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The effects of modulating $K^+_{(ATP)}$ channel activity in normal and 1-week denervated mouse EDL and soleus muscles

By

Wadih Y. Matar

A thesis submitted to the School of Graduate Studies and
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Department of Cellular and Molecular Medicine
Faculty of Medicine
University of Ottawa

To mom and dad.

"And there are those who give and give it all.

These are the believers in life and the bounty of life, and their coffer is never empty. (...)

And there are those who give and know not pain in giving, nor do they seek joy, nor give with mindfulness of virtue;

They give as in yonder valley the myrtle breathes its fragrance into space.

Through the hands of such as these God speaks, and from behind their eyes He smiles upon the earth."

Kahlil Gibran on "giving"
The prophet.

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ABSTRACT

During exercise, the metabolic rate of muscle increases considerably, and hence the concentration of various metabolites is altered. Surprisingly, the concentration of ATP is not decreased significantly even at exhaustion. This suggests that the muscle may have a safety mechanism that shuts down force generation in order to avoid energy depletion, as the latter causes irreversible damage to the muscle cell. The $K^+_{(ATP)}$ channel, which is activated by decreased ATP levels, might be involved in this safety mechanism. According to its postulated mechanism, once activated the channel can contribute to decreasing force generation by decreasing the excitability of the muscle membrane. The scope of this thesis will deal with the physiological role of the $K^+_{(ATP)}$ channel in skeletal muscle during fatigue.

The main objectives of this study were to understand the physiological role of the $K^+_{(ATP)}$ channel and how this role is affected by 1-week denervation in mouse EDL and soleus muscles. $K^+_{(ATP)}$ channels were either blocked with 10 μM glibenclamide, or activated with 100 μM pinacidil, while muscles were fatigued by tetanic contractions every sec for 3 min. In normal EDL and soleus muscles (excised from non-denervated animals), pinacidil increased the rate but not the extent of fatigue, abolished the resting tension developed during fatigue in control muscle, enhanced force recovery, and increased $^{86}Rb^+$ fractional loss during fatigue. Pinacidil had no effect on the ATP and PCr contents of EDL muscle. However, in soleus muscle, the decrease in ATP content observed during fatigue was abolished in the presence of pinacidil. Glibenclamide on the other hand, significantly increased resting tension in both

EDL and soleus muscles, prolonged the repolarization phase of action potential, and caused greater ATP depletion in soleus muscle. Glibenclamide did not affect the rate and extent of fatigue, force recovery and $^{86}Rb^{+}$ fractional loss.

After 1-week denervation, Kir6.2 mRNA content (the protein subunit that constitutes the pore of the $K^{+}_{(ATP)}$ channel) did not change in EDL, whereas it decreased by 2.7 fold in soleus. In denervated EDL and soleus muscles, pinacidil had the same effects as those observed in normal muscles. However, the pinacidil effects on the decrease in tetanic force and the increase in $^{86}Rb^{+}$ fractional loss were significantly greater in denervated than normal muscle. Another difference was that pinacidil reduced the extent of ATP depletion during fatigue only in soleus for normal muscles, whereas it was observed only in EDL for denervated muscle. Glibenclamide also had significantly greater effects in denervated than normal muscles. It caused a slower force decrease during fatigue and impaired force recovery in denervated muscles, whereas these effects were not significant in normal muscle.

The pinacidil effects suggest that in both normal and denervated muscles, the $K^{+}_{(ATP)}$ channels can contribute to a decrease in force during fatigue, help in preserving energy and muscle function (force recovery). On the other hand, the glibenclamide effects suggest that in normal muscles few $K^{+}_{(ATP)}$ channels are activated to significantly alter the rate of fatigue and force recovery, whereas in denervated muscles, the channel becomes more important in protecting muscle function (i.e. reducing resting tension and improving force recovery).

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LIST OF ABBREVIATIONS

$[\,]_i$	intracellular concentration
$[\,]_o$	extracellular concentration
°C	degree Celsius
μCi	microcurie
ADP	adenosine-5'-diphosphate
AMP	adenosine-5'-monophosphate
ANOVA	analysis of variance
ATP	adenosine-5'-triphosphate
Ba^{2+}	barium ion
Ca^{2+}	calcium ion
Ca_{50}	concentration for half-maximal activation
$CaCl_2$	calcium chloride
Cl^-	chloride ion
CO_2	carbon dioxide
Cs^{2+}	caesium
DMSO	dimethyl sulfoxide
EC_{50}	half-maximal effect
EDL	extensor digitorum longus
EGTA	ethylene glycol-bis(aminoethyl ether) N,N,N',N'-tetraacetic acid
FDB	flexor digitorum brevis

F_{max}	maximum calcium-activated force
gli	glibenclamide
H^+	hydrogen ion
Hz	hertz
IC_{50}	half-maximal inhibition
K^+	potassium ion
$K^+_{(ATP)}$ channel	ATP-sensitive K^+ channel
$K^+_{(Ca^{2+})}$ channel	calcium-activated K^+ channel
KCl	potassium chloride
KCO	potassium channel opener
K_2CO_3	potassium carbonate
K_d	concentration for half-maximal activation
kHz	kilohertz
K_i	concentration for half-maximal inhibition
Kir	potassium inward rectifier
L.S.D.	least significant difference
MΩ	megaohm
MCT	Duncan multiple comparison test
Mg^{2+}	magnesium ion
MgADP	magnesium adenosine-5'-diphosphate
MgATP	magnesium adenosine-5'-triphosphate
$MgCl_2$	magnesium chloride

mV	millivolt
n	number of sample
Na^+	sodium ion
NaCl	sodium chloride
$NaHCO_3$	sodium hydrogen carbonate
NaH_2PO_4	sodium dihydrogen orthophosphate
N/cm^2	Newton per centimetre square
NIDDM	non-insulin dependent diabetes mellitus
O_2	oxygen
P	probability
pCa	- log $[Ca^{2+}]$
PCA	perchloric acid
PCr	phosphocreatine
pH_i	intracellular pH
P_i	inorganic phosphate
pin	pinacidil
P_{open}	open-state probability
pS	picosiemen
Rb^+	rubidium ion
S.E.M.	standard error of the mean
SUR	sulfonylurea receptor
TEA	tetraethylammonium
t-test	student t-test

V	volt
VDCC	voltage-dependent calcium channel
v/v	volume/volume
w/w	weight/weight

THESIS FORMAT

This thesis is written in the paper format. Chapter 1 is an overall introduction to the work where the theoretical background is discussed. Chapter 2 is a paper titled "Pinacidil suppresses contractility and preserves energy but glibenclamide has no effect during fatigue in skeletal muscle". This paper is published in the American Journal of Physiology 278: C404-16, 2000. Chapter 3 consists of a second manuscript titled "Denervation enhances the effects of pinacidil and glibenclamide during fatigue in EDL and soleus muscles." This paper has been submitted to the American Journal of Physiology (Cell. Physiol.). The last chapter of this thesis consists of an overall discussion of the work.

CHAPTER 1: GENERAL INTRODUCTION

Muscle fatigue is defined as "the decrease in force generating capacity", and should be distinguished from exhaustion, which occurs when "the required force can no longer be maintained" (Vollestad & Sejersted, 1988). The etiology of fatigue is multifactorial and is not to date fully understood. It can possibly span anywhere from the activation of the α-motoneurone to the actin-myosin reaction. In an early work, Merton (1954) showed that the reduced force production, resulting from a prolonged voluntary contraction, could not be improved via direct motor nerve stimulation. He concluded that reduced central drive and neuromuscular transmission were not important factors in the onset of fatigue. Accordingly, most works point at the muscle itself as the principal site where fatigue occurs.

During exercise, the metabolic rate of muscle increases considerably, and hence the concentration of various metabolites starts to change. Most early work tried to find a correlation between the decrease in the intracellular metabolites, mainly ATP and PCr, and the drop in force production during fatigue (for example see Hermansen *et al.*, 1967; Hultman & Bergström, 1967). Although some correlations were established (Dawson *et al.*, 1978; Le Rumeur *et al.*, 1990) it was soon realized that a decrease in ATP or PCr concentrations does not cause any decrease in force generated by skinned fiber preparations (Chaen *et al.*, 1981; Steinen *et al.*, 1988; Godt & Nosek, 1989). It is now established that among all the metabolites which concentrations change during fatigue, only the increases in intracellular hydrogen (H^+) and inorganic phosphate (P_i) cause significant decreases in force (Cooke & Pate, 1985; Renaud *et al.*, 1986; Godt & Nosek, 1989; Westerblad *et al.*, 1997). In an interesting work, Godt and Nosek (1989) showed that all the

changes in metabolites can account for only one third of the decrease in force observed during fatigue. Another intriguing factor is that many studies report no major depletion of ATP during fatigue even at exhaustion (Katz *et al.*, 1986; Miller *et al.*, 1988). The latter suggests, that the muscle may have a safety mechanism that shuts down force generation in order to avoid energy depletion, which causes irreversible damage to the muscle cell. This safety mechanism would act as a limiting step during the onset of fatigue. The limiting step can occur anywhere between the propagation of the action potential at the level of the cell membrane to the generation of force by the actin-myosin complex. However, there is now strong evidence pointing to a failure of calcium (Ca^{2+}) release by the sarcoplasmic reticulum as the major cause of fatigue (Vollestad & Sejersted, 1988; Fitts, 1994). This then implies that the site of this limiting step could either be the excitability of the membrane or the sarcoplasmic reticulum itself.

Fenn (1937) was the first to report an increased potassium (K^+) efflux from fatigued muscle. Since then many studies have confirmed this finding (for example see Sreter, 1963; Hnik *et al.*, 1986; Lindinger & Heigenhauser, 1988), and have shown that the resultant increase in extracellular K^+ concentration is a major factor that can contribute to the decreased membrane excitability (Clausen & Everts, 1991; Bouclin *et al.*, 1995; Cairns *et al.*, 1995; Nielsen *et al.*, 1998). K^+ can leave the cell via a variety of channels collectively referred to as potassium channels. These include the delayed rectifier during an action potential and the inward rectifier in the resting state. There are two other K^+ channels that are normally closed in resting conditions, but can be opened under certain anormaxic conditions. One channel is the Ca^{2+}-sensitive K^+ channel ($K^+_{(Ca^{2+})}$ channel), which is activated when intracellular Ca^{2+} levels increase (Moczydlowski & Latorre, 1983; Vergara & Latorre, 1983; Latorre *et al.*, 1989). The second channel is the ATP-sensitive K^+

channel ($K^+_{(ATP)}$ channel) which is activated when intracellular energy levels (i.e. ATP) decrease (Spruce *et al.*, 1987). This type of channel might be involved in limiting force development during fatigue and therefore acts as a cytoprotective mechanism.

The scope of this thesis will deal with the $K^+_{(ATP)}$ channel. The first objective consisted of studying the physiological role of this channel in skeletal muscle during fatigue. Our second objective aimed at investigating the effects of abolishing muscle activity (via denervation) on the expression and the physiological importance of the channel. In the first section of the introduction, the characteristics of the $K^+_{(ATP)}$ channel will be described in terms of its biophysical properties, regulation by metabolites, molecular biology and pharmacology. In the second section, the postulated function of the $K^+_{(ATP)}$ channel in different tissues will be described, with the emphasis put on its role in skeletal muscle. Finally, the last section will state the problem and the objectives of this thesis.

1) CHARACTERISTICS OF THE $K^+_{(ATP)}$ CHANNEL

ATP-sensitive potassium channels have been found in various tissues, such as cardiac, skeletal and smooth muscles (Noma, 1983; Spruce *et al.*, 1985; Standen *et al.*, 1989) as well as pancreatic β-cells and neurons (Cook & Hales, 1984; Ashford *et al.*, 1988). In all of the above mentioned tissues, the $K^+_{(ATP)}$ channel is characterized by its sensitivity to ATP at the cytoplasmic surface of the membrane, its 60-75 pS conductance as well as its bursting activity.

1-A) BIOPHYSICAL PROPERTIES

The channel is highly selective for K^+ with negligible permeability to sodium (Na^+). In skeletal muscle, the Na^+ permeability is 65 times less than that of K^+ ($P_{Na}/P_K = 0.015$) (Spruce *et al.*, 1987). This Na^+-K^+ permeability ratio is well within the typical range of other potassium channels (Standen, 1992). The $K^+_{(ATP)}$ channel also permeates rubidium (Rb^+) with comparable permeability to K^+ (Nichols & Lederer, 1991). Spruce *et al.* (1987) showed that the Rb^+-K^+ permeability ratio (P_{Rb}/P_K) in frog skeletal muscle is equal to 0.76, with an external $[Rb^+]_o$ of 60 mM.

$K^+_{(ATP)}$ channels are time and voltage-independent, because neither the opening nor the closure of the channel are modulated by membrane potential (Noma, 1983; Spruce *et al.*, 1985). In the absence of ATP, the channel displays a series of rapid openings and closures resulting in a bursting activity pattern (Ashcroft *et al.*, 1988). The channel's bursts periods are separated by relatively long interburst periods where the channel is closed. Occasionally, the burst period may exhibit many shorter duration periods where the channel returns to its original closed-state level. The latter led Ashcroft *et al.* (Ashcroft *et al.*, 1988) to suggest that there are two types of channel

closures: a short closure that occurs within a burst and a longer closure accounting for interbursts closures. The mean closure time within a burst ranges from 0.3 to 0.8 msec whereas the mean open time varies from 2 to 3 msec. The longer interburst closure ranges from 100 to 300 msec (Ashcroft & Ashcroft, 1990). The characteristics of opening and closure of the channel are the same for the channels present in β-cells (Ashcroft *et al.*, 1988), cardiac (Zilberter *et al.*, 1988) and skeletal muscles (Spruce *et al.*, 1987).

The channel's current-voltage relation shows a weak inward rectification when the channel is exposed to roughly symmetrical concentrations of K^+; the outward currents being significantly smaller than inward (Ashcroft, 1988; Aguilar-Bryan *et al.*, 1998). This rectification is caused by a voltage-dependent block of outward currents by intracellular cations, mainly Na^+ and magnesium (Mg^{2+}) (Horie *et al.*, 1987). Polyamines are also believed to play a role in the inward rectification by blocking the channel at levels above the reversal potential (Lopatin *et al.*, 1995). The current-voltage relation of $K^+_{(ATP)}$ channel in frog skeletal muscle is also affected by changes in external potassium concentrations, $[K^+]_o$ (Spruce *et al.*, 1987). Spruce *et al.* (1987) reported that when $[K^+]_o$ is 60 mM, the estimated channel's conductance is 42 pS. A drop of $[K^+]_o$ to 2.5 mM, a concentration closer to physiological levels, decreased the conductance to 14 pS. In a different study, Woll *et al.* (1989) estimated the single channel conductance of the channel in mouse skeletal muscle to be 74 pS with 160 mM K^+ on each side of the membrane.

1-B) REGULATION OF CHANNEL ACTIVITY BY METABOLITES

1-B-i) ATP

The $K^+_{(ATP)}$ channel is closed by the binding of ATP on the intracellular side of the cell membrane (Spruce *et al.*, 1987). Extracellular ATP has no effect on channel activity. Under patch clamp conditions, ATP closes the channel with a half-maximal inhibition (Ki) at $[ATP]_i$ of 20-100 μM (Spruce *et al.*, 1987; Standen, 1992). The ability of ATP to close the channel does not appear to result from the molecule entering the pore and blocking the passage of K^+. Instead, evidence suggests that the inhibition by ATP is thought to be a result of direct binding of nucleotide to a specific site on the channel (see Molecular Biology section; Ashcroft, 1988). Further evidence show that Mg^{2+} is not required for the ATP effect, and the inhibition can be mimicked by non-hydrolysable ATP analogues, as well as AMP and ADP in the absence of Mg^{2+} (Ashcroft & Ashcroft, 1990; Shyng *et al.*, 1997). The channel's inhibition requires at least one phosphate, and the inhibition potency increases from ATP > ADP > AMP. The adenosine moiety of the nucleotide seems to be a major importance to the inhibitory effect since pyrimidine and guanosine triphosphate do not inhibit the channel (Aguilar-Bryan *et al.*, 1998). The number of ATP molecules required for channel inhibition remains unclear however, since Hill coefficient between 1 and 2 have been reported (Cook & Hales, 1984; Spruce *et al.*, 1987).

In intact tissue, intracellular ATP concentration never falls below the millimolar range, even under severe metabolic stress conditions such as ischemia in heart, and fatigue or metabolic inhibition in skeletal muscle (Sjogaard, 1991). The Ki value of ATP measured under patch clamp (20-100 μM) has therefore raised the question as to whether $K^+_{(ATP)}$ channels are activated in intact

tissues. Many studies have now shown an activation of the $K^+_{(ATP)}$ channel in intact tissue even though $[ATP]_i$ has not fallen to activation levels reported under patch clamp (see for example Deutsch *et al.*, 1991; Gramolini & Renaud, 1997). One possible explanation for this paradox is that the Ki values measured in excised membrane patches do not reflect the Ki values in intact cell membranes as it has been reported for the channel's pharmacological properties (see Pharmacology section). Another possibility is that ATP is compartmentalized in muscle fiber, with a specific membrane pool mainly linked to the Na^+-K^+ pump (Proverbio & Hoffman, 1977; Mercer & Dunham, 1981). This pool of ATP can decrease substantially during exercise, when the pump is working at a high rate. The $K^+_{(ATP)}$ channel may mainly "see" the ATP concentration of this pool and hence become activated. A third and most plausible explanation for the channel's activity under normal physiological conditions arises from the fact that the activity of the $K^+_{(ATP)}$ channel is regulated by a variety of other intracellular metabolites. Intracellular changes in these metabolites, which include ADP, lactic acid and H^+ ions alter the channel's activity (Spruce *et al.*, 1987; Ashcroft & Ashcroft, 1990; Keung & Li, 1991; Davies *et al.*, 1992).

1-B-ii) ADP

In the absence of ATP, ADP closes the $K^+_{(ATP)}$ channel with Ki values that are much higher than those reported for ATP inhibition. Studies, using patch clamp conditions reported Ki values at $[ADP]_i$ that range from 0.44 to 3.1 mM (Spruce *et al.*, 1987; Forestier & Vivaudou, 1993). The latter indicates that, under normal physiological conditions, ADP is a very weak inhibitor of the channel, since $[ADP]_i$ does not increase to more than 200 μM during fatigue (Fitts, 1994).

A more important role of ADP may actually be at the level of activation of the $K^+_{(ATP)}$ channel. In the presence of Mg^{2+}, MgADP activates the channel in the presence of inhibitory concentration of ATP, in both β-cell and cardiac muscle. The latter observation led Ashcroft and Ashcroft (1990) to suggest that the ATP/MgADP ratio may be more important than $[ATP]_i$ itself, in regulating the $K^+_{(ATP)}$ channel activity. However, the changes in ATP and ADP concentrations as well as changes in ATP/MgADP ratio that have previously been reported still do not explain the activation of the $K^+_{(ATP)}$ channel during ischemia in the heart or fatigue in skeletal muscle (Gasser & Vaughan-Jones, 1990; Nakaya *et al.*, 1991; Venkatesh *et al.*, 1991; Light *et al.*, 1994).

1-B-iii) Lactate and H^+ ions

Two other intracellular metabolites, namely lactate and H^+ ions, which concentrations increase during ischemia and fatigue, also activate the channel. In cardiac muscles, Keung & Li (1991) showed that at 20-40 mM, lactic acid activates the $K^+_{(ATP)}$ channel even in the presence of inhibiting concentration of ATP (2-5 mM). They also showed that lactate, at 20 mM, shortens the action potential duration of intact ventricular myocytes. This lactate effect is interesting because it mimics the shortening of the action potential observed during ischemia; an effect that is blocked by glibenclamide (a $K^+_{(ATP)}$ channel blocker) and therefore is attributed to $K^+_{(ATP)}$ channel activation (Keung & Li, 1991). Thus, the latter suggests that lactate may be an important activator of $K^+_{(ATP)}$ channels during ischemia, in the heart at least.

Davies (1990) studied the effects of varying the intracellular pH (pHi) from 7.2 to 6.3, on $K^+_{(ATP)}$ channel activity in excised patches from frog skeletal muscle. In the presence of 0.5 mM ATP, the drop in pHi increased markedly the open-state probability (P_{open}) of the $K^+_{(ATP)}$ channel

and reduced the inhibitory effect of ATP. Ki for ATP was 17 μM at pHi 7.2 and increased to 260 μM at pHi 6.3 (Davies *et al.*, 1992). They suggested that H^+ either affects the ATP binding site or the relative concentrations of the ionized forms of free ATP (Ashcroft & Ashcroft, 1990; Davies *et al.*, 1992). More importantly, the pHi values that they used were similar to those reported by Renaud (1989) in unfatigued (pHi 7.2) and fatigued (pHi 6.3) sartorius muscle fibers. The latter implies that the decrease in pHi during muscle fatigue may be an important factor that activates the $K^+_{(ATP)}$ channel. This is further confirmed by the fact that a NH_3-induced decrease in pHi from 7.2 to 6.3 activates the $K^+_{(ATP)}$ channels in intact resting sartorius muscle fiber (Standen *et al.*, 1992).

1-C) MOLECULAR BIOLOGY

Recent works demonstrated that $K^+_{(ATP)}$ channels are composed of two protein subunits: a Kir6.0 subunit, $\underline{K}^+$ inward rectifier, and a SUR subunit, sulfonylurea receptor. The Kir6.0 protein belongs to the inwardly rectifying potassium channel superfamily, and has the regular characteristics of this superfamily; that is: two transmembrane domains (M1 and M2) flanking a pore loop (H5) believed to be important in K^+ selectivity (Aguilar-Bryan *et al.*, 1998; Seino, 1999). Kir6.0 has an ATP binding site and makes up the pore of the channel (Aguilar-Bryan *et al.*, 1995; Inagaki *et al.*, 1995). Subsequent studies have shown the existence of two isoforms, Kir6.1 and Kir6.2. Kir6.1 is a 424 amino acid, 48 kDa protein that is expressed ubiquitously throughout the body. It is believed to be mainly present in the inner membrane of the mitochondria (Suzuki *et al.*, 1997). Mouse Kir6.2 is a 390 amino acid, 43.5 kDa protein that has 71% homology to Kir6.1 (Inagaki *et al.*, 1995). Kir6.2 is present at high levels in pancreatic islets and at low levels in heart, skeletal muscle and brain.

The SUR subunit was first purified and cloned based on its affinity for glibenclamide and is believed to be a regulatory component of the $K^{+}_{(ATP)}$ channel (Aguilar-Bryan *et al.*, 1995). SUR is a member of the ATP-binding cassette (ABC) superfamily. The members of this superfamily are characterized by having several transmembrane domains as well as three very distinct motifs (Aguilar-Bryan *et al.*, 1998; Seino, 1999). The predicted topology of SUR consists of 13 transmembrane domains as well as two nucleotides binding folds (NBFs) on the intracellular side. Each NBFs contains Walker A and B motifs and a third motif, lying between the two Walker motifs, thought to play a role in transducing the effects of nucleotide binding (Inagaki *et al.*, 1996; Shyng *et al.*, 1997; Aguilar-Bryan *et al.*, 1998).

Thus far, two SUR isoforms have been cloned: SUR1 and SUR2. SUR1 exhibits high-affinity sulfonylurea-binding activity (Aguilar-Bryan *et al.*, 1995). Hamster SUR1 is a 1582 amino acid, 177 kDa protein that has a high expression in pancreatic islets and a very low expression in rodent brain (Inagaki *et al.*, 1995). The second isoform of SUR, SUR2, actually comprises several isoforms that are generated by alternative splicing of the SUR2 gene. Rat SUR2A is a 1545 amino acid, 174 kDa protein that shares 68% homology with SUR1. It is predominantly present in heart and skeletal muscles (Inagaki *et al.*, 1996; Isomoto *et al.*, 1996). SUR2B has the same amino acid sequence as the SUR2A, except for the last 42 amino acids (Isomoto *et al.*, 1996). SUR2B is predominantly found in smooth muscle cells of the vascular bed. Other SUR2 isoforms have also been found, however their characterization is not to date complete (Aguilar-Bryan *et al.*, 1998; Seino, 1999).

The two Kir6.0 isoforms and the several SUR isoforms give the $K^+_{(ATP)}$ channel a great degree of structural diversity. This can be further complicated by the fact that the $K^+_{(ATP)}$ channel is heteromultimeric complex of four Kir6.0 subunits and four SUR subunits, that yields a (SUR-Kir)4 stochiometry (Inagaki *et al.*, 1997; Shyng & Nichols, 1997). It is therefore not surprising to see different subunit combinations of $K^+_{(ATP)}$ channels in different tissues. Thus far, some Kir/SUR combinations have been found to be predominant in certain tissues. For example, the predominant form of the $K^+_{(ATP)}$ channel in cardiac and skeletal muscles is the Kir6.2/SUR2A form, whereas the most abundant form in pancreatic β-cells is Kir6.2/SUR1 (Seino, 1999). The Kir6.2/SUR2B combination is found in most smooth muscle except in vascular smooth muscle where the Kir6.1/SUR2B combination predominates. The molecular biology data is immensely helpful in understanding the pharmacological diversity of the $K^+_{(ATP)}$ channel observed in different tissues.

1-D) PHARMACOLOGY

$K^+_{(ATP)}$ channel are modulated by a variety of pharmacological agents. Sulfonylureas, such as glibenclamide and tolbutamide, are channel blockers that close the channel (Ashcroft & Ashcroft, 1992; Seino, 1999). A variety of other drugs, such as diazoxide, nicorandil, levcromakalim and pinacidil, are collectively referred to as potassium channel openers (KCOs) as they open the channel (Aguilar-Bryan *et al.*, 1998; Babenko *et al.*, 1998). The pharmacology of these compounds is complex and differs greatly amongst different tissues mainly because of the tremendous diversity amongst $K^+_{(ATP)}$ channel subunit combinations. For example, pancreatic ß-cells are 500 times more sensitive to glibenclamide than cardiac cells, and it is now accepted that this difference is due to the different sensitivity of SUR1 (ß-cell) and SUR2A (cardiac cell) (Seino,

1999). Therefore, in this section, the discussion is limited to the pharmacology of the $K^{+}_{(ATP)}$ channel found in skeletal muscle.

1-D-i) Channel blockers

$K^{+}_{(ATP)}$ channels are blocked by a variety of K^{+} channel blockers such as tetraethylammonium (TEA), barium (Ba^{2+}) and caesium (Cs^{2+}) (Cook & Quast, 1999). However, these blockers often do not completely block the channel and are highly non-specific as they block other types of K^{+} channels. More specific $K^{+}_{(ATP)}$ channel blockers include phenothiazines and sulfonylureas. Of these two classes, only sulfonylureas are effective in blocking $K^{+}_{(ATP)}$ channels in skeletal muscle (Benton & Haylett, 1992).

Barrett-Jolley and McPherson (1998) investigated the effects of the best known sulfonylurea compounds, namely glibenclamide and tolbutamide, in intact rat skeletal muscle. They showed that both glibenclamide and tolbutamide were more potent in inhibiting intact muscle channel activity than channels in excised membrane patches as previously reported (Woll *et al.*, 1989; Allard & Lazdunski, 1993). The half-maximal inhibition constant, Ki, value of glibenclamide was calculated to be 5 nM, compared to 190 nM found in patches (Allard & Lazdunski, 1993). Tolbutamide was also more effective in blocking the $K^{+}_{(ATP)}$ channel in intact muscle, as the Ki was close to 10 μM. This Ki is 6-fold more potent than previously reported by Woll *et al.* (1989) in membrane patches. Barrett-Jolley and McPherson (1998) speculated that the lower Ki values in intact muscle might be due to the presence of intracellular Mg^{2+}-nucleotides which affect the efficiency of glibenclamide. The blocker's potency is also affected by ADP as increases in ADP levels diminish the blocking effects of glibenclamide.

Glibenclamide is the most widely used channel blocker to study the physiological role of the $K^+_{(ATP)}$ channel because its effectiveness and specificity are well characterised in skeletal muscle. Glibenclamide (at 10 μM) blocks most (>95%) $K^+_{(ATP)}$ channels under patch clamp conditions and during metabolic inhibition (Allard & Lazdunski, 1993; Allard *et al.*, 1995). It is highly specific for skeletal muscle $K^+_{(ATP)}$ channels. Glibenclamide has no effect on voltage-sensitive K^+ channels and $K^+_{(Ca^{2+})}$ channels incorporated into lipid bilayers (Light & French, 1994). It also has no effect on whole-cell currents under voltage clamp of rat skeletal muscle, on action potentials, and on the inward rectifier of frog skeletal muscle at rest (Davies *et al.*, 1992; Comtois *et al.*, 1993; Light *et al.*, 1994). There is however one effect of glibenclamide that must be considered for physiological experiments. Duty and Allen (1995) reported that glibenclamide at 50-100 μM shifts the pCa-force curve of intact muscle fiber of mouse flexor digitorum brevis (FDB) toward lower Ca^{2+} concentration; i.e. glibenclamide increases the Ca^{2+} sensitivity of the contractile component.

1-D-ii) Channel openers

Over the years, a variety of $K^+_{(ATP)}$ channel openers have been developed. Their overall effect appears to lower the channel's sensitivity towards ATP (Nichols & Lederer, 1991). In the presence of a KCO, the K_i value of ATP is shifted towards higher levels. The sensitivity of skeletal muscle $K^+_{(ATP)}$ channel from isolated membrane patches to KCO was found to be similar to that of cardiac cells. Interestingly, under patch clamp, KCOs are ineffective in activating the $K^+_{(ATP)}$ channel when ATP concentration is in the millimolar range (Fan *et al.*, 1990; Hussain *et al.*, 1994). The reported half-maximal activation constant, K_d, of pinacidil (K_d = 125 μM) and

cromakalim (K_d = 220 μM) required the presence of low concentrations of cytoplasmic ATP (approximately 500 μM) under patch clamp conditions (Allard & Lazdunski, 1993).

Despite these characteristics under patch clamp conditions, KCOs are effective in activating the channel in intact skeletal muscle fiber when the $[ATP]_i$ is in the millimolar range. In rat skeletal muscle, Barrett-Jolley and McPherson (1998) estimated that pinacidil has a K_d of 31.5 μM with a maximal effect at 100 μM. In comparison, the K_d of levcromakalim (an enantiomer of cromakalim) is 186 μM with no maximal effect at 400 μM (the maximum concentration that can be dissolved in water). These values are significantly lower than those found by Allard and Lazdunski (1993) in isolated membrane patches. Diazoxide, an extremely potent $K^+_{(ATP)}$ channel activator in smooth muscle and pancreatic β-cell, is ineffective in activating the channel in intact skeletal muscle (Barrett-Jolley & McPherson, 1998). This difference in diazoxide potency is because it activates the Kir6.2/SUR2B channels (predominant form in smooth muscle) and Kir6.2/SUR1 channels (predominant form in pancreatic β-cell), while it is ineffective on the Kir6.2/SUR2A combination (predominant form in skeletal muscle) (Seino, 1999). Overall, pinacidil is the most potent activator of $K^+_{(ATP)}$ channel in skeletal muscle. It is highly specific for the $K^+_{(ATP)}$ channel as it does not affect any other K^+ channel (Barrett-Jolley & McPherson, 1998), and is one of the most widely used KCO in physiological studies in skeletal muscle.

2) PHYSIOLOGICAL ROLE OF THE $K^+_{(ATP)}$ CHANNEL

As discussed above, the activity of the $K^+_{(ATP)}$ channel is affected by metabolites, especially ATP, ADP, H^+ and lactic acid. Thus, the channel couples cellular metabolism to electrical activity of the cell. This coupling has different roles in different tissues, such as the β-cell of pancreas, heart, and skeletal muscle.

2-A) INSULIN SECRETION

The role of $K^+_{(ATP)}$ channels is most understood in pancreatic β-cells. At normal glucose concentrations, the channel is active and maintains hyperpolarization. At high concentration, glucose enters the ß-cell via the glucose transporter (Glut2), and is metabolized to produce ATP. The increase in $[ATP]_i$ closes $K^+_{(ATP)}$ channels, resulting in membrane depolarization. The depolarization in turn, causes the opening of voltage-dependent calcium channels (VDCC), allowing Ca^{2+} entry. The rise in Ca^{2+} concentration in the β-cell triggers the secretion of insulin containing granules by exocytosis (Ashcroft *et al.*, 1988; Seino, 1999).

Accordingly, the $K^+_{(ATP)}$ channel, acting as a sensor for ATP, is the key molecule in the regulation of glucose-induced insulin secretion. Blocking $K^+_{(ATP)}$ channels by sulfonylureas, such as glibenclamide, mimics the effect of increasing $[ATP]_i$ following glucose absorption, and therefore stimulates insulin secretion from the pancreatic β-cells (Ashcroft *et al.*, 1988; Seino, 1999). The latter makes sulfonylureas a very important class of drugs in the treatment of non-insulin dependent diabetes mellitus (NIDDM).

2-B) CARDIAC AND SKELETAL MUSCLE

2-B-i) Postulated function and mechanism of action

Based on the characteristics of the $K^+_{(ATP)}$ channel, Noma (1983) postulated that the channel protects muscle against deleterious energy depletion and function impairment. This protection, referred to as the cytoprotective role of the $K^+_{(ATP)}$ channel, occurs at two different levels. Firstly, $K^+_{(ATP)}$ channels can increase energy delivery to the muscle and secondly, they can increase energy conservation within muscle fibers (Figure 1-1).

The opening of the $K^+_{(ATP)}$ channel of cardiac and skeletal muscle increases K^+ efflux down its concentration gradient (Gasser & Vaughan-Jones, 1990; Venkatesh *et al.*, 1991; Shivkumar *et al.*, 1997). This leads to a rise in interstitial fluid K^+ concentration, which then promotes vasodilatation within the arteriole resulting in hyperemia (Skinner & Powell, 1967; Vanelli *et al.*, 1994). The latter increases blood flow and hence energy (O_2, glucose) delivery to the muscle. Furthermore, an increase in plasma K^+ (up to 6 mM) stimulates carotid body chemoreceptors, which via the respiratory center causes an increased ventilation, hence respiration during exercise (Band & Linton, 1986; Burger *et al.*, 1988). $K^+_{(ATP)}$ channels are also present in the smooth muscle cells of blood vessels. Activation of these channels by endogenous vasodilators such as adenosine, has also been shown to cause vasodilatation (Dart & Standen, 1993; Nakhostine & Lamontagne, 1993; Quayle *et al.*, 1994). Once activated, the $K^+_{(ATP)}$ channel hyperpolarizes the membrane leading to a decrease in the open-state probability of the VDCC. This reduces the

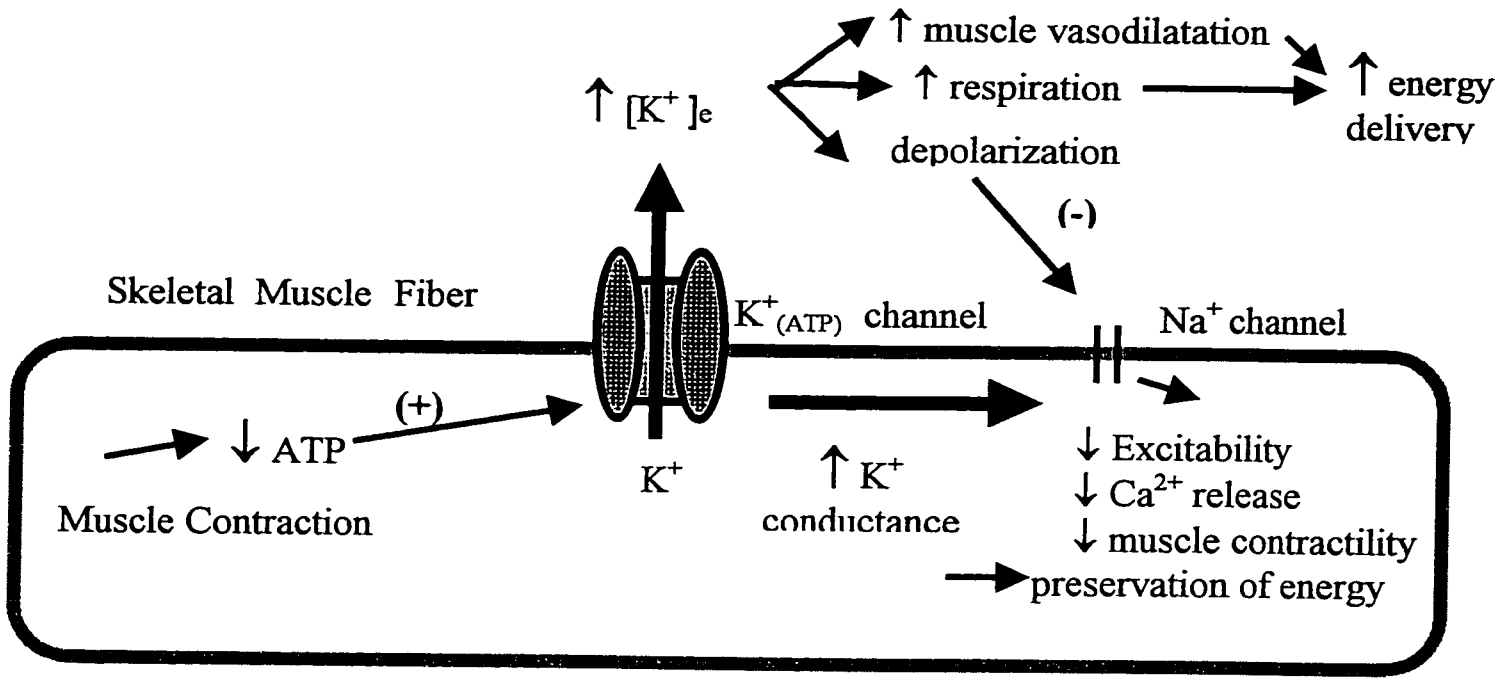

Figure 1-1: Postulated mechanism of action of the $K^+_{(ATP)}$ channel in skeletal muscle.

intracellular free Ca^{2+} concentration and therefore results in a relaxation of the vascular smooth muscle. So, the opening of both skeletal muscle and smooth muscle $K^{+}_{(ATP)}$ channels can protect the muscle by increasing its blood flow and energy delivery.

The second cytoprotective role of the $K^{+}_{(ATP)}$ channel is intrinsic to the muscle itself, as the channel is thought to decrease force production (Noma, 1983; Standen, 1992). The increased extracellular K^{+} concentration caused by the opening of the $K^{+}_{(ATP)}$ channel following a series of action potentials depolarizes the cell membrane and therefore inactivates Na^{+} channels (Hodgkin & Huxley, 1952; Adrian, 1956; Hodgkin & Horowicz, 1959). In addition, the $K^{+}_{(ATP)}$ channel increases K^{+} conductance causing a shorter action potential duration (Gasser & Vaughan-Jones, 1990; Nakaya *et al.*, 1991; Sauviat *et al.*, 1991). Together the inactivation of the Na^{+} channels and the shortening of the action potential duration reduce membrane excitability leading to a decrease in Ca^{2+} release and force production. When the amount of Ca^{2+} release and force decrease, the Ca^{2+}-ATPase pump and the myosin ATPase use less energy. Thus, the primary effect of the $K^{+}_{(ATP)}$ channel within the muscle fiber is to reduce energy utilization.

The $K^{+}_{(ATP)}$ channel is also present in the mitochondria where it is thought to regulate ATP synthesis. Mitochondrial $K^{+}_{(ATP)}$ channels have similar activation characteristics, in terms of ATP and ADP effects, to the $K^{+}_{(ATP)}$ channels found in the cell membrane (Inoue *et al.*, 1991; Garlid, 1996; Szewczyk, 1996). The mitochondrial $K^{+}_{(ATP)}$ channel is believed to control K^{+} influx in order to regulate mitochondrial volume (Garlid, 1996; Holmuhamedov *et al.*, 1998; Jaburek *et al.*, 1998). The latter is important to maintain the activity of the electron transport chain associated with ATP synthesis (Szewczyk, 1996). Studies have now shown that a modulation of

mitochondrial $K^+_{(ATP)}$ channel affects ATP synthesis (Holmuhamedov *et al.*, 1998). More importantly, there is evidence that the cytoprotective effect of $K^+_{(ATP)}$ channel during ischemia in heart is in part related to an activation of mitochondrial $K^+_{(ATP)}$ channels (Garlid *et al.*, 1997; Holmuhamedov *et al.*, 1998).

2-B-ii) Cardiac muscle

Under normal conditions, cardiac muscle $K^+_{(ATP)}$ channels are closed since the addition of sulfonylureas has no effect on cardiac current-voltage relationship and action potential (Fosset *et al.*, 1988). The same does not hold for cardiac muscles that are exposed to ischemia, anoxia or low glucose. Under those conditions, several events occur at the level of the cell membrane, including an increased K^+ conductance causing a shortening of the action potential duration from 400 msec to 100 msec (Faivre & Findlay, 1990; Gasser & Vaughan-Jones, 1990; Nakaya *et al.*, 1991). These effects can be mimicked by $K^+_{(ATP)}$ channel openers and are blocked by sulfonylureas suggesting that they are caused by an activation of $K^+_{(ATP)}$ channels (Nakaya *et al.*, 1991; Weiss *et al.*, 1992). Exposing cardiac muscle to $K^+_{(ATP)}$ channel openers also causes faster decrease in force as expected from the postulated function of the channel (McPherson *et al.*, 1993). Interestingly, blocking $K^+_{(ATP)}$ channels does not slow the force decrease during fatigue as expected (McPherson *et al.*, 1993). Thus, despite the fact that an activation of $K^+_{(ATP)}$ channels affects force, a blockage does not, an effect that remains to be explained.

Even though blocking $K^+_{(ATP)}$ channels does not affect force, it does have two important effects. Firstly, blocking the $K^+_{(ATP)}$ channel during ischemia or hypoxia causes greater ATP and PCr depletions than in control non-treated muscles (McPherson *et al.*, 1993; Decking *et al.*, 1995).

Secondly, the presence of the $K^+_{(ATP)}$ channel blocker impairs contractile function recovery during reperfusion of an ischemic heart (Grover *et al.*, 1991; Grover, 1994). Conversely, in the presence of channel openers, ATP and PCr depletions are lesser and function recovery is improved when compared to control. Thus, studies in cardiac muscles have demonstrated that $K^+_{(ATP)}$ channels can be important in preserving energy and protecting muscle function as originally proposed by Noma (1983). However, the $K^+_{(ATP)}$ channel's function in cardiac muscle has only been demonstrated under extreme metabolic stresses, such as ischemia and hypoxia. The role of the $K^+_{(ATP)}$ channel in cardiac muscle, under normoxic conditions, has yet to be investigated.

2-B-iii) Skeletal muscle

There are much fewer studies on the physiological role of the $K^+_{(ATP)}$ channel in skeletal muscle than in cardiac muscle. So far, studies have shown that $K^+_{(ATP)}$ channels are closed in resting, unfatigued conditions and they become activated during metabolic poisoning that causes large ATP depletions. One important aspect of the $K^+_{(ATP)}$ channel in skeletal muscle, however, is whether they have a role in the etiology of muscle fatigue. Light *et al.* (1994) and Comtois *et al.* (1993) showed that both glibenclamide (100 μM) and tolbutamide (2 mM) significantly increased the half-repolarization time of the action potential in frog sartorius muscle fibers fatigued with intermittent tetanic contractions every sec for 3 min. They went on to suggest that the $K^+_{(ATP)}$ channel was indeed activated during fatigue and that they contribute to the repolarization phase of the action potential in fatigued muscle. They also noted that force recovery following fatigue was also significantly impaired in the presence of the $K^+_{(ATP)}$ channel blockers. This impairment of force recovery following fatigue is similar to the impairment observed in cardiac muscle following

ischemia, which further supports Noma's notion (1983) that the $K^+_{(ATP)}$ channel plays an important cytoprotective role. However, neither glibenclamide nor tolbutamide significantly altered the rate of fatigue measured from the decrease in tetanic force during the fatigue stimulation. These results do not support the hypothesis that the $K^+_{(ATP)}$ channel contributes to the decrease in force.

Gramolini and Renaud (1997) also studied the role of the $K^+_{(ATP)}$ channel in frog sartorius muscle. In this study, they tried to determine the metabolic conditions that must be reached in order to activate a sufficient number of $K^+_{(ATP)}$ channels to contribute to a decrease in force. They exposed frog sartorius muscle to cyanide (2 mM) and iodoactate (1 mM) to induce metabolic inhibition. Under these conditions, $K^+_{(ATP)}$ channels were activated within 10 min of exposure, a time when the decrease in ATP concentration was only 10% from the initial concentration. Interestingly, the decrease in ATP that was sufficient to activate $K^+_{(ATP)}$ channels was smaller than the change in ATP observed during fatigue. When glibenclamide (100 μM) was added 8 min into the metabolic inhibition period, tetanic force increased during the next 10 min, and then decreased at a faster rate than in control non-treated muscles. If glibenclamide was added 60 min prior to metabolic inhibition, the decrease in tetanic force was faster than in control throughout the metabolic inhibition period. In both cases, the faster decrease in force observed in the presence of glibenclamide was accompanied by excessive depolarization, greater increase in resting tension, greater depletion of ATP and PCr contents, and greater increase in ADP content when compared to control non-treated muscle. Gramolini and Renaud concluded that $K^+_{(ATP)}$ channels can contribute to a decrease in force, but that most of the time blocking $K^+_{(ATP)}$ channels impairs muscle function. Furthermore, this impairment counteracted the expected slower decrease in force in the presence of glibenclamide.

The role of $K^+_{(ATP)}$ channel in skeletal muscle has also been studied in mammalian muscles. In one of the earlier works, Weselcouch *et al.* (1993) studied the effects of different $K^+_{(ATP)}$ channel openers and blockers on twitch force in the rat EDL muscle. Under normoxic conditions, cromakalim (at 300 μM) and pinacidil (at 100 μM) failed to affect the decrease in twitch force when muscles were stimulated at 0.2 Hz. However, under anoxic conditions, the KCOs accelerated the decrease in force and improved force recovery upon reoxygenation following fatigue. These effects were blocked by glibenclamide (1 and 10 μM) suggesting that they were caused by an activation of the $K^+_{(ATP)}$ channel. It's important to note that glibenclamide by itself (i.e. without KCOs) again did not have an effect on force under both normoxic and anoxic conditions. However, no attempt was made in this study to determine whether the $K^+_{(ATP)}$ channels were activated during the stimulation period. It is therefore possible that the lack of a glibenclamide effect was due to a lack of $K^+_{(ATP)}$ channel activation.

So far, only one study has showed an effect of glibenclamide during fatigue. Using mouse FDB fibers, Duty and Allen (1995) showed that glibenclamide (50-100 μM) added during fatigue causes a transient increase in Ca^{2+} release and tetanic force. This increase was then followed by a much faster decrease in tetanic force when compared to control muscle. The latter result is similar to the effect of glibenclamide on force observed in frog sartorius muscle when glibenclamide is added during the metabolic inhibition (Gramolini & Renaud, 1997). It is important to note, that the glibenclamide effect in the Duty and Allen (1995) study was only noted in 3 out of the 6 fibers tested; the authors did not give an explanation for this observation. Furthermore, the glibenclamide effect was observed at a concentration of 50-100 μM. At these concentrations, the authors themselves showed that glibenclamide has an effect on the Ca^{2+} sensitivity of the contractile

apparatus. In other words, the glibenclamide effect may have been related to a non-specific effect and not to a blocking of the $K^+_{(ATP)}$ channel *per se*.

3) STATEMENT OF PROBLEM AND OBJECTIVES

Thus far, the physiological role of the $K^+_{(ATP)}$ channel during fatigue of skeletal muscle, under normoxic conditions is not well understood. This has arisen in part from the widespread use of metabolic stress models (such as anoxia or metabolic inhibition) which do not represent a true model of fatigue. Furthermore, some studies used glibenclamide concentrations (>50 μM) that affect not just the $K^+_{(ATP)}$ channel but also the contractile apparatus (Duty & Allen, 1995). Finally, one study used a low frequency stimulation (0.2 Hz) that probably did not activate $K^+_{(ATP)}$ channels.

The overall objective of this thesis was therefore to better understand the physiological role of the $K^+_{(ATP)}$ channel in skeletal muscle. Three specific aims were pursued. The first aim was to test the hypothesis that during fatigue, $K^+_{(ATP)}$ channels can contribute to the decrease in force, and help in preserving energy and muscle function. For this, pinacidil was used to activate $K^+_{(ATP)}$ channels and the following parameters were measured: tetanic force during fatigue, $^{86}Rb^+$ fractional loss (to determine the activity level of the channel), ATP and PCr contents (to determine the effect on energy preservation) and tetanic force during the recovery period (to determine how the channel protects muscle function). The second aim was to determine if the fatigue model used, i.e. one tetanic contraction every sec for 3 min, activated the $K^+_{(ATP)}$ channel, and whether this activation affects tetanic force during fatigue and recovery as well as the changes in energy contents during

fatigue. In order to do so, we used glibenclamide to block the channel. The experiments resulting from these two aims are presented in Chapter 2.

It is well documented that denervation influences many biochemical and biophysical properties in skeletal muscles. Prior studies have shown that the expression of several proteins, including ionic channels, is altered significantly following denervation (Harris & Thesleff, 1971; Rogart & Regan, 1985). Potassium channels are no exception, as several reports show an altered expression following denervation. To date, no studies were preformed on the effects of denervation on the $K^+_{(ATP)}$ channel's expression. Thus, the third specific aim was to determine the effect of muscle activity on the channel's expression and physiological importance. Denervation was used as a model to completely abolish muscle activity and neurotrophic factors. The results of these experiments are presented in Chapter 3.

For each of the three specific aims, two important factors were taken into consideration. The first factor was the efficiency and specificity of pinacidil and glibenclamide. In mouse skeletal muscle, pinacidil is the most effective KCO with a maximal effect at 100 μM (Barrett-Jolley & McPherson, 1998). At 10 μM, glibenclamide blocks most (> 95%) $K^+_{(ATP)}$ channels under patch clamp (Allard & Lazdunski, 1993) and during metabolic inhibition in intact muscle fiber (Allard *et al.*, 1995). As discussed in the Pharmacology section, at those concentrations, neither pinacidil nor glibenclamide affect other channel the $K^+_{(ATP)}$ channel. Evidence is also given in Chapter 2 that at those concentrations pinacidil and glibenclamide do not affect the Ca^{2+} sensitivity of contractile apparatus. The second factor was the fact that skeletal muscle differ in the fiber type composition, metabolic profile, contractile and fatigue characteristics. Thus in this study, two muscles from CD-

1 mouse were used. The EDL muscle is primarily composed of type IIB (68%), IIX (20%), and IIA (12%) fibers (Rosenblatt & Parry, 1992). It is a fast-twitch, fatigable muscle with a high glycolytic and low oxidative capacity. The soleus muscle is composed of type I (40%) and type IIA (60%) fibers (Wigston & English, 1992). It is a slow-twitch muscle with a high fatigue resistance, low glycolytic and high oxidative capacity.

CHAPTER 2

PINACIDIL SUPPRESSES CONTRACTILITY AND PRESERVES ENERGY BUT GLIBENCLAMIDE HAS NO EFFECT DURING FATIGUE IN SKELETAL MUSCLE

by

W. Matar, T.M. Nosek[1], D. Wong, J.M. Renaud

University of Ottawa, Department of Cellular and Molecular Medicine, 451 Smyth Rd., Ottawa Ontario, Canada, K1H 8M5; [1]Medical College of Georgia, Department of Physiology and Endocrinology, Augusta, Georgia, USA (present address: Case Western Reserve University, School of Medicine, 10900 Euclid Avenue, Cleveland, Ohio 44106-4963)

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The skinned fiber experiments were done by Dr J.M. Renaud, in Dr T.M. Nosek's laboratory. Mr. D. Wong was involved in the $^{86}Rb^{+}$ fractional loss experiments. Dr J.M. Renaud helped in the enzymatic quantification of ATP and PCr.

ABSTRACT

The effects of 10 μM glibenclamide, a $K^+_{(ATP)}$ channel blocker, and 100 μM pinacidil, a channel opener, were studied to determine how the $K^+_{(ATP)}$ channel affects mouse EDL and soleus muscle during fatigue. Fatigue was elicited with 200 msec long tetanic contractions every sec. Glibenclamide did not affect rate and extent of fatigue, force recovery or $^{86}Rb^+$ fractional loss. The only effects of glibenclamide during fatigue were: an increase in resting tension (EDL and soleus), a depolarization of the cell membrane, a prolongation of the repolarization phase of action potential, and a greater ATP depletion in soleus. Pinacidil, on the other hand, increased the rate but not the extent of fatigue, abolished the normal increase in resting tension during fatigue, enhanced force recovery and increased $^{86}Rb^+$ fractional loss in both EDL and soleus. During fatigue, the decreases in ATP and PCr of soleus muscle were less in the presence of pinacidil. The glibenclamide effects suggest that fatigue elicited with intermittent contractions activates few $K^+_{(ATP)}$ channels which affect resting tension and membrane potentials but not tetanic force, while opening the channel with pinacidil causes a faster decrease in tetanic force, improves force recovery and helps in preserving energy.

KEYWORD $K^+_{(ATP)}$ channel, tetanic force, resting potential, action potential, $^{86}Rb^+$, ATP, PCr.

INTRODUCTION

$K^+_{(ATP)}$ channels are ligand-sensitive and voltage-insensitive K^+ channels that are closed by intracellular ATP (37;38). They become activated during ischemia, hypoxia or metabolic inhibition in cardiac and skeletal muscle fibers when the concentration of ATP decreases (12;21;29) and when the concentrations of ADP (42), lactate (cardiac muscle; 28), H^+ (skeletal muscle, 39), and adenosine (5) increase. Noma (34) postulated that the function of $K^+_{(ATP)}$ channels is to protect muscles against deleterious energy depletion and irreversible function impairment. This function may be important during ischemia or muscle fatigue because $K^+_{(ATP)}$ channels provide a mechanism that 1) detects low energy levels in muscle fiber and 2) reduces force production to preserve energy.

There are two mechanisms by which opening $K^+_{(ATP)}$ channels could reduce force. First, opening the channels increases K^+ conductance, which shortens action potential duration (19;21). Second, opening the channels increases K^+ efflux which increases extracellular K^+ concentration (11;19), depolarizes the membrane (24) and inactivates Na^+ channels (1). Together, shortening of the action potential and inactivation of Na^+ channels reduce membrane excitability, decrease Ca^{2+} release from intracellular stores and decrease force production (13;19). As the amount of Ca^{2+} released and force decrease, Ca^{2+}-ATPase and myosin ATPase utilize less energy. Indeed, blocking $K^+_{(ATP)}$ channels with glibenclamide has been shown to cause larger decreases in ATP and PCr levels during ischemia in cardiac muscle (31) and during metabolic inhibition in skeletal muscle (21).

The role of the $K^+_{(ATP)}$ channel during fatigue in skeletal muscle is still not well understood. We have previously reported that blocking $K^+_{(ATP)}$ channel with glibenclamide impaired force recovery of frog sartorius muscle following fatigue even though it did not affect the decrease in force during fatigue (29). Van Lunteren *et al.* (41) also found that glibenclamide did not affect force production of diaphragm muscle during continuous stimulation at 5 or 100 Hz. In these two studies, however, the glibenclamide concentration was 100 μM, a concentration that increases the Ca^{2+} sensitivity of contractile components (13); i.e., glibenclamide may have had some non-specific effects that masked the expected slowing of the rate of fatigue.

In another study, glibenclamide did not affect force of rat EDL (extensor digitorum longus) muscle continuously stimulated at 0.2 Hz (43). However in this case the glibenclamide concentration was only 1 μM, which is not large enough to block all $K^+_{(ATP)}$ channels (2;3). Finally, Duty and Allen (13) reported that glibenclamide (50-100 μM) affects the Ca^{2+} transient and force during fatigue, but only in 3 out of 6 single fibers from mouse FDB (flexor digitorum brevis) muscle. It is thus not clear whether $K^+_{(ATP)}$ channels contribute to the decrease in force during fatigue.

The overall objective of this study was to determine how modulating $K^+_{(ATP)}$ channel activity with both a channel agonist (pinacidil) and antagonist (glibenclamide) during fatigue affects muscle function (tetanic force, resting and action potential, $^{86}Rb^+$ efflux, ATP and PCr contents). Two muscles were used for this study: the mouse soleus muscle which is predominantly composed of slow fiber types (40% type I and 60% IIA fibers; 44), and the mouse EDL muscle which is predominantly composed of fast fiber types (68% type IIB , 20% IIX and 12% IIA fibers; 36).

The strategy of this study was to use concentrations of glibenclamide and pinacidil that are effective in blocking or activating $K^+_{(ATP)}$ channels respectively (see Methods), and which had no effect on the contractile apparatus (see Results). The effects of pinacidil observed in this study suggest that activating $K^+_{(ATP)}$ channels can preserve energy and protect muscle function as originally proposed by Noma (34). However, the effects of glibenclamide suggest that a significant number of $K^+_{(ATP)}$ channels are not activated when mouse EDL and soleus are fatigued with one tetanic contraction every sec.

METHODS AND MATERIALS

ANIMALS AND MUSCLE PREPARATION

CD-1 mice (2-3 month old) weighing 20-30 g were fed *ad libidum*. Mice were anesthetized with sodium pentobarbitol (Somnotol), delivered intraperitoneally at a dose of 0.8 mg/10 g body weight before EDL and soleus muscles were excised. EDL muscles weighed between 9 and 12 mg, while soleus muscles weighed between 7 and 9 mg. Mice were kept according to the guidelines of CCAC (Canadian Council for Animal Care), and the Animal Care Committee of the University of Ottawa approved all experimental protocols.

PINACIDIL AND GLIBENCLAMIDE AS MODULATORS OF $K^+_{(ATP)}$ CHANNEL ACTIVITY

Pinacidil (100 μM) was used to activate $K^+_{(ATP)}$ channels because it is the most effective channel opener in skeletal muscle. Pinacidil has a K_d of 31.5 μM and a maximum effect near 100 μM (6). Other channel openers such as diazoxide have no effect in skeletal muscle, whereas levcromakalim has a K_d of 186 μM with no maximum effect at 400 μM. Pinacidil also does not affect other K^+ channels than the $K^+_{(ATP)}$ channel (6). Glibenclamide was used to block $K^+_{(ATP)}$ channel since its effectiveness and specificity are well characterized in skeletal muscle. In most experiments, glibenclamide was used at 10 μM because at that concentration it blocks most (>95%) $K^+_{(ATP)}$ channels under patch clamp conditions (2) and during metabolic inhibition (3). A concentration of 100 μM was used for action potentials measurements (see below). At 100 μM, glibenclamide has no effect on voltage-sensitive K^+ channels, $K^+_{(Ca2+)}$ channels and whole-cell currents in voltage clamp (-60 mV to +32 mV) experiments on rat skeletal muscle (30) (6). Duty

and Allen (13) have reported that 50-100 μM glibenclamide shifts the pCa-force curve of intact muscle fiber of mouse FDB muscle toward lower Ca^{2+} concentrations. Glibenclamide is highly soluble in lipid membranes and has a poor solubility in water (14;16). It is thus not possible to ascertain what the intracellular concentration of glibenclamide was that affected the contractile components in those experiments.

MEASUREMENT OF FORCE IN SKINNED MUSCLE FIBERS IN THE PRESENCE OF GLIBENCLAMIDE AND PINACIDIL

Membrane disruption was accomplished by exposing small bundles of EDL and soleus muscle fibers to a skinning solution for 30 min at 22°C. The skinning solution contained 0.1% w/w Triton X-100, 1.0 mM Mg^{2+}; 5.0 mM MgATP; 15 mM PCr; 140.0 mM potassium methanesulfonate (KMS); 50.0 mM imidazole, 200 ionic strength, 10.0 mM EGTA, pCa > 8.5, pH 7.0 at 22°C. The skinning solution also contained a cocktail of protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM leupeptin, 1.0 mM benzamidine, 10 μM aprotinin, and 1.0 mM dithiothreitol). Skinned fibers prepared this way were used on the same day the animal was sacrificed.

Single EDL and soleus muscle fibers (ca. 4000 x 25-50 μm) were then isolated by holding one end of the muscle with a pair of forceps and pulling on the other end to free single fibers. Single fibers were mounted between an optoelectric force transducer (Scientific Instruments GMBH, Heidelberg, Germany) and a movable arm by wrapping the fibers several times around small stainless steel wires followed by a brief exposure to pCa 4.0 to secure the fibers to the wire. The length of the fiber between the wires after mounting was approximately 2 mm. The fibers

were bathed in solutions contained in 2.5 ml troughs milled in a spring-loaded Plexiglas plate. The composition of all solutions was calculated using a computer program (Borland International, Scotts Valley, CA) employing the stability constants commonly used (20). To avoid any possible source of transitional metals contaminating the bathing media, all solutions were prepared with nanopure water run through a chelex column.

Force vs. pCa Relationship

Skinned fibers were exposed to solutions of varying Ca^{2+} concentrations (pCa 8.5 to 4.0) in order to determine the force vs. pCa relationship with and without added drugs. All measurements were carried out at 22°C. Glibenclamide- and pinacidil-containing solutions were prepared by first dissolving the drugs in DMSO and then adding the proper volume to the different bathing solutions. The DMSO concentration was 0.1% (v/v) in all bathing solutions, including control solutions. Each force vs. pCa relationship was analyzed as described previously (8). Maximum calcium-activated force (F_{max}) was recorded and normalized to the cross sectional area of each fiber. Because the fibers were cylindrically shaped, cross sectional area was determined by measuring the diameter of the fiber via a micrometer attached to the eye piece of the microscope binocular. A computer program (Origin, Microcal) was used to fit the force vs. pCa curve for each fiber before and after exposure to glibenclamide to the Hill equation; i.e., $\% F_{max} = 100[Ca^{2+}]^n/((Ca_{50})^n + [Ca^{2+}]^n)$. Ca_{50} (the Ca^{2+} concentration producing half-maximal activation) was used as an index of Ca^{2+} sensitivity. The steepness of the curve was reflected in n, the Hill coefficient.

FORCE MEASUREMENTS IN INTACT EDL AND SOLEUS MUSCLE

Muscle chamber and solutions

Force measurements from intact EDL and soleus muscle were carried out using a chamber that was 0.9 cm wide, 1.7 cm long and 1.0 cm deep. One muscle tendon was tied to a lightweight stainless steel wire that was hooked to a force transducer. The other tendon was held in place between 2 Teflon clamps. The flow of fresh physiological saline solution was 15 ml/min through tubing located in both Teflon clamps. This allowed the solution to flank the top and bottom of the muscle alongside its length (as observed with a blue dye). The composition of the physiological saline solution for control condition contained (in mM): 118.5 NaCl, 4.7 KCl, 2.4 $CaCl_2$, 3.1 $MgCl_2$, 25 $NaHCO_3$, 2 NaH_2PO_4; 95% O_2: 5% CO_2, 5.5 d-glucose, pH 7.4. Glibenclamide- and pinacidil-containing solutions were prepared by first dissolving the drugs in DMSO and then adding the proper volume to the control solution. The final DMSO concentration was 0.1% (v/v) in all solutions, including control solution. The experimental temperature was 37°C at all time.

Muscle stimulation

Muscles were stimulated by passing a current between parallel platinum wires located on opposite sides of the muscle. A stimulator and isolation unit (Grass S88 and SIU5, USA) provided the electrical stimuli. Twitch contractions were elicited with single 0.3 msec long rectangular pulses of 8 V (supramaximal voltage). Unless specified otherwise, tetanic contractions were elicited with 200 msec long train of the same pulses at 140 Hz for soleus muscle, and 200 Hz for EDL muscle.

Force Measurement

Force was measured with a Cambridge ergometer (model 300, USA), and digitized with a Keithley Metrabyte A-D board (model DAS50, USA). Sampling rates were 20 kHz for twitches and 5 kHz for tetani. Twitch and tetanic force, defined as the maximum force developed during twitch and tetanic contractions, were later analyzed on computer as the difference between the maximum value and the baseline. Resting tension, defined as the amount of tension exerted by a muscle without any external stimulation, was measured from the baseline 5 msec before stimulation.

Measurements of twitch force and force-frequency curve

Muscle length was first adjusted to give maximum tetanic force. Muscles were then allowed a 30 min equilibration period during which twitches were elicited every 2 min. A force-frequency curve was measured by increasing the frequency of stimulation from 1 to 200 Hz. For these measurements, train duration was 200 msec for EDL and 400 msec for soleus muscle. After stabilization of twitch force (i.e., the disappearance of post-tetanic potentiation in EDL muscle), muscles were exposed to 0 (control), 10 or 100 μM glibenclamide for 120 min during which twitch contractions were recorded every 2 min. After the 120 min period, another force-frequency curve was measured.

Measurement of the rate of fatigue and force recovery

For these experiments, EDL and soleus muscles were always used in pairs. One muscle was exposed to either 10 μM glibenclamide or 100 μM pinacidil while the contralateral muscle was used as the control (no drug). Muscle length was adjusted for maximal tetanic force. Muscles were allowed to equilibrate in the absence or presence of glibenclamide or pinacidil for 30 min. During that time one tetanic contraction was elicited every 2 minutes. Fatigue was elicited with one 200 msec long tetanic contraction every sec for 3 min. Following fatigue, muscles were stimulated at 10, 20, 100, 200 sec, 5 min, and every 5 min thereafter until 30 min to measure the recovery of tetanic force.

MEASUREMENT OF RESTING AND ACTION POTENTIAL

The muscle chamber, solutions, muscle stimulation and fatigue protocol were all as described for the measurement of force. The experimental temperature was 37°C. Membrane potentials were measured using conventional microelectrodes as described by Light *et al.* (29). Briefly, microelectrodes (with tip potentials less than 5 mV and tip resistance varying between 10 and 20 MΩ) and reference electrodes (tip resistance of 1 MΩ) were filled with 3 M KCl. Action potentials were elicited by passing a small current between two fine platinum wires placed along the surface fibers in order to stimulate a small number of fibers. Action potentials were digitized at a sampling rate of 200 kHz. Resting potential was measured from the baseline of the action potential. Overshoot was measured from the action potential peak. Half-repolarization time was measured as the time interval between the time of the overshoot and the time 50% of the repolarization had occurred.

$^{86}RB^{+}$ EFFLUX MEASUREMENTS

Measurement of $K^{+}_{(ATP)}$ channel current using the voltage clamp or patch clamp technique is impossible during muscle contraction because of possible damage to the cell membrane by microelectrodes. We therefore measured the $^{86}Rb^{+}$ efflux prior to, during and after fatigue to estimate the activity of $K^{+}_{(ATP)}$ channels. $^{86}Rb^{+}$ is qualitatively a satisfactory marker for K^{+} movements through several K^{+} channels, including the $K^{+}_{(ATP)}$ channel (9).

Solutions, muscle stimulation and the fatigue protocol were all as described for force measurements. The muscle chamber was a methylacrylate cuvette in which muscles were attached vertically at one end to a hook and the other to a Grass FT03 force transducer. Tetanic forces were recorded on a Grass Polygraph (Model 7D, U.S.A.). Muscles were immersed throughout the experiment in 1.7 ml of solution constantly bubbled with 95% O_2: 5% CO_2 and maintained at 37°C. Muscles were loaded with $^{86}Rb^{+}$ (4-8 μCi/ml) for 60 min (with a change of fresh solution after 30 min). The $^{86}Rb^{+}$ loading was followed by an initial 40 min washout in a series of 3 cuvettes (15, 15 and 10 min) containing 1.7 ml of nonradioactive saline solution (preliminary washout experiments showed that a constant $^{86}Rb^{+}$ efflux was reached after 40 min, results not shown). Glibenclamide or pinacidil were added during the initial washout so muscles would be exposed to either drug 30 min prior to fatigue as described for the experiments on force measurements described above.

The basal $^{86}Rb^{+}$ efflux was measured prior to fatigue by changing the cuvette 3 times every 5 min. During the 3 min fatigue period and the first 3 min of recovery, cuvettes were changed every min. The last two cuvettes were used to measure $^{86}Rb^{+}$ efflux between the 3rd and 5th, and

between the 5th and 10th min of recovery. From each cuvette, 1.0 ml of incubation fluid was added to 10.0 ml of Biodegradable Counting Scintillant (CBS, Amersham, USA) for radioactive counting. The $^{86}Rb^+$ content remaining in muscles were determined by homogenizing muscles in 2 ml of 6% perchloric acid (PCA). The resulting solution was centrifuged at 10,000 g for 10 min and 1.0 ml of the supernatant was added to the Counting Scintillant. $^{86}Rb^+$ counting was done using a WinSpectral liquid scintillation counter (model 1414, Wallac Instruments, USA). Quenching was corrected by counting 1 μCi of $^{86}Rb^+$ in 1 ml of physiological solution and another 1 μCi in ml of 6% PCA.

ATP AND PCr MEASUREMENTS

Solutions, fatigue protocol, muscle stimulation and experimental chamber were all as described for $^{86}Rb^+$ efflux measurements. All muscles were first allowed to equilibrate 30 min in the absence (control) or presence of glibenclamide or pinacidil. Muscles were then freeze clamped in liquid nitrogen immediately after the 30 min equilibrium or after the 3 min fatigue period. All muscles were stored at –80°C until analyzed. The extraction of ATP and PCr was as described by Passoneau and Lowry (35) with some modifications. Briefly, muscles were freeze dried overnight with a freeze drier (Freezemobile 6, Virtis, USA). Tendons were then removed and muscle tissues broken in small pieces under a microscope. A known amount of dried muscle tissue (0.8-2.0 mg) was added to 400 μl of ice cold 6% PCA. The solution was then sonicated with a Microson ultrasonic cell disruptor (Heat System Ultrasonic Inc., USA) at maximum power for 15 sec. After centrifugation at 10,000 g and 4°C for 30 min, the supernatant was neutralized with ice cold 3 M K_2CO_3. K^+ salt was precipitated at 10,000 g and 4°C for 15 min. ATP and PCr content was measured from the supernatant according to the enzymatic test described by Passoneau and Lowry

(35) using a spectrophotometer (model DU 640, Beckman, USA). All assays contained 10 μM P^1,P^5-di(adenosine-5')pentaphosphate to inhibit any myokinase that resisted the PCA precipitation (35).

STATISTICAL ANALYSIS

ANOVA were used to determine significant differences. Split plot designs were used when muscles were tested at all levels of a treatment (e.g., time effect during fatigue and recovery). ANOVA calculations were made using the GLM (General Linear Model) procedures of the Statistical Analysis Software (SAS Institute Inc., Cary, NC USA). When a main effect or an interaction was significant, the least square difference (L.S.D.) was used to locate any significant difference (40). The word "significant" refers only to a statistical difference ($P<0.05$).

RESULTS

EFFECTS OF PINACIDIL AND GLIBENCLAMIDE ON CONTRACTILE COMPONENTS

Neither 100 μM pinacidil nor 10 μM glibenclamide had an effect on the pCa-force curve of skinned soleus muscle fibers (Fig. 2-1A, B). However at 100 μM glibenclamide, the pCa-force curve was shifted toward a lower Ca^{2+} concentration. The Ca_{50} values in the absence and presence of 100 μM glibenclamide were respectively 1.5 and 1.0 μM. The effects of 100 μM pinacidil, 10 and 100 μM glibenclamide were the same in EDL skinned muscle fibers (results not shown). To determine the concentration at which glibenclamide affects the contractile components, we measured a dose-response curve while soleus skinned muscle fibers were exposed at pCa 6.2. Glibenclamide had no significant effect at 10 μM, but it significantly increased force when its concentration equaled or exceeded 20 μM (Fig. 2-1C).

The above experiments were carried out at 22°C because of the instability of skinned fibers at 37°C (the temperature used for the fatigue experiments). To confirm that 10 μM glibenclamide has no effect on the contractile apparatus of intact EDL and soleus muscle at 37°, we measured the glibenclamide effect on twitch force and force-frequency curve over a 2 hour period. $K^{+}_{(ATP)}$ channels are inactive in unfatigued muscle (3). Thus, any increase in twitch force or shift in the force-frequency curve toward lower frequencies would be evidence that glibenclamide increases the Ca^{2+} sensitivity of the contractile components. Glibenclamide at 10 μM had no effect on twitch force, while at 100 μM it caused the expected significant increase in both EDL (Fig. 2-2) and soleus muscle (results not shown). Glibenclamide at 10 μM also had no effect on the force-

FIGURE 2-1

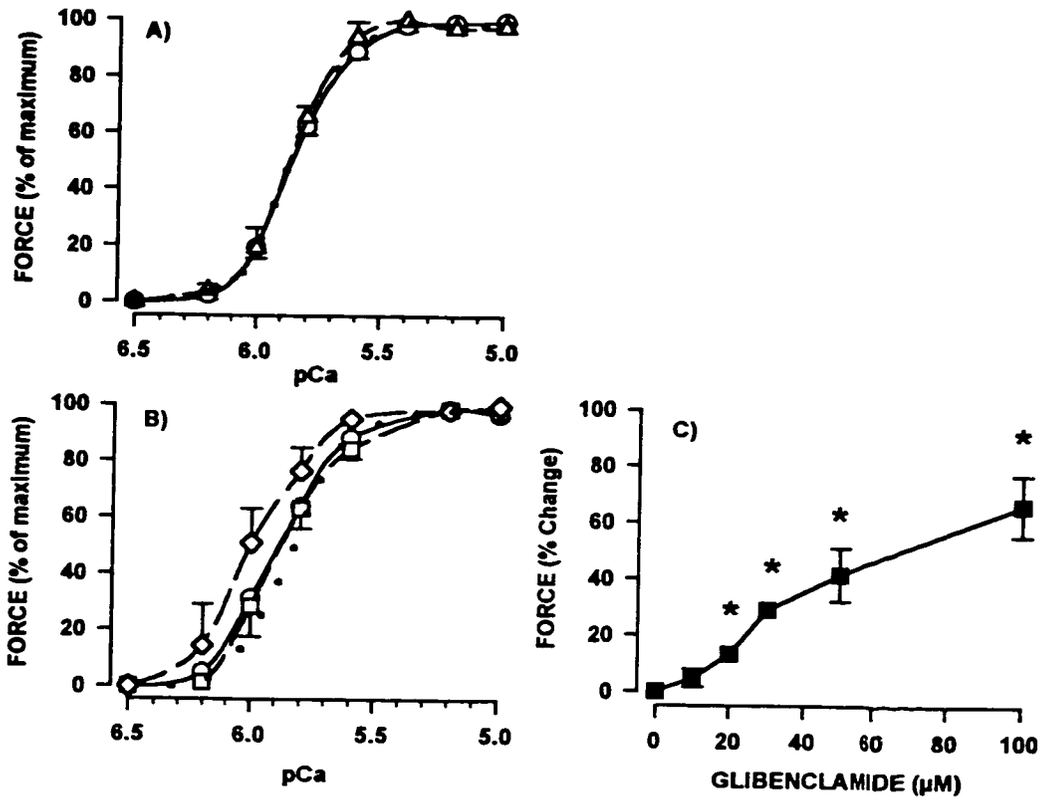

Figure 2-1. Effect of pinacidil and glibenclamide on the pCa-force curve of skinned soleus muscle fiber. Three pCa-force curves were measured for each muscle fiber at 22°C: the first in the absence of a drug (control), the second in the presence of a drug and the third after washout. A) Effect of pinacidil: O, control; Δ, 100 μM pinacidil; ••••, washout. Force is expressed as a percent of the force at pCa 5.0. B) Effect of glibenclamide: O, control; □, 10 μM glibenclamide; ◇, 100 μM glibenclamide; ••••, washout. C) Effect of glibenclamide on force development. Force in the presence of glibenclamide at pCa 6.2 is expressed as a percent of the force measured in the absence of the drug. Vertical error bars represent the S.E.M. of 5 muscle fibers (not shown if smaller than symbols). * Force in the presence of glibenclamide was significantly greater than in the absence of glibenclamide; ANOVA and L.S.D., $P<0.05$.

FIGURE 2-2

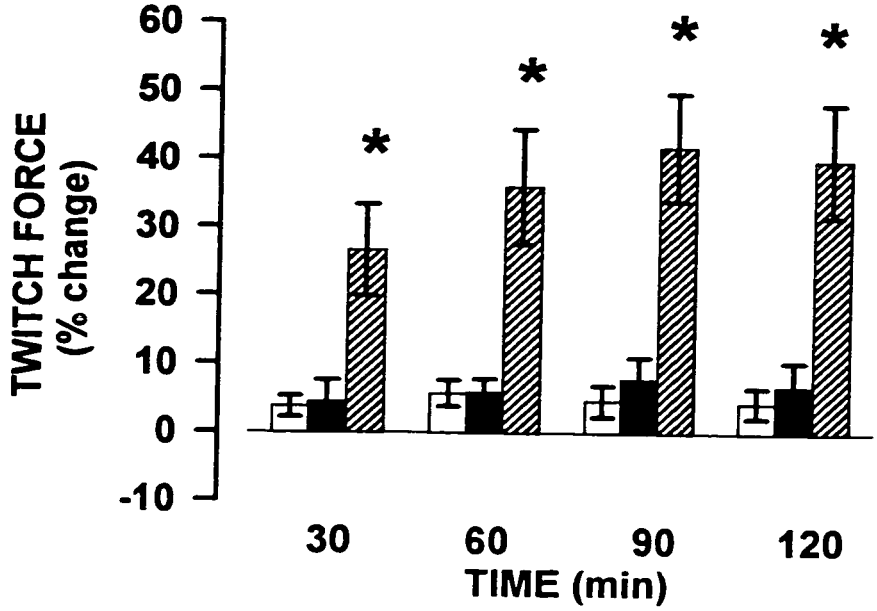

Figure 2-2. Effect of glibenclamide on the twitch force of intact EDL muscle. Muscles were exposed at 0, 10 or 100 μM glibenclamide at time 0. Twitch force is defined as the maximum force measured during a twitch, and is expressed as a percent change of the initial force at time 0. Experimental temperature was 37°C. Bars: open, control; filled, 10 μM glibenclamide; hatched, 100 μM glibenclamide. Vertical error bars represent the S.E.M. of 5 muscles. * Change in twitch force was significantly different from 0%; ANOVA and L.S.D., $P<0.05$.

frequency curves of unfatigued EDL (Fig. 2-3) and soleus muscle (results not shown). Thus, the results from Fig. 2-1, 2-2 and 2-3 demonstrate that 10 μM glibenclamide to block and 100 μM pinacidil do no affect the contractile apparatus of EDL and soleus muscle.

EFFECTS OF GLIBENCLAMIDE AND PINACIDIL ON CONTRACTILITY DURING FATIGUE

Effect prior to fatigue

Mean tetanic forces, measured at the beginning of each experiment, of control EDL and soleus muscles were respectively 36.5 $\pm$ 0.5 and 30.1 $\pm$ 0.2 N/cm^2 (n=10). Mean tetanic forces of the pinacidil and glibenclamide groups were not significantly different from those of controls (data not shown). The tetanic force of control EDL muscles decreased by 2.18 $\pm$ 0.36% (n=10) during the 30 min equilibrium period prior to fatigue. In the presence of 10 μM glibenclamide or 100 μM pinacidil the decreases were respectively 4.82 $\pm$ 2.62% and 4.21 $\pm$ 2.65% (n=5). For soleus muscles the decreases were 0.10 $\pm$ 0.55% (control), 2.31 $\pm$ 1.05% (glibenclamide) and 0.50 $\pm$ 0.75% (pinacidil). None of those decreases were significant (ANOVA $P > 0.05$).

Rate of fatigue

The rate of fatigue was measured from the decrease in tetanic force when muscles were stimulated with one tetanic contraction every sec. The rate of fatigue was faster in EDL muscle than in soleus muscle. For example, after one min of stimulation, the tetanic force of soleus muscle was 74.1% of the pre-fatigue force compared to 9.3% for EDL muscle (Fig. 2-4). The extent of fatigue was also greater in EDL: at the end of the 3 min fatigue period, tetanic force of EDL

FIGURE 2-3

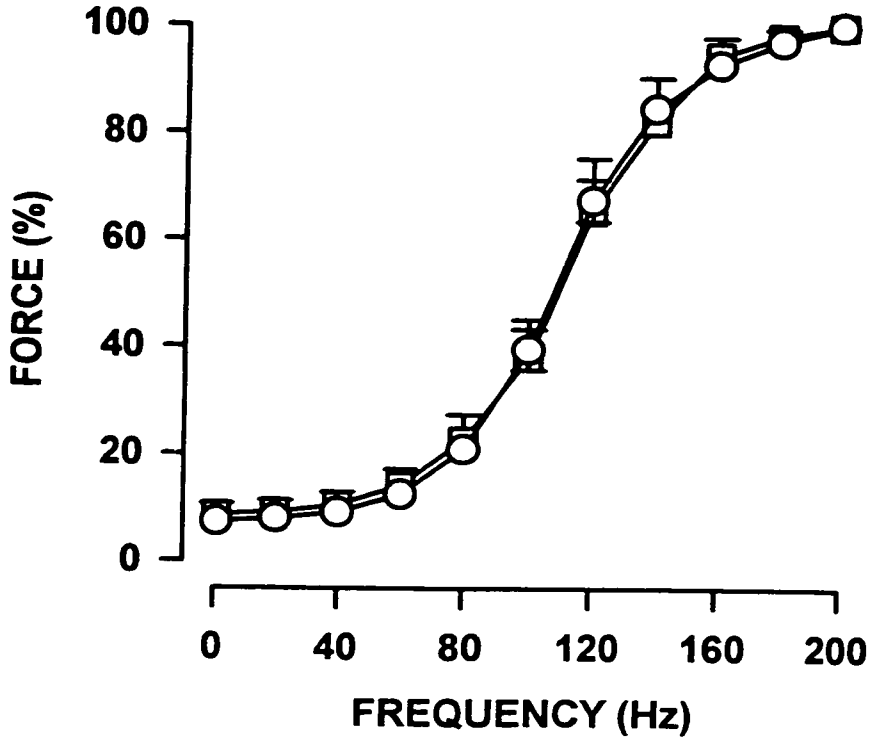

Figure 2-3. Effect of glibenclamide on the force-frequency curve of EDL muscle. A force-frequency curve was measured at the beginning of the experiment and after a 2 hour incubation in the absence (O) or presence (□)of 10 μM glibenclamide (muscles used here were the same as those for the data of Fig. 2-2). Experimental temperature was 37°C. Vertical error bars represent the S.E.M. of 5 muscles. The force-frequency curves of control EDL after 2 hours were not significantly different from those measured at the beginning of the experiments (data not shown, ANOVA, $P > 0.05$). The force-frequency curve in the presence of glibenclamide was not significantly different from the control curve (ANOVA, $P > 0.05$).

FIGURE 2-4

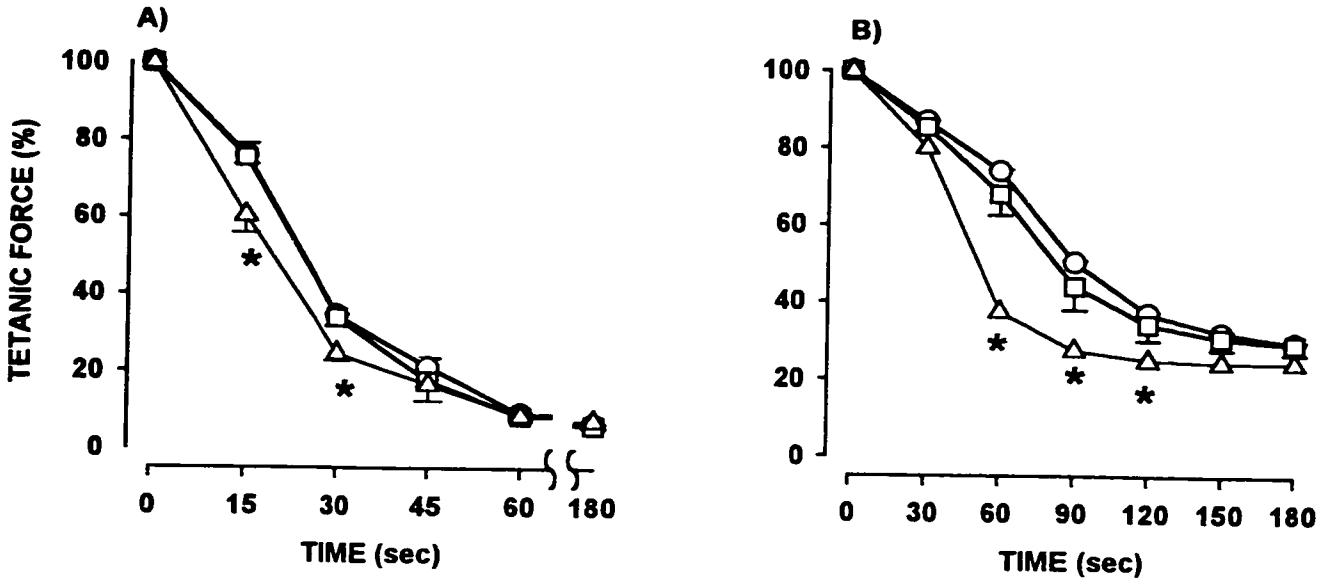

Figure 2-4. Effects of glibenclamide and pinacidil on the rate of fatigue in A) EDL and B) soleus muscles. Fatigue was induced with one 200 msec long tetanic contraction (200 Hz for EDL and 140 Hz for soleus) every sec for 3 min. Glibenclamide or pinacidil was added 30 min prior to fatigue. Experimental temperature was 37°C. The rate of fatigue was measured from the decrease in tetanic force, which is expressed as a percent of the tetanic force measured prior to fatigue (t=0). For clarity, data are shown at every 15 or 30 sec (also notice the break between 60 and 180 sec in A). Symbols: O, control; □, 10 μM glibenclamide; Δ, 100 μM pinacidil. Vertical error bars represent the S.E.M. of 5 muscles (absent when smaller than symbols). * Mean tetanic force in the presence of pinacidil was significantly different from mean tetanic force in control muscle during the same time period; ANOVA, L.S.D., $P < 0.05$.

muscle was 6.7% compared to 29.7% for soleus muscle. Glibenclamide at 10 μM had no effect on the decrease in tetanic force during fatigue for both EDL and soleus. 100 μM pinacidil, on the other hand, caused a faster decrease in tetanic force when compared to control muscles. In EDL muscle, the pinacidil effect was significant only during the first 30 sec of stimulation (Fig. 2-4A). Mean tetanic forces of pinacidil-exposed EDL after 15 and 30 sec were respectively 15.7% and 8.55 less than the mean forces of control EDL.

Although pinacidil had similar effects on soleus, there were also two major quantitative differences when compared to EDL muscle. First, pinacidil caused a significant increase in the rate of fatigue after 60 sec of stimulation in soleus, compared to 15 sec in EDL. Second, the difference in tetanic force between control and pinacidil-exposed muscle was larger in soleus than in EDL: the maximum difference was 37.3% after 60 sec of stimulation for soleus compared to 15.7% after 15 sec for EDL.

Resting tension

An increase in resting tension developed during fatigue due to the failure of muscles to completely relax between contractions. The increase in resting tension was quite variable and not observed in all control muscles. Despite the variability, two noticeable effects were observed in the presence of glibenclamide and pinacidil. To best analyze these effects, we pooled together data from all muscles used in this study (fatigue kinetics of Fig. 2-4, $^{86}Rb^{+}$ fractional loss in Fig. 2-8, ATP and PCr content in Fig. 2-9).

The first effect was on the proportion of muscles that developed resting tension during fatigue. For EDL muscle, 30% of 33 control muscles developed elevated resting tension compared to 90% of 20 glibenclamide-exposed muscles. This represented a 3-fold increase. For soleus muscles, 45% of 31 control muscles generated elevated resting tension, while the proportion was 63% of 19 glibenclamide-exposed muscles. A total of 21 EDL and 20 soleus muscles were exposed to 100 μM pinacidil and none of them developed elevated resting tension during fatigue.

The second effect was a greater increase in resting tension in glibenclamide-exposed muscles. Figure 2-5 shows the mean values calculated only from muscles that generated an elevated resting tension. In control EDL muscle, the increase in resting tension was less than 1% of the pre-fatigue tetanic force and was not significant, while a significant 4.9% increase was observed in the presence of 10 μM glibenclamide (Fig. 2-5A). Control soleus muscle developed greater resting tension than control EDL muscle (Fig. 2-5B). In control soleus, the increase in resting tension became significant after 90 sec and reached a maximum of 13.5% of the pre-fatigue tetanic force after 180 sec. The increase was again greater in the presence of glibenclamide, but a significant difference between control and glibenclamide-exposed muscle was observed only after 180 sec.

Recovery of tetanic force

Tetanic force increased toward its pre-fatigue value when the fatigue stimulation was stopped. The initial rate and extent of force recovery was greater in control soleus muscles than in control EDL muscle (Fig. 2-6). After 200 msec of recovery, the tetanic force of EDL muscle was

FIGURE 2-5

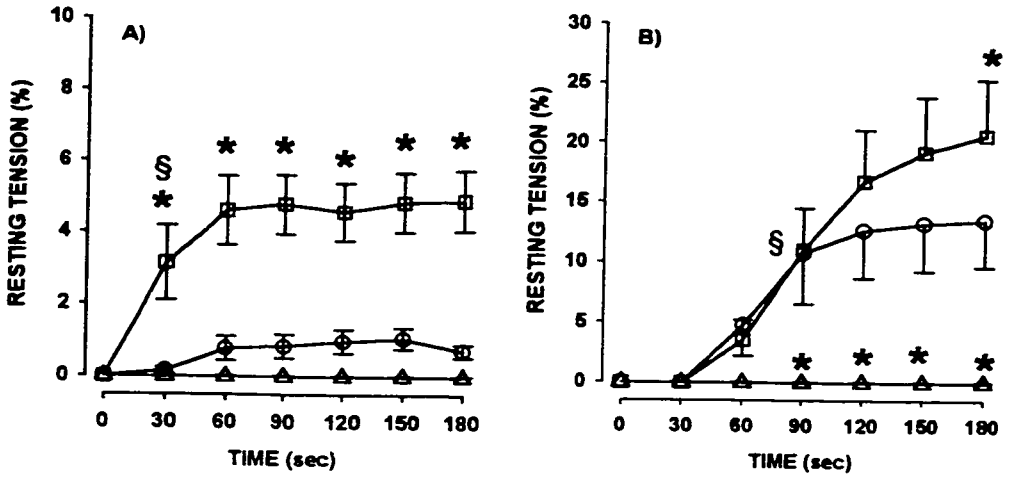

Figure 2-5. Effects of glibenclamide on the resting tension of A) EDL and B) soleus muscles during fatigue. Fatigue was induced with one 200 msec long tetanic contraction (200 Hz for EDL and 140 Hz for soleus) every sec for 3 min. Glibenclamide was added 30 min prior to fatigue. Experimental temperature was 37°C. Resting tension is defined as the tension measured 5 msec prior to a tetanic stimulation, and is expressed as a percent of the tetanic force measured prior to fatigue. Mean resting tensions were calculated only from muscles that developed a resting tension during fatigue (see text for the proportion of muscles that developed a resting tension). None of the muscles exposed to pinacidil developed a resting tension (data not shown). Symbols: O, control; □, 10 μM glibenclamide. Note the difference in the scale of resting tension between A and B. Vertical error bars represent S.E.M. * Mean resting tension in the presence of glibenclamide or pinacidil was significantly different from control muscle at that same time period; ANOVA, L.S.D., $P < 0.05$. § Indicate the time when mean resting tension became significantly different from time 0; ANOVA, L.S.D., $P < 0.05$.

FIGURE 2-6

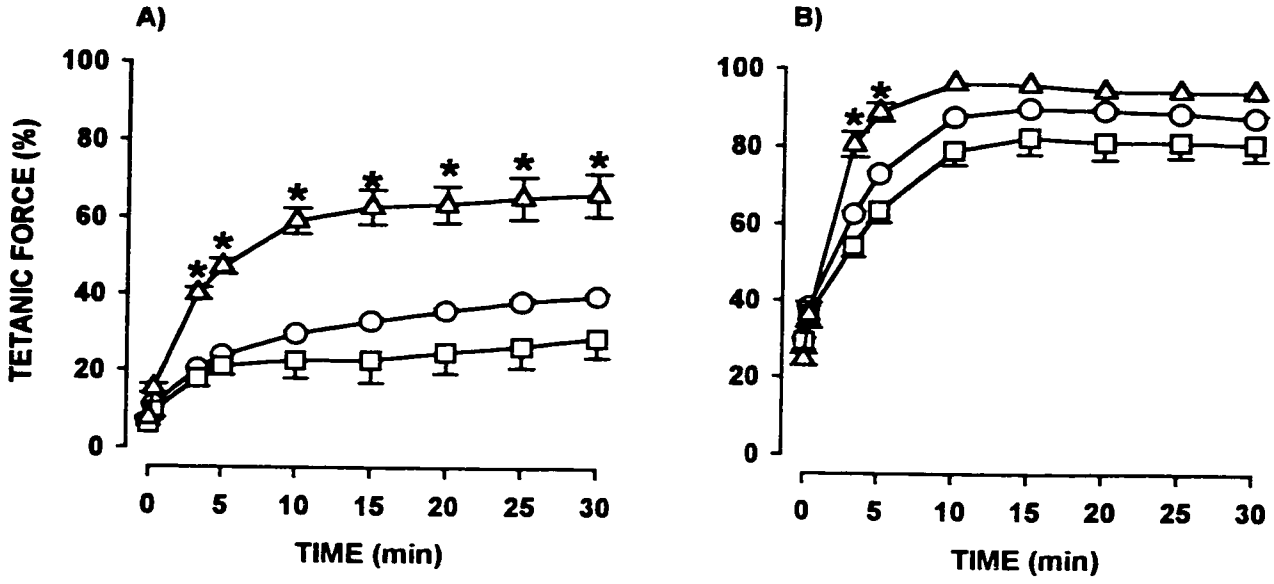

Figure 2-6. Effects of glibenclamide and pinacidil on tetanic force recovery of A) EDL and B) soleus muscles. Force recovery was measured after fatigue had been elicited with one 200 msec long tetanic contraction (200 Hz for EDL and 140 Hz for soleus) every sec for 3 min. Glibenclamide or pinacidil was added 30 min prior to fatigue and remained present during recovery. Experimental temperature was 37°C. Tetanic force is expressed as a percent of the tetanic force measured prior to fatigue. Symbols: O, control; □, 10 μM glibenclamide; Δ, 100 μM pinacidil. Vertical error bars represent the S.E.M. of 5 muscles (absent when smaller than symbols). * Mean tetanic force in the presence of pinacidil was significantly different from mean tetanic force in control muscle during the same time period; ANOVA, L.S.D., $P < 0.05$.

20.3% of the pre-fatigue force compared to 62.5% in soleus muscle. Tetanic force reached a maximum of 89.9% after 15 min of recovery in soleus compared to 39.8% after 30 min of recovery in EDL muscle. For both EDL and soleus muscle, the recovery of tetanic force in the presence of 10 μM glibenclamide was slightly less than in control. This difference was not significant.

Pinacidil increased both the initial rate and the extent of force recovery in EDL muscle, while it increased only the initial rate in soleus muscle. After 200 msec of recovery, the tetanic force of pinacidil-exposed EDL was 40.2% compared to 20.3% for control muscle. This 20% difference in force observed at 200 msec was still observed after 30 min of recovery as the values were 39.8% and 66.2% for control and pinacidil-exposed muscle, respectively. The maximum difference in force recovery in soleus muscle was observed after 200 msec when tetanic forces of control and pinacidil-exposed muscles were respectively 62.5% and 80.5%, an 18% difference. Thereafter, the difference in tetanic force between the two groups of soleus muscles decreased and was no longer significant after 10 min of recovery.

EFFECTS OF GLIBENCLAMIDE AND PINACIDIL ON MEMBRANE POTENTIAL

To better understand how glibenclamide and pinacidil affected tetanic force and resting tension, we determined their effects on action potentials (this section) and $^{86}Rb^{+}$ fractional loss (next section). When measuring action potentials, soleus muscles were exposed to 100 μM glibenclamide in the last 30 sec of the fatigue period. This allowed fatigue to proceed as it did under control conditions and to rapidly block any active $K^{+}_{(ATP)}$ channels in fibers located at the surface of muscles before fibers were penetrated with microelectrodes.

In control soleus, the repolarization phase of the action potential was shorter and the overshoot smaller after fatigue, compared to action potentials measured before fatigue (Fig. 2-7A). The shortening of the repolarization phase and the decrease in overshoot that occurred during fatigue were abolished when 100 μM glibenclamide was added 30 sec before the end of fatigue. On average, the effect of glibenclamide on the repolarization phase became significant after 5 min of recovery (Fig. 2-7B). At that time, the mean half-repolarization times in the absence and presence of glibenclamide were respectively 0.229 and 0.264 msec.

Mean overshoots of control soleus before and after fatigue were respectively 24.2 $\pm$ 1.8 mV (n=4/21, number of muscles/fibers) and 19.2 $\pm$ 2.8 mV (n=4/9), a 5.0 mV difference. For the glibenclamide-exposed group, mean overshoots were 28.5 $\pm$ 0.9 mV (n=4/19) before fatigue and 26.6 $\pm$ 1.8 mV (n=4/12) after fatigue, a difference of 2.1 mV. A significant 5.6 mV hyperpolarization occurred during fatigue as mean resting potentials of control soleus were -77.8 $\pm$ 1.5 mV prior to and -83.4 $\pm$ 2.3 mV after fatigue (ANOVA $P < 0.05$). This hyperpolarization was abolished in the presence of glibenclamide as the mean values before and after fatigue were respectively -78.0 $\pm$ 1.7 and -80.3 $\pm$ 1.8 mV.

To determine if activation of $K^+_{(ATP)}$ channels by pinacidil was large enough to alter membrane potentials, we studied how pinacidil affects resting and action potential in soleus muscle at rest. Pinacidil significantly reduced the mean overshoot by 8.8 mV (Table 2-1), but did not cause a shortening of the action potential. In fact, the mean half-repolarization time was significantly longer by 0.019 msec in the presence of pinacidil. These effects were observed

FIGURE 2-7

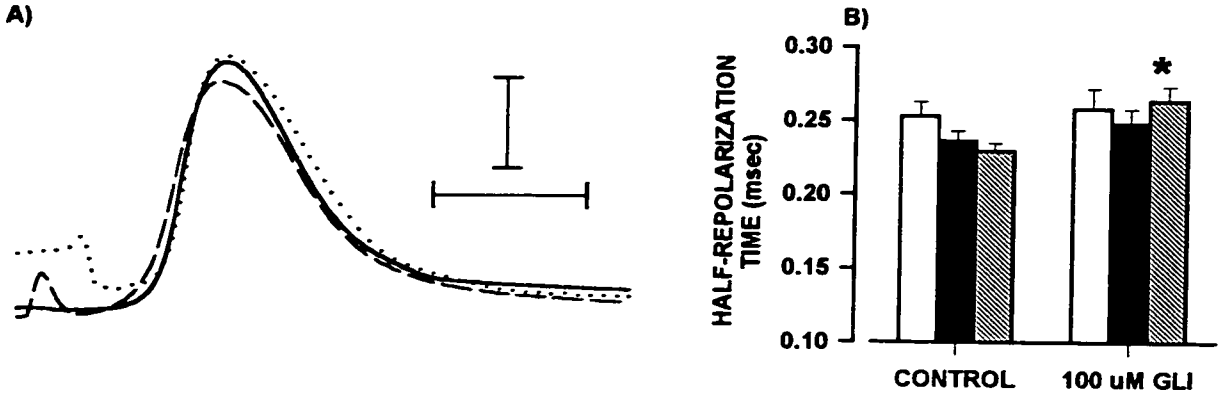

Figure 2-7. Effect of glibenclamide on the action potential of soleus muscle. A) An action potential was measured before fatigue (—) and immediately after fatigue in the absence (----) or in the presence (••••) of 100 μM glibenclamide added 30 sec before the end of the fatigue period. Fatigue was elicited with one 200 msec long tetanus (140 Hz) every sec for 3 min. Experimental temperature was 37°C. Horizontal bar represents 1 msec, vertical bar 40 mV. B) Half-repolarization time was measured as the time interval between the peak and 50% repolarization. Bars: open, before fatigue; filled, 0-2 min of recovery; hatched, 5-7 min or recovery. Vertical error bars represent the S.E.M. * Mean value in the presence of glibenclamide was significantly different from the mean value of control muscle at the same time period, t-test $P < 0.05$.

TABLE 2-1

EXPERRIMENTAL CONDITION	RESTING POTENTIAL (mV)	OVERSHOOT (mV)	HALF-REPOLARIZATION TIME (msec)	n
A) Control	-74.8 ± 1.9	23.4 ± 1.6	0.222 ± 0.008	4/24
100 µM Pinacidil	-76.6 ± 4.1	14.6 ± 1.4*	0.241 ± 0.004*	4/25
Washout	-77.4 ± 1.1	19.2 ± 2.0	0.225 ± 0.001	4/18
B) Control	-76.4 ± 1.9	19.2 ± 1.1	0.219 ± 0.008	3/12
10 µM Glibenclamide 100 µM Pinacidil	-77.3 ± 1.8	18.7 ± 1.4	0.236 ± 0.003*	3/15
Washout	-74.7 ± 2.0	20.4 ± 1.5	0.231 ± 0.013	3/16

Table 2-1. Effect of pinacidil on resting and action potentials of soleus muscle. Action potentials were first measured in the absence of pinacidil and glibenclamide (Control). In A, action potentials were measured 20 min after the addition of 100 μM pinacidil. In B, action potentials were measured after exposing muscle 20 min to 10 μM glibenclamide, and then 20 min to 10 μM glibenclamide and 100 μM pinacidil. Action potentials were also measured 2 hours after drugs had been washed out. Experimental temperature was 37°C. Data are expressed as MEAN ± S.E.M.; 'n' represents the number of muscles/number of fibers. * Mean value was significantly different from the 'Control value', t-test $P<0.05$.

while the resting potential was unaffected by pinacidil. In the presence of 10 μM glibenclamide, pinacidil did not affect the overshoot, while the half-repolarization time was still prolonged.

Action potentials were not measured in EDL because the glibenclamide and pinacidil effects were small in soleus. Instead, we measured $^{86}Rb^+$ fractional loss in order to better estimate $K^+_{(ATP)}$ channel activity during fatigue.

EFFECT OF GLIBENCLAMIDE AND PINACIDIL ON $^{86}Rb^+$ EFFLUX

$^{86}Rb^+$ fractional losses of control EDL and soleus muscles at rest were quite similar, being respectively 0.0075 $\pm$ 0.0005 (n=9, number of muscles) and 0.0073 $\pm$ 0.0004 (n=7). When control EDL muscles were stimulated to fatigue, $^{86}Rb^+$ fractional loss increased by 0.0136/min during the first min (Fig. 2-8A). Thereafter, $^{86}Rb^+$ fractional loss decreased slightly, but remained significantly greater than the value at rest. In control soleus muscle, $^{86}Rb^+$ fractional loss increased by 0.0100 during the first min of fatigue (Fig. 2-8B). It continued to increase slightly during the last 2 min of the fatigue period. In both EDL and soleus muscle, $^{86}Rb^+$ fractional loss returned to pre-fatigue levels within 2 min after the fatigue period.

The $^{86}Rb^+$ fractional losses at rest (results not shown) and during fatigue (Fig. 2-8) were not affected by 10 μM glibenclamide. Even at 100 μM, glibenclamide failed to reduce $^{86}Rb^+$ fractional loss during fatigue (results not shown). Pinacidil also had no effect on $^{86}Rb^+$ fractional losses in resting EDL (0.0079 $\pm$ 0.0005, n=7) and soleus (0.0078 $\pm$ 0.0007, n=8) muscle. However, it significantly increased the losses during fatigue. In EDL muscle, $^{86}Rb^+$ fractional loss during the first min was 1.3-fold greater in the presence of pinacidil than in control muscles (Fig. 2-8A). The

FIGURE 2-8

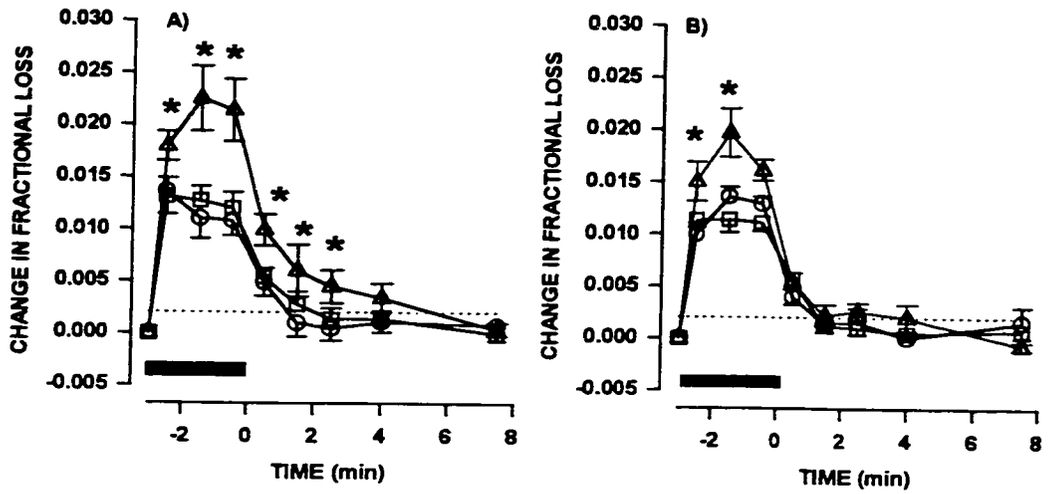

Figure 2-8. Effects of glibenclamide and pinacidil on $^{86}Rb^{+}$ fractional loss from A) EDL and B) soleus muscles. Muscles were loaded one hour with 4-8 μCi $^{86}Rb^{+}$ at 37°C. After 4 initial washouts (15, 15, 10 and 5 min), the $^{86}Rb^{+}$ fractional loss was measured at rest from 3 successive periods of 5 min. Glibenclamide or pinacidil were added during the initial period, so muscles would be exposed to them 30 min prior to fatigue. Muscles were fatigued at time –3 min. Washout periods were one min long during fatigue and during the first 3 min of recovery; the last two washouts were 2 and 5 min long. Data are given as the change in fractional losses. Symbols are plotted in the middle of the time period when washouts were taken. Symbols: O, control; □, 10 μM glibenclamide; Δ, 100 μM pinacidil; ■■, fatigue period. Vertical error bars represent the S.E.M. of 7-9 muscles. * Mean $^{86}Rb^{+}$ fractional loss in the presence of glibenclamide or pinacidil was significantly greater control value at the same time period; ANOVA and L.S.D., $P < 0.05$. .... Indicates the level of the $^{86}Rb^{+}$ fractional loss that is significantly different from zero; ANOVA and L.S.D., $P < 0.05$.

difference increased to 2.0-fold for the last two min of the fatigue period. $^{86}Rb^+$ fractional losses in the presence of pinacidil also continued to remain above those of control until the 3rd min of recovery. In soleus muscle, pinacidil significantly increased $^{86}Rb^+$ fractional losses by 1.4-1.5 times above those of control only during the first 2 min of fatigue. Thereafter, the $^{86}Rb^+$ fractional losses were similar in control and pinacidil-exposed muscle.

EFFECTS OF GLIBENCLAMIDE AND PINACIDIL ON ATP AND PCr CONTENT

Prior to fatigue, ATP and PCr contents of control EDL muscle were respectively 29.2 and 108.7 μmoles/g dry weight (Fig. 2-9A, B). The contents of both metabolites decreased significantly during fatigue; the values after fatigue being 15.0 μmoles/g dry weight for ATP and 15.3 μmoles/g dry weight for PCr. Neither 10 μM glibenclamide nor 100 μM pinacidil affected the ATP and PCr content of EDL muscle at rest or the decrease during fatigue.

The ATP and PCr contents of soleus muscle at rest were respectively 19.3 and 74.2 μmoles/g dry weight (Fig. 2-9C, D). Thus, ATP and PCr contents were smaller in soleus than EDL muscle. Such differences in ATP and PCr contents between fast-twitch muscle (like EDL) and slow-twitch muscle (like soleus) are in agreement with previous studies (32;33). During fatigue, the PCr content decreased significantly to 23.4 μmoles/g dry weight, while the decrease in ATP content to 15.8 μmoles/g dry weight was not significant.

Contrary to the situation observed in EDL muscle, both glibenclamide and pinacidil significantly altered ATP and PCr content of soleus muscle. Glibenclamide had no effect prior to fatigue, but caused a significantly greater depletion of ATP during fatigue. After fatigue, the ATP

FIGURE 2-9

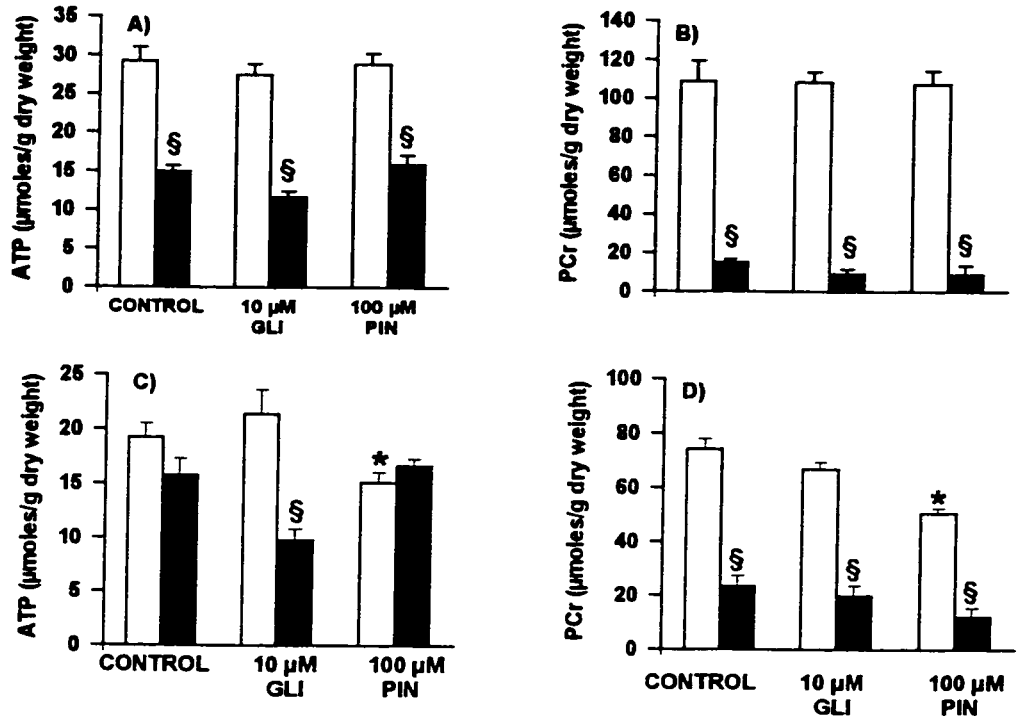

Figure 2-9. The effects of glibenclamide and pinacidil on A,C) ATP and B,D) PCr content of A,B) EDL and C,D) soleus muscles. Muscles were incubated for 30 min in the absence or presence of glibenclamide or pinacidil. Muscles were freeze clamp immediately after the 30 min incubation (muscles at rest) or immediately after fatigue. Fatigue was induced with one 200 msec long tetanic contraction (200 Hz for EDL and 140 Hz for soleus) every sec for 3 min. Experimental temperature was 37°C. Bars: open, ATP and PCr content in muscle at rest; filled, ATP and PCr content after fatigue. Vertical error bars represent S.E.M. of 6-8 muscles. * Mean ATP or PCr contents at rest were significantly different in the presence of glibenclamide or pinacidil than in control muscle; ANOVA and L.S.D., $P < 0.05$. § Mean ATP or PCr content after fatigue was significantly different than before fatigue; ANOVA and L.S.D., $P < 0.05$.

content of control soleus muscle was 3.5 μmole/g dry weight lower than rest value, whereas for glibenclamide-exposed muscle the decrease was 11.6 μmole/g dry weight. Prior to fatigue, the ATP and PCr contents of pinacidil-exposed muscles were respectively 4.2 and 23.7 μmole/g dry weight less than in control soleus. During fatigue PCr content decreased by 38.4 μmole/g dry weight in the presence of pinacidil compared to 50.1 μmole/g dry weight in control muscles. For ATP, the content increased by 1.6 μmole/g dry weight in the presence of pinacidil, while it decreased by 3.8 μmole/g dry weight in control soleus.

DISCUSSION

The major findings of this study are that during fatigue, glibenclamide-exposed muscles rarely showed any physiological difference compared to control EDL and soleus muscles, whereas pinacidil had several effects. During fatigue, glibenclamide caused i) greater increase in resting tension, ii) greater depletion of ATP content (only in soleus) and iii) abolished the cell membrane hyperpolarization and shortening of action potential duration. Glibenclamide did not affect $^{86}Rb^{+}$ fractional loss, rate of fatigue and force recovery. Pinacidil i) increased the rate of fatigue; ii) abolished the increase in resting tension; iii) increased the rate of force recovery, iv) increased $^{86}Rb^{+}$ fractional loss; v) reduced the action potential overshoot and vi) reduced the ATP and PCr depletion. The effects of pinacidil then suggest that $K^{+}_{(ATP)}$ channels can influence tetanic force during fatigue. However, the effects of glibenclamide suggest that they do not influence tetanic force when mouse EDL and soleus muscles are fatigued with one tetanic contraction every sec.

SPECIFICITY OF GLIBENCLAMIDE AND PINACIDIL

So far, there is no evidence that glibenclamide (10 and 100 μM) and pinacidil (100 μM) affect channels other than the $K^{+}_{(ATP)}$ channel in mammalian skeletal muscle (6;30). Although, glibenclamide increases the Ca^{2+} sensitivity of contractile components of intact muscle fibers (13), the current study, using skinned muscle fibers, shows that this effect occurs only when the glibenclamide concentration is greater than 10 μM (Fig. 2-1C). At 10 μM, glibenclamide did not affect twitch force (Fig. 2-2) or the force-frequency curve (Fig. 2-3) of intact EDL or soleus muscles. Pinacidil also had no effect on the pCa-force curve of EDL or soleus skinned muscle

fibers (Fig. 2-1A). Therefore, the effects of glibenclamide and pinacidil observed in this study are believed to be related to a modulation of $K^+_{(ATP)}$ channel activity; i.e., a block by glibenclamide and an activation by pinacidil.

ACTIVATION OF $K^+_{(ATP)}$ CHANNELS DURING FATIGUE AND BY PINACIDIL

Activity of $K^+_{(ATP)}$ channels during fatigue was estimated from the effect of glibenclamide and pinacidil on membrane potentials and $^{86}Rb^+$ fractional loss (as a marker for K^+ movement across the channel, 9). Here, we shall discuss the activation of $K^+_{(ATP)}$ channels during fatigue and by pinacidil, and in a later section, the importance of this activation on force.

Activation of $K^+_{(ATP)}$ channels during fatigue in control muscle

A hyperpolarization and shorter repolarization phase were observed in fatigued soleus muscle fibers when compared to unfatigued fibers (Fig. 2-7). This is contrary to most *in vitro* studies, which have reported the reverse (see for example Balog *et al.* (4) and Juel (27)). Although there is no study reporting shorter repolarization phase in fatigued muscle, there is at least one study reporting a hyperpolarization in rat soleus muscle (23). In the latter study, evidence was provided for a contribution of the Na^+-K^+ pump to the hyperpolarization. The current study now shows that both the hyperpolarization and prolongation of action potential repolarization phase caused by fatigue were abolished by glibenclamide. These glibenclamide effects can be explained by a block of $K^+_{(ATP)}$ channels that decreases K^+ conductance. A reduction in K^+ conductance prevents the hyperpolarization by reducing the outward K^+ current that maintains a negative resting potential (which means that both Na^+-K^+ pump (23) and $K^+_{(ATP)}$ channel are important in the

hyperpolarization process). A smaller K^+ conductance also reduces the K^+ current that contributes to the repolarization phase.

The reversal of the fatigue effects on membrane potential by glibenclamide suggests that $K^+_{(ATP)}$ channels were activated during fatigue. However, the glibenclamide effects on resting and action potentials were very small, and in some cases significant only after 5 min of recovery (e.g., the repolarization phase). Furthermore, 10 μM glibenclamide did not affect the increase in $^{86}Rb^+$ fractional loss when EDL and soleus muscles were stimulated to fatigue (Fig. 2-8). The lack of an effect on $^{86}Rb^+$ fractional loss can not be explained on the basis that glibenclamide is ineffective at 10 μM for two reasons. First, at that concentration, glibenclamide blocks most $K^+_{(ATP)}$ channels under patch clamp conditions (2) and during metabolic inhibition (3). Second, even at 100 μM, glibenclamide did not affect $^{86}Rb^+$ fractional loss (results not shown). Thus, our results then suggest that fatigue elicited with one tetanic contraction every sec does not activate a large number of $K^+_{(ATP)}$ channels that physiologically alters membrane potential and $^{86}Rb^+$ fractional loss.

Activation of $K^+_{(ATP)}$ channels by pinacidil

Contrary to the effects of fatigue stimulation, pinacidil at 100 μM is effective in activating $K^+_{(ATP)}$ channels. Three observations support this conclusion. First, Barrett-Jolley and McPherson (6) reported maximal activation of $K^+_{(ATP)}$ current by pinacidil at 100 μM in intact resting flexor digitorum brevis muscle fibers. Second, we observed that pinacidil reduced the action potential overshoot in resting soleus muscle fiber, an effect that was blocked by glibenclamide (Table 2-1). The pinacidil effect on the overshoot can be explained by an activation of $K^+_{(ATP)}$ channel that

increases K^+ conductance, which in turn reduces the depolarization effect of Na^+ channels and overshoot. Third, we observed that during fatigue, pinacidil significantly increased $^{86}Rb^+$ fractional loss to a greater extent than observed in control muscles (Fig. 2-8).

PHYSIOLOGICAL ROLE OF $K^+_{(ATP)}$ CHANNELS DURING FATIGUE

Muscle contractility

The rate of fatigue, as measured from the decrease in tetanic force (Fig. 2-4), and the recovery of force following fatigue (Fig. 2-6) were not affected by 10 μM glibenclamide. This is not surprising considering that glibenclamide had few effects on membrane potential and none on $^{86}Rb^+$ fractional loss. Glibenclamide, however, affected the development of resting tension during fatigue; it increased the proportion of muscles that generated resting tension as well as the level of resting tension. The increase in resting tension can not be due to an effect on contractile components because 10 μM glibenclamide does not affect the pCa-force curve of skinned muscle fibers (Fig.2-1). Pinacidil had the opposite effect of glibenclamide as it completely abolished the increase in resting tension. Together, the glibenclamide and pinacidil effects suggest that modulating $K^+_{(ATP)}$ channel significantly alters the capacity of fatigued muscle to relax between contractions. Taking into account that glibenclamide affected resting tension but not tetanic force, we hypothesize that 1) few $K^+_{(ATP)}$ channels are activated during fatigue and 2) the number of active channels is large enough to affect resting tension, but was too small to affect tetanic force.

Although $K^+_{(ATP)}$ channels did not contribute to the decrease in force in our fatigue model, it does not imply that these channels are not important in skeletal muscle during other metabolic

stresses. We found that an activation of $K^+_{(ATP)}$ channels by pinacidil increased the rate of fatigue, abolished the increase in resting tension and increased the rate of force recovery. The pinacidil effects support Noma's (34) hypothesis that the channel can contribute to the decrease in force (rate of fatigue) and protect muscle function (lower resting tension and faster force recovery). However, more studies will be necessary to determine what physiological conditions activate a sufficient number of $K^+_{(ATP)}$ channels to affect muscle contractility and protect muscle function.

Mechanisms of action of $K^+_{(ATP)}$ channel

In the Introduction, two mechanisms of action were described for $K^+_{(ATP)}$ channels. One mechanism involves a shortening of the action potential duration, and the other an increase in K^+ efflux and extracellular K^+ that decrease membrane excitability. An activation of $K^+_{(ATP)}$ channels by pinacidil resulted in an 8 mV decrease in overshoot and a 0.020 msec increase in half-repolarization time of action potential (Table 2-1). Such changes in action potential are not expected to greatly affect the development of tetanic force. This constitutes a major difference between cardiac and skeletal muscle because activation of $K^+_{(ATP)}$ channels in cardiac muscle (by a channel opener or metabolic stress) reduces action potential duration by 2- to 4-fold, and reduces force development (12;19).

If in skeletal muscle an effect on action potential is not important, then an effect on K^+ efflux must be a major mechanism of action when $K^+_{(ATP)}$ channels were activated by pinacidil. This is indeed supported by the fact that pinacidil significantly increased $^{86}Rb^+$ fractional loss during fatigue (Fig. 2-8). However, the pinacidil effect on $^{86}Rb^+$ fractional loss (or K^+ efflux) was

greater in EDL. This can not explain how the rate of fatigue in the presence of pinacidil was increased to a greater extent in soleus muscle (Fig. 2-4), unless other events are considered.

First, the intracellular K^+ concentration (27) and the stimulation frequency (see Methods) are greater in EDL than soleus muscle. Consequently, the K^+ efflux associated with the electrical activity of cell membrane (or K^+ delayed rectifiers during the repolarization phase) should be greater in EDL muscle. A higher K^+ efflux in EDL muscle is further supported by the fact that during the first min of fatigue, $^{86}Rb^+$ fractional loss was 0.0136 in EDL compared to 0.0100 in soleus. Second, K^+ must reach a critical extracellular concentration before it suppresses muscle contractility (7). Third, several other factors such as decreases in Ca^{2+} release or increases in inorganic phosphate and H^+ can contribute to the decrease in force (15).

During the first min of fatigue, the tetanic force of EDL muscle decreased by more than 90%; during that time one can expect that several factors (including K^+) have reached a level that suppresses force. Under those conditions, an activation of $K^+_{(ATP)}$ channels appears to only have a relatively small effect on the rate of fatigue. In control soleus muscle, on the other hand, tetanic force decreased by only 25% during the first min. It is thus unlikely that K^+ has reached its critical concentration at which it suppresses force. Under those conditions, an activation of $K^+_{(ATP)}$ channels with pinacidil can increase the K^+ efflux and extracellular concentration resulting in a large and rapid decrease in tetanic force as we observed between the 30th and 60th sec of stimulation (Fig. 2-4B). Thus, in skeletal muscle, the major mechanism by which $K^+_{(ATP)}$ channels suppress force would involve a greater increase in extracellular K^+ concentration and not a shortening of action potential duration (as it does it cardiac muscle).

Effects of $K^+_{(ATP)}$ channel on energy content

The decrease in ATP and PCr contents during fatigue was not affected by pinacidil in EDL muscle, whereas in soleus muscle it was smaller in the presence of pinacidil when compared to control muscle (Fig. 2-9). Thus, the better preservation of energy during fatigue in soleus muscle in the presence of pinacidil correlates well with the larger decrease in tetanic force (Fig. 2-4) and resting tension (Fig. 2-5). Glibenclamide also had no effect on ATP and PCr contents of EDL, which is not surprising since it did not affect tetanic force.

Glibenclamide and pinacidil, however, each had an effect in soleus that can not be explained by an effect on contractility. First, the decrease in ATP content during fatigue was greater in the presence of glibenclamide even though the rate of fatigue had not been affected. Second, pinacidil caused significant decreases in ATP and PCr content prior to fatigue, while it had no effect on tetanic force. These two effects can not be explained from our results. These observations suggest that they may involve mitochondria $K^+_{(ATP)}$ channels. First, $K^+_{(ATP)}$ channels are present in the inner membrane of mitochondria (26). Second, mitochondrial $K^+_{(ATP)}$ channels are blocked by glibenclamide (17) and activated by pinacidil (25). Third, the effects were observed in soleus muscle, which has a high mitochondrial content, and not in EDL muscle, which has a low mitochondrial content (36;44). Mitochondria $K^+_{(ATP)}$ channels are believed to maintain mitochondrial volume to maximize ATP synthesis (18). It can then be suggested that blocking these channels with glibenclamide reduces ATP synthesis during fatigue leading to greater ATP depletion than in control muscle. Pinacidil, on the other hand, may have caused a sudden and large activation of mitochondrial $K^+_{(ATP)}$ channels resulting in an imbalance of K^+ flux that was

detrimental to ATP synthesis. Thus, future studies will be necessary to better understand the physiological role of mitochondrial $K^+_{(ATP)}$ channel on ATP and PCr contents in muscle.

In summary, the results from the effects of pinacidil, a $K^+_{(ATP)}$ channel opener, suggest that in EDL and soleus muscle, $K^+_{(ATP)}$ channels can contribute to a decrease in force, reduce the depletion of ATP and PCr, and protect muscle function (by reducing resting tension and improving force recovery following fatigue) as originally postulated by Noma (34). However, too few $K^+_{(ATP)}$ channels are activated to accomplish this role when EDL and soleus muscles are fatigue with one tetanic contraction per sec for 3 min.

ACKNOWLEDGMENTS

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CHAPTER 3

DENERVATION ENHANCES THE EFFECTS OF PINACIDIL AND GLIBENCLAMIDE DURING FATIGUE IN EDL AND SOLEUS MUSCLES

by

W. Matar, J.A. Lunde, B.J. Jasmin and J.M. Renaud

University of Ottawa, Department of Cellular and Molecular Medicine, 451 Smyth Rd., Ottawa Ontario, Canada, K1H 8M5

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RT-PCR experiments (Figure 3-3) were done by Dr B.J. Jasmin and Mr. J.A. Lunde.

ABSTRACT

The objective of this study was to determine if a loss of muscular activity/and or neurotrophic factors following denervation alters the expression and the physiological importance of the $K^+_{(ATP)}$ channel in EDL and soleus muscles during fatigue. Fatigue was induced with one tetanic contraction every sec for 3 min. After 1-week denervation, Kir6.2 mRNA content did not change in EDL, whereas it decreased by 2.7 fold in soleus. In both muscles, 100 μM pinacidil, increased the rate of fatigue, abolished resting tension and increased the extent of recovery. It also increased $^{86}Rb^+$ fractional loss and caused smaller ATP depletion during fatigue. The latter effect was however observed only in EDL muscle. Glibenclamide, at 10 μM, did not affect the decrease in tetanic force during fatigue, but increased resting tension and impaired force recovery. Glibenclamide also caused slower decrease in total force (tetanic force + resting tension) in soleus but not in EDL. When these effects of pinacidil and glibenclamide on denervated muscles are compared to those reported by Matar *et al.* (2000) in normal muscle, the data suggests that the role of $K^+_{(ATP)}$ channels is greater in denervated muscle.

KEYWORD $K^+_{(ATP)}$ channel, Kir6.2, force recovery, tetanic force, resting tension, $^{86}Rb^+$, ATP, PCr.

INTRODUCTION

The expression of several K^+ channels in skeletal muscle is altered by neurotrophic factors and muscle activity. The low-conductance Ca^{2+}-activated K^+ channel (or SK^+ channel) is present in myotubes during development, and its expression becomes suppressed following innervation (23). In adult muscle denervation increases its levels of expression (21).

The expression of K^+ channels decreases following denervation. These include: the inwardly rectifying K^+ channel (IRK1 or Kir2.1) (24), the voltage-gated K^+ channel (Kv3.4) (31), and the large conductance Ca^{2+}-activated K^+ channel (or BK^+ channel) (9). These three potassium channels have important roles in adult skeletal muscle. The IRK1 is responsible for maintaining membrane potential at rest (24). The Kv3.4 channel is a fast-inactivating channel that may be involved in the modulation of Ca^{2+} inward current (31). The BK^+ channel is thought to be important in reducing membrane excitability when intracellular Ca^{2+} levels are elevated during repeated muscle contraction (27). Thus, for three K^+ channels that regulate muscle activity, denervation reduces their expression.

The $K^+_{(ATP)}$ channel is another potassium channel that has an important role in adult skeletal muscle where it couples the muscle cell's metabolism to its electrical activity. The channel is activated during muscle activity that leads to a drop in energy levels; i.e. decrease in ATP, or increase in ADP (2;7). Once activated, the channel contributes to a decrease in force and Ca^{2+} (4;8). The importance of this channel is to provide muscle with a mechanism that reduces energy utilization when energy levels are low, which eventually protects muscle against deleterious energy

depletion and function impairment (19;28). Indeed, blocking $K^+_{(ATP)}$ channel significantly impairs the capacity of cardiac and skeletal muscle to recover force following a metabolic stress, such as ischemia or fatigue (15;11).

The objective of this work was to study whether denervation affects the expression and the physiological effects of $K^+_{(ATP)}$ channel in mouse skeletal muscle. The $K^+_{(ATP)}$ channel, like the IRK1, Kv3.4 and BK^+ channels, is important in active muscle. We thus hypothesized that the expression and physiological effect of $K^+_{(ATP)}$ channel decrease when muscle becomes inactive following denervation as reported for the other three K^+ channels. We determined the mRNA content of Kir6.2, the protein subunit that constitutes the pore of the $K^+_{(ATP)}$ channel (12;22). To test if the physiological effects of the $K^+_{(ATP)}$ channel are altered following denervation, we determined the effect of pinacidil, a channel opener, and glibenclamide, a channel blocker, on tetanic force, $^{86}Rb^+$ fractional loss, ATP and PCr during fatigue. Our results showed that the Kir6.2 mRNA content decreases following denervation in soleus, but not in EDL muscle. They also showed that the effects of pinacidil and glibenclamide during fatigue are enhanced following denervation, despite the changes in mRNA (in soleus muscle at least).

METHODS AND MATERIALS

ANIMALS AND MUSCLE DENERVATION

All experimental procedures were approved by the Animal Care Committee of the University of Ottawa. Two-three month old female CD-1 mice weighing 25-30 g were obtained from Charles River and housed according to the guidelines of CCAC (Canadian Council for Animal Care). The animals were fed *ad libidum*. Before surgery, animals were injected with 0.01 ml of the analgesic buprenorphine (0.03 mg/ml). Muscle denervation was performed under anesthesia with halothane. The left hindlimb was denervated by excision of a 5 mm segment of the sciatic nerve at the thigh region. Subsequently, two more analgesic injections were administered to the animal within 24 hours after the operation. Prior to any experiment, mice were tested for any reinnervation occurrences. The test consisted of suspending the animal by its tail and checking for the absence of a reflex extension of the foot and spreading of the toes.

TOTAL mRNA EXTRACTION, REVERSE-TRANSCRIPTION AND POLYMERASE-CHAIN REACTION

The mRNA content of Kir6.2 was quantified using a semi-quantitative RT-PCR method. For all samples, total RNA was extracted from EDL and soleus muscles using Trizol as recommended by Gibco (Burlington, Canada). Total RNA was redissolved into 20 µl of Rnase-free water. For each sample, the RNA was further diluted to a final concentration of 50 ng/µl measured using a GeneQuant II RNA/DNA spectrophotometer. Reverse transcription of 2 µl of this dilution was carried out as previously described by Jasmin *et al.* (13), except for the following changes.

The primer sequences for amplification of Kir6.2 cDNAs were based on the sequence from Inagaki *et al.* (12), and were as follows: primer A 5'-TCACCCTGCGCCATGGCCGC-3' at 697 bp and primer B 5'-TAGCGGCCGTCCTCCTCGGC-3' at 1108 bp. Each cycle of amplification consisted of 1 min at 94°C, 1 min at 60°C, 1 min at 72°C and 10 min extension at 72°C. The number of cycles was 34 and was within the linear range of amplification. The RT-PCR products were visualized on 1.5% agarose gels containing ethidium bromide. Quantification was performed by separating the PCr products in agarose gel containing the fluorescent dye VistraGreen (Amersham), and the labeling intensity was measured using a Storm phosphor imaging system (Molecular Dynamics).

FATIGUE EXPERIMENT

Muscle preparation and solutions

Mice were anesthetized with an intraperitonal injection of sodium pentobarbitol (Somnotol), delivered at a dose of 0.8 mg/10 g body weight. EDL and soleus muscles were excised and both tendons were tied with surgical silk (6.0) to allow attachment of muscle to the experimental apparatus.

Muscles were always immersed in physiological saline solution containing (in mM): 118.5 NaCl, 4.7 KCl, 2.4 $CaCl_2$, 3.1 $MgCl_2$, 25 Na_2HCO_3, 2 NaH_2PO_4; 5.5 d-glucose. The solution was continuously bubbled with 95% O_2: 5% CO_2, and had a pH 7.4. The temperature of all experiments was 37°C. Glibenclamide and pinacidil containing solutions were prepared by first

dissolving the drugs in DMSO before adding the proper volume to the saline solution. The final concentration of DMSO (including control solutions) was 0.1% (v/v).

Glibenclamide, at 10 μM, was used to block $K^+_{(ATP)}$ channel since its effectiveness and specificity are well characterized in skeletal muscle. At that concentration, glibenclamide blocks most (>95%) $K^+_{(ATP)}$ channel under patch clamp condition (1) and during metabolic inhibition (2). Glibenclamide has no effect on other K^+ channels (16) and on the pCa-force curve of skinned muscle fibers (17). Pinacidil, a $K^+_{(ATP)}$ channel opener, was used at a concentration of 100 μM. At this concentration, Barrett-Jolley & McPherson (3) showed that pinacidil has a near maximal effect in activating $K^+_{(ATP)}$ channels in intact flexor digitorum muscle fibers, and that it has no effect on any other channel than the $K^+_{(ATP)}$ channel.

Force measurements

Measurements of tetanic force was carried out as described by Matar *et al.* (17). Briefly, tetanic force was measured with a Cambridge ergometer (model 300, USA), and digitized with a Keithley Metrabyte A-D board (model DAS50, USA). Sampling rate was 5 kHz. Muscles were stimulated by passing a current between parallel platinum wires located on opposite sides of the muscle. Tetanic contractions were elicited with 200 msec long train of 0.3 msec long rectangular pulses of 6 V (supramaximal voltage). Stimulation frequencies were 140 Hz for soleus muscle, and 200 Hz for EDL muscle.

Fatigue and recovery protocols

Muscle length was adjusted to give maximal tetanic force and allowed a 30 min equilibration period. During this period, tetanic contractions were elicited every 2 min. For all pinacidil- and glibenclamide-exposed muscles, drugs were added at the beginning of the equilibration period (i.e. 30 min before fatigue) and were present throughout the remainder of the experiment. After equilibration, fatigue was elicited with one tetanic contraction every sec for 3 min. Following fatigue, muscles were stimulated at 10, 20, 100, 200 sec, 5 min, and every 5 min thereafter until 30 min to measure the recovery of tetanic force.

ATP AND PCR MEASUREMENTS

These measurements were done as described by Matar *et al.* (17). Briefly, muscles were freeze clamped in liquid nitrogen immediately after the equilibration period to establish rest-state values, or immediately after fatigue to obtain fatigued values. Muscles were stored at -80 °C until analyzed. The extraction of ATP and PCr was as described by Passoneau & Lowry (20) with some modifications as described in Matar *et al.* (17).

$^{86}RB^{+}$ FRACTIONAL LOSS MEASUREMENTS

The methodology was as described by Matar *et al.* (17). Briefly, we used $^{86}Rb^{+}$ fractional loss to estimate the activity of $K^{+}_{(ATP)}$ channel, as the latter permeates $^{86}Rb^{+}$ at a similar permeability as K^{+} (18). Muscles were loaded with $^{86}Rb^{+}$ (4-8 μCi/ml) for 60 min (with a change of fresh solution at 30 min). The $^{86}Rb^{+}$ loading was followed by 4 washout periods (15, 15 , 5 and 5 min). The basal $^{86}Rb^{+}$ fractional loss was then obtained from three 5 min washout periods.

During fatigue and the first 3 min of recovery, washout periods were 1 min long. $^{86}Rb^{+}$ fractional loss was also measured between the 3rd and 5th, and between the 5th and 10th min of recovery. At the end of the experiment, muscles were homogenized in 2 ml 6% perchloric acid. The homogenate was centrifuged at 10,.000 g for 30 min. $^{86}Rb^{+}$ counting was done using a WinSpectral liquid scintillation counter (model 1414, Wallac Instruments, USA), and quenching was corrected by counting 1 μCi of $^{86}Rb^{+}$ in 1 ml of physiological solution and another 1 μCi of $^{86}Rb^{+}$ in 1 ml of 6% perchloric acid.

STATISTICAL ANALYSIS

ANOVA was used to determine significant differences. Split plot designs were used when muscles were tested at all levels of a treatment (e.g., time effect during fatigue and recovery). In all other cases, a two way ANOVA design was used. ANOVA calculations were made using the GLM (General Linear Model) procedures of the Statistical Analysis Software (SAS Institute Inc., Cary, NC USA). When a main effect or an interaction was significant, the least square difference (L.S.D.) was used to locate the significant differences (29). The word "significant" refers only to a statistical difference ($P < 0.05$).

RESULTS

EFFECT OF 1- AND 2-WEEK DENERVATION ON EDL AND SOLEUS MUSCLES

Muscle weight

Denervation caused significant decrease in muscle weight in both EDL and soleus muscles (ANOVA, $P < 0.05$). After 1-week denervation, the contralateral innervated EDL muscle weighed 9.38 $\pm$ 0.23 mg (n=15, number of muscles), while denervated muscle weighed 8.31 $\pm$ 0.24 mg (n=15). After 2-weeks denervation, the respective values were 8.94 $\pm$ 0.25 mg (n=5) and 6.44 $\pm$ 0.19 mg (n=5). The decreases in weight between innervated and denervated muscles were greater after 2-weeks (28.0%) than after 1-week denervation (11.4%).

Similar decreases in weight were observed for the soleus muscle, where after 1-week denervation, the contralateral innervated soleus muscle weighed 7.13 $\pm$ 0.23 mg (n=15), while denervated muscle weighed 5.10 $\pm$ 0.24 mg (n=15). The respective values were 7.58 $\pm$ 0.45 mg (n=5) and 5.14 $\pm$ 0.48 mg (n=5), after 2-weeks denervation. The differences in weight between innervated and denervated muscles were similar between 1- and 2-weeks denervation periods (respective values being 28.5% and 32.2%).

Initial tetanic force

Denervation had no significant effect on the normalized initial tetanic forces (ANOVA, $P > 0.05$). After 1-week denervation, initial tetanic forces were 40.6 $\pm$ 4.8 and 39.4 $\pm$ 4.3 N/cm^2 for

innervated and denervated EDL muscles respectively (n=5). Soleus muscles developed lesser force compared to the EDL: initial tetanic force of soleus muscles were 32.6 ± 4.0 N/cm² (n=5) for innervated muscle and 33.1 ± 3.2 N/cm² (n=5) for 1-week denervated muscle. Initial tetanic forces following 2-week denervation for both EDL and soleus muscles were similar to those measured after 1-week denervation (data not shown). For all the above-mentioned experimental conditions, tetanic force did not change significantly over the 30 min equilibration period prior to fatigue (data not shown).

Kinetics of fatigue

Compared to the contralateral innervated EDL muscle, 1-week denervated EDL had significant slower initial rate and lesser extent of fatigue (Fig. 3-1A). For example, tetanic force after 30 sec of stimulation decreased to 46% of pre-fatigue force in denervated EDL compared to 35% in innervated muscle. At the end of the fatigue period, the tetanic force of innervated EDL dropped to 8% of pre-fatigue force, whereas the force of denervated EDL dropped to 15%. The longer denervation period of 2 weeks did not affect the kinetics of fatigue as they were similar to those reported for 1- week denervation.

Contrary to EDL, the decrease in tetanic force during the first 60 sec of stimulation was significantly greater in 1-week denervated than innervated soleus muscle (Fig. 3-1B). For example, at 60 sec, tetanic force of denervated soleus was 44% of pre-fatigue value compared to 65% for innervated soleus; a difference of 21%. Thereafter, there was no significant difference between tetanic forces of innervated and denervated muscles; i.e. the extent of fatigue was the same, as

FIGURE 3-1

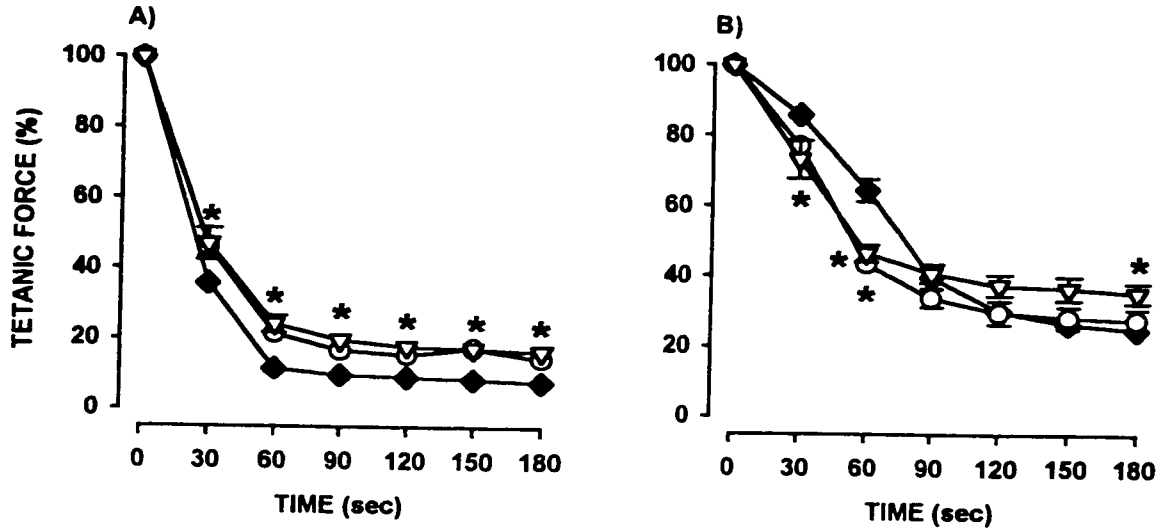

Figure 3-1. Effects of 1- and 2-week denervation on tetanic force during fatigue of A) EDL and B) soleus muscles. Fatigue was induced with one 200 msec long tetanic contraction (200 Hz for EDL and 140 Hz for soleus) every sec for 3 min. Experimental temperature was 37°C. Tetanic force is the maximum force developed during a 200-msec tetanus and is expressed as a percent of the tetanic force measured prior to fatigue (Time 0 sec). Data from contralateral innervated muscles excised from 1- and 2- week denervated animals were not significantly different and were therefore pooled together for clarity. Symbols: ◆, innervated; O, 1-week denervated, ∇, 2-week denervated. Vertical error bars represent the S.E.M. of 10 innervated or 5 denervated muscles (absent when smaller than symbols). * Mean tetanic force following 1- and 2-week denervation was significantly different from mean tetanic force in innervated muscle during the same time period; ANOVA, L.S.D., $P < 0.05$.

tetanic force of both muscles decreased to about 30% of the initial force. Even though, 1- and 2-week denervation showed the same decrease in tetanic force during the first 60 sec of stimulation, the 2-week denervated soleus had a significant lesser extent of fatigue after 180 sec of stimulation.

Kinetics of force recovery

Both denervated EDL and soleus muscles showed greater initial rate as well as extent of force recovery, when compared to their respective innervated controls (Fig. 3-2). At the end of the recovery period, innervated EDL recovered to 46% of the pre-fatigue force, whereas the force recovery of 1-week denervated EDL was 68% (Fig. 3-2A). Although the longer denervation period of 2 weeks showed a tendency for further force recovery, the differences between 1- and 2-week denervation was not significant. The effect of denervation was similar in soleus muscle, except that the differences between innervated and denervated muscles were smaller in soleus and both 1- and 2-week denervated muscle recovered to the same extent (Fig. 3-2B).

Overall, there were few differences between the effects of 1- and 2-week denervation on muscle weight, kinetics of fatigue and recovery. Thus, the remainder of the experiments were conducted using 1-week denervated mice.

EFFECTS OF 1-WEEK DENERVATION ON THE $K^+_{(ATP)}$ CHANNEL

Kir6.2 mRNA content

Kir6.2 is the protein subunit that forms the pore of the $K^+_{(ATP)}$ channel (12). The ethidium bromide- stained agarose gel did not show any apparent difference in Kir6.2 mRNA content

FIGURE 3-2

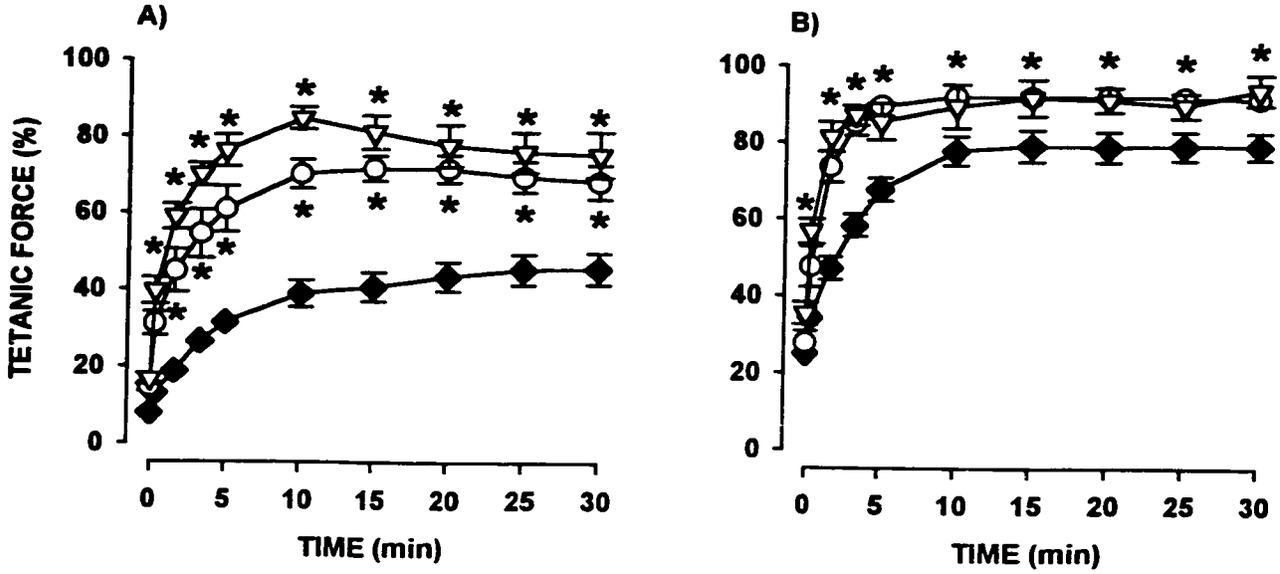

Figure 3-2. Effects of 1- and 2-week denervation on tetanic force recovery of A) EDL and B) soleus muscles. Fatigue was induced with one 200 msec long tetanic contraction (200 Hz for EDL and 140 Hz for soleus) every sec for 3 min. Experimental temperature was 37°C. Tetanic force is expressed as a percent of the tetanic force measured prior to fatigue. Data from contralateral innervated muscles excised from 1- and 2- week denervated animals were not significantly different and were therefore pooled together for clarity. Symbols: ♦, innervated; ○, 1-week denervated, ▽, 2-week denervated. Vertical error bars represent the S.E.M. of 10 innervated or 5 denervated muscles (absent when smaller than symbols). * Mean tetanic force following 1- and 2-week denervation was significantly different from mean tetanic force in innervated muscle during the same time period; ANOVA, L.S.D., $P < 0.05$.

between innervated and denervated EDL muscle (Fig. 3-3). Denervated soleus, on the other hand, showed a decreased mRNA content. Quantification of Kir6.2 mRNA on a VistaGreen-stained agarose gel showed that the content in denervated soleus was 2.7-fold less than that of innervated soleus, while the ratio of control to denervated EDL was 1.07.

Effects of glibenclamide and pinacidil during fatigue

Denervated muscles for each of the 3 experimental conditions (i.e., non-treated denervated, pinacidil- and glibenclamide-exposed muscles) were excised from three different groups of mice. For all measurements (tetanic force, resting tension, $^{86}Rb^{+}$ fractional loss, ATP and PCr contents), the contralateral innervated muscles were never exposed to either pinacidil or glibenclamide and were used as control. Data from the three groups of innervated EDL and soleus muscles were then compared to ensure that there was no difference among the different groups of mice. This was indeed the case for all measurements (data not shown), except for the $^{86}Rb^{+}$ fractional loss in EDL (which will be further described later).

During the 30 min equilibration prior to fatigue, neither 10 μM glibenclamide nor 100 μM pinacidil had an effect on tetanic force (data not shown). Glibenclamide did not affect the fatigue kinetics of denervated soleus (Fig. 3-4A). Pinacidil, on the other hand, caused significantly faster drop in force during the first 60 sec of stimulation. The decrease was the largest at 30 sec when the difference in tetanic force between non-treated and pinacidil-exposed muscles was 17%. The extent of fatigue was not however altered by pinacidil, as the decrease in force throughout the remainder of the fatigue stimulation was similar between the two conditions.

FIGURE 3-3

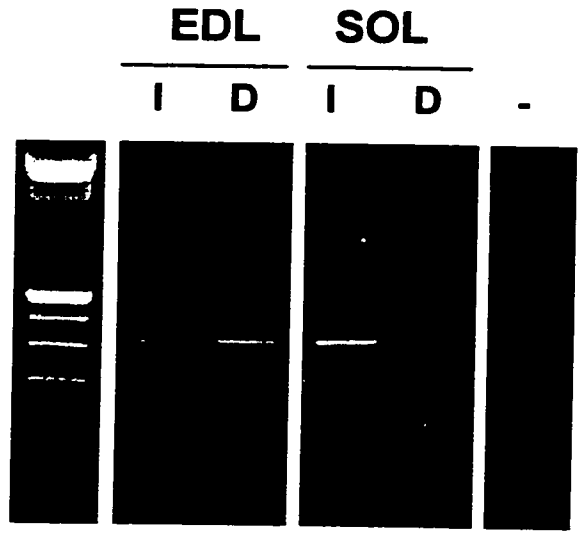

Figure 3-3. Effect of 1-week denervation on Kir6.2 mRNA content of EDL and soleus muscles. RT-PCR products were ran on 1.5% agarose gel and visualized with ethidium bromide. The bands represent the pooled sample of 10 denervated (D) and 10 innervated (I) EDL and soleus muscles. Negative control (-) was carried out using RNase free water.

FIGURE 3-4

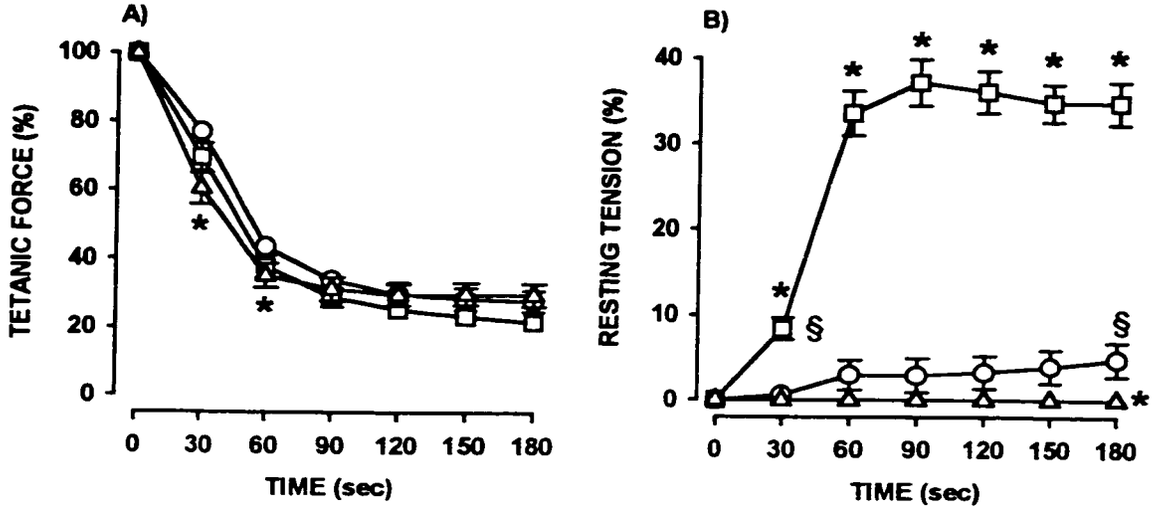

Figure 3-4. Effects of glibenclamide and pinacidil on A) tetanic force and B) resting tension during fatigue of 1-week denervated soleus muscle. Fatigue was induced with one 200 msec long tetanic contraction (140 Hz for soleus) every sec for 3 min. Experimental temperature was 37°C. Glibenclamide or pinacidil was added 30 min prior to fatigue and remained throughout the experiment. Tetanic force is expressed as a percent of the tetanic force measured prior to fatigue (Time 0 sec). Resting tension is defined as the tension measured 5 msec prior to a tetanic stimulation, and is expressed as a percent of the tetanic force measured prior to fatigue. Symbols: O, non-treated; □, 10 μM glibenclamide; Δ, 100 μM pinacidil. Note the difference in the scale between A and B. Vertical error bars represent the S.E.M. of 5 muscles (absent when smaller than symbols). * Mean tetanic force or resting tension in the presence of glibenclamide or pinacidil was significantly different from values of 1-week denervated muscle during the same time period; ANOVA, L.S.D., $P < 0.05$. § Indicate the time when mean resting tension became significantly different from zero; ANOVA, L.S.D., $P < 0.05$.

Denervated soleus muscle had a 4.8% increase in resting tension at the end of the fatigue stimulation (Fig. 3-4B). This increase became significantly greater in the presence of glibenclamide after 30 sec of stimulation. After 60 sec of stimulation, the glibenclamide-exposed muscle had a resting tension of 35% that was maintained throughout the fatigue stimulation. None of the soleus muscles exposed to 100 μM pinacidil showed an increase in resting tension.

Considering the large effect of glibenclamide on resting tension, we determined the effect of glibenclamide and pinacidil on total force. The latter was calculated as the sum of the force present in the absence of stimulation (resting tension) and the force developed during a contraction (tetanic force). When compared to non-treated denervated soleus muscle, total force became significantly greater in the presence of glibenclamide after 60 sec of stimulation. Pinacidil significantly decreased total force, but only during the early part of fatigue (30-60 sec, Fig. 3-5B).

Most of the glibenclamide and pinacidil effects during fatigue in denervated EDL muscle were similar to those observed for the soleus. That is, pinacidil caused faster decrease in tetanic force during the first 60 sec of stimulation, whereas glibenclamide had no effect (data not shown). Pinacidil also abolished the increase in resting tension during fatigue, while resting tension increased to 10.8 $\pm$ 1.1% (n=5) of pre-fatigue tetanic force in the presence of glibenclamide compared to 4.3 $\pm$ 1.2% (n=5) in non-treated denervated EDL. There was, however, one major difference between denervated EDL and soleus where glibenclamide did not affect the decrease in total force in EDL (Fig. 3-5A).

FIGURE 3-5

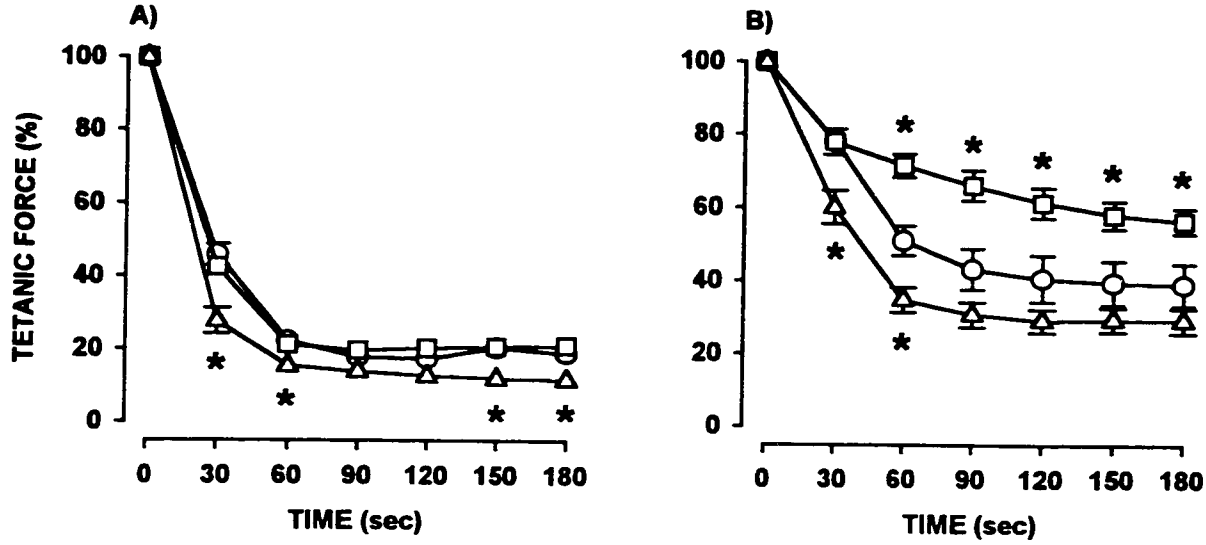

Figure 3-5. Effects of glibenclamide and pinacidil on total force during fatigue of 1-week denervated A) EDL and B) soleus muscles. Fatigue was induced with one 200 msec long tetanic contraction (200 Hz for EDL and 140 Hz for soleus) every sec for 3 min. Experimental temperature was 37°C. Glibenclamide or pinacidil was added 30 min prior to fatigue and remained throughout the experiment. Total force was calculated by adding the amount of force present in the absence of stimulation (resting tension) and the force developed during a contraction (tetanic force). It is expressed as a percent of the total force measured prior to fatigue (Time 0 sec). Symbols: O, non-treated; □, 10 μM glibenclamide; Δ, 100 μM pinacidil. Vertical error bars represent the S.E.M. of 5 muscles (absent when smaller than symbols). * Mean total force in the presence of glibenclamide or pinacidil was significantly different from mean total force of 1-week denervated muscle during the same time period; ANOVA, L.S.D., $P < 0.05$.

Effects of glibenclamide and pinacidil on force recovery

In denervated EDL, 10 μM glibenclamide significantly reduced the extent of force recovery. Denervated EDL muscles recovered 68% of pre-fatigue tetanic force after 30 min, whereas in the presence of glibenclamide the muscles only recovered to 44% (Fig. 3-6A). Glibenclamide had a similar effect on denervated soleus muscle, but the decrease in force recovery was less than in EDL (Fig. 3-6B). Furthermore, the difference was only significant between the 3rd and 10th min of recovery.

Pinacidil had similar effect on denervated EDL and soleus muscle where it slightly improved the extent of tetanic force recovery when compared to non-treated muscle (Fig. 3-6). However, this increase was significant between the 1st and 5th min of recovery in soleus, which showed a maximal (100%) recovery (Fig. 3-6B).

ATP and PCr contents

In EDL muscle, both rest-state ATP and PCr contents were significantly lower following 1-week denervation (Fig. 3-7A, B). ATP content decreased by 25% and PCr content dropped by 32%. Fatigue caused significant decreases in both ATP and PCr levels of innervated and denervated EDL. The decreases were, however, smaller in denervated muscle. Following fatigue, innervated EDL had a drop of 22 μmole/g dry weight of ATP compared to 14 μmole/g dry weight for the denervated EDL. PCr levels dropped by 73 μmole/g dry weight in innervated EDL compared to 49 μmole/g dry weight in denervated muscle.

FIGURE 3-6

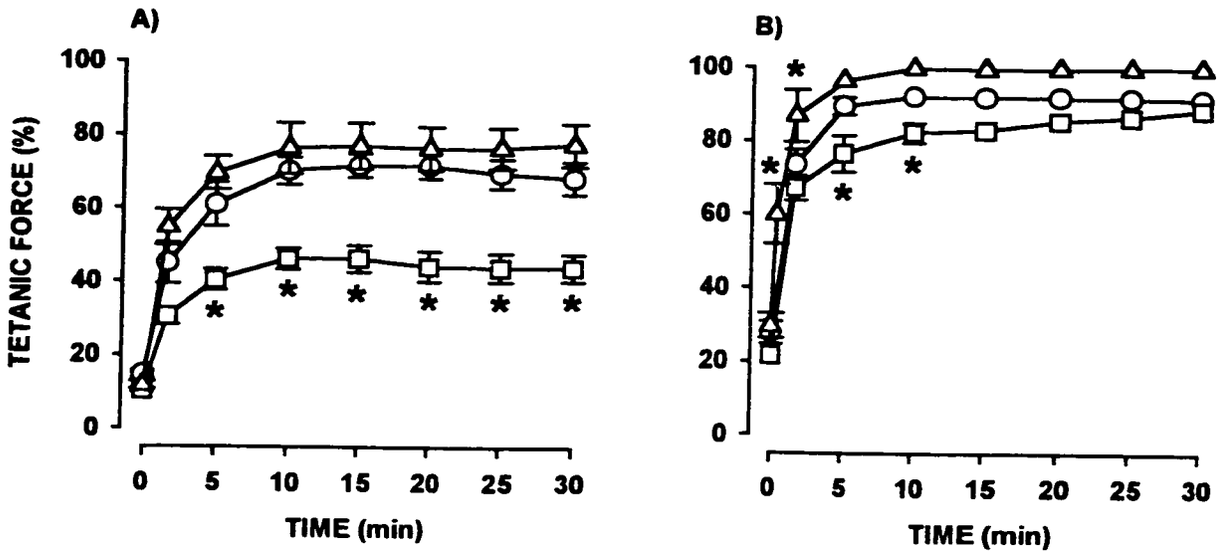

Figure 3-6. Effects of glibenclamide and pinacidil on tetanic force recovery of 1-week denervated A) EDL and B) soleus muscles. Fatigue was induced with one 200 msec long tetanic contraction (200 Hz for EDL and 140 Hz for soleus) every sec for 3 min. Experimental temperature was 37°C. Glibenclamide or pinacidil was added 30 min prior to fatigue and remained present during recovery. Tetanic force is expressed as a percent of the tetanic force measured prior to fatigue. Symbols: ○, non-treated; □, 10 μM glibenclamide; Δ, 100 μM pinacidil. Vertical error bars represent the S.E.M. of 5 muscles (absent when smaller than symbols). * Mean tetanic force in the presence of glibenclamide or pinacidil was significantly different from mean tetanic force of control 1-week denervated muscle during the same time period; ANOVA, L.S.D., $P < 0.05$.

FIGURE 3-7

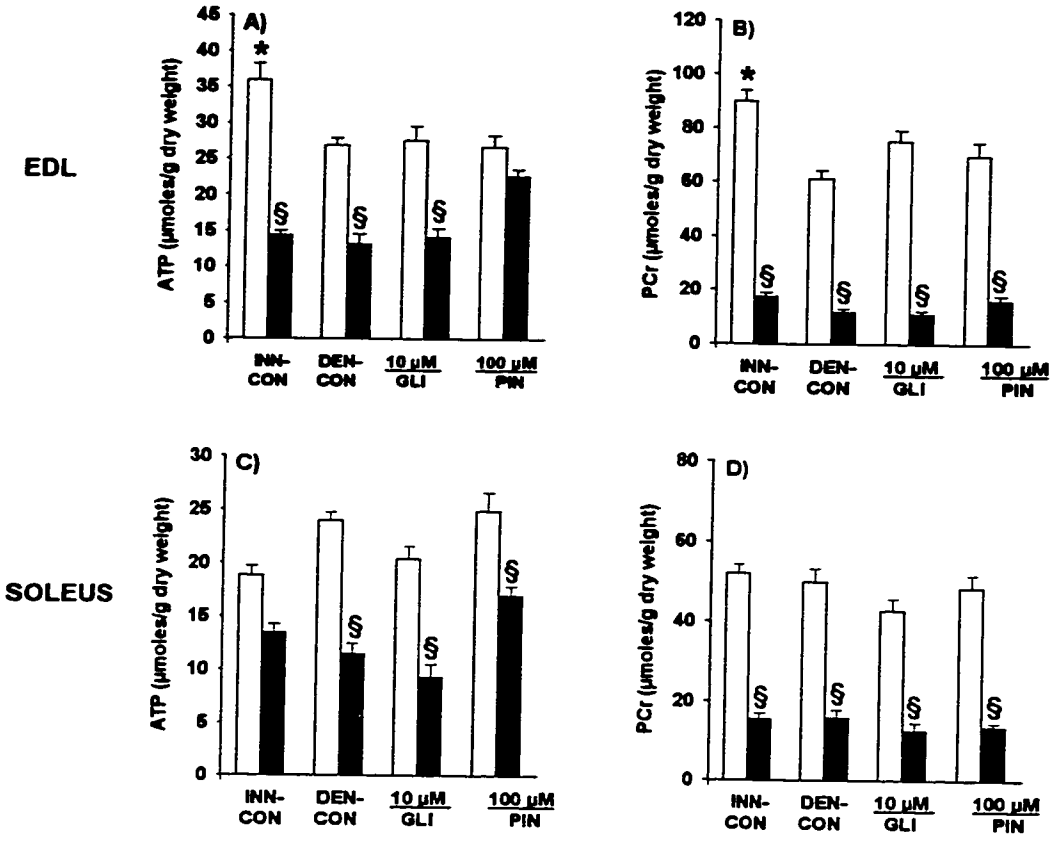

Figure 3-7. The effects of glibenclamide and pinacidil on A,C) ATP and B,D) PCr content of 1-week denervated A,B) EDL and C,D) soleus muscles, at 37°C. Muscles were incubated for 30 min in the absence or presence of glibenclamide or pinacidil. Fatigue was induced with one 200 msec long tetanic contraction (200 Hz for EDL and 140 Hz for soleus) every sec for 3 min. Symbols: □, ATP and PCr content in muscle at rest; ■, ATP and PCr content after fatigue. Note the difference in the scales. Vertical error bars represent the S.E.M. of 8-10 muscles. * Mean ATP or PCr contents at rest were significantly different from the rest values of 1-week denervated muscles; ANOVA and L.S.D., $P < 0.05$. § Mean ATP or PCr content was significantly less after fatigue; ANOVA and L.S.D., $P < 0.05$.

Contrary to EDL, denervated soleus muscle showed higher rest-state ATP content than innervated soleus (Fig. 3-7C). However the difference was not significant. The decrease in ATP during fatigue was significant in denervated soleus, being 13 μmole/g dry weight, but not in innervated soleus where the decrease was 6 μmole/g dry weight. Both rest-state and after fatigue PCr values were the same between innervated and denervated soleus (Fig. 3-7D).

In both denervated EDL and soleus muscles, the presence of glibenclamide or pinacidil did not affect the rest-state ATP and PCr values (Fig. 3-7). Glibenclamide did not affect metabolite decreases during fatigue. On the other hand, pinacidil had one substantial effect: ATP content of non-treated denervated EDL muscle dropped significantly by 14 μmole/g dry weight during fatigue, which was much greater than the 4 μmole/g dry weight decrease observed in the pinacidil-exposed EDL.

$^{86}Rb^{+}$ fractional loss

Basal $^{86}Rb^{+}$ fractional losses of innervated and denervated EDL muscles were similar as the values were respectively 0.0092 $\pm$ 0.0007/min and 0.0091 $\pm$ 0.0006/min. Basal $^{86}Rb^{+}$ fractional losses for soleus muscles were similar to those of EDL (data not shown). In EDL, $^{86}Rb^{+}$ fractional loss increased significantly during fatigue, but there were no differences between innervated and denervated muscles (Fig. 3-8A). Denervated soleus muscle, on the other hand, showed significantly greater $^{86}Rb^{+}$ fractional loss than innervated soleus (Fig. 3-8B). During the first min of fatigue, fractional losses of innervated and denervated soleus were respectively 0.0155/min and

FIGURE 3-8

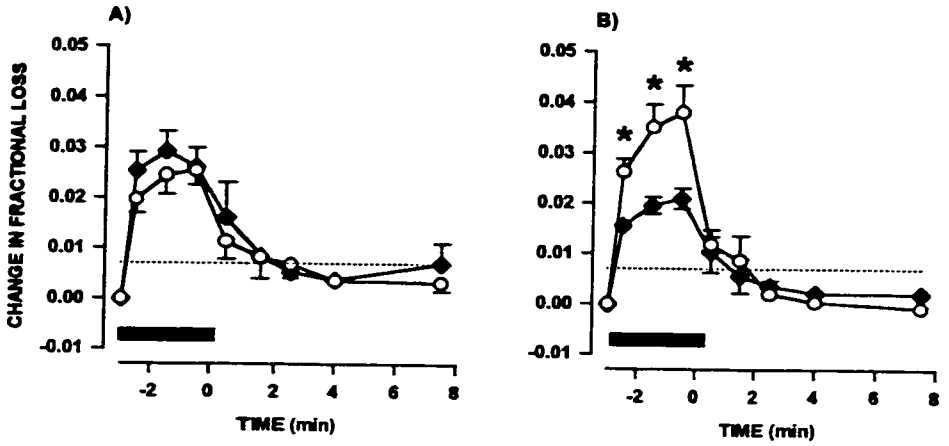

Figure 3-8. Effects of 1-week denervation on $^{86}Rb^{+}$ fractional loss from A) EDL and B) soleus muscles. Muscles were loaded one hour with 4-8 μCi $^{86}Rb^{+}$. After 4 initial washouts, the $^{86}Rb^{+}$ fractional loss was measured at rest from 3 successive periods of 5 min. Muscles were then fatigued (at time –3 min). Washout periods were one min long during fatigue and during the first 3 min of recovery; the last two washouts were 2 and 5 min long. Experimental temperature was 37°C. Data are given as the change in fractional losses measured during fatigue and recovery (basal $^{86}Rb^{+}$ fractional losses measured before fatigue are given in the text). Symbols are plotted in the middle of the time period when washouts were taken. Symbols: ◆, innervated; O, 1-week denervated; ■■, fatigue period. Vertical error bars represent the S.E.M. of 7-8 muscles (absent when smaller than symbols). * Mean $^{86}Rb^{+}$ fractional loss of denervated muscle was significantly greater than in innervated muscle at the same time period; ANOVA and L.S.D., $P < 0.05$. .... Indicates the level of $^{86}Rb^{+}$ fractional loss that was significantly greater than zero; ANOVA and L.S.D., $P < 0.05$.

0.0263/min, a difference of 0.0108/min. This difference increased to 0.0156 and 0.0170/min during the 2nd and 3rd min of fatigue respectively.

The mean $^{86}Rb^{+}$ fractional loss of the three groups of contralateral innervated EDL muscles that were used as control for the three experimental conditions (i.e. non-treated denervated, glibenclamide- and pinacidil-exposed muscles) were slightly but not significantly different (data not shown). This prevented us from directly comparing $^{86}Rb^{+}$ fractional losses between the three conditions. Instead, we calculated the differences in $^{86}Rb^{+}$ fractional losses between denervated and innervated muscles. This method allowed us to correct for any discrepancies existing amongst the innervated controls. Although the problem was not observed with innervated soleus muscles, the data are presented the same way.

Glibenclamide did not affect the difference in $^{86}Rb^{+}$ fractional loss between denervated and innervated EDL and soleus muscles (Fig. 3-9). However, pinacidil had a significantly increased $^{86}Rb^{+}$ fractional loss on both muscles. For example, in soleus muscle the increase in $^{86}Rb^{+}$ fractional loss caused by denervation was doubled in the presence of pinacidil.

FIGURE 3-9

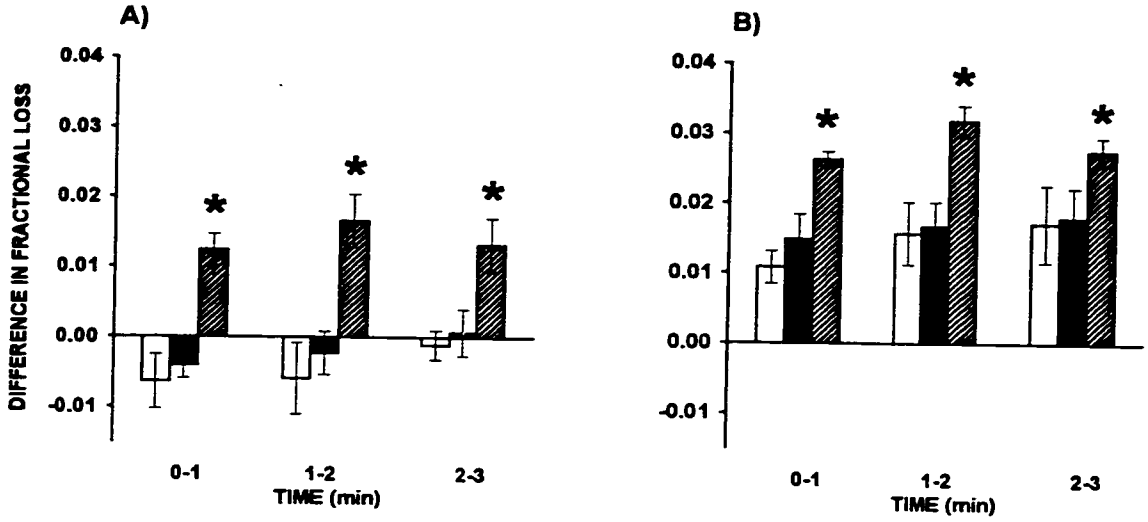

Figure 3-9. Effects of glibenclamide and pinacidil on $^{86}Rb^{+}$ fractional loss from 1-week denervated A) EDL and B) soleus muscles. Experimental protocols were as described in Figure 3-8. Data are expressed as a difference in $^{86}Rb^{+}$ fractional loss between denervated muscles (non-treated, glibenclamide and pinacidil) and their respective contralateral innervated muscles. A positive value means that the $^{86}Rb^{+}$ fractional loss was greater in the denervated than innervated muscles. Symbols: □, non-treated; ■, 10 μM glibenclamide; □, 100 μM pinacidil. Vertical error bars represent the S.E.M. of 7-8 muscles. * Mean difference in $^{86}Rb^{+}$ fractional loss in the presence of glibenclamide or pinacidil was significantly different from the difference in non-treated denervated muscle; ANOVA and L.S.D., $P < 0.05$.

DISCUSSION

In this paper, we used denervation as a model to abolish muscle activity and/or neurotrophic factors in order to study their effects on the $K^+_{(ATP)}$ channel's expression and physiological role. RT-PCR measurements showed a decreased Kir6.2 mRNA content for the soleus, but an unchanged content for the EDL following denervation. The channel's mRNA expression was not the only factor changed by denervation, as a comparison between denervated muscles (this study) and muscles excised from non-operated animals (17) showed that denervation also changed the effect of glibenclamide, a $K^+_{(ATP)}$ channel blocker, and pinacidil, a $K^+_{(ATP)}$ channel opener, on EDL and soleus muscles.

EFFECTS OF DENERVATION

Denervation itself had significant effects on the kinetics of fatigue and recovery, ATP and PCr content, as well as $^{86}Rb^+$ fractional loss. Both 1-and 2-week denervated EDL muscles had slower decrease in tetanic force during fatigue, whereas the reverse was observed in denervated soleus muscle (Fig. 3-1). These results are in agreement with those of Webster & Bressler (32). This study also shows that denervation enhances the recovery of tetanic force following fatigue (Fig. 3-2). Such an observation is interesting considering the significant muscle weight loss that is associated with denervation.

One week denervation also affected ATP and PCr contents, but the effects differed between EDL and soleus muscles. In EDL, the metabolite contents decreased following denervation, while denervated soleus muscle showed a slight increase in ATP content and an unchanged PCr content

(Fig. 3-7). Furthermore, the decreases in ATP and PCr caused by fatigue were smaller in denervated EDL compared to innervated EDL, whereas the decreases in ATP were greater in denervated soleus compared to innervated soleus. The latter two observations correlate with force measurements. That is, the decrease in tetanic force, ATP and PCr contents were all lesser in denervated EDL, while they were greater in innervated soleus.

K^+ is another factor that contributes to the onset of fatigue when its concentration increases in the interstitial fluid as a result of a net increase in K^+ efflux (26). In this study, $^{86}Rb^+$ fractional loss was measured to estimate K^+ efflux as $^{86}Rb^+$ is a qualitatively good marker for K^+ movement across cell membrane (5;10). One week denervation did not change $^{86}Rb^+$ fractional loss during fatigue in EDL, while it significantly increased it in soleus (Fig. 3-8). So, even though Kotsias & Venosa (14) have previously shown that the K^+ permeability at rest decreases following denervation, our results indicate that the latter does not hold during fatigue.

EFFECTS OF MODULATING $K^+_{(ATP)}$ CHANNEL ACTIVITY IN DENERVATED MUSCLE

The postulated function of $K^+_{(ATP)}$ channel is to reduce force development when energy levels decrease in order to preserve energy and protect muscle function. To test this function, Matar *et al.* (17) studied the effects of pinacidil in EDL and soleus muscles excised from non-operated mice (referred to as normal muscle). We showed that 100 μM pinacidil was effective in activating $K^+_{(ATP)}$ channels as it increased $^{86}Rb^+$ fractional loss during fatigue. We also showed that pinacidil-induced activation of the $K^+_{(ATP)}$ channel i) causes faster decrease in tetanic force, ii) preserves energy as ATP depletion during fatigue was smaller than in control muscle, and iii)

protects muscle function as the increase in resting tension during fatigue was abolished and force recovery improved. These pinacidil effects on $^{86}Rb^{+}$ fractional loss (Fig. 3-9), tetanic force (Fig. 3-4A), resting tension (Fig. 3-4B), force recovery (Fig. 3-6) and ATP (Fig. 3-7) were all observed in denervated EDL and soleus muscles. This therefore suggests that $K^{+}_{(ATP)}$ channel has the same function in denervated and normal muscles.

Based on these results, blocking $K^{+}_{(ATP)}$ channels should cause slower decrease in tetanic force, greater increase in resting tension during fatigue, and a smaller and/or slower force recovery. Indeed, 10 μM glibenclamide had those effects in denervated muscle on resting tension and force recovery, but not on the decrease in tetanic force during fatigue. The lack of a glibenclamide effect on tetanic force during fatigue is consistent with other studies (6;33).

This study showed for the first time that glibenclamide increases the amount of force during fatigue as expected. This was observed when the force generated during a contraction (tetanic force) was added to the force present between contractions (resting tension) to give what we defined as total force. It was then observed that glibenclamide caused slower decrease in total force when compared to non-treated muscles. This glibenclamide effect was mainly due to the fact that in denervated soleus muscle glibenclamide caused a large increase in resting tension, which at the end of the fatigue period was equivalent to 37% of the pre-fatigue tetanic force (Fig. 3-4B).

Contrary to soleus muscle, glibenclamide did not cause slower decrease in total force in denervated EDL (Fig. 3-5A). One explanation for this result is that in the presence of glibenclamide denervated EDL developed much less resting tension (10.8%) than denervated soleus

(37.0%). Another explanation is that blocking $K^+_{(ATP)}$ channel impairs muscle function (15). Indeed, the presence of a $K^+_{(ATP)}$ channel blocker impaired force recovery by 24% when compared to non-treated denervated EDL (Fig. 3-6A). This function impairment may have then prevented denervated EDL muscle to develop greater total force.

In denervated EDL and soleus muscles, glibenclamide did not affect the rest-state and after fatigue ATP and PCr levels (Fig. 3-7A, B). The latter suggests that the glibenclamide effects on resting tension and force recovery are not due to greater depletions of these metabolites. Glibenclamide also failed to reduce $^{86}Rb^+$ fractional loss in both denervated EDL and soleus muscle (Fig. 3-9). A decrease in $^{86}Rb^+$ fractional loss was expected if the glibenclamide effect on total force and resting tension during fatigue were due to a blocking of $K^+_{(ATP)}$ channel. However, this is not the first time that glibenclamide at 10 μM fails to affect either K^+ or Rb^+ loss under conditions known to activated $K^+_{(ATP)}$ channels. In cardiac muscle, 10 μM glibenclamide prevented the shortening of action potential that occurs during hypoxia, while it had no effect on K^+ efflux unless its concentration was increased to 100 μM (30). Furthermore, we have now observed during fatigue under hypoxic conditions larger increase in resting tension in muscles excised from $K^+_{(ATP)}$ channel-deficient muscles (Kir6.2 knockout mouse) than in muscles from wild type mice, whereas $^{86}Rb^+$ fractional losses were the same for both muscle groups (unpublished results). Shivkumar *et al.* (25) has recently given evidence that the K^+ or Rb^+ efflux during hypoxia depends primarily on an intracellular Na^+ accumulation, and that blocking $K^+_{(ATP)}$ channels was not sufficient in reducing K^+ or Rb^+ efflux under those conditions. It would thus appear that the same occurs in denervated muscle. We therefore suggest that the glibenclamide effect on force in

denervated muscles is not be due to a non-specific effect, but rather due to an effect of glibenclamide on $K^+_{(ATP)}$ channel.

THE IMPORTANCE OF $K^+_{(ATP)}$ CHANNEL IN NORMAL AND DENERVATED MUSCLES

In the Introduction we hypothesized that denervation reduces the expression and physiological effect of $K^+_{(ATP)}$ channel. The Kir6.2 mRNA content was 2.7-fold less in denervated than normal soleus, whereas the EDL mRNA content remained unchanged (Fig. 3-3). This is not the first time that denervation has different effects on the expression of a K^+ channel in different muscles. For example, denervation also reduces the Kv3.4 mRNA content in mouse EDL while it increased it in soleus (31). Thus, the abolishment of neurotrophic factors and/or muscle activity via denervation can decrease Kir6.2 mRNA content as initially hypothesized (see Introduction), but only in soleus and not in EDL muscles, suggesting a muscle specific effect.

To determine if denervation also reduces the physiological importance of $K^+_{(ATP)}$ channel, we compared the effects of glibenclamide and pinacidil in denervated muscle (this study) to those measured in normal muscle from the study of Matar *et al.* (17). Figure 3-10 shows the effects on the contractile parameters. Most of the pinacidil effects were greater in denervated than normal muscles (Fig. 3-10A). The differences were especially noticeable for the tetanic force in soleus and total force in EDL. In both cases, pinacidil had no effect in normal muscle, while it augmented the decrease in tetanic force by 17% in denervated soleus and by 7% for the total force in EDL.

FIGURE 3-10

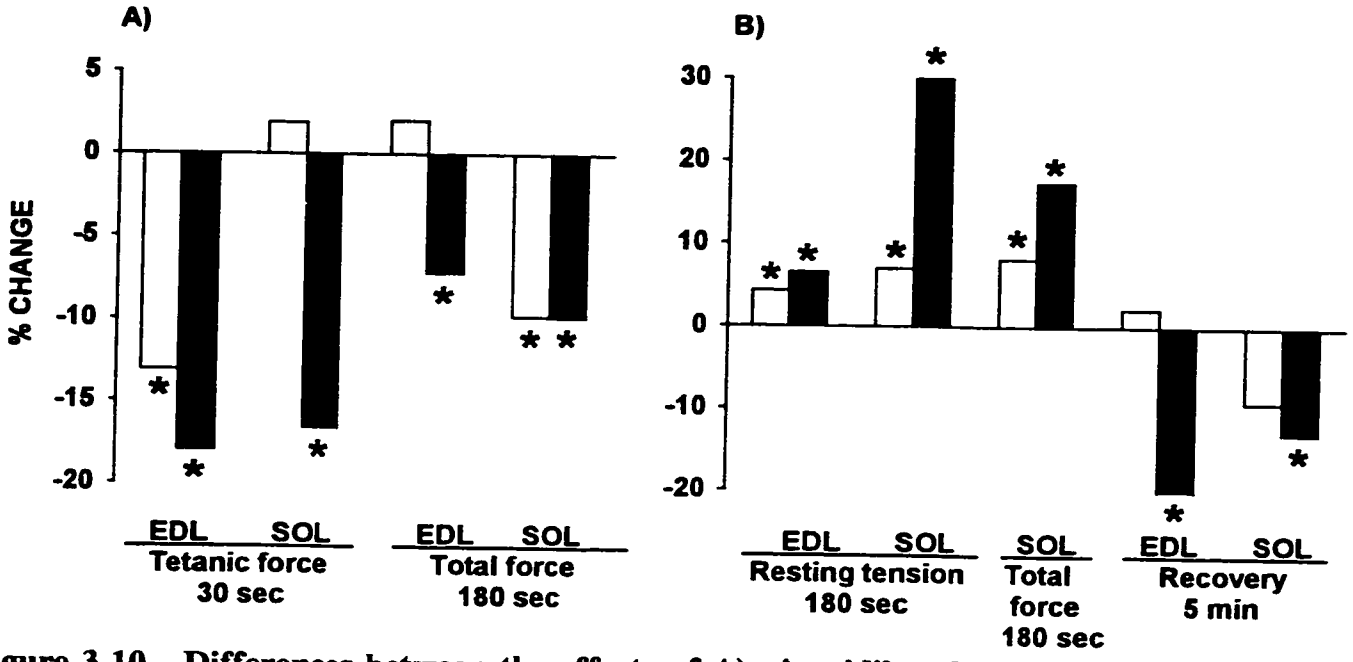

Figure 3-10. Differences between the effects of A) pinacidil and B) glibenclamide on tetanic force, resting tension, total force and recovery of normal and 1-week denervated EDL and soleus muscles. Normal muscle data is obtained from Matar *et al.* (17). Denervated muscle data is obtained from Fig. 3-4, 3-5 and 3-6. Data is presented as the difference between the value obtained from the drug treated muscle and that of the non-treated muscle. The data selection for this figure was based on two criteria. First, data were used only when pinacidil or glibenclamide had a significant effect in either normal or denervated muscle. Second, data were excluded if the mean values were 0% or 100% as the differences can not give a true index of the drug's effect. Symbols: □, normal muscle; ■, 1-week denervated muscle. Note the difference in the scale between A and B. * Effect was significantly different when compared to its proper control; ANOVA, L.S.D., $P < 0.05$.

We have previously reported that glibenclamide had no effect on the rate of fatigue and force recovery, while it caused significant increase in resting tension of normal muscle (17). The situation is quite different in denervated muscles as glibenclamide had several effects (Fig. 3-10B). The increase in resting tension caused by glibenclamide was much greater in denervated muscle, especially in soleus where the difference was 4.3-fold. Force was also influenced as total force was 17.4% greater in the presence of glibenclamide in denervated soleus and only 8.1% in normal soleus. Finally, glibenclamide significantly impaired force recovery in both denervated EDL and soleus, but not in normal muscles.

While glibenclamide failed to affect $^{86}Rb^+$ fractional loss, pinacidil was able to increase it in both normal and denervated EDL and soleus muscles. Again, as for the force measurements, the pinacidil effect is greater in denervated muscle. In normal soleus, pinacidil increased $^{86}Rb^+$ fractional loss by 0.0050/min and 0.0061 during the 1st and 2nd min of fatigue respectively. The effect of pinacidil was respectively 3.4 and 2.6-fold higher in denervated soleus. The same effect was noted with the EDL. For example, $^{86}Rb^+$ fractional loss was increased by 0.0043/min during the 1st min of fatigue of normal muscle, compared to 0.0160 in denervated soleus; an increase of 3.7-fold. These results suggest that pinacidil is more effective in activating $K^+_{(ATP)}$ channels in denervated than normal muscles.

In conclusion, our results show that most of the effects of pinacidil, a $K^+_{(ATP)}$ channel opener, and glibenclamide, a $K^+_{(ATP)}$ channel blocker, on tetanic force, and $^{86}Rb^+$ fractional loss during fatigue and recovery are greater in denervated than innervated EDL and soleus muscles. This suggests, that on a relative scale, $K^+_{(ATP)}$ channels become more important following

denervation even though the mRNA content of Kir6.2, the protein that forms the channel, is not affected in EDL and decreases in soleus.

ACKNOWLEDGMENTS

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CHAPTER 4: GENERAL DISCUSSION

The $K^+_{(ATP)}$ channel is activated when the ATP concentration decreases or the ADP concentration increases. It is therefore a channel that can be activated during any metabolic stress that depletes cell energy. Indeed, there are many studies that have previously reported an activation of the $K^+_{(ATP)}$ channel during ischemia, hypoxia, anoxia and metabolic poisoning (Castle & Haylett, 1987; Gasser & Vaughan-Jones, 1990; Deutsch *et al.*, 1991; McCullough *et al.*, 1991; Benndorf *et al.*, 1992; McPherson *et al.*, 1993; van Winkle *et al.*, 1994; Decking *et al.*, 1995; Gramolini & Renaud, 1997). Under these conditions, it has been shown that the $K^+_{(ATP)}$ channel causes faster decrease in force and has a cytoprotective effect as it protects muscle against deleterious energy depletion and irreversible function impairment.

The above-mentioned conditions, that were shown to activate the channel, represent extreme metabolic stresses that are not necessarily physiologically relevant. Muscle fatigue is a well-characterized physiological phenomenon and is defined as the decrease in force generating capacity when a muscle is repeatedly stimulated. Fatigue is a multifactorial event that can involve different sites within the muscle (Vollestad & Sejersted, 1988; Fitts, 1994). The most important sites include: 1) propagation of action potential down the t-tubule, 2) Ca^{2+} release by the sarcoplasmic reticulum, 3) Ca^{2+} activation of the contractile apparatus, 4) cross-bridge cycle, and 5) the supply of high energy phosphates. It is now well established that the decline in force that characterizes fatigue arises, in large part, from a failure of the sarcoplasmic reticulum to release Ca^{2+}, leading to a sub-maximal myoplasmic free Ca^{2+} concentration during contractions (Allen *et al.*, 1989; Lee *et*

al., 1991; Baker *et al.*, 1993). The cause of the decreased Ca^{2+} release is not to date fully understood, but one possibility is the reduction of the amplitude and duration of action potentials in t-tubules (Westerblad *et al.*, 1991). The latter can be caused by an increased extracellular K^+ concentration, which depolarizes cell membrane leading to an inactivation of Na^+ channels. It is at this site that $K^+_{(ATP)}$ channels are expected to contribute to fatigue because they can allow for an increase in extracellular K^+ concentration and they directly affect the action potential repolarization phase. The latter will decrease force production and hence conserve energy, giving therefore to the $K^+_{(ATP)}$ channel a cytoprotective role. In this study, three objectives were pursued: 1) to verify if the channel can perform its cytoprotective role during fatigue elicited by intermittent tetanic stimulation by testing the effects of the channel opener pinacidil; 2) to test if the channel is activated by the same fatigue protocol by using glibenclamide to block the channel; and 3) to study the effects of the abolishment of muscle activity (via denervation) on the channel's expression and physiological importance.

CAN THE $K^+_{(ATP)}$ CHANNEL PERFORM ITS POSTULATED MECHANISM?

One effect of pinacidil was observed at the level of force development. In both normal and denervated muscles, the rate of fatigue was faster in the presence of pinacidil when compared to control non-treated muscle. Pinacidil, however, did not affect initial tetanic force before fatigue or the extent of fatigue. This is in agreement with two other studies where $K^+_{(ATP)}$ channel openers do not affect twitch force and Ca^{2+} release in unfatigued muscle fibers (Weselcouch *et al.*, 1993; Burton & Smith, 1997). This suggests that the $K^+_{(ATP)}$ channel does not constitute a primary mechanism of force decrease, but rather a modulatory mechanism which can increase the rate of fatigue when energy levels are too low.

This study also suggests that contrary to the situation in cardiac muscle, the main mechanism of action of the $K^+_{(ATP)}$ channel in skeletal muscle is an increase in K^+ efflux, and not a shortening of the action potential. Firstly, pinacidil only causes a small 8.8 mV decrease in the overshoot and a slight prolongation of the action potential duration in unfatigued muscle. Such changes are not expected to affect Ca^{2+} release in muscle, a fact that has now been confirmed by Burton & Smith (1997). Secondly, pinacidil caused a large increase in $^{86}Rb^+$ fractional loss, which was used as a marker for K^+ movement across the cell membrane. Thus, the increase in $^{86}Rb^+$ fractional loss indicates that an activation of the $K^+_{(ATP)}$ channel increases K^+ efflux. The higher K^+ efflux increases extracellular K^+ concentration, which further depolarizes cell membrane leading to a decreased membrane excitability, Ca^{2+} release and force development.

Pinacidil also had two other important effects as it abolished resting tension development during fatigue and improved force recovery following fatigue. These two effects are in agreement with the notion that the $K^+_{(ATP)}$ channel is cytoprotective. This is especially important in regard to the abolishment of resting tension. Resting intracellular Ca^{2+} levels are higher in fatigued compared to unfatigued muscle fibers (Lee *et al.*, 1991). It is therefore possible that the increase in resting tension is due to an increase in intracellular Ca^{2+} levels. Then, an abolishment of resting tension in the presence of pinacidil suggests that an activation of the $K^+_{(ATP)}$ channel helps in reducing Ca^{2+} levels between contractions, and hence the deleterious effects associated with Ca^{2+} (Leonard & Salpeter, 1979; Neerunjun & Dubowitz, 1979). Thus, one mechanism explaining the improvement of force recovery in the presence of pinacidil might involve lower intracellular Ca^{2+} levels between contractions.

It is interesting to note that despite the fact that pinacidil always abolished resting tension in both EDL and soleus muscles, it did not always reduce the ATP depletion during fatigue. Furthermore, no correlation between ATP preservation during fatigue and improvement of force recovery was ever established in the presence of pinacidil. For example, in denervated EDL muscle, pinacidil caused smaller ATP depletion, but did not improve force recovery. Conversely, in normal EDL muscle, pinacidil did not affect ATP depletion, but significantly improved force recovery. Thus, this study suggests that a preservation of ATP is not an important mechanism by which pinacidil improves force recovery.

Overall, the effects of pinacidil confirm the cytoprotective role of the $K^+_{(ATP)}$ channel where they can decrease force development, improve muscle function and in some cases prevent energy depletion during fatigue.

ROLE OF THE $K^+_{(ATP)}$ CHANNEL DURING FATIGUE IN NORMAL MUSCLE

After establishing the effects of an activation of the $K^+_{(ATP)}$ channel, the next question concerns whether the channel is activated during fatigue in normal muscle, and hence performs its postulated function. For this, glibenclamide was used to block $K^+_{(ATP)}$ channel activity. The results indicate that in normal muscle, the $K^+_{(ATP)}$ channel is not activated to a very large extent during fatigue. Blocking the channel had no effect on the rate of fatigue, force recovery and $^{86}Rb^+$ fractional loss. However, glibenclamide did have one major effect as it significantly increased resting tension during fatigue in both EDL and soleus muscles. This effect of blocking the $K^+_{(ATP)}$ channel with glibenclamide on resting tension has also been previously reported in a number of studies (see for example McPherson *et al.*, 1993; Gramolini & Renaud, 1997). The increase in

resting tension during fatigue is also greater in $K^+_{(ATP)}$ channel-deficient muscles excised from Kir6.2 knockout mice, when compared to the increase in resting tension in the wild type (Gong & Renaud; unpublished results). The latter suggests that the glibenclamide effect on resting tension is not due to a non-specific effect, but rather to a block of $K^+_{(ATP)}$ channels. This result along with the pinacidil effect on resting tension support the notion that the $K^+_{(ATP)}$ channel is an important modulator of resting tension during fatigue. Thus when EDL and soleus muscles are fatigued with repeated tetanic contractions, the only apparent role of the $K^+_{(ATP)}$ channel is a decrease in resting tension.

However, one can still ask the question as to how the glibenclamide effect on resting tension is explained by a blocking of the $K^+_{(ATP)}$ channel when little or no channel activity was detected. That is, blocking $K^+_{(ATP)}$ channels did not affect $^{86}Rb^+$ fractional loss and had only a very small effect on membrane potential. One possible explanation for the glibenclamide effect on resting tension might involve $K^+_{(ATP)}$ channels located in t-tubules (Coronado & Parent, 1989; Light & French, 1994). An activation of these $K^+_{(ATP)}$ channels can not be detected by our $^{86}Rb^+$ fractional loss measurements because the t-tubule lumen space is narrow (Sjogaard, 1991) limiting the diffusion of $^{86}Rb^+$ out of the t-tubules (Almers, 1980).

THE EFFECTS OF MUSCLE ACTIVITY ON THE EXPRESSION AND PHYSIOLOGICAL ROLE OF THE $K^+_{(ATP)}$ CHANNEL

Contrary to normal muscle, glibenclamide had many significant effects during fatigue of 1-week denervated EDL and soleus muscles. In soleus muscle, glibenclamide significantly reduced the decrease in total force during fatigue when compared to non-treated soleus. This effect was

mainly due to a large increase in resting tension. Furthermore, glibenclamide significantly impaired force recovery in both denervated EDL and soleus muscles, whereas it had no effect in normal muscles. Pinacidil also had greater effects in denervated muscles when compared to normal muscles. The $K^+_{(ATP)}$ channel opener decreased tetanic force in denervated soleus and total force in denervated EDL to greater extents than it did in normal muscles. Furthermore, the increases in $^{86}Rb^+$ fractional losses were greater in denervated muscles suggesting that the $K^+_{(ATP)}$ channel can play a more predominant role in K^+ efflux in the denervated muscle.

These results suggest that the effects of $K^+_{(ATP)}$ channels are enhanced by denervation. This was observed despite the fact that the Kir6.2 mRNA content in denervated soleus was 2.7-fold less than in normal. One possible explanation would be that the decrease in mRNA content does not reflect the change in Kir6.2 protein content (a parameter that could not be assessed because of the unavailability of antibody or drugs that specifically bind Kir6.2). Another possibility is that the expression of several K^+ channels decreases after denervation (Escobar *et al.*, 1993; Shin *et al.*, 1997; Vullhorst *et al.*, 1998). It is then possible that on a relative scale, the decrease in $K^+_{(ATP)}$ channel expression is less than the other K^+ channels, making the $K^+_{(ATP)}$ channel more important. Overall, these results show that a decreased muscle activity and/or neurotrophic factors can influence the expression of the $K^+_{(ATP)}$ channel (at least Kir6.2 mRNA content in soleus) and that it also enhances the physiological effect of the $K^+_{(ATP)}$ channel.

In conclusion, the $K^+_{(ATP)}$ channel provides a cytoprotective mechanism in skeletal muscle. In normal muscle, this cytoprotective effect is limited to a decrease in resting tension when mouse EDL and soleus muscles are fatigued with one tetanic contraction every sec. The cytoprotective

effect is enhanced in denervated muscle as $K^{+}_{(ATP)}$ channels not only reduce resting tension (especially in soleus muscle), but are also important for better force recovery following fatigue.

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