that mimicked intense exercise and loss of cellular integrity (34; 55). Improved contractility was associated with membrane hyperpolarization (34; 55). Thus, the increase in catecholamines observed during exercise appears to contribute to maintaining cell excitability and contractility through sensitization of the Na\(^+-\)K\(^+\) ATPase to Na\(^+\).

**Insulin Regulation of Na\(^+-\)K\(^+\)-ATPase Function**

Peptide hormones are another major class of Na\(^+-\)K\(^+\) ATPase regulators, the most studied of these being insulin. Insulin has been reported to stimulate Na\(^+-\)K\(^+\) ATPase activity in rat skeletal muscle and to induce hyperpolarization of the cell (14). Studies in rat skeletal muscle cell lines have associated this effect with activation of PKC and dephosphorylation of the \(\alpha_1\) subunit, mediated by a Ser-Thr protein phosphatase (14). In HEK-293 cells, insulin induced rat \(\alpha_1\) isoforms to undergo translocation (72). Evidence suggests this translocation involves decreasing serine phosphorylation via PKC and phosphatidylinositol-3-kinase (PI3K) (72).

The major mechanism of insulin action in skeletal muscle is through the triggering of pump translocation from intracellular stores to the plasma membrane (73). Using fractionation and immunohistochemistry, it has been shown that \(\alpha_2\) and \(\beta_1\) subunits increase in abundance at the muscle surface with insulin stimulation (38; 53). The differentiation between translocation to the sarcolemma and T-tubule has not been determined. The time course for this action is 15-30 minutes (70). In rat skeletal muscle this effect appears to be specific to oxidative fibres (type I and IIA) and to the \(\alpha_2\) and \(\beta_1\) isoforms (49).

Other mechanisms of insulin action have also been identified. Insulin indirectly stimulates Na\(^+-\)K\(^+\) ATPase activity through insulin-induced opening of Na\(^+\) transport.
channels and elevation in intracellular Na\(^+\) (70). Also, insulin has been reported to increase the Na\(^+\)-K\(^+\) ATPase affinity for Na\(^+\) in kidney cortical tubules; however, this finding has not been extrapolated to muscle (73). Such a mechanism was proposed by McKenna et al (54) to occur in skeletal muscle after they failed to find evidence of insulin-stimulated pump translocation using the \(^3\)H-ouabain binding technique (54). Ouabain, which is a cardiac glycoside that binds irreversibly to the \(\alpha\) subunit in a 1:1 molecular stoichiometry (14), is used to measure total Na\(^+\)-K\(^+\) pump content (\(\beta_{max}\)) in resting muscle (22). The assumption with this measure is that ouabain binding is selective to the \(\alpha\) isoforms in the sarcolemma (69). However, it is possible that ouabain penetrates to the subsarcolemmal space; it has been shown that ouabain causes the internalization of \(\alpha\) subunits within one hour of ouabain exposure (75). Thus, this measure may not be appropriate for detecting changes in Na\(^+\)-K\(^+\)-ATPase cellular distribution. Given growing evidence supporting translocation and the lack of evidence for insulin-induced increases in Na\(^+\) affinity in muscle, the assessment of pump translocation as the major mechanism of insulin action in skeletal muscle seems appropriate.

The rapid action of insulin is triggered through the insulin receptor signaling cascade (70). The insulin receptor tyrosine kinase stimulates atypical PKC activation, which results in the transient phosphorylation of the Na\(^+\)-K\(^+\) ATPase \(\alpha\) subunit (13). Chibalin et al (13) reported transient translocation and increased phosphorylation of Na\(^+\)-K\(^+\) pumps by PKC with insulin treatment of rat Sol (13). Since the insulin-mediated modifications to the Na\(^+\)-K\(^+\) ATPase resulted in increased \(V_{max}\) in plasma membrane fractions but not in muscle homogenate, this suggests that insulin did not alter the catalytic properties of the pump (13). Phosphoamino acid analysis of Sol revealed that phosphorylation of the \(\alpha\) subunit occurred primarily on Ser residues, with a small amount of Thr phosphorylation (13). Tyrosine
phosphorylation was detected in Sol when incubated with insulin (13). Based on the time course of phosphorylation, it was hypothesized that tyrosine (Tyr) residue phosphorylation was responsible for the translocation effect with the \( \alpha_2 \) isoform but not with the \( \alpha_1 \) isoform, and specifically the Tyr-543 residue was postulated as a probable target for PKC (13). It was hypothesized, based on sequence analysis, that tyrosine phosphorylation of the \( \alpha_2 \) subunit at Tyr-543 may prevent endocytosis of pumps by blocking a clathrin mediated endocytic motif (13). A role for PLM in mediating insulin-dependent PKC influences on pump function in skeletal muscle has not yet been considered. Given recent evidence that PLM mediates pump stimulation by PKC in cardiac myocytes (33), studies that investigate a role for PLM in the regulation of insulin-dependent stimulation of pump activity are needed.

In summary, evidence suggests that translocation is specific to the \( \alpha_2 \) isoform of the \( \text{Na}^+-\text{K}^+ \) ATPase; however, a role for the \( \alpha_1 \) and \( \beta_1 \) isoforms cannot yet be discounted. It remains unclear what isoforms undergo translocation, the exact mechanism through which translocation occurs, and whether it is individual isoforms or functional heterodimers that translocate. Apart from PKC, roles for Protein Kinase A (PKA) (70) and PLM in mediating translocation cannot be discounted. Downstream of the insulin receptor tyrosine kinase, a role for PKC, protein phosphatase activation, and pump phosphorylation have all been implicated in insulin mediated regulation of \( \text{Na}^+-\text{K}^+ \) pump activity (73).
Summary of Na\(^+\)-K\(^+\)-ATPase Regulation

The elucidation of regulatory influences on Na\(^+\)-K\(^+\) ATPase function remains a growing concern. The precise mechanisms of Epi and insulin action through PKA and PKC pathways are incomplete. Specific isoforms of PKC involved in insulin-induced translocation are speculative. Furthermore, it is unknown if there are muscle fibre type differences with regards to the different isoforms of signaling molecules involved in the messenger pathways and the sensitivities of these pathways to hormone stimulation. Given the apparent fibre type specific response of insulin-induced translocation, it is very feasible to hypothesize that Na\(^+\)-K\(^+\) pump regulation is both isoform and fibre type specific.

Substrate Utilization by, and Energy Supply of, the Na\(^+\)-K\(^+\) ATPase
It appears that the properties of the Na\(^+\)-K\(^+\) ATPase, specifically \(V_{\text{max}}\), correlate with the oxidative potential of muscle fibres more so than with contractile speed of the fibres (22). It has been shown that both the \(\alpha\) subunit content and the oxidative potential of the muscle fibre are upregulated within days following the onset of regular activity (30; 31). Given this relationship, it is inviting to suggest that the Na\(^+\)-K\(^+\) ATPase derives its energy from oxidative phosphorylation. However, evidence suggests that the energy requirements of the Na\(^+\)-K\(^+\) ATPase are supplied preferentially through glycolysis (41; 42).

Increased lactate production is evidence of increased glucose metabolism by the glycolytic pathway and has been reported in the presence of oxygen (41). Decreased skeletal muscle membrane potential and increased intracellular Na\(^+\) concentrations have been described in experimental models of sepsis and shock, conditions in which lactate production is abnormally high (41). It has been shown that the rate of lactate production can be reduced in the presence of ouabain (41). Co-localization between the glycolytic enzymes and the Na\(^+\)-K\(^+\) pumps has been proposed to account for this association (41). The coupling of Na\(^+\)-K\(^+\) ATPase activity to glycolysis has been observed in glioma cells, brain astrocytes, diaphragm, and smooth muscle (41). Incubation of Sol and EDL muscles in media designed to increase intracellular Na\(^+\) stimulated glycolysis and increased lactate production in proportion to the increase in Na\(^+\)-K\(^+\) ATPase activity (41). When glucose was replaced with pyruvate as the sole energy source no increase in lactate production was observed, indicating that glucose was essential for stimulation of the Na\(^+\)-K\(^+\) pumps (41).

When the effects of Epi on Na\(^+\)-K\(^+\) ATPase activity and glycolysis were examined, it was reported that Epi treatment significantly increased lactate production and decreased intracellular Na\(^+\) in a dose dependent manner in both Sol and EDL (42). Both lactate
production and Na⁺-K⁺ transport were inhibited by ouabain (42). These measures were of whole muscle lactate; it would be ideal to measure subsarcolemmal lactate production to identify the coupling of glycolysis to Na⁺-K⁺ pump function. Epi also caused a significant reduction in muscle glycogen in both Sol and EDL that was significantly inhibited by ouabain (42). Also, a study investigating the role of Na⁺-K⁺ pumps in insulin-induced lactate release by skeletal muscle reported that stimulation of Na⁺-K⁺ ATPase activity by hyperinsulinemia was associated with increased lactate production in skeletal muscle (58).

Direct support for a protective role of glycogen in maintaining fibre excitability was reported in mechanically skinned fibres, in which higher glycogen content conferred an increased ability to respond to T-system depolarization (3). This demonstrates the Na⁺-K⁺ ATPase dependence on glucose for ATP. At rest, this dependence was shown to be specifically through glycolysis and not oxidative phosphorylation in a study that used oxidative and glycolytic inhibition to show that oxidative inhibition did not alter intracellular Na⁺ or K⁺ content while blocked glycolysis dramatically increased intracellular Na⁺ (59). This suggests that, normally, glycolysis is required to fuel the Na⁺-K⁺ ATPase.

Fatigue associated with prolonged sub-maximal exercise has been shown to correlate with muscle glycogen depletion (6). Furthermore, carbohydrate (CHO) ingestion increases exercise time to fatigue during prolonged exercise (45). Since glycolysis is required for Na⁺-K⁺ pump function and muscle glycogen stores are limited in the muscle, it is possible that supplements containing glucose could prolong exercise time through protection of glycogen stores. This effect has been shown during running exercise at 70% VO₂peak (74) when glucose supplementation began immediately before exercise. However, it was recently reported that glucose supplementation during prolonged cycle exercise at 60% VO₂peak
humans had no glycogen sparing effect in skeletal muscle (19). Glucose supplementation started after 30 min of exercise (19). Thus, it appears as though there may be an intensity and timing dependency for a glycogen sparing effect of glucose supplementation.

In rat plantaris (mixed fibre type composition), glucose infusion during prolonged indirect electrical stimulation in situ did not protect muscle glycogen stores even though fatigue was attenuated (45). Instead, the attenuation of fatigue was associated with better maintenance of M-wave characteristics, suggesting that the beneficial effect of glucose infusion could at least partly be due to a better maintenance of the electrical properties of the sarcolemma (45). This observation allows one to speculate that the increased plasma glucose could have increased the Na\(^+\)-K\(^+\) ATPase activity by providing ATP produced by glycolysis (45). There was also a marked increase in plasma insulin concentration with the glucose infusion, which introduces the possibility that the protection of cell excitability was through insulin stimulation of Na\(^+\)-K\(^+\) ATPase activity (45). However, a subsequent study that increased insulin concentration independent of plasma glucose during the same prolonged indirect electrical stimulation protocol did not observe the same attenuation of muscle fatigue as with glucose infusion (46). Thus, it was concluded that the high plasma glucose concentration and/or its delivery to the muscle protected sarolemmal excitability (46).

Given the biochemical differences between type I and type II fibres, it was hypothesized that glucose infusion could protect both muscle function and muscle glycogen in Sol (52). However, even in type I fibres there was no glycogen sparing effect of glucose infusion; glucose infusion during prolonged indirect electrical stimulation of rat Sol muscle in situ did not attenuate glycogen utilization despite attenuating muscle fatigue (52). Attenuation of muscle fatigue was greater in Sol than in plantaris (52). Together, this data
suggests that a role may exist for the Na\(^+\)-K\(^+\) ATPase in attenuating muscle fatigue during glucose infusion through glucose stimulated increases in Na\(^+\)-K\(^+\) ATPase activity to protect M-wave characteristics. However, since glycogen utilization is not altered with this stimulation protocol, this suggests that the infused glucose is not being used as the primary substrate for ATP production. Infused glucose may act as the substrate for increased ATP production to accommodate glucose-induced increases in Na\(^+\)-K\(^+\) ATPase activity. Na\(^+\)-K\(^+\) ATPase activity has not been directly measured with this protocol; thus, it is purely speculation that the glucose stimulated an increase in Na\(^+\)-K\(^+\) ATPase activity. Promising results in this regard have been obtained in humans where glucose supplementation during prolonged cycle exercise resulted in a transient increase in Na\(^+\)-K\(^+\) ATPase activity (32).

**Relationship between Na\(^+\)-K\(^+\)-ATPase and GLUT4 regulation**

The glucose transporter 4 (GLUT4) protein is the major transporter of glucose into the muscle cell (28). This protein is a facilitated transporter of glucose. It is concentrated in intracellular membranes but is rapidly translocated to the plasma membrane during both exercise and insulin stimulation (28). Interestingly, the regulation of the Na\(^+\)-K\(^+\)-ATPase (specifically the \(\alpha_2\) subunit) and the GLUT4 transporter is remarkably similar. These proteins are found both in sarcolemmal and intracellular membranes, and exercise and insulin stimulation induce translocation of both proteins from intracellular sites to the sarcolemmal membrane (27; 40; 53). However, there is segregation of the GLUT4 and Na\(^+\)-K\(^+\)-ATPase intracellular pools (47). Given common responses to insulin stimulation, growing evidence that the Na\(^+\)-K\(^+\)-ATPase depends greatly on glucose as a substrate, and the tightly regulated nature of glucose metabolism, it may be hypothesized that the Na\(^+\)-K\(^+\)-
ATPase and GLUT4 proteins share common and/or co-regulated signaling pathways. Evidence for such a link may be drawn from a study by Ramlal et al (64), which involved transgenic mice over-expressing human GLUT4. An increase in GLUT4 content in both intracellular and sarcolemmal membranes, a greater insulin response, and a higher glucose load tolerance were observed. This was associated with a decreased Na\(^+\)-K\(^+\)-ATPase content but a higher translocation of \(\alpha_2\) and \(\beta_1\) subunits (64). Furthermore, in animal models of diabetes, decreased Na\(^+\)-K\(^+\)-ATPase activity and altered isoform expression are commonly observed. These conditions are not always reversed through insulin therapy (71). This suggests that the Na\(^+\)-K\(^+\)-ATPase may also display insulin resistance. Such a condition could become implicated in diabetic complications such as obesity and neuropathy since a decrease in pump response to insulin would result in lower activity and less ATP consumption (71).

The mechanism of GLUT4 transport in response to insulin stimulation has been investigated to a greater extent than that of the Na\(^+\)-K\(^+\)-ATPase. It has been demonstrated that GLUT4 responds to insulin stimulation via 2 independent pathways: one that induces translocation and one that increases intrinsic activity (28). The translocation pathway, including the mapping of vesicular trafficking for GLUT4, has been a topic of intense investigation. One regulatory element that appears to be shared for both proteins is PI3 kinase (71). However, downstream of PI3 kinase, it is not clear if signaling molecules are of shared or divergent pathways. Although upstream signals may be shared, there is likely a divergence of downstream pathways specific to each protein.

The pathway leading to increased intrinsic activity of GLUT4 is less understood. For some time insulin activation of GLUT4 intrinsic activity was believed to be directed by
mitogen activated protein kinase (MAPK) (28). However, decreased insulin-stimulated glucose uptake was observed in the presence of drugs that inhibit MAPK, even in the presence of drug resistant MAPK (1). Thus, although there appears to be 2 distinct pathways of insulin stimulation of GLUT4, the mechanism through which insulin increases intrinsic activity remains unclear. However, the existence of such a pathway suggests a similar mechanism may be present for the Na\(^+\)-K\(^+\)-ATPase.

**Rationale for this Study**

The aim of this study is to investigate the acute regulation of the Na\(^+\)-K\(^+\) ATPase in muscles of different fibre type composition in response to insulin. Some work done with *in vivo* (49) and *ex vivo* (13) systems suggest that insulin stimulates Na\(^+\)-K\(^+\)-ATPase activity solely through a translocation mechanism in skeletal muscle. This effect occurs within 15-30 min of insulin exposure (70). These previous studies examined only \(V_{\text{max}}\) and a thorough analysis of Na\(^+\)-K\(^+\)-ATPase behaviour has yet to be performed. The focus of this thesis is to determine the effects of acute insulin treatment on the kinetic characteristics of Na\(^+\)-K\(^+\)-ATPase activity *in vitro*. The use of an *in vitro* model allows for the control of all experimental conditions so that the specific effects of insulin can be isolated without speculation on possible confounding variables.

Specific consideration will be given to the possibility that insulin stimulates increased intrinsic pump activity. To-date, no studies have supported such a finding in skeletal muscle; however, insulin has been shown to increase Na\(^+\) affinity in kidney cortical tubules (73). It is possible that acute stimulation of intrinsic Na\(^+\)-K\(^+\)-ATPase activity occurs in a shorter time frame than that of translocation. Thus, a mechanism to increase activity
with insulin stimulation both immediately (intrinsic activation) and over longer durations (translocation) would be in place. Experiments involving insulin exposure of 15-30 min would thus not detect increases in intrinsic activity. Intrinsic activity may also be increased throughout insulin exposure. Because previous studies detected increases in $V_{\text{max}}$ in sarcolemmal fractions following insulin exposure, but not in muscle homogenate, it has been concluded that the increased activity was due to a translocation mechanism not detectable in homogenate (13). This infers that the population of pumps in the endosomal membranes is active. Since $V_{\text{max}}$ did not increase in homogenate, insulin must not have altered intrinsic activity. However, it is possible that insulin had an isoform specific effect that was masked by the stimulation of all pumps; increases in intrinsic activity may have been masked by the large population of pumps being measured versus small increases in intrinsic activity. Thus, the increase in $V_{\text{max}}$ reported in sarcolemmal fractions may in fact be a combination of increased intrinsic activity and translocation. Evidence for such a possibility has been recently found in a model that uses cyclic stretch of cultured skeletal muscle cells to increase pump activity (76). This study detected increased $V_{\text{max}}$ and translocation in homogenates using the 3-O-MFPase assay (76). Furthermore, since previous studies have only investigated insulin-induced increases in $V_{\text{max}}$, it is possible that insulin alters the sensitivity of the pumps.

Using fractionation techniques and in vitro insulin exposure, the effect of insulin on Na$^+$$-K^+$-ATPase activity in the absence of any translocation event can be examined. The possibility of translocation is eliminated through fractionation, which separates the sarcolemmal and endosomal pools of Na$^+$$-K^+$ pumps. Acute insulin exposure can then be administered. In this way it can be determined if the role of insulin in Na$^+$$-K^+$-ATPase
regulation is purely through translocation or through a combination of increases in intrinsic activity and translocation. Treatment of muscle homogenates with insulin prior to fractionation will be used to mimic previous *in vivo* and *ex vivo* studies of insulin exposure. This condition can be used to determine if the *in vitro* insulin treatment induces translocation. Na⁺-K⁺-ATPase α₂ and GLUT4 content will be probed in each fraction for evidence of translocation. Furthermore, it can be examined whether translocation induces an increase in Na⁺-K⁺-ATPase activity in the sarcolemmal fraction as previously reported. Acute insulin exposure in these fractions can then determine whether or not insulin stimulates further increases in Na⁺-K⁺-ATPase activity over and above any increases that result from homogenate incubation with insulin prior to fractionation. In this way, the contributions of translocation and increased intrinsic activity can be evaluated.

In order to hypothesize a possible mechanism for insulin action on the Na⁺-K⁺-ATPase, α₂ tyrosine phosphorylation will be probed. It has been previously reported that Sol α₂ isoform undergoes tyrosine phosphorylation with insulin exposure *ex vivo* (13). Homogenates treated with insulin can be probed for α₂ tyrosine phosphorylation to determine if *in vitro* insulin exposure also increases α₂ tyrosine phosphorylation.

Thus, using *in vitro* techniques to assess Na⁺-K⁺-ATPase function, the ability of insulin to stimulate Na⁺-K⁺-ATPase activity through intrinsic activation and translocation can be evaluated. Furthermore, a role for α₂ tyrosine phosphorylation in the signaling of these processes can be investigated. Also, given differences in oxidative potential and isoform distribution in muscles of different fibre types (22), the effect of insulin to stimulate Na⁺-K⁺-ATPase will be examined in Sol, RG, and WG muscles.
Statement of the Problem

The purpose of this study is to examine the *in vitro* effects of insulin on Na\(^+\)-K\(^+\)-ATPase activity in skeletal muscles of different fibre types.

Subsidiary Problem

A secondary objective is to examine the role of the *in vitro* protocol in stimulating intrinsic activity and possible mechanisms through which insulin stimulation of the Na\(^+\)-K\(^+\)-ATPase occurs.

Hypotheses

1. It is hypothesized that insulin will stimulate an increase in Na\(^+\)-K\(^+\)-ATPase activity in muscle homogenates, as measured with the 3-O-MFPase assay.
2. Fractionation will result in 2 active pools of Na\(^+\)-K\(^+\) pumps: a sarcolemmal and an endosomal pool.
3. Insulin exposure in sarcolemmal and endosomal fractions will result in increases in Na\(^+\)-K\(^+\)-ATPase activity.
4. Pre-treatment of homogenates with insulin prior to fractionation will result in a higher Na\(^+\)-K\(^+\)-ATPase activity in these insulin-treated fractions, when compared to non-treated fractions.
5. Pump activity in pre-treated fractions will not be further increased with acute insulin exposure.
6. \(\alpha_2\) tyrosine phosphorylation will be associated with translocation. As such, it was expected that \(\alpha_2\) tyrosine phosphorylation will increase in muscle homogenates with insulin incubation.
Chapter Two

Methods

Animals

Twelve week old male Sprague Dawley rats (n=14) were used for all experimental procedures. The animals, weighing 378 ± 4.98 g (mean ± SE), were provided with food and water ad libitum and maintained on reverse light-dark cycles. Sampling was performed in the mid-morning. Animals were anaesthetized with a dose of sodium pentobarbital (−0.1mL/100g body wt) (Somnotol, MTC Pharmaceuticals, Cambridge, ON, Canada) and soleus (Sol), red gastrocnemius (RG), and white gastrocnemius (WG) muscles were excised. Careful consideration was taken to excise only pure red and pure white regions of the gastrocnemius. These regions contain predominately Type I/IIA and Type IIB fibres, respectively (18). Soleus is composed of predominately Type I fibres (18). Connective tissue was removed and muscles were frozen in liquid nitrogen until further analysis.

Experimental Design

Three different experiments were conducted in order to examine the role of insulin in stimulating Na⁺-K⁺-ATPase activity. The experimental design is summarized in Figure 3.

In Experiment 1, pump activity was measured in muscle homogenates with (Insulin – Ins) and without (Control – Con) 5 min insulin exposure at 37°C. These experiments were designed to determine if the 3-O-MFPase assay could detect changes in activity with short insulin incubation.

In Experiment 2, the populations of pumps found in the plasma and endosomal membranes were separated using fractionation techniques. In this way, insulin-induced
translocation of the Na\textsuperscript{+}-K\textsuperscript{+}-ATPase could not be a mechanism of increased pump activity. Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity was measured, in both plasma membrane (SLP) and endosomal (EN) fractions, with (Ins) and without (Con) 5 min insulin (75\textmu M) exposure at 37°C. Any increase in activity due to insulin incubation was due solely to insulin stimulation of intrinsic pump activity.

In Experiment 3, muscle homogenates were pre-treated with insulin (625\textmu M) for 5 min at 37°C prior to fractionation of SLP and EN membranes. As in the second experiment, 3-O-MFPase activity was then measured on SLP and EN fractions with (Ins) and without (Con) 5 min insulin (75 \textmu M) exposure at 37°C. This experiment was designed to examine possible mechanisms of insulin stimulated Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity. The activity of insulin-treated fractions (Experiment 3 - Con) was compared with that of non-treated fractions (Experiment 2 – Con). This comparison determined whether or not the 5 min pre-incubation with insulin stimulated increases in pump activity. The addition of insulin to fractions already pre-treated with insulin (Experiment 3 – Ins) determined if insulin could stimulate further increases in pump activity. This condition gave insight into the time dependence of the insulin-induced changes in pump activity.

Western blots were performed for the \(\alpha_2\) subunit of the Na\textsuperscript{+}-K\textsuperscript{+}-ATPase and GLUT4 protein in the SLP and EN fractions from both non-treated (Experiment 2 - Con) and insulin-treated (Experiment 3 - Con) homogenates. This data determined if any increase in pump activity in insulin-treated SLP fractions could be attributed to translocation. Also, tyrosine phosphorylation (Tyr-P) of the \(\alpha_2\) subunit was measured in homogenates of non-treated and insulin-treated samples in order to examine a role for Tyr-P in an insulin-induced effect on Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity.
Figure 3: Overview of the experimental design. Procedures were performed on soleus (Sol), red gastrocnemius (RG), and white gastrocnemius (WG) muscle samples (Con=Control; Ins=Insulin (75 μM); SLP=sarcolemmal enriched fraction; EN=endosomal fraction; Tyr-P=tyrosine phosphorylation).

Whole Homogenate Preparation

Whole homogenates were prepared by homogenizing portions of previously frozen samples (~40 mg) in 20 vol of ice-cold buffer containing (in mM) 10 trometamol, 2-amino-2-(hydroxymethyl)-1-3-propanediol (Tris base), 25 EDTA, and 250 sucrose (pH 7.4), and a commercially prepared combination of protease inhibitors – inhibits serine, cysteine, metalloproteases, and calpains (Roche Diagnostics, Indianapolis, IN). Samples were homogenized on ice with a glass-on-glass hand homogenizer. All samples were aliquoted
and quickly frozen in liquid nitrogen until further analysis. These samples were used to assess K⁺-stimulated Na⁺-K⁺-ATPase activity in Experiment 1 (n = 10 for each of Sol, RG, and WG).

**Lowry Protein Assay to Determine Total Protein Concentration**

Each sample was run in triplicate. Fifty μL of homogenate, previously diluted to a total of 1000x, was added to a 12 x 75mm culture tube. A volume of (0.5 mL) alkaline copper reagent (in grams: 0.05 CuSO₄·5H₂O, 0.1 potassium sodium tartrate, 2.0 NaOH, and 10 Na₂CO₃) was added to all tubes, mixed well, and left to stand at room temperature for 10 min. Next, 2 mL of phenol reagent (5 mL of 2N into 80 mL H₂O) was added and mixed immediately in each tube. Samples were incubated at 55°C for 5 min. Samples were removed from the heat and cooled for 1 min in tap water before being read on a spectrophotometer at a wavelength 650 nm. Fifty μL of water served as a “blank” and 50 μL of standards with protein concentrations of (in mg/mL) 0.5, 0.25, and 0.125 were used to generate a standard curve to check linearity.

**K⁺-stimulated 3-O-methylfluorescein phosphatase Activity Assay**

The K⁺-stimulated 3-O-methylfluorescein phosphatase activity (3-O-MFPase) was assessed fluorometrically with a SpectraMax GeminiXS microplate fluorometer (Molecular Devices, Sunnydale, CA). This procedure was adapted from Fraser and McKenna (26) and modified by our laboratory (69). The sample preparation was as follows. Samples were diluted 5x in cold homogenate buffer containing 0.1% deoxycholate. 5 μL of this was added to microcentrifuge tubes containing 300 μL of various KCl (pH 7.40) concentrations made up
in assay medium (in mM: 5 MgCl₂, 1.25 EDTA, 1.25 EGTA, 100 Tris base). The KCl concentrations used were (in mM) 0, 0.2, 0.5, 0.75, 1.0, 1.5, 3.0, and 5.0. Microcentrifuge tubes were mixed well and 250 μL of each KCl concentration was added to a well in the microplate before being incubated for 5 min at 37°C (Appendix A - Con). The reaction was started with the addition of 160 μM 3-O-methylfluorescein phosphate (3-O-MFP). The metabolism of this substrate, and subsequent appearance of the fluorescing compound 3-O-MF, was measured over 3 min an excitation wavelength of 475nm and an emission wavelength of 515nm (SpectraMax GeminiXS microplate fluorometer, Molecular Devices, Sunnydale, CA). The resulting slope was corrected against a known standard of 3-O-MF, and the K⁺-stimulated 3-O-MFPase activity was determined as the difference between the slopes generated from samples incubated in medium containing the different concentrations of KCl and “blank” samples incubated in medium without KCl. Values were expressed relative to homogenate total protein concentration as determined by the Lowry protein assay. 

\[ Y = Y_{\text{max}} + \frac{X}{k_m + X} \]

where \( Y \) is the activity of the sample at a specific substrate concentration (X), \( Y_{\text{max}} = V_{\text{max}} \), and \( k_m \) is Michaelis-Menten constant. In cases when 3-O-MFPase activity decreased at high KCl concentrations and skewed calculations, these values were removed from the calculation.
**Generation of Dose-Response Curve**

A dose-response curve for 3-O-MFPase activity at 5 mM KCl with varying concentrations of insulin was generated in order to determine 1) if insulin exhibited a dose response and 2) the optimal insulin concentration for *in vitro* activation of 3-O-MFPase activity. Given the *in vitro* nature of all experiments, supra physiological insulin concentrations were chosen in order to avoid a null effect of insulin on activity measures due to a lack of receptor saturation in the preparation. The samples were read as described above under the 3-O-MFPase activity assay with the following modifications. Five mM KCl was used in all samples, as it was only the concentration of insulin that was manipulated. Concentrations of (in μM) 0, 25, 50, 75, 375, 500, and 625 insulin were tested. The measured activity values were corrected against a “blank” (0 mM KCl; 0 μM Ins) and compared to the value obtained with 5 mM KCl and 0 μM insulin. This experimental setup is depicted in **Appendix B**; the concentration dependency curves can be found in **Appendix C** (Sol N=7; RG N=4; WG N=4).

**Time Dependency for the Response of 3-O-MFPase Activity to Insulin**

In order to determine the time required for a maximal insulin effect on *in vitro* 3-O-MFPase activity, incubations at 37°C of 5, 10, 15, and 20 min in 5 mM KCl and 75 μM insulin prior to the addition of 3-O-MFP were examined. An additional incubation was added consisting of 20 min in 5 mM KCl followed by the addition of 75 μM insulin and an additional 5 min incubation. This extra condition served to determine if the same insulin effect achieved after 5 min could be detected after 5 min insulin incubation 20 min later. A control trial containing 5 mM KCl and 0 μM insulin was run with each incubation time. Following
analysis of these time dependencies, additional time dependencies were examined with 1) 5mM DTT and 2) 1% PPase inhibitor present in the homogenate buffer in an attempt to prevent decreases in 3-O-MFPase activity with incubation time. A summary of these findings can be found in Appendix D (Sol N=4; RG N=4; WG N=3).

**K⁺-dependent 3-O-MFPase Activity for Sol, RG, and WG Homogenates Incubated with 75 μM Insulin**

The 3-O-MFPase activity was measured as described above. Sixteen wells of the microplate were read at one time for each trial. All samples (n=10) were run in triplicate. One set of 8 wells served as a control (Con); this set contained the 8 different KCl concentrations (in mM: 0, 0.2, 0.5, 0.75, 1, 1.5, 3, and 5). The second set of 8 wells served as the insulin (Ins) condition. This set contained the same 8 KCl concentrations; however, wells 2-8 also contained 75 μM insulin. The kinetic curves obtained for the control and insulin treated conditions were compared to determine if insulin treatment affected Vₘₐₓ or kₘ. On a separate occasion a series of “blanks” were measured with and without 75 μM insulin in the “blank” to determine the influence of insulin on the background 3-O-MFPase activity in the assay. The diagram for this setup is found in Appendix A.

**Enriched Sarcolemmal Fraction Preparation**

Muscle samples were separated into two membrane fractions: a sarcolemmal particulate fraction (SLP) and an endosomal fraction (EN). This procedure was conducted as described by Sandiford et al (69), and adapted from Fuller et al (27). As stated by Sandiford et al (69), this isolation protocol was favored over other methods because only two fractions are
generated from the tissue samples and only a small amount of the membranes are discarded. The SLP fraction, at least in cardiac tissue, contains in excess of 85% of the ouabain-sensitive ATPase activity (27).

Samples from each muscle (Sol, RG, WG) were cut and weighted into ~140 mg pieces. For this it was necessary to pool tissue. Initially, 5 soleus samples were pooled in each of 2 fractions. Four more soleus samples were added to increase the N. Three RG samples and 3 WG samples were each pooled into each of 3 fractions. Four more samples were added to each of these groups. All steps in the fractionation procedure were carried out at 4°C. Samples were minced and incubated for 30 min at 4°C in a salt solution containing 0.5 M NaCl and 20 mM HEPES, pH 7.4. The high-salt solution detaches the membranes from the myofilaments (myofilaments are then pelleted at low speed and discarded) (27). The samples were then homogenized in 10x the volume of homogenate buffer containing (in mM) 250 sucrose, 1 EDTA, 20 HEPES (pH 7.4), and a commercially prepared combination of protease inhibitors (Roche Diagnostics). Homogenates were centrifuged at 1,000 g for 5 min and the pellets resuspended. Next, a second 1,000 g spin for another 5 min was performed and the supernatants of both 1000 g spins were combined. These supernatants were then centrifuged at 100 g for 10 min. The supernatants from this step were then centrifuged at 5,000 g for 10 min. The pellets from this spin were stored on ice, and the resulting supernatants were centrifuged at 20,000 g for 30 min. Pellets from the 5,000 g and 20,000 g spins were combined, resuspended in ~400μL of homogenate buffer, aliquoted, and frozen in liquid nitrogen and stored at -80°C until use. This was the SLP fraction. The supernatant from the 20,000 g spin represented the EN fraction. The EN fraction was suspended in the same buffer as described for SLP, aliquoted, frozen, and stored under
similar conditions (see Appendix E for flow diagram). The two resulting fractions (SLP and EN) were assayed to determine the K\(^+\)-stimulated 3-O-MFPase activity of the Na\(^+\)-K\(^+\)-ATPase as well as to perform Western blotting using antibodies against the \(\alpha_2\) subunit of the Na\(^+\)-K\(^+\)-ATPase, GLUT4, and tyrosine phosphorylated residues. 3-O-MFPase activity was performed as described above, where a set of KCl concentrations were assayed both with and without exposure to 75\(\mu\)M insulin in order to determine acute insulin effects on activity in these fractions (Appendix A).

Two groups of fractions were made: non-treated (Experiment 2) and insulin-treated (Experiment 3). Non-treated fractions were made exactly as above. For the insulin-treated group, samples were incubated for 5 min at 37°C in 625\(\mu\)M insulin following homogenization and frozen in liquid nitrogen immediately. The insulin concentration was determined in pilot work; the same insulin concentration was used as that determined to stimulate maximam Tyr-P. Samples were frozen in liquid nitrogen to capture any effect of insulin at the conclusion of 5 min. This procedure mimics those previously performed in whole soleus muscle (13; 69). These 2 conditions allowed for the assessment of insulin treatment to induce both subunit redistribution and stimulation of intrinsic activity.

**Imunoprecipitation of Tyrosine Phosphorylated Residues**

All Tyr-P proteins were immunoprecipitated and probed for the presence of the \(\alpha_2\) subunit of the Na\(^+\)-K\(^+\)-ATPase. This procedure was performed on homogenates of non-treated and insulin-treated groups (n=5). An insulin concentration of 625\(\mu\)M was determined to be optimal in stimulating Tyr-P of the \(\alpha_2\) subunit of the Na\(^+\)-K\(^+\)-ATPase. Samples were incubated for 5 min at 37°C before being frozen in liquid nitrogen to capture any effect of
insulin at the conclusion of 5 min. A sub-sample of SLP and EN non-treated and insulin-treated fractions were also probed for Tyr-P.

This procedure was conducted as described by Sandiford et al (69). Samples were homogenized (2 x 10-15mg per sample) in 20x homogenate buffer containing (in mM) 10 Tris base (pH 7.4), 25 EDTA, 250 sucrose, and a commercially prepared combination of protease inhibitors (Roche Diagnostics, Indianapolis, IN). A volume of homogenate corresponding to 750μg of protein was aliquoted from each sample. Samples were incubated at 37°C for 5 min, with or without 625μM insulin before being frozen in liquid nitrogen. Then, samples were thawed and incubated for 1 h at 4°C in 3x ice cold lysis buffer containing (in mM) 20 Tris (pH 8.0), 135 NaCl, 10 Na₄P₂O₇, 10 NaF, 1 Na₃VO₄, 10% glycerol, and 1% Triton-X. Following lysis buffer treatment, samples were centrifuged at 300 g for 10 min at 4°C and the supernatants treated with 60μL of a 50% protein A-agarose bead slurry (KPL, Gaithersburg, MD) for 1 h at 4°C. Samples were then centrifuged at 14000 g for 20 sec and the supernatant incubated with 50μL of anti-phosphotyrosine (PY69) antibody (BD Biosciences Canada) and 100μL of the protein A-agarose slurry for 4 h at 4°C. Samples were once again centrifuged at 14000 g for 20 sec and the supernatant saved for contamination profiling. The pellets were washed 4x with lysis buffer, once with wash 1 (in M: 0.1 Tris pH 8.0, 0.5 LiCl), once with wash 2 (in mM: 10 Tris pH 7.6, 150 NaCl, 1 EDTA), and once with wash 3 (in mM: 20 HEPES pH 7.4, 5 MgCl₂, 1 DTT). Samples were then suspended in 20μL of each homogenate buffer and 4x SDS-PAGE sample buffer and stored at 4°C overnight. The next morning samples were heated to 95°C for 5 min to elute the antigen from the antibody-agarose complex and the Tyr-P samples were probed via
Western blotting for the presence of the α2 subunit of the Na⁺-K⁺-ATPase (see Appendix F for a flow diagram).

**Western Blotting Procedures**

Samples containing either 25 μg protein (fractions that probed for α2, GLUT4) or 35μL (Tyr-P samples that probed for α2) were electrophoresed on 7.5% sodium dodecyl polyacrylamide gels (Bio-Rad Mini-PROTEAN III) according to the general procedures previously published from our laboratory (22). Briefly, a biotinylated ladder was used as a molecular weight standard (Cell Signaling Technology, Beverly, MA). Proteins were transferred to polyvinylidene difluoride membranes (PVDF membrane, Bio-Rad) and blocked for 1 h in 5% nonfat milk (GLUT4) or 5% BSA (α2) in Tris-buffered saline (TBS, pH 7.5). Membranes were incubated with primary monoclonal antibodies against GLUT4 (1:200) or polyclonal antibodies against α2 (1:500) overnight (Upstate Biotechnology, Lake Placid, NY). After being washed 6 x 5 min in 0.1% TBS Tween-20 (TBS-T), membranes were incubated for 60 min in goat anti-mouse secondary antibody (GLUT4) or goat anti-rabbit secondary antibody (α2) (Chemicon International, Temecula, CA) diluted to 1:3000 (GLUT4) and 1:1000 (α2) in TBS-T. An enhanced chemiluminescence procedure was used for antibody identification (Amersham, Buckinghamshire, UK). Blots were analyzed by use of a Chemi Genius2 model bio imaging system (SynGene, Frederick, MD). Protein expression was expressed relative to a brain standard for α2 and α-actin (Sigma Chemical, St. Louis, MO) for GLUT4. Supernatants from the Tyr-P procedure were blocked overnight in 5% nonfat milk in TBS (pH 7.5) and incubated for 2 h with primary monoclonal antibodies against Tyr-P diluted to 1:1000. After a 30 min wash out period, membranes were incubated
with goat anti-mouse secondary antibody for 1 h (1:1000 dilution), washed, and detected using the enhanced chemiluminescence procedure.

**Maximal CitrateSynthase (CS) Activity**

Frozen tissue (~5mg each, n=8 for each of Sol, RG, WG) was homogenized in a phosphate buffer (pH 7.4) containing 16mM Na₂HPO₄, 4mM KH₂PO₄, 0.02% bovine serum albumin (BSA), 5mM β-mercaptoethanol, and 0.5mM EDTA and diluted (1:100) in 20 mM imidazole buffer with 0.02% BSA. The maximal activity of CS was determined fluorometrically, as described in Appendix G.

**K⁺-dependencies for 3-O-MFPase Activity for Sol, RG, and WG Homogenates Incubated with 7mM Glucose**

Given evidence suggesting a protective role for glucose supplementation on membrane excitability, the effect of glucose on Na⁺-K⁺-ATPase activity independent of insulin was investigated. This experiment was designed exactly as that for the K⁺ dependent 3-O-MFPase activity with 75μM insulin except that insulin was replaced by 7mM glucose solution made up in assay medium (n=5). This glucose concentration was selected during pilot work in which a concentration dependency of glucose was conducted using 5mM KCl during the 3-O-MFPase assay (n=3). Appendices A and B display the setup for these experiments. The concentration dependencies can be found in Appendix H. This data is presented in Appendix I.

**Statistical Analysis**
All values are presented as mean ± SE. Statistical analysis was performed using Statistica version 5 software (Statsoft, Tulsa, OK, 1996). A one-way, repeated measures ANOVA was used to analyze maximal CS activity to determine if differences in muscle oxidative potential existed. Two-way, repeated measures ANOVA were used to identify differences in $K^+$-stimulated activity dependencies between 1) Con and Ins conditions in homogenate and fractions and 2) non-treated and insulin-treated experiments in fractions. Two-way, repeated measures ANOVA were also used to analyze Western blot and Tyr-P data to detect differences in $\alpha_2$ subunit distribution and $\alpha_2$ subunit Tyr-P between non-treated and insulin-treated experiments. Significance was set at P<0.05. Post hoc analysis of mean values was performed using a Newman-Keuls test. A student t-test was used to analyze differences in $V_{\text{max}}$ and $k_m$ between Con and Ins in each experiment and between non-treated and insulin-treated experiments, with significance set at P<0.05.

In Appendix H, two-way, repeated measures ANOVA were used to identify differences in $K^+$-stimulated activity dependencies between Con and GLU conditions in skeletal muscle homogenates. Significance was set at P<0.05. Post hoc analysis of mean values was performed using a Newman-Keuls test.
Chapter Three

Results

Citrate Synthase Activity

In order to confirm that the muscles differed in oxidative potential, maximal citrate synthase activity was measured (Figure 4). As expected, the oxidative potential of the muscles ranked RG>Sol>WG (P<0.01).

![Figure 4: Maximal citrate synthase (CS) activity in skeletal muscle homogenates of different muscle types (Sol = soleus; RG = red gastrocnemius; WG = white gastrocnemius). Values are mean ± SE (n=8). * Significantly different from Sol; # Significantly different from RG.](image)

Insulin-stimulated Na\(^+\)-K\(^-\)-ATPase Activity in Skeletal Muscle Homogenates – Experiment 1

Kinetic curves for K\(^-\)-dependent 3-O-MFPase activity were determined in Sol, RG, and WG muscle homogenates (Figure 5). \(V_{\text{max}}\) and \(k_m\) data are summarized in Table 4. Insulin exposure caused a 16 and 34% increase in \(V_{\text{max}}\) in Sol and RG, respectively. In WG, \(V_{\text{max}}\) was unaltered by insulin exposure but \(k_m\) increased 53%.
Table 4: Summary of $V_{\text{max}}$ and $k_m$ properties for Na\(^+\)-K\(^+\)-ATPase activity in skeletal muscle homogenates of different muscle types, with and without 75\(\mu\)M insulin, for 5 min at 37\(^\circ\)C

<table>
<thead>
<tr>
<th></th>
<th>Sol</th>
<th></th>
<th>RG</th>
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<th>WG</th>
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<tr>
<td></td>
<td>Con</td>
<td>Ins</td>
<td>Con</td>
<td>Ins</td>
<td>Con</td>
</tr>
<tr>
<td>$V_{\text{max}}$</td>
<td>221 ± 17</td>
<td>256 ± 21*</td>
<td>190 ± 14</td>
<td>256 ± 18*</td>
<td>104 ± 4.6</td>
</tr>
<tr>
<td>$k_m$</td>
<td>0.76 ± 0.07</td>
<td>0.73 ± 0.11</td>
<td>0.68 ± 0.04</td>
<td>0.75 ± 0.12</td>
<td>0.38 ± 0.07</td>
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</table>

Values are means ± SE (n=10). * Significantly different from Con (P<0.05). $V_{\text{max}}$, maximal activity (nmol·mg prot\(^{-1}\)·h\(^{-1}\)); $k_m$, K\(^+\) activity required for 50% of maximal Na\(^+\)-K\(^+\)-ATPase activity; Sol = Soleus; RG = red gastrocnemius; WG = white gastrocnemius.

In Sol, main effects of KCl concentration (0<0.2<0.5<0.75<1.0<1.5<3.0=5.0) and condition (Con<Ins) were found (P<0.01). In RG, an interaction between KCl concentration and condition was observed (P<0.02). Na\(^+\)-K\(^+\)-ATPase activity was higher in the Ins condition at all KCl concentrations. An interaction between KCl concentration and condition was also observed in WG (P<0.01). Although there was no difference between Con and Ins at $V_{\text{max}}$, pump activity was lower in Ins than in Con at lower KCl concentrations (0.5, 0.75, 1.0 and 1.5 mM). The difference between Con and Ins could be attributed to an insulin-stimulated increase in non-specific activity that was not present in Sol or RG.
Figure 5: $K^+$-dependent $Na^+\text{-}K^+$-ATPase activity in skeletal muscle homogenates of A) soleus, B) red gastrocnemius (RG), and C) white gastrocnemius (WG) muscles, with and without 75μM insulin for 5 min at 37°C (Con = control, Ins = insulin). Values are means ± SE (n=10). In A, main effects ($P<0.05$) for KCl (0<0.2<0.5<0.75<1.0<1.5<3.0=5.0) and condition (Con<Ins) were found. In B and C, interactions ($P<0.05$) between KCl concentration and condition were found. * Significantly different from Con; # Significantly greater than 1.5 mM KCl. In C, a significant difference was also detected in $k_m$, Con<Ins.

A comparison between muscles shows that, for $V_{max}$ in Con, WG<RG=Sol ($P<0.01$). However, a trend exists for Sol>RG ($P<0.07$). There was no difference in $V_{max}$ between Sol
and RG in the Ins condition, with WG<Sol=RG (P<0.01). The \( k_m \) was lower in WG Con than in Sol or RG Con (P<0.01). No difference in \( k_m \) was observed between muscles in the Ins condition.

**Insulin-stimulated Na\(^+\)-K\(^+\)-ATPase Activity in Non-Treated SLP and EN Fractions – Experiment 2**

\( K^+ \)-dependent 3-O-MFPase activity was then assessed in non-treated SLP and EN fractions (Figures 6 and 7, respectively). \( V_{\text{max}} \) and \( k_m \) data are summarized in Table 5. In SLP fractions, \( V_{\text{max}} \) increased by 15 and 12% in Sol and RG, respectively. In Sol SLP, \( k_m \) also decreased by 22% with insulin exposure. No change in \( V_{\text{max}} \) or \( k_m \) was observed with insulin exposure in WG SLP. In EN fractions, no change in \( V_{\text{max}} \) or \( k_m \) was observed in Sol or WG. However, in RG EN, a 24% increase in \( V_{\text{max}} \) was detected with insulin exposure.

Table 5: Summary of \( V_{\text{max}} \) and \( k_m \) properties for Na\(^+\)-K\(^+\)-ATPase activity in non-treated fractions of different muscle types, with and without 75\( \mu \)M insulin, for 5 min at 37°C

<table>
<thead>
<tr>
<th></th>
<th>Sol</th>
<th>RG</th>
<th>WG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Con</td>
<td>Ins</td>
<td>Con</td>
</tr>
<tr>
<td>SLP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( V_{\text{max}} )</td>
<td>1710 ± 186</td>
<td>1970 ± 231*</td>
<td>1476 ± 128</td>
</tr>
<tr>
<td>( k_m )</td>
<td>0.86 ± 0.15</td>
<td>0.67 ± 0.12 *</td>
<td>1.52 ± 0.21</td>
</tr>
<tr>
<td>EN</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( V_{\text{max}} )</td>
<td>300 ± 45</td>
<td>352 ± 73</td>
<td>246 ± 38</td>
</tr>
<tr>
<td>( k_m )</td>
<td>0.31 ± 0.08</td>
<td>0.44 ± 0.15</td>
<td>0.54 ± 0.11</td>
</tr>
</tbody>
</table>

Values are means ± SE (Sol, n=6; RG, WG, n=7). * Significantly different from Con (P<0.05). \( V_{\text{max}} \), maximal activity (nmol·mg prot\(^{-1}\)·h\(^{-1}\)); \( k_m \), \( K^+ \) activity required for 50% of maximal Na\(^+\)-K\(^+\)-ATPase activity; Sol = Soleus; RG = red gastrocnemius; WG = white gastrocnemius; SLP = plasma membrane fraction; EN = endosomal membrane fraction

In Sol SLP fractions, main effects of KCl concentration \((0<0.2<0.5<0.75=1.0<1.5<3.0=5.0)\) and condition (Con<Ins) were observed (P<0.01). From Figure 7A, it is evident that the Sol EN fraction contains a population of pumps that is active. In Sol EN, no significant difference was observed between Con and Ins, but a main effect of KCl.
concentration was found (0<0.2<0.5<1.5=3.0, 1.5>5.0). It can be seen that, at high KCl concentrations, KCl exhibits an inhibitory effect on pump activity in the Sol EN fraction.

In RG SLP, an interaction between KCl concentration and conditions was observed (P<0.03). There was no difference between Con and Ins conditions at low KCl concentrations; however, Con<Ins at high concentrations of KCl. In Figure 7B, it can be seen that the RG EN fraction also contains an active population of Na⁺-K⁺-ATPase. In RG EN, a main effect for KCl concentration was found (0<0.2<0.5<0.75<1.5=3.0=5.0, 1.0<3.0). However, unlike in Sol EN, a trend (P<0.10) was observed for a condition effect, Con<Ins.

In WG SLP fractions, main effects of KCl concentration (0<0.2=0.5<0.75<1.0=1.5<3.0<5.0) and condition (Con<Ins) were detected (P<0.05). In WG EN fractions, a main effect of KCl was observed (0<0.2<0.5<1.0=1.5=3.0>5.0, 0.75<1.5) (P<0.05). A decrease in pump activity was observed at 5.0 mM KCl. As in Sol and RG, the WG EN fraction contained an active population of Na⁺-K⁺-ATPase.
Figure 6: K⁺-dependent Na⁺-K⁺-ATPase activity in non-treated sarcolemmal enriched (SLP) fractions of A) soleus, B) red gastrocnemius (RG), and C) white gastrocnemius (WG) muscles, with and without 75μM insulin for 5 min at 37°C (Con = control, Ins = insulin). Values are means ± SE [n = 6 (Sol), 7 (RG, WG)]. In A, main effects (P<0.05) for KCl (0<0.2<0.5<0.75<1.0<1.5<3.0<5.0) and condition (Con<Ins) were found. In C, main effects (P<0.05) for KCl (0<0.2=0.5<0.75<1.0=1.5<3.0 <5.0) and condition (Con<Ins) were found. In B, an interaction between KCl and condition was observed (P<0.05). * Significantly different from Con; † Significantly different from 1.5mM KCl; †† Significantly different from 3mM KCl. In A, a significant difference was also observed in kₗₗₗ, Ins<Con.
Figure 7: K⁺-dependent Na⁺-K⁺-ATPase activity in non-treated endosomal (EN) fractions of A) soleus, B) red gastrocnemius (RG), and C) white gastrocnemius (WG) muscles, with and without 75μM insulin for 5 min at 37°C (Con = control, Ins = insulin). Values are means ± SE [n = 6 (Sol), 7 (RG, WG)]. A main effect for KCl (P<0.05) was found in A, B, and C (P<0.05). In A, 0<0.2<0.5<1.5=3.0=5.0; in B, 0<0.2<0.5<0.75<1.5=3.0=5.0; and in C, 0<0.2<0.5<1.0=1.5=3.0>5.0, 0.75<1.5. No significant differences were detected between Con and Ins.
In non-treated SLP fractions, a comparison between muscles shows that, for $V_{\text{max}}$, \( \text{WG} < \text{RG} = \text{Sol} \) (P<0.01). However, a trend exists for Sol>RG (P<0.07). The $V_{\text{max}}$ in Ins was also greater than that in Con (P<0.01). The $k_m$ was higher in RG than in Sol or WG (P<0.01). No difference in $k_m$ was observed between Con and Ins conditions.

In non-treated EN fractions, a comparison between muscles shows that, for $V_{\text{max}}$, \( \text{WG} < \text{RG} = \text{Sol} \) (P<0.02). There was no difference between Con and Ins. For $k_m$, Sol=WG<RG (P<0.03).

A within-muscle comparison between SLP and EN fractions showed that $k_m$ was lower in EN when compared to SLP in Sol (P<0.05) and in RG (P<0.01). A trend for the $k_m$ to be lower in EN when compared to SLP was observed in WG (P<0.06).

**Insulin-stimulated Na$^+$-K$^+$-ATPase Activity in Insulin-Treated SLP and EN Fractions – Experiment 3**

Next, K$^+$-dependent 3-O-MFPase activity was assessed in the insulin-treated SLP and EN fractions (Figures 8 and 9, respectively). $V_{\text{max}}$ and $k_m$ data are summarized in Tables 7 and 8. In SLP fractions, an increase in $V_{\text{max}}$ (24\%) with insulin exposure was only observed in RG. Insulin exposure caused a 15\% increase in $k_m$ in Sol SLP. In EN fractions, no change in $V_{\text{max}}$ or $k_m$ was detected in any muscle; however, a trend (P<0.10) for an increase in $V_{\text{max}}$ was detected in RG EN.
Table 6: Summary of $V_{\text{max}}$ and $k_m$ properties for Na$^+$-K$^+$-ATPase activity in insulin-treated fractions of different muscle types, with and without 75μM insulin, for 5 min at 37°C

<table>
<thead>
<tr>
<th></th>
<th>Sol</th>
<th></th>
<th>RG</th>
<th></th>
<th>WG</th>
<th></th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Con</td>
<td>Ins</td>
<td>Con</td>
<td>Ins</td>
<td>Con</td>
<td>Ins</td>
</tr>
<tr>
<td>$V_{\text{max}}$</td>
<td>1461 ± 92</td>
<td>1569 ± 141</td>
<td>1145 ± 119</td>
<td>1426 ± 150</td>
<td>966 ± 83</td>
<td>1092 ± 119</td>
</tr>
<tr>
<td>$k_m$</td>
<td>0.66 ± 0.12</td>
<td>0.76 ± 0.15</td>
<td>0.79 ± 0.11</td>
<td>1.01 ± 0.21</td>
<td>0.46 ± 0.06</td>
<td>0.60 ± 0.12</td>
</tr>
</tbody>
</table>

**Values** are mean ± SE (Sol n=6; RG, WG n=7). * Significantly different from Con (P<0.05). $V_{\text{max}}$, maximal activity (nmol·mg prot$^{-1}$·h$^{-1}$); $k_m$, K$^+$ activity required for 50% of maximal Na$^+$-K$^+$-ATPase activity; Sol = Soleus; RG = red gastrocnemius; WG = white gastrocnemius; SLP = plasma membrane fraction; EN = endosomal membrane fraction.

In both Sol SLP and Sol EN fractions, no difference in pump activity was observed between Con and Ins. However, main effects of KCl concentration were detected in both SLP (0<0.2<0.5<0.75<1.0<1.5<3.0=5.0) and EN (0<0.2<0.5<0.75<1.5 & 3.0) fractions (P<0.01).

In RG SLP fractions, as observed in RG homogenate and non-treated SLP fractions, an interaction between KCl concentration and condition was observed (P<0.01). 3-O-MFPase activity was greater in Ins than in Con at higher concentrations of KCl. In RG EN fractions, an interaction was also observed between KCl concentration and condition (P<0.02). 3-O-MFPase activity was greater in Ins than in Con at 1.5 and 3mM KCl.

In WG SLP fractions, a main effect of KCl concentration (0<0.2<0.5<0.75<1.0<1.5<3.0=5.0) was observed (P<0.01). No difference was detected between Con and Ins. In EN fractions, an interaction was observed between KCl concentration and condition (P<0.01). 3-O-MFPase activity was greater in Ins than in Con at 5mM KCl; however, maximal activity was reached at 1.5mM KCl and there were no differences between conditions at this concentration.
Figure 8: $\mathrm{K}^+$-dependent $\mathrm{Na}^+\mathrm{K}^+$-ATPase activity in insulin-treated sarcolemmal enriched (SLP) fractions of A) soleus, B) red gastrocnemius (RG), and C) white gastrocnemius (WG) muscles, with and without 75 $\mu$M insulin for 5 min at 37°C (Con = control, Ins = insulin). Values are means ± SE [n = 6 (Sol), 7 (RG, WG)]. A main effect for KCl concentration ($P<0.05$) was found in A and C ($0<0.2<0.5<0.75<1.0<1.5<3.0=5.0$). In B, an interaction between KCl concentration and condition was observed ($P<0.05$). * Significantly different from Con; # Significantly different from 1.5 mM KCl; † Significantly different from 3 mM KCl.
Figure 9: K⁺-dependent Na⁺-K⁺-ATPase activity in Ins-treated endosomal (EN) fractions of A) soleus, B) red gastrocnemius (RG), and C) white gastrocnemius (WG) muscles, with and without 75μM insulin for 5 min at 37°C (Con = control, Ins = insulin). Values are means ± SE [n = 6 (Sol), 7 (RG, WG)]. A main effect for KCl concentration (P<0.05) was found in A (0<0.2<0.5<0.75<1.5 and 3.0). In B and C, an interaction between KCl concentration and condition (P<0.05) was observed. * Significantly different from Con; # Significantly different from all other KCl concentrations; † Significantly different from 3 mM KCl.
In insulin-treated SLP fractions, a comparison between muscles shows that, for $V_{\text{max}}$, WG$<$Sol ($P<0.02$) and Con$<$Ins. Also, a trend exists for RG$>$WG ($P<0.07$). The $k_m$ was lower in WG when compared to RG ($P<0.05$). No difference in $k_m$ was observed between Con and Ins conditions.

In insulin-treated EN fractions, a comparison between muscles shows that, for $V_{\text{max}}$, WG$<$RG=Sol ($P<0.01$). There was no difference between Con and Ins. Differences in $k_m$ were not found between muscles or between Con and Ins.

A within muscle comparison between SLP and EN fractions showed that $k_m$ was lower in EN when compared to SLP in RG ($P<0.01$). A trend for the $k_m$ to be lower in EN when compared to SLP was observed in WG ($P<0.07$). No difference in $k_m$ between fractions was detected in Sol.

Effect of Insulin-Treatment on Na$^+$$\cdot$K$^+$-ATPase Activity in SLP and EN Fractions – Comparison between Experiment 2 Con and Experiment 3 Con

Comparisons were made between the Na$^+$$\cdot$K$^+$-ATPase activity in non-treated and insulin-treated fractions in order to determine if the insulin treatment caused an increase in pump activity. In Sol SLP fractions, an interaction between KCl concentration and experimental condition was observed ($P<0.05$), non-treated $>$ insulin-treated at 3 and 5 mM KCl (Figure 10A). No difference between non-treated and insulin-treated experiments was observed in the EN fractions. For $k_m$, insulin-treated was 23% lower than non-treated in SLP while no difference was observed in EN fractions.

In RG SLP fractions, an interaction between KCl concentration and experimental condition was observed ($P<0.01$) (non-treated $>$ insulin-treated at 3 and 5 mM KCl, Figure
10B). An interaction was also observed in RG EN fractions between KCl concentration and experimental condition (P<0.01) (insulin treated > non-treated at 0.2, 0.5, and 1.0 mM KCl, Figure 10C). Insulin-treatment caused 48 and 39% reductions in $k_m$ in SLP and EN fractions, respectively, when compared to non-treated fractions.

In WG, no differences in activity were observed between non-treated and insulin-treated experiments, in either the SLP or the EN fractions. However, $k_m$ was 48% lower in insulin-treated SLP fractions when compared to non-treated SLP fractions.
Figure 10: Comparison of the K⁺-dependent Na⁺-K⁺-ATPase activity between non-treated (Exp. 2 Con) and insulin-treated (Exp. 3 Con) experiments in A) Soleus (Sol) sarcolemmal enriched (SLP) fractions, B) Red gastrocnemius (RG) SLP fractions, and C) RG endosomal (EN) fractions. Values are means ± SE [n = 6 (Sol), 7 (RG, WG)]. An interaction between KCl concentration and Experimental condition was observed in A, B, and C. * Significantly different from Exp. 2 Con; # Significantly different from 1.5mM KCl.
Western-Blotting Data from SLP and EN Fractions

The $\alpha_2$ subunit distribution was measured in non-treated (Exp. 2 Con) and insulin-treated (Exp. 3 Con) SLP and EN fractions of all muscles (Figure 11). No difference was found in $\alpha_2$ subunit distribution between non-treated and insulin-treated groups or between muscles in the SLP fractions. However, in EN fractions, a main effect (P<0.03) of muscle was observed, with $\alpha_2$ subunit distribution ranking WG<RG=Sol. A main effect of condition was also observed in EN fractions, Control > Insulin-treated (P<0.02). A comparison between SLP and EN fractions revealed a significant difference, SLP>EN (P<0.01), in all muscles.

![Bar chart A](image1)

![Bar chart B](image2)

**Figure 11:** $\alpha_2$ subunit distribution in A) sarcolemmal enriched (SLP) and B) endosomal (EN) fractions of non-treated (Exp. 2 Con) and insulin-treated (Exp. 3 Con) experiments (Sol = soleus; RG = red gastrocnemius; WG = white gastrocnemius). Values are means ± SE [n = 6 (Sol), 7 (RG, WG)]. In SLP fractions, no differences were detected between muscles or conditions. In EN fractions, main effects (P<0.05) of muscle (WG < RG = Sol) and experimental condition (non-treated > insulin-treated) were observed. A main effect (P<0.05) was also observed between fractions (SLP>EN).
GLUT4 distribution showed a similar response to insulin-treatment as the $\alpha_2$ subunit (Figure 12). No difference was found in GLUT4 distribution between non-treated and insulin-treated groups in SLP fractions, but a decrease in GLUT4 distribution was detected in the insulin-treated when compared to the non-treated condition in EN fractions ($P<0.05$). In both SLP and EN fractions, GLUT4 content appears to be greatest in RG when compared to Sol and WG. A comparison between SLP and EN fractions was not performed due to the nature of the GLUT4 content calculations. GLUT4 content was normalized to $\alpha$-actin, which was different between the SLP and EN fractions.

Figure 12: GLUT4 distribution in A) sarcolemmal enriched (SLP) and B) endosomal (EN) fractions of non-treated (Exp. 2 Con) and insulin-treated (Exp. 3 Con) experiments (Sol = soleus; RG = red gastrocnemius; WG = white gastrocnemius). Values are means ± SE [n = 2 (Sol), 3 (RG, WG)]. In SLP fractions, a main effect ($P<0.05$) of muscle (Sol=WG<RG) was detected. In EN fractions, a main effect ($P<0.05$) of experimental condition (non-treated > insulin-treated) was observed.
$\alpha_2$ subunit Western-Blotting Data from Homogenates Following Immunoprecipitation of Tyrosine Phosphorylated (Tyr-P) Residues

Tyr-P residues of muscle homogenates from non-treated and insulin-treated experiments were immunoprecipitated and Western blots for the detection of the $\alpha_2$ subunit were performed (Figure 13). The presence of $\alpha_2$ subunits in these blots reveals that the $\alpha_2$ subunits are phosphorylated on tyrosine residues. No difference in $\alpha_2$ subunit Tyr-P was detected between muscles or conditions. However, within muscle comparisons detected an increase in $\alpha_2$ subunits Tyr-P in WG, insulin treated > non-treated ($P<0.05$).

Figure 13: Tyrosine phosphorylation of the Na$^+$-K$^+$-ATPase $\alpha_2$ subunit in muscle homogenates of non-treated and insulin-treated experiments (Sol = soleus; RG = red gastrocnemius; WG = white gastrocnemius). Values are means ± SE (n=5). No significant differences were found between muscles or conditions.
Chapter Four

Discussion

Insulin-stimulated Na\(^+\)-K\(^+\)-ATPase Activity in Muscle Homogenates – Experiment I

The present study offers new insights into the role of insulin in stimulating Na\(^+\)-K\(^+\)-ATPase activity. In agreement with our first hypothesis, insulin stimulated increases in $V_{\text{max}}$, but only in Sol and RG homogenates (Experiment 1). Increased Na\(^+\)-K\(^+\)-ATPase activity was also observed at lower KCl concentrations. This novel finding, observed using the 3-O-MFPase assay, is contrary to those observations reported previously (13; 38; 49; 53). These earlier studies have provided evidence that the effect of insulin is limited to involve translocation of $\alpha_2$ (13; 38; 49; 53) and $\beta_1$ (38; 49) subunits from intracellular membranes to the plasma membrane. In particular, insulin-induced $\alpha_2$ subunit translocation in the absence of increased $V_{\text{max}}$ in Sol homogenate (13) has provided strong evidence for this hypothesis. However, a recent study has shown that cyclic stretch in cultured skeletal muscle cells not only induces translocation of $\alpha_2$ subunits from intracellular membrane to the plasma membrane, but also increases $V_{\text{max}}$ in the homogenate (76). Inhibition of translocation significantly attenuated, but did not abolish, the increase in $V_{\text{max}}$ associated with cyclic stretch (76). The relevance of this study is that the authors used the 3-O-MFPase assay to detect changes in $V_{\text{max}}$ (76). This observation suggests that the 3-O-MFPase assay can detect changes in $V_{\text{max}}$ associated with a translocation mechanism in homogenate. In the current study, the increased $V_{\text{max}}$ observed in homogenates from Sol and RG exposed to insulin cannot be attributed solely to translocation or intrinsic activity; it may be a combination of both.
In WG homogenate, 5 min insulin exposure decreased Na\(^+\)-K\(^+\)-ATPase activity at lower KCl concentrations; the \(k_{in}\) was increased by 53% in the Ins conditions. Before concluding that insulin decreases pump sensitivity in WG further attention is warranted. Insulin stimulated an increase in background activity in WG homogenates that was not observed in Sol or RG homogenates. This larger background, once subtracted, accounts for the decrease in pump activity. The source of this non-specific activity needs to be identified in order to determine the true nature of insulin’s effect in WG homogenate.

The observation that insulin affects muscles composed of mostly oxidative fibres, and not muscles of glycolytic fibres, has been previously reported (49). Lavoie et al (49) reported that insulin-induced translocation of the \(\alpha_2\) subunit and subsequent increases in \(V_{\text{max}}\) in plasma membrane fractions were specific to oxidative fibres. The null effect of insulin on \(V_{\text{max}}\) in WG homogenate observed in this study supports the hypothesis that insulin-induced changes in Na\(^+\)-K\(^+\)-ATPase distribution and activity are fibre type specific.

*Insulin-stimulated Na\(^+\)-K\(^+\)-ATPase Activity in Control SLP and EN Fractions – Experiment 2*

In Experiment 2, the plasma membrane (SLP) and endosomal (EN) enriched pools of Na\(^+\)-K\(^+\)-ATPase were separated. The fractionation protocol employed has been reported to isolate 70% of Na\(^+\)-K\(^+\)-ATPase immunoreactivity and 86% of the ouabain sensitive ATPase activity to the SLP fraction, at least in cardiac tissue (27). By using these fractions, it was possible to assess the insulin-induced influence on pump intrinsic activity, without the confounding effect of translocation. Thus, it could be investigated whether the increase in \(V_{\text{max}}\) observed in Sol and RG homogenates was due to translocation or intrinsic activity, or both. As
hypothesized, an active pool of Na\(^+\)-K\(^+\)-ATPase was detected in both non-treated SLP and non-treated EN fractions. Insulin exposure increased \(V_{\text{max}}\) in Sol and RG, but not in WG, non-treated SLP fractions. However, an increase in \(V_{\text{max}}\) in non-treated EN fractions was only observed in RG. This evidence suggests that insulin regulates Na\(^+\)-K\(^+\)-ATPase differentially in the plasma and endosomal membranes.

Although evidence suggests that insulin-induced translocation is specific to the \(\alpha_2\) (13; 38; 49; 53) and \(\beta_1\) (38; 49) subunits, it is unclear whether individual isoforms or functional heterodimers translocate. Indirect evidence would suggest that it is functional heterodimers that undergo translocation. Chibalin et al (13) showed no change in \(V_{\text{max}}\) in Sol muscle homogenate following 20 min insulin incubation, but did report increases in \(V_{\text{max}}\) in plasma membrane fractions. This implies that the intracellular stores of pumps were in a fully active state; if only \(\alpha_2\) subunits underwent translocation and formed functional heterodimers on the plasma membrane with previously embedded \(\beta\) subunits, an increase in \(V_{\text{max}}\) in homogenate should have been detected following incubation with insulin. Evidence in the present study supports the assumptions of Chibalin et al (13) that the EN pool of pumps does display activity.

In Sol non-treated SLP fractions, insulin exposure caused a 15% increase in \(V_{\text{max}}\), almost identical to that in Sol homogenate. A similar pattern of insulin-induced increases in pump activity was also observed at lower KCl concentrations. This suggests that the increase in 3-O-MFPase activity observed in muscle homogenate was due to an insulin-stimulated increase in intrinsic activity. However, a 12% decrease in \(k_m\) was also detected in the Ins condition, suggesting that insulin can also increase pump affinity for K\(^+\). Insulin failed to cause a significant increase in pump activity in Sol non-treated EN fractions, which
suggests that the insulin stimulated increase in pump activity detected in Sol homogenate was due solely to activation of pumps on the plasma membrane, and not to increased pump abundance mediated by translocation.

The data from RG non-treated fractions also suggests that the increase in insulin-stimulated activity in RG homogenate was due to increases in intrinsic activity; insulin caused a 34% increase in $V_{\text{max}}$ in homogenate and a 36% increase in $V_{\text{max}}$ in the non-treated fractions (SLP+EN). Neither the non-treated SLP nor EN fractions showed higher activity at KCl concentrations below 1mM. This suggests that an additive effect of insulin in SLP and EN fractions contributed to the change in RG homogenate.

In WG non-treated SLP fractions, there was a trend ($P<0.07$) for an insulin-induced increase in $V_{\text{max}}$. A main effect, Con<Ins, was also observed. This suggests that an insulin-induced increase in Na$^+$-K$^+$-ATPase activity was masked in homogenate by an unknown factor causing high non-specific activity. Although it was suggested that a null effect of insulin in WG homogenate agrees with previous evidence (49) of fibre type specific responses to insulin-induced translocation, this observation suggests a more complicated scenario in which WG may respond to insulin stimulation. It is possible some regulatory factor that inhibits insulin-induced pump stimulation in homogenate became dissociated from the Na$^+$-K$^+$-ATPase during fractionation procedures. Since the population of pumps in the WG non-treated EN fraction displayed no response to insulin, it can be hypothesized that such a factor is associated only with the plasma membrane.

A comparison between muscles showed that $V_{\text{max}}$ was greater in Sol and RG than in WG (Con homogenates, non-treated SLP fractions, and non-treated EN fractions). This observation agrees with previous work that suggests $V_{\text{max}}$ correlates with oxidative potential.
The measurement of maximal CS activity in this study confirmed that the muscles of different fibre type possessed different oxidative potential (RG>Sol>WG). Interestingly, the $k_m$ was WG<RG=Sol in homogenate and WG=Sol<RG in non-treated SLP and EN fractions. Furthermore, $k_m$ was lower in EN fractions when compared to SLP fractions. Differences in isoform distribution, $\alpha\beta$ heterodimers, and total pump content [ranking RG>Sol=WG (22)] likely contribute to differences in $V_{\text{max}}$ and $k_m$. However, independent from differences due to the intrinsic properties of the isoforms present in the muscles of different fibre type, rationale for the observed differences in $V_{\text{max}}$ and $k_m$ between muscle types can be hypothesized from evidence of differential phospholipid fatty acid composition between Type I and Type II muscle fibres (9). The membrane environment has been reported to be a determining factor of $\text{Na}^+\text{-K}^+$-ATPase molecular activity (enzyme activity/enzyme number) in homogenate (20). It is also possible that the populations of pumps in the SLP and EN membranes exist in different phospholipid environments, which in turn influences catalytic properties and molecular activity. If molecular activity could be measured in each of the SLP and EN fractions, it could be determined if the 2 populations of pumps are in the same active state. If pumps in the SLP fraction have a greater molecular activity, this could explain how translocation could be detected in muscle homogenates; translocation of pumps from EN to SLP would theoretically shift them into a more active state and thus contribute to increased 3-O-MFPase activity. Thus, it is possible that differing membrane environments in the plasma and endosomal membranes contributes to differences in activity between SLP and EN populations of pumps.
Insulin-stimulated Na\textsuperscript{+}-K\textsuperscript{+}-ATPase Activity in Insulin-treated SLP and EN Fractions – Experiment 3

In Experiment 3, K\textsuperscript{+}-dependent 3-O-MFPase activity was measured in insulin-treated SLP and EN fractions. Prior to fractionation, homogenates were treated with insulin (625 μM) for 5 min at 37°C. The plasma membrane (SLP) and endosomal (EN) enriched pools of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase were then separated, exactly as performed in Experiment 2. Assessment of 3-O-MFPase activity was performed on insulin-treated SLP and EN fractions under both Con and Ins conditions. In the Ins condition, fractions were exposed to 75 μM insulin for 5 min at 37°C prior to the measurement of activity.

The experimental procedure mimicked those procedures from in vivo and ex vivo studies (13; 38; 49; 53). Samples were incubated with insulin, frozen quickly, and then thawed and subjected to analysis at a later time. This experiment was performed in order to examine the possibility of α\textsubscript{2} subunit translocation in response to 5 min in vitro insulin incubation. The goal was to determine if translocation could have contributed to the increases in pump activity observed in homogenates. Although data from Experiment 2 suggests that increases in intrinsic activity can explain the increased activity in homogenate, this experiment was required to confirm this hypothesis. If no evidence of translocation was detected and pump activity was higher in insulin-treated Con fractions vs non-treated Con fractions, it could have been concluded that 5 min in vitro insulin incubation of homogenates was not sufficient to stimulate translocation; instead, increases in pump activity observed in muscle homogenates in Experiment 1 were due entirely to insulin-induced increases in intrinsic activity. Also, the exposure of the insulin-treated fractions to 75 μM insulin, as
performed in Experiment 2, could determine if further increases in pump activity were possible with subsequent insulin exposure.

Contrary to our hypothesis, an increase in pump activity was not observed in insulin-treated Con fractions when compared to the activity in non-treated Con fractions; in fact, $V_{\text{max}}$ was actually reduced in insulin-treated Sol and RG SLP Con fractions. However, $k_m$ was lower in insulin-treated SLP Con fractions of all muscles (Sol, RG, WG) and in insulin-treated RG Con EN fractions when compared to non-treated Con fractions. Since an insulin-induced decrease in $k_m$ was only observed in non-treated Sol Ins SLP in Experiment 2, this effect of insulin on $k_m$ is puzzling. It is possible that the higher concentration of insulin used in the insulin treatment of homogenates in Experiment 3 was responsible for the differing effect.

Western blotting data indicates that the insulin treatment was not sufficient to induce translocation of $\alpha_2$ subunits from EN membranes to SLP membranes. Although a decrease in $\alpha_2$ subunit abundance was observed in the EN fractions treated with insulin, no increase in $\alpha_2$ subunit abundance was observed in the respective SLP fractions. As well, no change in GLUT4 distribution was observed in SLP or EN fractions with insulin treatment. Actin filament remodeling plays a key role in regulating the translocation of GLUT4 from intracellular membranes to the plasma membrane (67). GLUT4 translocation to the plasma membrane is almost completely abolished if actin remodeling is disrupted (67). The high salt treatment performed prior to homogenization in preparation of fractionation detaches the myofilaments from membranes (27). It is possible that this treatment inhibits GLUT4 translocation. If the $\alpha_2$ subunit possesses a similar mechanism of translocation, this too may be inhibited. A decrease in EN membranes may still be observed if translocation is initiated
and subunits are moved into vesicles for trafficking. These vesicles may be lost during low speed centrifugation steps in the fractionation procedure. Thus, it can be concluded that the insulin-treatment of homogenates prior to fractionation did not result in translocation of $\alpha_2$ subunits (or GLUT4) from EN to SLP membranes.

In the absence of translocation, data from Experiment 2 suggests that the 5 min insulin incubation prior to fractionation should have caused increases in intrinsic pump activity in insulin-treated Con SLP fractions. It is possible that, since the membranes in the homogenate were not permeated prior to insulin incubation, the insulin treatment did not activate the insulin signaling cascade. If vesicles formed inside-out during homogenization, could insulin bind to the insulin receptor? Also, if the insulin-receptor tyrosine kinase was located on the inside of intact vesicles, then it may not be able to activate the downstream regulatory elements necessary to illicit an insulin response. The use of membrane permeating agents or freeze thawing could address these concerns.

It was originally hypothesized that pump activity in insulin-treated fractions would not be increased further with the 75$\mu$M insulin exposure. However, given insulin’s lack of effect on pump activity in insulin-treated Con fractions, the hypothesis may be made that subsequent insulin exposure should induce increases in pump activity, as observed in Experiment 2. Also, since deoxycholate is used to permeate membranes prior to insulin exposure, an increase in pump activity would provide indirect evidence that the insulin signaling cascade was not activated in the insulin-treated Con fractions.

In Sol and WG tissues, insulin exposure of the insulin-treated SLP fractions did not yield an increase in pump activity as observed in the non-treated fractions; the Sol SLP Ins condition even showed an increase in $k_m$ as opposed to the decrease in $k_m$ observed in non-
treated Sol SLP Ins. As in the non-treated EN fractions, pump activity in the Sol and WG insulin-treated EN fractions was not stimulated by insulin. Compared to Con, an increase in $V_{\text{max}}$ was observed in insulin-treated RG SLP Ins. Furthermore, the $K^+$-dependent 3-O-MFPase activity in insulin-treated RG SLP Con and Ins conditions, although of lower magnitude, was of almost identical pattern to that in the non-treated RG SLP Con and Ins conditions. Insulin also had a stimulatory effect on pump activity at the high concentrations of KCl in insulin-treated RG EN. Another observation consistent with that in non-treated fractions is that $k_m$ was lower in EN fractions when compared to SLP fractions.

Given some similarities between non-treated and insulin-treated fractions responses to insulin exposure, it is unknown why Sol and WG insulin-treated SLP Con and Ins conditions did not show the same relationship as that in non-treated SLP Con and Ins conditions. It appears as though the conditions under which insulin-treatment prior to fractionation altered the ability of the Na$^+$-K$^+$-ATPase in Sol and WG insulin-treated SLP fractions to respond to subsequent insulin exposure. However, insulin-treated RG SLP fractions displayed the ability to retain the response to insulin exposure, suggesting some regulatory element that allows for the insulin induced increase in Na$^+$-K$^+$-ATPase activity to be preserved following insulin-treatment of homogenates prior to fractionation. In all 3 experiments, RG was most responsive to insulin, followed by Sol and then WG. This suggests a relationship between oxidative potential of the fibre and insulin-stimulated pump activity. This hypothesis is supported by the maximal CS activity data in this study, which ranked, highest to lowest, RG>Sol>WG. Previous studies have also identified a relationship between both oxidative potential and pump activity (22) and oxidative potential and insulin-induced increases in $V_{\text{max}}$ (49).
Possible Mechanisms for Insulin-induced Increases in Na⁺-K⁺-ATPase Activity

The role of Tyr-P in the stimulation of intrinsic activity by insulin could not be evaluated in this study. Although no increase in α₂ Tyr-P was observed in muscle homogenates following insulin treatment, the fact that no increase in translocation or intrinsic pump activity was detected in insulin-treated fractions when compared to non-treated fractions indicates that the insulin treatment was not effective in stimulating pump activity. Chibalin et al (13) reported increases in Tyr-P in muscle homogenate following ex vivo insulin treatment of Sol in association with the translocation of α₂ subunits. This suggests that Tyr-P is specific to translocation. A hypothesis that Tyr-P is not associated with insulin-induced increases in intrinsic Na⁺-K⁺-ATPase activity would parallel observations with the GLUT4 protein. GLUT4 is stimulated by insulin to both increase intrinsic activity and translocate from intracellular membranes to the plasma membrane (28). However, the signaling pathways for these two events diverge somewhere along the insulin signaling cascade (28). Thus, it is possible that the Na⁺-K⁺-ATPase undergoes similar regulation with respect to insulin stimulation.

Given a role for PKC in the insulin-induced stimulation of pump activity, serine phosphorlyation should also be considered as a possible mechanism of insulin-induced increases in Na⁺-K⁺-ATPase activity. Ser-23 of the Na⁺-K⁺-ATPase has been identified as a target for PKC (10); however, PKC phosphorylation of the pump generally leads to inhibition of activity (12). The possibility exists that the increase in intrinsic activity is not mediated by direct phosphorylation. The regulatory protein PLM may be implicated. A PKC dependent mechanism of Na⁺-K⁺-ATPase regulation through PLM phosphorylation has been
reported in cardiac myocytes (33). In fact, PKC phosphorylation of PLM on Ser-63 and Ser-68 causes an increase in $V_{\text{max}}$ without altering $\text{Na}^+$ affinity (33). Although PKA phosphorylation of PLM is associated with increases in pump affinity of $\text{Na}^+$ (33), no effect on apparent $\text{K}^+$ affinity was detected in *Xenopus* oocytes (7). Thus, it is unlikely that PKA phosphorylation of PLM mediated changes in $k_m$ that were observed in this study. The absence of PKC-mediated increases in pump activity in PLM-knockout mice suggests that PKC-dependent effects on the pump are mediated primarily by PLM rather than direct pump phosphorylation (33). Furthermore, it has been recently reported that PKC phosphorylation of PLM increases the intrinsic activity of the $\text{Na}^+-\text{K}^+$-ATPase $\alpha_2\beta_1$ heterdimer in the *Xenopus* oocyte expression system (7). The possibility of an increase in cell surface expression was eliminated (7). The phosphorylation of PLM did not result in a complete dissociation from the $\text{Na}^+-\text{K}^+$-ATPase (7). Thus, such a mechanism may be possible in skeletal muscle in which PLM phosphorylation mediates an increase in catalytic activity as stimulated by PKC downstream of insulin receptor tyrosine kinase.

The observation that insulin did not increase $\text{Na}^+-\text{K}^+$-ATPase activity in WG muscle homogenate may indicate a fibre type specific effect of insulin. Although insulin stimulated activity in WG non-treated SLP fractions, this may simply indicate that some regulatory mechanism was removed during fractionation. Given the diversity in isoform properties and muscle specific expression of these isoforms, differential regulation in response to hormonal stimuli seems appropriate. The specificity of insulin-induced translocation of $\alpha_2\beta_1$ to oxidative fibres is an example of such differential regulation (49). Previous work has shown that WG expresses higher amounts of the $\beta_2$ subunit (22). The prevalence of $\alpha_2\beta_2$ heterdimers in WG may be involved in this differential response to insulin stimulation in
muscle homogenates. However, simple isoform diversity cannot explain why WG non-treated SLP Ins fractions show an increase in pump activity. Ultimately, the source of the insulin-induced non-specific activity in WG homogenates must be determined before the functional significance of insulin-induced increases in pump activity in WG SLP fractions can be interpreted.

Comparison of “in vitro” Model to “in vivo” and “ex vivo” Models

Some key differences exist that may explain the discrepancy in findings between this study and those performed previous. In studies utilizing an *in vivo* model, animals were injected with insulin and left for 30 min (38; 49; 53); of these studies, only Lavoie et al (49) measured $V_{\text{max}}$, and the measures were restricted to membrane fractions of red and white fibres. Chibalin et al (13) used an *ex vivo* (whole muscle excised and incubated in media supplemented with nutrients) model in which whole Sol muscle was incubated in a solution containing insulin for 30 min. $V_{\text{max}}$ was measured through a $^{32}$P-radiolabelled assay that required 15 min incubation (13). Chibalin et al (13) reported an increase in $V_{\text{max}}$ in Sol plasma membrane fractions, but no change in $V_{\text{max}}$ in Sol homogenate, following insulin treatment (13). This observation implies that the entire population of pumps in the muscle was active in the homogenate. As a result, translocation would not lead to a change in $V_{\text{max}}$ in the homogenate. Any change (or lack there of) in $V_{\text{max}}$ would be an indication of insulin influence on intrinsic activity. Lavoie et al (49) detected increased $V_{\text{max}}$ in plasma membrane fractions of red muscle fibres using the 3-O-MFPase assay. However, no measures of $V_{\text{max}}$ in homogenate were performed in the study (49).
Both the *in vivo* and the *ex vivo* studies observed an increase in $\alpha_2$ subunit abundance at the plasma membrane with insulin treatment (13; 38; 49; 53). As concluded by Chibalin et al (13), the observed increase in $V_{\text{max}}$ was a result of $\alpha_2$ subunit translocation to the plasma membrane. However, these previous studies used long incubation times with insulin (13; 38; 49; 53). Furthermore, the duration of the assay used by Chibalin et al (13) to measure $V_{\text{max}}$ was considerably longer than the one employed in the present study. It is possible that these previous experiments (13; 38; 49; 53) were not suited to detect possible influences of insulin on intrinsic pump activity. A short, possibly transient, effect of insulin on intrinsic activity could serve to increase pump activity as a mechanism to maintain Na$^+$ and K$^+$ homeostasis until the translocation pathway can be fully activated. Such a hypothesis is supported by our data, which shows insulin stimulated increases in $V_{\text{max}}$ in Sol and RG after 5 min incubation at 37°C. Also, insulin-incubation time dependencies show that $V_{\text{max}}$ decreases with longer incubation times (Appendix D). A short insulin exposure, as performed in this study, may not be of sufficient time to induce translocation of $\alpha_2$ subunits from intracellular stores to the plasma membrane. However, it cannot be assumed that translocation did not occur, as this phenomenon may occur more quickly *in vitro*.

The use of an *in vitro* model allows for control of the experimental conditions in a way not possible with *in vivo* studies. With *in vivo* studies, the contribution of confounding variables that may influence the desired experimental perturbation is a concern. For example, stress of the animals may increase catecholamine levels, which in turn influences dependent measures. Also, if blood flow distribution to the muscle is not homogeneous, then measurements in whole muscle homogenate may dilute an effect of the treatment. With the use of an *ex vivo* model, is the whole muscle exposed to the surrounding fluid environment?
The muscle is incubated in an oxygenated medium containing nutrients and any treatment (ex. hormone, drug). Since there is not perfusion of the muscle, are fibres in the interior of the muscle exposed to the same concentration of supplements in the media as those in direct contact with the media?
Chapter 5

Summary and Conclusions

Summary

This is the first study to show insulin-induced increases in Na\(^+\)-K\(^+\)-ATPase activity in skeletal muscle homogenates. The fractionation of plasma membrane and endosomal populations of Na\(^+\)-K\(^+\)-ATPase allowed for the assessment of pump activity without the possibility of translocation. Insulin stimulated increases in pump activity in the SLP fractions, providing evidence that insulin can stimulate increases in intrinsic activity. However, fibre type differences in the response to insulin stimulation suggest differential regulation with respect to oxidative potential. Given differences in isoform distribution and αβ heterodimer combinations in muscles of different fibre types, such regulation is not surprising. Also, differences in catalytic properties between SLP and EN membranes suggest that the membrane environment of the pump may play an intricate role in determining pump activity. Insulin-treatment of homogenates in vitro may allow for further investigation into the mechanism of insulin-induced stimulation of intrinsic Na\(^+\)-K\(^+\)-ATPase activity and the possible role of Tyr-P in this stimulation.

Limitations

One limitation of the present study is the concentration of insulin investigated. During pilot work, insulin concentration dependency curves (Appendix C) were performed in each muscle in order to determine optimal insulin-stimulated increases in V\(_{\text{max}}\). Since insulin incubation was in vitro, we wanted to ensure insulin receptor saturation. With muscle homogenization, membranes form vesicles and become interspersed in the homogenate. As
such, the organization of hormone receptors in close vicinity to hormone delivery (i.e. localized on plasma membrane with close capillarization and short diffusion distances for hormones) is not maintained and the probability of stimulating signaling cascades may be decreased. We did not want to observe a null effect of insulin due to inadequate dosage. Our study appears to be the first attempt to assess insulin influence on Na\(^+\)-K\(^+\)-ATPase activity in vitro using the 3-O-MFPase assay. The goal was to determine whether or not it was possible to detect an insulin effect, not to simulate physiological conditions. This insulin activation of 3-O-MFPase activity was shown to be specific to the Na\(^+\)-K\(^+\)-ATPase: insulin stimulation of background activity was measured and subtracted in each muscle preparation. Also, trials conducted in Sol homogenate with a Ca\(^{2+}\)-ATPase inhibitor, thapsigargin, showed no influence of thapsigargin on 3-O-MFPase activity (unpublished observations). The specificity of the assay to the Na\(^+\)-K\(^+\)-ATPase could not be determined with conventional ouabain incubations. The incubation of muscle homogenate with 5mM ouabain for 20 min inhibits >90% of the K\(^+\)-stimulated 3-O-MFPase activity (69). Given optimal insulin exposure was 5 min, with subsequent decreases in \(V_{\text{max}}\) with longer incubations (Appendix D), the use of ouabain to measure the specificity of insulin-induced increases in \(V_{\text{max}}\) was inappropriate. Despite the high insulin concentrations utilized, the observed effect of insulin is intriguing. These measurements should be performed with physiological concentrations of insulin to increase the impact of the current findings; it must be determined if this in vitro model can still detect insulin-stimulated increases in pump intrinsic activity with physiological insulin concentrations.

Second, the procedure in Experiment 3 prevented the investigation of some hypotheses. The absence of an insulin-induced increase in pump activity in fractions
following homogenate incubation with insulin prevented the investigation of a role for Tyr-P in mediating changes in pump activity. Future experiments may circumvent this issue by using membrane permeating procedures prior to insulin incubation. Also, translocation may have been abolished due to treatment of homogenates with high salt solution. However, an experimental model in which translocation is inactivated may prove to be useful. In this way, any changes in activity observed with insulin incubation must be attributed to changes in intrinsic activity. Tyr-P could then be measured to determine its role in mediating this change. Thus, this model may very well provide an ideal method of investigating the mechanisms of insulin-induced changes in intrinsic activity.

Third, in Experiment 3, there was no control condition in which samples were incubated at 37°C without insulin-treatment. In Experiment 2, non-treated homogenates were not incubated at 37°C for 5 min prior to fractionation. These samples were treated the same as homogenates in Experiment 1 in order to determine if increases in intrinsic activity could account for changes in activity observed in homogenates. Thus, the conditions under which insulin-treatment occurred may have altered the response of the Na⁺-K⁺-ATPase to insulin stimulation.

Fourth, in Experiment 2 it was suggested that insulin differentially regulates Na⁺-K⁺-ATPase in SLP and EN membranes. This was postulated because insulin did not increase pump activity in EN fractions as it did in SLP fractions. This suggests that some regulatory element(s) was missing. It is also possible that the necessary signaling proteins to illicit an insulin response were not present in EN fractions. In fact, since the exact insulin signaling pathway is not known, the presence of all the necessary proteins to illicit an insulin response could not be confirmed in either fraction. It is possible that insulin had a direct effect on the
Na\textsuperscript{+}-K\textsuperscript{+}-ATPase. To test this, experiments in which downstream proteins in the insulin signaling cascade are inhibited and pump activity measured are required.

\textit{Future Direction}

The findings from this study provide a basis for further investigations into the effects of insulin on Na\textsuperscript{+}-K\textsuperscript{+}-ATPase function. First, Experiments 1 and 2 should be completed using physiological concentrations of insulin in order to determine if the \textit{in vitro} model is a valid method to measure insulin-induced influences on pump activity at these concentrations. Second, as suggested above, inhibitors of proteins in the insulin signaling cascade could be used to investigate the pathway through which insulin is influencing Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity in the \textit{in vitro} model. Third, modification to the insulin-treatment in Experiment 3 may lead to a novel model in which translocation mechanisms are inhibited and insulin-induced changes in activity can be isolated. Using this model, the mechanism of insulin-stimulation of pump activity can be investigated. For example, probing of Tyr-P in each of SLP and EN fractions could investigate if Tyr-P has a role in insulin-induced effects and if these two membrane sources of pumps are differentially modified by insulin.

The findings from \textit{in vitro} studies can also be applied to an \textit{in vivo} animal model. Previous experiments have shown that glucose supplementation attenuates fatigue without sparing muscle glycogen stores (32; 45; 52). Furthermore, Karelis et al (45; 52) have demonstrated protection of cell excitability as measured by the M-wave in rat Sol and plantaris muscles. Recent work by Green et al (32) provides evidence of Na\textsuperscript{+}-K\textsuperscript{+} ATPase activation during glucose supplementation in human VL muscle. It may be hypothesized that Na\textsuperscript{+}-K\textsuperscript{+}-ATPase is activated by the elevated insulin during glucose supplementation. Using
electrical stimulation in the anaesthetized rat with glucose infusion, the role of translocation, Tyr-P, and intrinsic activation of the Na\(^+\)-K\(^+\)-ATPase can be investigated. If steps in the mechanism of insulin activation are known from \textit{in vitro} studies, these steps can be probed in muscle following the \textit{in vivo} treatment to determine their role, if any, in maintaining membrane excitability.
   *Endocrinology* 146: 3773-3781, 2005.


25. **Fraser SF, Li JL, Carey MF, Wang XN, Sangkabutra T, Sostaric S, Selig SE, Kjeldsen K and McKenna MJ.** Fatigue depresses maximal in vitro skeletal muscle


62. **Petersen AC, Murphy KT, Snow RJ, Leppik JA, Aughey RJ, Garnham AP, Cameron-Smith D and McKenna MJ.** Depressed Na+\(^+\)-K+\(^+\)-ATPase activity in skeletal muscle at fatigue is correlated with increased Na+\(^+\)-K+\(^+\)-ATPase mRNA expression following intense exercise. *Am J Physiol Regul Integr Comp Physiol* 289: R266-R274, 2005.


Appendix A: A black, flat bottom micro-plate was loaded with 250μL of sample in each well. A set of 8 wells was used for each of control (Con) and insulin (Ins). The blank well contained 0 mM KCl.

<table>
<thead>
<tr>
<th></th>
<th>Con</th>
<th>Ins or GLU</th>
</tr>
</thead>
<tbody>
<tr>
<td>blank</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.2mM KCl</td>
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<td></td>
</tr>
<tr>
<td>0.5mM KCl</td>
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<td></td>
</tr>
<tr>
<td>0.75mM KCl</td>
<td></td>
<td>75 μM Ins</td>
</tr>
<tr>
<td>1.0mM KCl</td>
<td></td>
<td>OR</td>
</tr>
<tr>
<td>1.5mM KCl</td>
<td></td>
<td>7 mM glucose</td>
</tr>
<tr>
<td>3mM KCl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5mM KCl</td>
<td></td>
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</tr>
</tbody>
</table>

Appendix A: A black, flat bottom micro-plate was loaded with 250μL of sample in each well. A set of 8 wells was used for each of control (Con) and insulin (Ins). The blank well contained 0 mM KCl.
Appendix B:
Set-up of the micro-plate to generate a dose response curve in the measure of 3-O-MFPase activity

<table>
<thead>
<tr>
<th></th>
<th>Ins dose response</th>
<th>GLU dose response</th>
</tr>
</thead>
<tbody>
<tr>
<td>blank</td>
<td>○</td>
<td>○</td>
</tr>
<tr>
<td>0μM</td>
<td>○</td>
<td>0 mM</td>
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<tr>
<td>25μM</td>
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<td>5 mM</td>
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<td>12 mM</td>
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<tr>
<td>500μM</td>
<td>○</td>
<td>15 mM</td>
</tr>
<tr>
<td>625μM</td>
<td>○</td>
<td>20 mM</td>
</tr>
</tbody>
</table>

Appendix B: A black, flat bottom micro-plate was loaded with 250μL of sample in each well. A set of 8 wells was used to generate a dose response curve. Blanks contained 0 mM insulin (Ins) or GLU (glucose) and 0 mM KCl. Five mM KCl was present in wells 2-7.
Appendix C: Insulin-concentration dependent Na\(^+\)-K\(^+\)-ATPase activity in muscle homogenates of different muscle types (Sol = soleus; RG = red gastrocnemius; WG = white gastrocnemius). Valued are mean ± SE (Sol n=7; RG n=4; WG n=4). Maximal insulin-stimulated activity was observed at 75μM insulin.
Appendix D
Time dependence of 3-O-MFPase activity, with and without 75μM Insulin

Appendix D: Time-dependent Na⁺-K⁺-ATPase activity in muscle homogenates, with and without 75μM insulin at 37°C, of A) Soleus (Sol); B) red gastrocnemius (RG); and C) white gastrocnemius (WG). Valued are mean ± SE (Sol n=4; RG n=4; WG n=3). In Sol, main effects of time, 20min<10min<5min, and condition, Con<Ins, were found. In RG, main effects of time, 20min=15min=10min<5min, and condition, Con<Ins, were found. In WG, a main effect of condition, Con<Ins, was found. Also, a trend (P<0.07) was detected for an effect of time.
Appendix E
Fractionation of Sarcolemmal enriched and Endosomal membranes

Homogenate
1000g for 5 min

supernatant  pellet
resuspend 1000g for 5 min

supernatant  pellet
100g for 10 min

supernatant  pellet
100g for 10 min

supernatant  pellet
5000g for 10 min
pellet (store on ice)

20000g for 30 min

En fraction (500-600 μL)

freeze in LN2 and store at -80 degrees C

SLP fraction

combine and resuspend in ~400 μL

freeze in LN2 and store at -80 degrees C

Appendix E: Flow diagram of fractionation procedure
Appendix F: Immunoprecipitation of Tyrosine Phosphorylated Proteins

100 uL homog

- Ice cold lysis buffer (3x) 1h @ 4°C
  - 300g for 10 min
  - Pellet

- 60 uL of 50% protein A-agarose bead slurry; 1h @ 4°C
  - 14000 g for 20 sec
  - Pellet

- 50 uL of PY69; 100 uL of the protein A-agarose slurry; 4h @ 4°C
  - 14000 g for 20 sec
  - Pellet

- Wash 4x with lysis buffer
  - Supernatant
  - Save for contamination profiling

- Wash 1:
- Wash 2:
- Wash 3:
  - Suspended in 20 uL of each homog buffer
  - Store at 4°C
  - Heat at 95°C for 5

Western blotting for α2 subunit

Appendix F: Flow diagram of Tyr-P immunoprecipitation protocol
Appendix G
Maximal Citrate Synthase Activity Assay

Reaction 1

* 10 µl of 1:2500 dil. homog. (4 µg muscle) is added to 100 µl reagent 1.
- the above step requires a 50x (10 ul sample added to 490 ul buffer) dilution of the original 1:50 homogenate using diluting media.
- 3.33, 6.67 and 10 nmoles citric acid / 10 µl used as standards.
- this is allowed to react for 1 hour at room temp.
* stopped by adding 10 µl of 0.5 N NaOH and heating at 95°C for 5 min.
  (this also destroys any excess oxaloacetate)

__________________________________________________________________________

Reaction 2

* 1 ml of reagent 2 is added to the product of the first reaction.
- leave for 20 min at room temp.
* add 60 ul of 1 N HCl, leave 10 min at room temp.
* add 100 ul aliquot to 1 ml of 6 N NaOH/Imidazole
- heat at 60°C for 20 min.
- a fluorescing NAD⁺ by product is measured.

__________________________________________________________________________

Enzymes, Substrates and Products

Reaction 1

Citrate Synthase
Oxaloacetate + S-Acetyl-CoA → Citrate
EC 4.1.3.7
add 0.5 N NaOH and heat to 95°C to destroy excess oxaloacetate

Reaction 2

Citrate Lyase
Citrate → Acetate + Oxaloacetate
EC 4.1.3.6

Malate Dehydrogenase
Oxaloacetate + NADH + H⁺ → Malate + NAD⁺
EC 1.1.1.37
**Reagent Preparation** (citrate synthase)

### Reagent 1 (100 µl / sample)

<table>
<thead>
<tr>
<th>Stock</th>
<th>for 5 ml</th>
<th>Final Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Tris-HCl</td>
<td>1M</td>
<td>250µl</td>
</tr>
<tr>
<td>2. Acetyl CoA</td>
<td>FW 809.6</td>
<td>1.62mg</td>
</tr>
<tr>
<td>(Sigma A-2056)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. Oxalacetate</td>
<td>FW 132.1</td>
<td>0.33mg</td>
</tr>
<tr>
<td>added just prior to the start of the assay</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. BSA</td>
<td>10%</td>
<td>125µl</td>
</tr>
</tbody>
</table>

MIX ITEMS 1 AND 4 WITH 4ml OF H2O, BRING TO VOL. WITH dH2O, ADD ITEM 2. pH TO 8.1.

### Reagent 2 (1 ml / sample)

<table>
<thead>
<tr>
<th>Stock</th>
<th>for 30 ml</th>
<th>Final Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Tris-HCl</td>
<td>1M</td>
<td>3ml</td>
</tr>
<tr>
<td>2. ZnCl2</td>
<td>100mM</td>
<td>30µl</td>
</tr>
<tr>
<td>3. BSA</td>
<td>10%</td>
<td>30µl</td>
</tr>
<tr>
<td>4. NADH</td>
<td>FW 709.4</td>
<td>0.64mg</td>
</tr>
<tr>
<td>5. Citrate Lyase</td>
<td>0.625U/ml</td>
<td>150µl</td>
</tr>
<tr>
<td>(2.5mg into 1ml 100mM Tris)</td>
<td>(BM 354 074)</td>
<td></td>
</tr>
<tr>
<td>6. Malate dehydrogenase</td>
<td>5mg/ml</td>
<td>15µl</td>
</tr>
<tr>
<td>(BM 127 914)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(1200 U/mg)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

MIX ITEMS ITEMS 1,2,3 AND 4 WITH 15ml OF dH2O, BRING TO VOL., ADD ITEMS 5 AND 6. pH TO 7.5.
Standard Preparation

Citric acid - MW 192.1
- 2.666, 5.333 and 8 nmole of standard are required in the 10 µl of standard to be added.
- place 19.21 mg of citric acid into 10 ml of dH2O (100 nmoles / 10 µl). Freeze in small aliquots (500 µl) in eppendorf tubes and store at -50°C or lower.
- bring 500 µl of the concentrated standard prepared above to 5 ml using 4.5 ml of dH2O (10 n mole / 10 µl).
- add 333 and 667 µl of this solution to 667 and 333 µl of dH2O respectively.
- this yields 2 additional standards of 3.33 and 6.67 n moles / 10 µl.

Calculations (sample only! absolute units of fluorescence will vary)

Highest standard: 8 nmole s / 10 µl
   Fluorescence per 8 nmole is 19.75/hr
   Fluorescence per nmole is 2.469/hr/nmole

Sample: a 4 ug sample of tissue yields a fluorescence of 170 units.
# nmole s converted per 4ug of tissue : 8 / 2.469 = 3.240 nmole s/hr/4 ug tissue
   or 0.810 µmole s/hr/µg tissue
   or 0.810 m mole s/hr/g tissue

Assuming 15 % protein:
# m mole s/hr/g protein: 0.810 /.15 = 5.400 m mole s/hr/g prot
   or 5.400 moles/hr/kg prot

Method adapted with small changes from

American Journal of Physiology. 244 (Cell Physiology 13): C276-C287, 1983
Appendix H: Glucose-concentration dependent Na\(^+\)-K\(^+\)-ATPase Activity

Appendix G: Glucose-concentration dependent Na\(^+\)-K\(^+\)-ATPase activity in muscle homogenates of different muscle types (Sol = soleus; RG = red gastrocnemius; WG = white gastrocnemius). Valued are mean ± SE (n=3). No significant differences in glucose-stimulated activity were observed; however 7mM glucose was chosen for experimentation because it is in the higher range of physiological glucose concentrations and there appears to be a slight spike in activity at this glucose concentration.
Appendix I: Glucose stimulation of Na\(^+\)-K\(^+\)-ATPase Activity

Appendix H: K\(^+\)-dependent Na\(^+\)-K\(^+\)-ATPase activity in skeletal muscle homogenates of A) soleus, B) red gastrocnemius (RG), and C) white gastrocnemius (WG) muscles, with and without 7mM glucose (GLU) for 5 min at 37°C. Values are means ± SE (n=5). In A, main effects (P<0.05) for KCl (0<0.2<0.5<0.75<1.0=1.5=3.0=5.0) and condition (Con<GLU) were found. In B, an interaction between KCl concentration and condition was observed (P<0.05). In C, a main effect (P<0.05) of KCl
concentration (0<0.2<0.5<0.75<1.5=3.0=5.0, 1.0<3) was observed. * Significantly different from Con; "
Significantly different from 1.5 mM KCl.

Given evidence that glucose stimulates Na\(^+\)-K\(^+\)-ATPase activity, the effect of 7mM glucose on the K\(^+\)-stimulated 3-O-MFPase activity was assessed in Sol, RG, and WG muscle homogenates. In this experiment, glucose was not used as a substrate. Thus, it can be examined whether glucose has any effect on Na\(^+\)-K\(^+\)-ATPase activity as a signaling molecule. This data suggests that, at least in Sol, GLU may serve a role as a signaling molecule in stimulating pump activity.