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Impairment of Force Development in K_{ATP} Channel Deficient Skeletal Muscle Involves Ca^{2+} Influx Through L-Type Ca^{2+} Channels

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Impairment of force development in K_{ATP} channel deficient skeletal muscle involves Ca^{2+} influx through L-type Ca^{2+} channels

By

Carlo Cifelli

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ABSTRACT

ATP-sensitive potassium (K_{ATP}) channels link membrane excitability to metabolism. They are regulated by intracellular nucleotides and other factors, and have been shown to play a role in development of skeletal muscle force, but controversy surrounds their role during fatigue. The aim of this research project was to determine the role of K_{ATP} channel under conditions that allow for better assessment of changes in force during fatigue, by virtue of using a smaller whole muscle model less subject to anoxia. Thus, the first objective was to determine the effect of the loss of K_{ATP} channel activity on force during fatigue in small FDB muscle bundles. K_{ATP} channel deficient fibers had faster and greater decreases in peak tetanic force during fatigue, developed greater resting tension, and had lower force recovery following fatigue compared to control wild type muscles. The second objective was to determine whether the functional impairment in skeletal muscle without K_{ATP} channel activity was due to an increase in Ca^{2+} influx. When $[Ca^{2+}]_e$ was reduced or L-type Ca^{2+} channels partially blocked, Kir6.2$^{-/-}$ FDB muscle had slower fatigue development, less resting tension, and had an improved force recovery.

A novel phenomenon was observed while studying the effect of K_{ATP} channel activity *in vitro*. During a second bout of fatigue the decrease in peak tension was significantly lower than the decrease during the first bout of fatigue. Furthermore, the deleterious effects of the loss of K_{ATP} channel activity during an initial fatigue were absent during the second fatigue in FDB exposed to glibenclamide.

It is concluded i) that the K_{ATP} channel is important to prevent impairment of function during fatigue, ii) that this impairment of function is due to an increase in Ca^{2+}

influx through L-type Ca^{2+} channels, causing Ca^{2+} overload, and iii) that fatigue resistance increases while the dependency on the K_{ATP} channel to prevent function impairment and fiber damage decreases following one fatigue bout at 37°C; a phenomenon here termed fatigue pre-conditioning.

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LIST OF ABBREVIATIONS

$[\ ]_i$	intracellular concentration
$[\ ]_e$	extracellular concentration
°C	degree Celsius
A_1	adenosine receptor 1
A_2	adenosine receptor 2
ABC	ATP-binding cassette
ACh	acetylcholine
ADP	adenosine-5'-diphosphate
AMP	adenosine-5'-monophosphate
ANOVA	analysis of variance
ATP	adenosine-5'-triphosphate
Ba^{2+}	barium ion
BCl	big conductance chloride channel
BK_{Ca}	Ca^{2+}-sensitive K^+ channel
Ca^{2+}	calcium ion
Ca_{50}	concentration for half-maximal activation
CFTR	cystic fibrosis transmembrane conductance regulator
CK	creatine kinase
Cl^-	chloride ion
CON	control
delFAT1	delayed first fatigue

DMSO	dimethyl sulfoxide
EDL	extensor digitorum longus
FAT1	first fatigue
FAT2	second fatigue
FDB	flexor digitorum brevis
F_{max}	maximum calcium-activated force
glib	glibenclamide
GTP	guanosine triphosphate
H^+	hydrogen ion (proton)
HMT	heavy metal transporter
Hz	hertz
IC_{50}	half-maximal inhibition
K^+	potassium ion
K_{ATP} channel	ATP-sensitive K^+ channel
KCO	potassium channel opener
K_d	concentration for half-maximal activation
K_i	concentration for half-maximal inhibition
Kir	potassium inward rectifier
K_v	voltage-sensitive K^+ channel
L.S.D.	least significant difference
MDR	multidrug resistance exporter
Mg^{2+}	magnesium ion
MgADP	magnesium adenosine-5'-diphosphate

MgATP	magnesium adenosine-5'-triphosphate
MHC	myosin heavy chain
mM	millimolar
MRP	multidrug resistance protein
mV	millivolt
n	number of sample
Na^+	sodium ion
NBD	nucleotide binding domain
NBF	nucleotide binding fold
N/cm^2	Newton per centimeter square
pCa	-log $[Ca^{2+}]$
PCr	phosphocreatine
pH_i	intracellular pH
P_i	inorganic phosphate
P_{open}	open-state probability
Rb^+	rubidium ion
SCl	small conductance chloride channel
S.E.	standard error
SR	sarcoplasmic reticulum
SUR	sulfonylurea receptor
TEA	tetraethylammonium
TMD	transmembrane domain
t-test	student t-test

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

CHAPTER 1

GENERAL INTRODUCTION

Muscle activity increases metabolic rate 20- to 100-fold depending on fiber type and activity intensity (Gibbs, 1987). Although skeletal muscles have mechanisms to increase ATP production, many muscular activities eventually lead to a situation in which ATP demand exceeds production (Fitts, 1994). If too large, an energy deficit will result in muscle function impairment, fiber damage and even cell death. Consequently, muscles have the ability to prevent large and damaging ATP depletion. One mechanism for this may involve the ATP-sensitive K^+ (K_{ATP}) channel. The K_{ATP} channels have been named as such as they close upon the binding of ATP. More importantly, they appear critical in preventing fiber damage in skeletal muscle during treadmill running (Thabet *et al.*, 2005).

The K_{ATP} channels could prevent fiber damage by their capacity to link the energy status of the cell to the electrical activity of the cell membrane. Once activated, the direct effect of the K_{ATP} channels is a reduction in action potential amplitude, especially during fatigue development in skeletal muscle (Gong *et al.*, 2003). As a consequence of lower action potential amplitude, less Ca^{2+} is released by the sarcoplasmic reticulum (Burton & Smith, 1997; Duty & Allen, 1995) and less force is generated by the contractile components (Gong *et al.*, 2003; Matar *et al.*, 2000). It has been suggested that the K_{ATP} channels contribute to the decrease in force during fatigue in order to prevent damaging ATP depletion by reducing the activity of Ca^{2+}-ATPase and myosin-ATPase (Seino & Miki, 2003).

Abolishing KATP channel activity should result in a slower rate of fatigue as the channel no longer affects action potentials. However, abolishing KATP channel activity leads to several function impairments. Compared to normal muscles, the impairments in KATP channel deficient muscle include fiber damage during treadmill running *in vivo* (Thabet *et al.*, 2005) and during fatigue *in vitro* (Bourassa & Renaud, unpublished results), large cell membrane depolarization (Gramolini & Renaud, 1997), greater resting tension, which increases when muscles fail to fully relax between contractions (Gramolini & Renaud, 1997), and lower capacity to recover force following fatigue (Gong *et al.*, 2000; Light *et al.*, 1994; Matar *et al.*, 2000). Together these impairments should give rise to faster decrease in force during fatigue when compared to control. Interestingly, most studies report no effect on the rate of fatigue as measured from the decrease in tetanic force when KATP channel activity is abolished pharmacologically, by exposing wild type muscle to glibenclamide (Light *et al.*, 1994; Matar *et al.*, 2000; Van Lunteren *et al.*, 1998; Weselcouch *et al.*, 1993) or genetically using Kir6.2$^{-/-}$ muscles, a KATP channel knockout (Gong *et al.*, 2000; Gong *et al.*, 2003). Being a sulphonylurea, glibenclamide binds to the SUR2 subunit and then acts upon the Kir6.2 subunit, closing the pore of the channel allosterically (Van Lunteren *et al.*, 1998). In regards to fatigue kinetics, Kir6.2$^{-/-}$ muscles behave like wild type muscle exposed to glibenclamide suggesting that no compensation occurs in these muscles from a chronic lack of KATP channel activity (Gong *et al.*, 2000; Matar *et al.*, 2000).

These studies raise the question as to whether the KATP channel really affects force during fatigue. It is argued later in the Introduction (see section entitled "Skeletal muscle,") that the apparent lack of an effect may have been in part because some studies

were carried out at 25°C and for those carried out at 37°C the muscle may have been too large to see any effect, because of a large anoxic core. On this basis, it would be an advantage to examine KATP channel activity and fatigue using small muscle bundles from the mouse flexor digitorum brevis (Bruton *et al.*, 1998).

In cardiac cells, KATP channels also protect against a Ca^{2+} overload, a phenomenon known to cause fiber damage. The action of the KATP channel in cardiac muscle is to reduce Ca^{2+} influx through the L-type Ca^{2+} channels (Lascano *et al.*, 2002). In skeletal muscle, abolishing KATP channel activity during fatigue results in large increases in both resting tension (Matar *et al.*, 2000; Gong *et al.*, 2000) and in resting intracellular [Ca^{2+}] ([Ca^{2+}]i) (Bourassa & Renaud, unpublished results). It remains to be determined if the greater resting [Ca^{2+}]i is due to greater Ca^{2+} influx and is responsible for fiber damage during fatigue in KATP channel deficient skeletal muscle. This question is directly addressed in objective 2 of this research project.

1) MUSCLE CONTRACTION AND MECHANISM OF MUSCLE FATIGUE

1-A) The contraction cycle

For muscles to generate force or do work, they must contract. Skeletal muscle contraction is an active process that involves three major steps: action potential generation at and propagation from the neuromuscular junction, excitation-contraction coupling and the contraction-relaxation process.

A voluntary muscle contraction begins in the cortex of the brain that sends information to the brain stem or spinal cord. From there, somatic α-motoneurons, which innervate muscle fibers, send action potentials down their axons bundled within peripheral nerves. At the neuromuscular junction (NMJ), a specialized synapse of the α-

motoneuron and the muscle fiber, the electrical signal is relayed to the muscle via the release of acetylcholine (ACh), a neurotransmitter. ACh binds to its receptor, which is a ligand-gated cation channel, on the motor end plate at the NMJ. Once ACh is bound, the channel opens and allows Na^+ to enter the fiber. As Na^+ moves in, the membrane depolarizes and after reaching a threshold, neighbouring voltage sensitive Na^+ channels are activated initiating an action potential. An action potential involves two cation fluxes: Na^+ influx through voltage-gated Na^+ channels to depolarize the membrane and K^+ efflux through the voltage-gated K^+ (Flagg *et al.*, 2004) channels to repolarize the membrane. The action potential then propagates along the surface of the muscle fiber and into the t-tubules, which are invaginations of the cell membrane.

The transmission of the electrical signal to the mechanical events of the contraction is called excitation-contraction coupling and involves an increase in $[Ca^{2+}]_i$. The t-tubule membrane contains voltage-sensors known as the dihydropyridine (DHP) receptors, which are also L-type Ca^{2+} channels (Silverthorn, 2004). These channels interact directly with the Ca^{2+} release channels located on the membrane of the sarcoplasmic reticulum, which are also known as ryanodine receptors (RyR). When the t-tubular membrane is depolarized during the action potential, the DHP receptors undergo a conformational change, which activates and opens RyR in the SR. Stored SR Ca^{2+} then diffuses down its electrochemical gradient into the cytosol.

In muscle, the contractile unit is the sarcomere, which is made up of thin filaments attached to the Z-line and thick filaments between the thin filaments (Silverthorn, 2004). The thin filaments contain the regulatory proteins, troponin and tropomyosin, and one of the contractile proteins, actin. The thick filament contains the

other contractile protein, myosin. When $[Ca^{2+}]_i$ increases, Ca^{2+} binds to the subunit C of troponin, which via an interaction with the subunit T causes the displacement of tropomyosin from the myosin binding sites on actin. This allows the myosin cross-bridges to interact with actin. After the binding to actin, the myosin cross-bridges execute a power stroke that pulls the thin filament toward the center of the sarcomere, allowing for the development of force (isometric contraction) or shortening (isotonic contraction). The SR actively pumps Ca^{2+} back in which returns Ca^{2+} to baseline levels once action potentials cease. As $[Ca^{2+}]_i$ decreases, Ca^{2+} detaches from troponin allowing tropomyosin to return to its inhibitory position. The muscle then relaxes after the detachment of myosin from actin.

1-B) Energy requirement during contraction

Muscles require a constant source of ATP to power the three ATPases during any contraction. The first ATPase is the myosin ATPase, located in the cross-bridge. It converts chemical energy into mechanical energy during the power stroke. The second ATPase is the Ca^{2+} ATPase, located in the SR membrane. It actively pumps Ca^{2+} back into the SR to allow muscle relaxation. The third ATPase is the Na^+K^+ pump, located in the cell membrane. It pumps 2 K^+ in and 3 Na^+ out of the cytosol in order to restore and maintain the physiological Na^+ and K^+ concentration gradients, required for action potential generation. The Na^+K^+ ATPase also generates a net outward current as one positive charge is transported out of the cytosol during each cycle; thus the Na^+K^+ ATPase contributes to the cell membrane potential. Together, the myosin and Ca^{2+} ATPases account for more than 99% of the ATP consumption during a muscular contraction (Gibbs, 1987).

Since the ATPases only use ATP as a direct source of energy, any muscular activity requires an increased ATP production to meet the increased energy demand. The primary sources of ATP are phosphocreatine, glycolysis, and oxidative phosphorylation (Silverthorn, 2004). In fact, the sudden increase in ATP demand is always met by increases in ATP production. All muscle fibers initially use phosphocreatine (PCr) to generate ATP until the glycolytic or oxidative phosphorylation pathways are fully activated. The processes depend on the intensity of muscular activity and the fiber types being recruited.

Skeletal muscle fibers are classified according to the myosin isoform they express. The speed at which a muscle fiber may contract is determined by the myosin isoform expressed in the fiber's thick filaments (Hamalainen & Pette, 1995). Muscle fibers were initially divided into two fiber types. Fast-twitch fibers complete multiple contractile cycles more rapidly than slow-twitch fibers. Their speed translates into faster force development or shortening velocities. Among the slow fibers, only one myosin isoform was found for skeletal muscle (Hamalainen & Pette, 1995). This myosin was designated type I, and by association, fibers expressing this myosin isoform are called type I fibers. In general, type I fibers are not only the slowest contracting fibers they also have moderate Ca^{2+} ATPase activity (Hamalainen & Pette, 1995). They contain numerous mitochondria and are well vascularized giving them a high oxidative phosphorylation capacity (Hamalainen & Pette, 1995). Due to their low myosin ATPase and Ca^{2+} ATPase activity concomitantly with a large capacity to generate ATP, these fibers are among the most fatigue resistant. These fibers are normally involved in the

maintenance of posture which requires constant muscle stimulation at very low frequencies.

For the fast fiber types, three myosin isoforms have been cloned and used to define IIA, IIB and IIX fibers. Type IIA fibers are also fatigue-resistant because they basically have the same oxidative capacity as those of type I fibers (Silverthorn, 2004). They are also the fibers showing large increases in oxidative capacity with endurance training (Russell *et al.*, 2003). Type IIA fibers are generally used during standing, walking and prolonged low level muscular activity such as running.

Type IIB fibers are, on the other hand, the most glycolytic and the least oxidative of all skeletal muscle fibers (Hamalainen & Pette, 1995). For that reason, they are easily fatigued because of the poor capacity of glycolysis to generate ATP combined with the highest rate of ATP utilization by the myosin- and Ca^{2+}-ATPase pump (Silverthorn, 2004). The IIB gene is highly expressed in rodents (Thabet *et al.*, 2005). Although the type IIB gene is present in man, the myosin IIB isoform is normally not expressed (Smerdu *et al.*, 1994). The third fast myosin isoform, type IIX, also gives rise to a fast-twitch, glycolytic fiber. In terms of oxidative capacity and fatigue resistance, type IIX fibers are intermediate between type IIA and IIB fibers (Parry, 2001). Type IIB and IIX fibers are the least used, and are recruited for large amount of force development and rapid shortening speed during activities such as a sprint, high jump, or power lifting (Silverthorn, 2004).

Despite the capacity of all muscle fibers to increase ATP production to meet the increased demand, all muscular activities eventually lead to a situation in which ATP production is insufficient to meet consumption. During a marathon, it can take hours

occurs once muscle glycogen drops below a critical mass (van der Vusse *et al.*, 1989). During a sprint or weight lifting, this occurs within seconds because the highly glycolytic type IIB or IIX fibers are used and because strong contractions intermittently stop the blood flow (Mauer *et al.*, 1999). If ATP levels decrease too much, the capacity of the fiber to maintain proper Na^+ and K^+ concentration gradient or to pump Ca^{2+} back into the SR becomes significantly impaired. Tissue damage is also another consequence of a lack of ATP (Ferrari *et al.*, 1993). For those reasons, mechanisms must then exist to protect muscle from ATP run down and the subsequent functional impairment and cell damage. In general, it is now accepted that muscle fatigue is actually such a protective mechanism (Noel, 2002).

1-C) Muscle fatigue

Muscle fatigue is defined as a decrease in force generating capacity, and is distinct from exhaustion which describes a condition in which muscle is no longer able to continue power output or exercise intensity (Vollestad & Sejersted, 1988). Fatigue may be due to a failure anywhere along the path of events from the central nervous system to the muscle itself as described in the previous section. However, early work has shown that reduced force production in human, resulting from prolonged voluntary contractions, could not be improved via direct motor nerve stimulation (Merton, 1954). It was concluded that reduced central drive and neuromuscular transmission were not important factors in the onset of fatigue, and that fatigue was an intrinsic property of the muscle itself.

Early hypotheses of muscle fatigue were centered on ATP depletion. Historically, it was believed that a decrease in ATP would reduce force development by the contractile

components because of a lack of energy. These hypotheses were shown to be wrong. Many studies reported substantial supplies of ATP in fatigued fibers, even at exhaustion (Lindinger *et al.*, 1987; Spriet *et al.*, 1987; Katz *et al.*, 1986; Miller *et al.*, 1988). When the cross-bridge cycle, or the interaction between myosin and actin, became understood, it then became clear that any decreases that have been reported during fatigue could not be a cause for the decrease in force at the level of the sarcomere for two reasons. First, within the physiological changes in concentration during fatigue, ATP has no effects on force production by the contractile components. Second, extremely large ATP decreases below 1 mM actually increases force (Godt & Nosek, 1989; Seow & Ford 1997; Wang & Kawai, 1997). It is now well accepted that ATP depletion is not a limiting factor in muscle fatigue, at least at the level of the sarcomere where force is generated.

During fatigue, many other metabolites change in concentration, including increases in ADP, lactic acid, inorganic phosphate and adenosine, as well as decreases in intracellular pH (Neerunjun & Dubowitz, 1979) and PCr. The exercise-induced accumulation of lactic acid and the resulting decrease in pHi have long been considered important factors contributing to the decrease in force (Westerblad *et al.*, 1991; Fitts, 1994). In an extensive study using skinned fiber preparations, Godt and Nosek (1989) demonstrated that among all the metabolites that change in concentration during fatigue, only the decreases in pHi and increases in inorganic phosphate (P_i, from the breakdown of phosphocreatine) cause significant decreases in the force developed by the sarcomere. Their findings also demonstrated that the metabolite changes could only account for one third of the decrease in force observed during fatigue.

It was then realized that both the pH and P_i effects are temperature dependent. Godt and Nosek (1989) had carried out their experiments at 25°C. When temperature is increased from 25°C to 32°C, pH has less effect on maximum force; it was even suggested that at 37°C, pH probably has a very minor effect on force (Westerblad *et al.*, 1997). A similar temperature dependency was eventually reported for P_i on maximum force (Debold *et al.*, 2006). Therefore, the mechanism of fatigue must be upstream of the contractile components. In fact, if fatigue is a protection against damaging ATP depletion, it makes better sense that fatigue involves a decrease in Ca^{2+} release because 40% of the ATP consumption during contractions comes from Ca^{2+} ATPases (Gibbs, 1987).

1-C-i) Role of Ca^{2+} in muscle fatigue

There is now strong evidence that one major mechanism of muscle fatigue is a decrease in Ca^{2+} release by the SR (Westerblad *et al.*, 1991; Allen *et al.*, 2002). When fatigue is elicited in single FDB fibers, the tetanic $[Ca^{2+}]_i$, or the maximum $[Ca^{2+}]_i$ during a tetanic contraction, increases by approximately 50%. It then slowly declines until it markedly decreases in the final stage of fatigue (Westerblad & Allen, 1991). The marked decrease in tetanic $[Ca^{2+}]_i$ is concomitant with a similar marked decrease in tetanic force (Allen *et al.*, 2002). The most important point is that the decrease is so large that it results in submaximal tetanic $[Ca^{2+}]_i$ that can no longer fully stimulate the contractile components, resulting in submaximal force development (Allen *et al.*, 1989; Lee & Allen, 1991; Baker *et al.*, 1993). Furthermore, while the effect of increased P_i on maximum force is small, the depression of force by P_i at submaximal $[Ca^{2+}]$ is quite substantial because of a large decrease in Ca^{2+} sensitivity of the contractile components

(Debold *et al.*, 2006). Thus, once tetanic $[Ca^{2+}]_i$ becomes submaximal, the decrease in force becomes substantial. It has been suggested that reduced tetanic $[Ca^{2+}]_i$ is either due to a failure of the SR to release Ca^{2+} or a reduced membrane excitability. In these studies, Ca^{2+} is the major factor for peak force production and not the force-length relationship, as the muscle bundle is kept at a constant length throughout the entire protocol.

1-C-ii) Failure of SR to release Ca^{2+} as a mechanism of muscle fatigue

There are three possible mechanisms by which the SR fails to release Ca^{2+}: (i) loss of Ca^{2+} from the SR, (Smerdu *et al.*, 1994) failure of the SR Ca^{2+} release process, or (iii) buffering of Ca^{2+} by P_i within the SR (Westerblad & Allen, 1991; Fryer *et al.*, 1995; Duke & Steele, 2000).

Both K^+ and caffeine induce a contracture in muscle. Exposing a fiber to 190 mM K^+ causes a large depolarization of the cell membrane, which directly activates the DHP receptor and Ca^{2+} release. Caffeine, on the other hand, directly activates the RyR. Compared to a normal electrically-induced contraction, which requires action potential generation, the amount of Ca^{2+} release is much greater during a K^+ or caffeine contracture. Following fatigue, the amount of Ca^{2+} released during a K^+ or caffeine contracture is less than in unfatigued fibers. However, the amount of Ca^{2+} released during those contractures after fatigue is still greater than the amount of Ca^{2+} released before fatigue during an electrically-induced tetanic contraction (Allen *et al.*, 1989). These findings suggest that Ca^{2+} is available for release in the SR but that there is not enough Ca^{2+} left in the SR at the end of fatigue to fully activate the contractile components (Allen *et al.*, 1989).

The failure of SR Ca^{2+} release may be related to a failure of the SR to respond to the action potential trigger. The activity of both DHP and RyR receptor depends on ATP levels where a decrease in activity occurs as ATP decreases (Blazev & Lamb, 1999). In the case of RyR the inhibitory effect of reduced ATP may also be potentiated by ATP hydrolysis products, including adenosine, ADP, AMP or IMP, which competitively antagonize the action of ATP on the RyR (Duke & Steele, 1998, Blazev & Lamb, 1999, Laver *et al.*, 2001). Therefore, a reduced activity of either the DHP receptor or RyR may explain in part the decrease in tetanic $[Ca^{2+}]_i$ during the final stages of fatigue.

The depletion of PCr during any muscular activity results in a P_i accumulation that can reach 25–50 mM depending on fiber type (Dawson *et al.*, 1980). It has been suggested that P_i diffuses in the SR and buffers Ca^{2+} so less free Ca^{2+} is available during excitation. Microinjection of P_i into unfatigued mouse fibers resulted in a 35% decrease in tetanic $[Ca^{2+}]_i$ (Westerblad & Allen, 1996). This is in agreement with lower Ca^{2+} release with increased P_i. However, several other effects of P_i-injection in unfatigued fibers differ markedly from the situation during fatigue. First, fatiguing stimulation is associated with a progressive rise in resting $[Ca^{2+}]_i$ while the reverse was observed following P_i microinjection. Second, during fatigue there is a slowing of Ca^{2+} uptake by the SR (Westerblad & Allen, 1991), while injection of P_i increases the rate of SR Ca^{2+} accumulation. Therefore reductions in the myoplasmic Ca^{2+} and increases in P_i act synergistically during fatigue to reduce muscular force (Debold *et al.*, 2006).

Another problem is the time at which P_i increases and tetanic Ca^{2+} decreases. The depletion of PCr and the increase in P_i occurs within seconds, while the decrease in tetanic Ca^{2+} occurs much later. If P_i is to affect Ca^{2+} release, its mechanism of action

must be delayed until such time arises that tetanic Ca^{2+} must decrease. A possible mechanism may involve a delayed P_i-entry in the SR. A big conductance chloride channel (BCl) and a small conductance chloride channel (SCl) have been characterized on the SR membrane. It has been proposed that they are also permeable to P_i and are a major pathway for P_i entry into the SR lumen (Laver *et al.*, 2001). Furthermore, these channels are ATP sensitive where a decrease in ATP increases the activity of the channel (Westerblad & Lannergren, 1995). Therefore, it was suggested that P_i entry into the SR lumen might be blocked at normal levels of ATP, but increase as ATP declines during the final stages of fatigue.

1-C-iii) Role of membrane excitability in the decrease in Ca^{2+} during fatigue

Studies using high-frequency stimulation have shown that during fatigue the action potential amplitude declines, the depolarization and repolarization phases slow down, and the membrane potential between action potentials becomes less negative reaching values around -50 mV from a normal value of -75 to -85 mV (Lännergren, 1986; Lännergren, 1987). When fatigue is elicited using intermittent stimulation similar changes are observed, except the membrane potential depolarized to only -70 mV (Balog *et al.*, 1994; Balog & Fitts, 1996). These effects on action potentials are attributed to the rise in $[Na^+]_i$ and $[K^+]_e$, which have been debated as major causes of fatigue.

During an action potential, Na^+ influx causes depolarization of the membrane and K^+ efflux repolarizes the membrane. When the Na^+K^+ pump becomes unable to transport enough Na^+ and K^+ to match the fluxes during action potentials, the result is a net increase in $[K^+]_e$ and $[Na^+]_i$ while $[K^+]_i$ decreases. $[Na^+]_e$, on the other hand, changes little because of water lost in the extracellular space to the intracellular space that

matches the Na^+ influx (Bouclin *et al.*, 1995; Cairns *et al.*, 1995). The net result of the changes in $[Na^+]_i$, $[K^+]_e$ and $[K^+]_i$ is a decrease in the Na^+ and K^+ concentration gradient that has profound effect on membrane potentials. Given that changing $[Na^+]_i$ experimentally is quite difficult, Cairns *et al.* (2003) mimicked the change in Na^+ concentration gradient by reducing $[Na^+]_e$. When the change in Na^+ concentration gradient during fatigue was mimicked in unfatigued soleus muscle they observed (i) an increased number of inexcitable fibers, (Smerdu *et al.*, 1994) a large reduction of action potential amplitude, and (iii) a lower capacity to generate action potentials to every stimulus during a tetanic stimulation. As a consequence of these Na^+ effects on membrane excitability, large decreases in force were observed with lowered $[Na^+]$ gradient supporting a role for Na^+ during fatigue.

For K^+, the immediate effect of lowered K^+ concentration gradient is on the resting membrane potential which experiences a depolarization. As the membrane depolarizes, the activity of the voltage-dependent Na^+ channels decrease because of an increased degree of inactivation (Juel, 1988; Ruff, 1996). As less Na^+ channels are available, large decreases in action potential amplitude and conduction velocity are observed (Juel, 1988; Yensen *et al.*, 2002). The reduced action potential amplitude then causes lower Ca^{2+} release from the SR (Cairns *et al.*, 1995; Clausen & Everts, 1991; Nielsen *et al.*, 1998). Therefore increased $[K^+]_e$ can potentially contribute to the etiology of muscle fatigue (Sjøgaard *et al.*, 1990; Ruff, 1996).

Intriguing, new and recent studies, however, have shown that both Na^+ and K^+ do not always depress membrane excitability and force development. Under some conditions they can both enhance contractility despite reduced membrane excitability. For example,

small increases in $[Na^+]_i$ increase the Na^+K^+ pump activity which then hyperpolarizes the membrane, counteracting the depolarization-induced inactivation of Na^+ channels when $[K^+]_e$ increases (Bouclin *et al.*, 1995). As well, small increases in $[K^+]_e$ potentiates twitch force; only large $[K^+]_e$ increases have depressive effects on force (Pedersen *et al.*, 2003). In studies using unfatigued mouse muscle, peak twitch force of EDL and soleus is greater at 7-12 mM $[K^+]_e$ than at 4.7 mM (Shyng & Nichols, 1998; Yensen *et al.*, 2002). Furthermore, Pedersen *et al.* (2003) reported that at 37°C the critical $[K^+]_e$ at which tetanic force declines in unfatigued muscle is about 11 mM, which is greater than the maximum 10 mM K^+ reported in the interstitial space during fatigue. Furthermore, a decrease in pH_i and an increase in catecholamines further increases this critical $[K^+]_e$ to 15 mM (Clausen & Everts, 1991). Such studies then raise the questions as to whether either Na^+ or K^+ really contributes to a decrease in force during fatigue.

In all the above studies, the effect of Na^+ and K^+ were studied individually, however strong interactive effects between ions must also be considered. For example, at 7 mM K^+ – a value well below the maximum 10 mM K^+ observed at the end of fatigue – has no effect on peak tetanic force of unfatigued frog muscle while it potentiates twitch force as long as the $[Na^+]_e$ is maintained. However, small decreases in $[Na^+]_e$ as little as 20 mM, which reduces the $[Na^+]$ gradient by only 17%, suddenly causes the effect of 7 mM K^+ to switch from a potentiating to a depressing effect on both peak twitch and tetanic force (Bouclin *et al.*, 1995). A similar interaction between Na^+ and K^+ has also been reported in mammalian muscle. Such studies demonstrate therefore that Na^+ and K^+ have dual roles in muscle; one role is to maximize muscle performance and one is to depress force development. Renaud (2002) suggested that the balance between these two

effects depends on the physiological state of muscle where at the beginning of any exercise the major function of Na^+ and K^+ is one of maximizing muscle performance. Then when an energy deficit develops or fatigue is triggered, a change occurs in which the effect of Na^+ and K^+ becomes depressive. The exact mechanism for these changes is unknown, but there is now evidence for a mitochondrial factor that is released when ATP synthesis decreases, and that factor decreases membrane excitability. In fact for Na^+ and K^+ to switch functions, something must affect the properties of the transporters and channels that affect the Na^+ and K^+ flux.

More recent studies are now focusing on how the activity of ion transporters and channels are affected during muscular activity, and how they modify the ion effects on muscle contractility. For example, catecholamines increase the Na^+K^+ pump activity and play an important role in preventing the K^+ depressing effect on force development. Another example is the Cl^- channel. Pedersen *et al.* (2005) demonstrated that the drop in membrane excitability and force at 11 mM $[K^+]_e$ can substantially be reduced when pH is reduced with lactic acid. During an action potential, Cl^- moves inside the cytosol counteracting the Na^+ depolarization and helping K^+ repolarization (Nielsen *et al.*, 2001; Pedersen *et al.*, 2003). In normal $[K^+]_e$, the removal of Cl^- from the extracellular space has little effect, but at 11 mM K^+ when many Na^+ channel are inactivated, the removal of Cl^- helps obtain greater depolarization as less Cl^- counters the Na^+ depolarization. Thus, in this case increases in Na^+K^+ pump activity and decreases in Cl^- can help maximize muscle performance at the beginning of exercise, but less is known concerning changes in transporter and channel activity leading to fatigue, but some may involve K^+ channels.

There are numerous K^+ channels within the sarcolemma which allow for K^+ fluxes across the cell membrane. As discussed earlier, the voltage-dependent K^+ (Flagg *et al.*, 2004) channels allow for K^+ efflux during the repolarization phase of the action potential. These channels are active only when the membrane depolarizes to -50 mV. In the resting state when resting membrane potential is between -70 and -80 mV, another K^+ channel, the strong inward rectifier Kir2.1 channel allows small outward currents responsible to maintain the resting membrane potential. This channel closes upon large depolarization to prevent a K^+ efflux that would counteract the Na^+ depolarization (Jongsma & Wilders, 2001). Interestingly, Kristensen *et al.* (2006) found that Kir2.1 channels are concentrated primarily in t-tubules and may also be important in allowing large K^+ influx when K^+ accumulates in the t-tubule during fatigue. They based this on the fact that $[K^+]$ increases in the t-tubules may become large enough to favour K^+ influx.

Two other K^+ channels that are generally closed in unfatigued muscle open under situations such as anoxia or fatigue and contribute to fatigue. One is the Ca^{2+}-sensitive K^+ channel (BK_{Ca}) and the other is the ATP-sensitive K^+ channel (K_{ATP}). The former channel is proposed to be activated by the binding of Ca^{2+} to specific sites on the cytoplasmic face of the channel. It has been suggested that the channel may be important to prevent Ca^{2+} overload as its activation increases K^+ efflux which then depresses action potential amplitude (Jacquemond & Allard, 1998; Kristensen *et al.*, 2006). The latter channel, the K_{ATP} channel, will now be fully described being the focus of this thesis.

2) K_{ATP} CHANNEL

The ATP-sensitive potassium (K_{ATP}) channels were first discovered in cardiac muscle and later in pancreatic β-cells (Cook & Hales, 1984; Noma, 1983). Since then

they have been found in many other tissues including skeletal muscle (Spruce *et al.*, 1985), neurons (Ashford *et al.*, 1988), and smooth muscle (Standen *et al.*, 1989). The K_{ATP} channel was named for the inhibitory effect of ATP, which acts to reduce channel activity. As the channel affects K^+ conductance, its role is to couple membrane potential to the metabolic state of the cell by sensing changes in intracellular ATP concentrations. Consequently, the channel responds to metabolic changes that occur during hypoxia, ischemia, fatigue, or changes in glucose concentration. In the following sections, the molecular structure, isoforms, regulation and function of the K_{ATP} channel will be discussed.

2-A) Molecular Structure of the K_{ATP} channel

Reconstitution experiments demonstrated that K_{ATP} channels form an octamer of two subunits in which four Kir6 and four SUR subunits assemble together (Aguilar-Bryan *et al.*, 1998; Seino & Miki, 2003). K_{ATP} channels are the first example of a heteromultimeric protein complex composed of two proteins that are structurally unrelated to each other (Seino, 1999) (Fig. 1-1). The Kir6 subunit is a member of the superfamily of potassium inward rectifiers (Kir) (Inagaki *et al.*, 1995). Like all members of the Kir superfamily, Kir6.x subunits have two transmembrane regions, M1 and M2, separated by the extracellular H5 loop located near the pore region of the channel (Fig. 1-1). The H5 loop is a very conserved sequence of amino acids in most K^+ channels, including the voltage-sensitive K^+ (K_V) channel, and has been identified as part of the potassium selectivity "filter" (Jan & Jan, 1992). Early studies have shown that the M2 region, or inner pore helix, forms the permeation pathway with a glycine- tyrosine-glycine (GYG) sequence. In the Kir6 subunit the tyrosine is replaced by phenylalanine

FIGURE 1-1

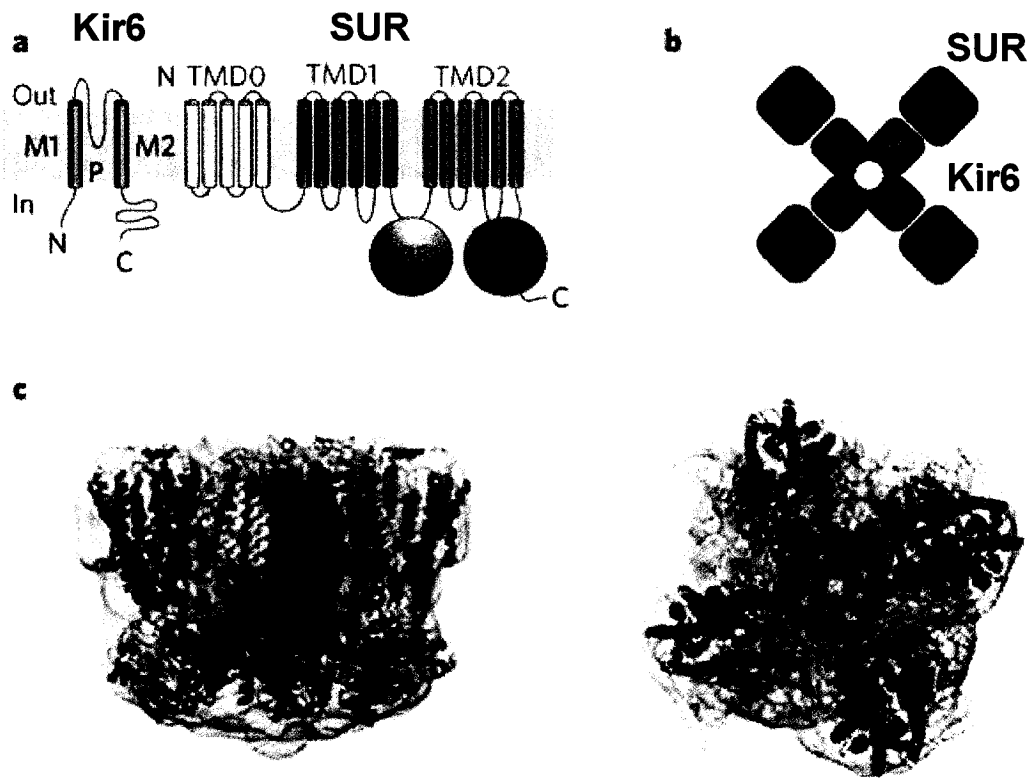

Figure 1-1. Molecular structure of the KATP channel. a, Kir6.x subunits generate the channel pore and SUR subunits generate the regulatory subunit. TMD, transmembrane domain; NBF, nucleotide-binding fold (or nucleotide-binding domain (NBD)); M1, M2, transmembrane helices; P, pore (H5 loop). **b**, The channel is a functional octamer of four Kir6.x subunits, and each subunit is associated with four SUR subunits. **c**, Images at 18 Å resolution of the entire KATP complex viewed in the plane of the membrane (left) or from above the membrane (right) (Nichols, 2006).

giving instead a GFG sequence. It is the lateral or twisting motions of these helices that possibly allow K^+ to move through the pore (Aguilar-Bryan & Bryan, 1999). Like for all K_V and Kir channels, the pore of the K_{ATP} channel is formed by 4 subunits, here the Kir6.*x*. A major difference among K^+ channels, however, is the fact that the Kir subunit has only two transmembrane domains lining the pore, whereas the K_V channel subunit is made of six transmembrane helices and an H5 loop between the transmembrane segments 5 and 6 (S5 and S6) that line and face the pore (Choe *et al.*, 1999).

To date, three Kir6 genes have been cloned and sequenced; the Kir6.1 and Kir6.2 in mouse, human and rat, and Kir6.3 in zebrafish. Kir6.1, a 424 amino acid and 48 kDa protein, is expressed ubiquitously in several tissues. Kir6.2, a 390 amino acid and 43.5 kDa protein, has 71% homology with Kir6.1 (Inagaki *et al.*, 1995). Most recently, Zhang *et al.* (2006) has identified and characterized a novel member of the K_{ATP} channel subunit family, Kir6.3, a protein of 432 amino acids that shares 66% identity with the mammalian Kir6.2 but differs considerably from the mammalian Kir6.1 and Kir6.2 in the C-terminus. The mammalian subunits contain an Arg-Lys-Arg (RKR) motif in the C-terminus. The RKR motif is an endoplasmic reticulum (ER) retention signal that prevents Kir6 proteins to reach the cell membrane in the absence of the SUR subunits (Aguilar-Bryan & Bryan, 1999). Kir6.3 in zebrafish lacks this RKR motif (Zhang, 2006).

The K_{ATP} channels are the only known members of the Kir superfamily for which the Kir6 subunits are associated with a regulatory subunit, SUR (Seino, 1999) (Fig. 1-1). The SUR name is derived from the sensitivity of the proteins to sulfonylurea compound, such as the anti-diabetic drugs tolbutamide and glibenclamide (Seino, 1999). The SUR subunits are much larger proteins compared to the Kir subunit (Aguilar-Bryan *et al.*,

1995). SURs are encoded by two genes, SUR1 and SUR2. SUR1 is a 1582 amino acid and 177 kDa protein. SUR2A is a 1545 amino acid and 174 kDa protein that shares 68% identity with SUR1. Both SUR1 and SUR2 genes give rise to several isoforms due to a number of splice variants, the two most studied ones being from the SUR2 gene: SUR2A and SUR2B (Seino & Miki, 2003).

The SUR subunits are members of the ATP-binding cassette (ABC) superfamily. This superfamily has a variety of proteins with very different functions such as the Multidrug Resistance Protein (MRP) and Exporter (MDR) involved in drug resistance, the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) channel which is a chloride channel and the Heavy Metal Transporter (HMT) Family (Dean, 2005). Like most members of the ABC binding cassettes, the SUR subunits have several transmembrane domains and nucleotide binding domains (NBD) or folds (NBF) The most accepted proposed topology for the SURs involves three transmembrane domains, TMD0, TMD1 and TMD2, with respectively 5, 6 and 6 transmembrane regions. The protein also has a very hydrophobic extracellular segment at the N terminus and intracellular C-terminus. The nucleotide binding domain, NBD1, is located in the intracellular loop between TMD1 and TMD2, while NBD2 in the C-terminal region. Each nucleotide binding domain contains two nucleotide binding motifs, the Walker A and Walker B sequence. The highly conserved amino acid sequence between the SUR proteins and the ABC family are the Walker A and B motifs, and a conserved (LSGGQ) sequence in the segment linking the two Walker motifs (Walker *et al.*, 1982).

2-B) Regulation of K_{ATP} channel activity

2-B-i) Inhibition of K_{ATP} Channel by ATP and ADP

As previously mentioned, the K_{ATP} channel was named for the inhibitory effect of ATP, which acts to reduce channel activity via an allosteric effect. Photoaffinity labeling has shown that ATP binds directly to the Kir6.2 subunit to close the pore of the channel. The ATP binding site is located in the upper part of the intracellular domain, approximately 17 Å below the plane of the membrane. The position of this site facilitates the access to cytosolic ATP (Antcliff *et al.*, 2005). The phosphate tail of ATP electrically and simultaneously interacts with two Kir6 subunits. The α-phosphate interacts with an arginine residue at position 201 (R201) and the β-phosphate interacts with a lysine residue at position 185 (K185) in the C-terminus of one Kir6 subunit. The γ-phosphate interacts with an arginine at position 50 (R50) in the N-terminus of the adjacent Kir6 subunit. The N6 atom of the adenine ring interacts with a glutamate residue at position 179 (E179) and an arginine at position 301 (R301) in the same subunit that interacts with the γ-phosphate. The ATP-binding site on Kir6.*x* is unusual in that it does not require Mg^{2+} (Ashcroft & Kakei, 1989) and shows high selectivity for the adenine base (Tucker *et al.*, 1998). As there are four Kir6.2 subunits forming the pore, there are also four ATP-binding pockets. Since ATP binds to two subunits and considering that each subunit interacts with two other subunits, the binding of one ATP can influence the relative conformation of two adjacent subunits. This explains why binding of a single ATP molecule is sufficient to close the pore (Markworth *et al.*, 2000). ADP also binds to the Kir6.2 subunit in the absence of Mg^{2+}, however ADP has a much lower affinity than ATP. Like for ATP, the binding of ADP closes the channel (Markworth *et al.*, 2000).

In reconstitution experiments, the ATP-sensitivity of Kir6.2 together with SUR1 is 10-20 fold greater than Kir6.2 alone (from $K_i \sim 100$ μM to $K_i \sim 10$ μM) (Tanabe *et al.*,

TABLE 1-1

K_{ATP} Channel	K_i for ATP (μM)
Kir6.2	100-200
Kir6.2/SUR1	10
Kir6.2/SUR2A	100
Kir6.2/SUR2B	68
Kir6.1/SUR2B	0.1

Table 1-1: Half-maximal inhibition of ATP. Half-maximal inhibition (K_i) values are shown for various K_{ATP} channel heteromultimers.

2000). Thus, the interaction with SUR increases the channel sensitivity to the inhibition by ATP. The ATP-sensitivity varies even more among tissues depending on combinations of Kir and SUR subunits (See Table 1-1). For example, the half-maximal inhibition of ATP for the Kir6.2/SUR2A in cardiac cells is 10-times greater than that of the Kir6.2/SUR1 complex in the pancreas. Consequently, 10-times more ATP is required to close 50% of K_{ATP} channels in cardiac muscle than those in the pancreas.

2-B-ii) Activation of K_{ATP} channel by MgADP

While the inhibitory effects of Mg^{2+}-independent ATP and ADP appear to be mediated via the pore-forming subunit, Kir6.2, the activation effect of Mg^{2+}-nucleotide diphosphates, such as MgADP, is conferred to the sulfonylurea receptor subunit of the channel (Tucker *et al.*, 1998). The binding of MgADP to SUR increases channel activity by reducing the inhibitory effect of ATP on the Kir subunit (Dunne *et al.*, 1986; Misler & Petersen, 1986). Interestingly, MgADP binds to the NBD2 while another ATP binds to the NBD1. When ATP/ADP ratio decreases, NBD1 binds ATP and NBD2 binds MgADP (Gribble *et al.*, 1997). In this conformational state, the interaction of SUR1 with Kir6.2 reduces the affinity of Kir6.2 to ATP, which allows the opening of the channel. In contrast, when the ATP/ADP ratio increases, the decrease in MgADP induces dissociation of bound MgADP from NBD2, which then results in the release of ATP from NBD1. As the release of MgADP and ATP occurs from SUR1, the Kir6.2 affinity for ATP increases (Ueda *et al.*, 1999).

In pancreatic β-cells, the regulation of the K_{ATP} channel by the ATP/ADP ratio appears sufficient (Aguilar-Bryan & Bryan, 1999). However, in other tissues, such as cardiac and skeletal muscles, the changes in ATP/ADP ratio appears insufficient to allow

for K_{ATP} channel activation. Since the intracellular ATP concentration is generally in the millimolar range, the open probability (P_{open}) of K_{ATP} channels in the resting state is normally very low. Furthermore, the intracellular concentration of ATP in muscle being 5–10 mM at rest, changes little during any metabolic stress such as sustained activity (Carlson & Siger, 1960). This concentration is several-fold higher than the intracellular ATP concentration required to have half the K_{ATP} channels close in skeletal muscle (0.1 mM) (Spruce *et al.*, 1987). Even a small increase in ADP is insufficient for any impact on activity, indicating that the P_{open} of K_{ATP} channels would be low even during sustained activity. Despite differences in the above-mentioned K_i values and physiological ATP levels, there is evidence that K_{ATP} channels are indeed activated during metabolic stress such as ischemia, metabolic inhibition, and fatigue.

2-B-iii) Regulation of K_{ATP} Channel by other metabolites

During muscular activity leading to fatigue, the intracellular pH (pH_i) of skeletal muscle fibers decreases as lactic acid is produced (Cady *et al.*, 1989). Cady *et al.* (1989) showed that pH_i in human muscle decreases from 7.0 to 6.5 during fatiguing exercise. In some studies decreases in pH_i of almost 1 pH unit have been reported (Pan *et al.*, 1988; Renaud, 1989). When this change in pH_i is mimicked in unfatigued frog muscle it results in the activation of K_{ATP} channels (Davies *et al.*, 1992). Under patch-clamp conditions, H^+ has no effect on P_{open} and channel conductance in the absence of ATP (Davies *et al.*, 1992). However, in the presence of 0.5 mM ATP, a drop in pH_i increased the P_{open} of the K_{ATP} channel. One mechanism for the H^+ effect is a reduction in affinity for ATP. The K_i for ATP at pH_i 7.2 is 17 μM and increases to 260 μM at pH_i 6.3 (Davies *et al.*, 1992). Therefore, lowering pH_i in resting muscle with normal ATP levels activates K_{ATP}

channels, giving evidence that one mechanism that activates the channel during fatigue is pH.

Lactic acid increases from 1-2 mM at rest to over 20 mM in fatigued muscle (Needham DM, 1971). It is not known if lactate affects the K_{ATP} channel activity in skeletal muscle. However, in cardiac muscle, lactate increases K_{ATP} channel activity (while pH_i is constant). At 20-40 mM, it activates K_{ATP} channels in the presence of inhibiting ATP concentrations (2-5 mM) (Keung & Li, 1991). Furthermore, lactate shortens the action potential duration of ventricular myocytes, which is an effect similar to those of K_{ATP} channel openers such as pinacidil and cromakalim (Nichols & Lederer, 1991). Finally, Keung & Li (1991) demonstrated that all lactate effects are abolished by the administration of glibenclamide, a K_{ATP} channel blocker. The mechanism of action for lactate on K_{ATP} channel activity has not been studied, but these studies demonstrate that lactate is, with pH, a second metabolite that can activate the channels in muscle during metabolic stress leading to a decrease in energy level.

During bouts of fatiguing exercise, interstitial adenosine increases significantly. Lott *et al.* (2001) showed resting concentrations of adenosine to be in the 0.25 μM range increasing to approximately 1.1 – 1.2 μM during work at 60% of maximum. Adenosine released from active muscle can open K_{ATP} channels (Comtois *et al.*, 1994). Adenosine receptors are ubiquitous throughout the human body and have been shown to be present in the sarcolemmal membrane of skeletal muscle fibers (Challis *et al.*, 1992). The activation of K_{ATP} channels by adenosine had been demonstrated in cardiac (Kirsch *et al.*, 1990) and skeletal muscle via both A_1 and A_{2A} receptors (Barrett-Jolley *et al.*, 1996). Activation of K_{ATP} channels by adenosine requires a G-protein signaling pathway, GTP,

and hydrolyzable ATP, suggesting that adenosine activation of KATP channels may involve a G-protein dependent phosphorylation (Barrett-Jolley *et al.*, 1996).

Overall, the regulation of the KATP channel activity involves a complex mechanism including several factors. Of importance to this study is the response of the channel to a change in energy level. ATP deficit during a metabolic stress such as muscle fatigue is always associated with increases in ADP, H^+, lactic acid and adenosine, all of which can activate the KATP channel under ATP inhibitory conditions. Most importantly, these activation characteristics allow for the coupling of the electrical activity of the cell membrane to the energy state of the cell.

2-C) Physiological Role of the KATP Channel

The coupling of electrical activity and energy state by KATP channels has different roles in various tissues. The functions of the KATP channel can be grouped into two major categories: 1) glucose homeostasis and ii) cytoprotection. In terms of glucose homeostasis, the KATP channel regulates insulin secretion by pancreatic β-cells (Miki & Seino, 2005), glucagon secretion by pancreatic α-cells (Miki & Seino, 2005) and also via the glucose-sensory neurons in the hypothalamus (Miki & Seino, 2005). It also modulates glucose uptake in skeletal muscle (Minami *et al.*, 2004). Here only the function of cytoprotection will be discussed as it is the focus of this thesis.

2-C) Cytoprotection

KATP channels are involved in protection of the cell against irreversible cell damage brought on by ATP depletion (Ferrari *et al.*, 1993). This myoprotection occurs at two different levels. The first level involves the KATP channel in vascular smooth muscle cells, that once activated allow for vasodilatation of blood vessels in active tissues

(Vanelli & Hussain, 1994). This gives rise to greater delivery of energy supplies. The second level of cytoprotection is at the cellular level in neurons, cardiac and skeletal muscle cells. KATP channels act here to shut down activity of the cellular machinery to preserve energy. Here the discussion will be limited to cardiac and skeletal muscle.

2-C-i-a) Cardiac Muscle

Cardiac KATP channels are formed by the Kir6.2 and SUR2A subunits (Table 1-2) (Ashcroft & Ashcroft, 1990). They open in the absence of ATP and are closed by micromolar concentrations of ATP (Nichols & Lederer, 1991). Opening the KATP channel increases permeability allowing for greater K^+ efflux. The direct and largest effect of the KATP channel is to shorten the action potential (AP) duration by accelerating the repolarization phase due to greater K^+ conductance (Gasser & Vaughan-Jones, 1990; Sauviat *et al.*, 1991). For example, in cardiac cells action potential duration lasts 400 msec, but can decrease to 100 msec upon activation of KATP channels (Faivre & Findlay, 1990; Gasser & Vaughan-Jones, 1990). Noma (1983) had suggested that KATP channels are important for the protection of cellular energy metabolism via a control of membrane excitability.

Since then other physiological roles for KATP channels have been found such as the maintenance of cardiac cellular homeostasis, especially in the adaptive reaction to stress. One of these functions involves the response to catecholamine surges during stress associated with treadmill running (Liu *et al.*, 2004; Zingman *et al.*, 2002). During treadmill running large amounts of epinephrine and norepinephrine are released by sympathetic neurons close to the heart and by the adrenal glands. One function of these

TABLE 1-2

Sulphonylurea Receptor	Inward Rectifier	Tissue
SUR1	Kir6.2	Pancreatic β-cell/neuronal
SUR2A	Kir6.2	Cardiac/skeletal muscle
SUR2B	Kir6.1	Vascular smooth muscle
	Kir6.2	Vascular smooth muscle

Table 1-2: KATP channel isoforms. KATP channels are composed of Kir6.*x* and SUR subunits. Shown are the major isoforms present in each tissue.

catecholamines is to increase heart rate. For the heart rate to increase, action potential duration must decrease. In Kir6.2$^{-/-}$ mice, which have no K_{ATP} channels in the heart, the reduction in action potential duration is not observed under the influence of catecholamines. This results in impaired cardiac performance and death in 73% of Kir6.2$^{-/-}$ mice during treadmill running because of lethal arrhythmia (Zingman *et al.*, 2002). It also appears that K_{ATP} channels are important in the Ca^{2+} handling of cardiac cells: Kir6.2$^{-/-}$ mice experience disrupted Ca^{2+} handling due to defective control of cardiac membrane excitability which results in Ca^{2+} overload and cardiac damage. Interestingly, when verapamil, a Ca^{2+} channel blocker, was used on Kir6.2$^{-/-}$ mice, mortality decreased from 73% in untreated Kir6.2$^{-/-}$ mice to 17% in verapamil-treated Kir6.2$^{-/-}$ mice (Zingman *et al.*, 2002). Furthermore, studies by Kane *et al.* (2004) demonstrated that the repetitive stress of swimming increased mortality in the Kir6.2$^{-/-}$ mice associated with pathologic Ca^{2+}-dependent structural damage in the heart and impaired cardiac performance. Therefore, under physiological stress conditions, the K_{ATP} channel plays an important role in the increased cardiac activity induced by catecholamines and in cytoprotection against damaging Ca^{2+} overload.

The K_{ATP} channel of cardiac muscle has also been extensively studied under hypoxia and ischemia. Under these conditions, several events occur at the level of the cell membrane, including an increased K^+ conductance causing a shortening of the action potential (Faivre & Findlay, 1990; Gasser & Vaughan-Jones, 1990). Contrary to the situation with catecholamines during exercise, shortening of action potential duration is important to reduce Ca^{2+} release from the SR and force production to preserve ATP. Indeed blocking K_{ATP} channels during ischemia reduces the extent of force decrease,

while opening the channels increase the extent (Weiss *et al.*, 1992; McPherson *et al.*, 1993). These effects can be mimicked by the addition of KATP channel openers. During ischemia, Ca^{2+} increases within the cell (Allen & Xiao, 2003). This increased Ca^{2+} load produces a strong hypercontracture of the cardiac muscle. Furthermore, Ca^{2+} together with damaging metabolites such as reactive oxygen species leads to mechanical damage of the myocardium (Ganote, 1983; Piper *et al.*, 2003; Piper *et al.*, 2004). Baczko *et al.* (2004) showed that activation of KATP channels can prevent this damaging Ca^{2+} overload via a mechanism that is dependent on hyperpolarization of diastolic membrane potential, as well as by reducing the action potential duration which reduces extracellular Ca^{2+} influx through L-type Ca^{2+} channels (Lascano *et al.*, 2002). Thus, under both physiological and pathological conditions, the KATP channel is vital in preventing fiber damage mainly from Ca^{2+} overload. Although some studies have shown some effects on ATP preservation it appears that Ca^{2+} regulation is the most important.

2-C-i-b) Skeletal Muscle

Skeletal muscle expresses mainly the SUR2A and Kir6.2 subunits of the KATP channel (Seino, 1999). This was further supported by the fact that Kir6.2$^{-/-}$ and SUR2$^{-/-}$ mice have no KATP channel activity in muscle (Seino *et al.*, 2000). A more recent study, however, has given evidence that some, but not all, muscle fibers also express other SUR subunits. All muscles expressed Kir6.1 and 6.2, and SUR2A and 2B mRNA transcripts. In most muscles, however, the Kir6.1 and SUR2B may be coming from vascular smooth muscle KATP channels, as drugs that specifically target SUR2B have no effect on membrane current in skeletal muscle fiber. However, some muscle expressed higher mRNA levels for SUR1 and SUR2B. The presence of SUR1 and SUR2B in some fibers

was confirmed with drugs that selectively target SUR1, SUR2A and SUR2B. Overall FDB expressed all three isoforms, SUR1, SUR2A and SUR2B. Evidence for a hybrid assembly of SUR2A and SUR2B was found in some but not all fibers of soleus and EDL muscle (Tricarico *et al.*, 2006). Consequently, different muscles will have different responses to ATP inhibition and metabolic stresses (Flagg & Nichols, 2005). Many of their properties, including ATP inhibition, reduction of ATP inhibition by ADP, activation by adenosine, kinetics, conductance, and sensitivity to KATP openers at relatively high concentrations, are quite similar to those of cardiac sarcolemmal KATP channels (Forestier *et al.*, 1996; Davies *et al.*, 1991; Allard & Lazdunski, 1993; Forestier & Vivaudou, 1993; Barrett-Jolley *et al.*, 1996).

As discussed in the section entitled "Regulation of KATP channel by other metabolites," skeletal muscle undergoes large changes in metabolites during fatigue and many of these changes activate KATP channels. That is when an energy deficit develops, a number of metabolites such as ADP, H^+, adenosine, and lactic acid levels increase in concentration. The KATP channels are almost entirely closed at rest, but open in response to these metabolic changes (Light *et al.*, 1994; Matar *et al.*, 2000). KATP channels, being located in the cell membrane, exert their immediate effects at the level of the cell membrane excitability. Two mechanisms exist by which KATP channels can affect membrane excitability. A first mechanism involves an increase in K^+ efflux (Matar *et al.*, 2000) resulting in an increase of $[K^+]_e$ (Renaud, 2002). However, as discussed in the role of membrane excitability in fatigue, it is no longer clear how an increase in $[K^+]_e$ affects muscle contraction.

The second mechanism is better understood. It involves an effect on the action potential itself. Upon activation, the KATP channel remains opened at all membrane potentials as its rectifying properties are very weak (Noma, 1983; Spruce *et al.*, 1987). So during an action potential, KATP channels counteract the depolarizing effect of Na^+ giving rise to lower action potential amplitude (Gong *et al.*, 2003). They also contribute to the repolarization phase, albeit a shortening of the action potential has only been reported during metabolic inhibition (Gramolini & Renaud, 1997). The decrease in action potential amplitude is the most likely mechanism by which Ca^{2+} release and hence force production is reduced in skeletal muscle. Indeed, activation of KATP channels with channel openers is associated with faster decrease in SR Ca^{2+} release in rat FDB muscle during fatigue and metabolic poisoning (Duty & Allen *et al.*, 1995; Burton & Smith, 1997). As less Ca^{2+} is released, the decrease in force during fatigue is also faster (Matar *et al.*, 2000; Gong *et al.*, 2003). Recent studies in this laboratory has now given evidence that these KATP channel effects may be important in preventing large ATP depletion (Li & Renaud, unpublished results), probably because of less activity of the Ca^{2+}- and myosin-ATPases.

Therefore, if activation of KATP channels increases the rate at which Ca^{2+} release and tetanic force drop during fatigue, blocking KATP channel activity must slow down the decline of Ca^{2+} release and force production during fatigue. Indeed, exposing muscles to channel blockers during fatigue and metabolic inhibition results in an immediate increase in Ca^{2+} release and force development (Duty & Allen, 1995; Gramolini & Renaud, 1997). These initial increases, however, are short-lived as they are followed by decreases that are faster in muscles with normal KATP channel activity. If glibenclamide is added prior to

metabolic inhibition, then the decrease in force is faster than in the absence of glibenclamide (Gramolini & Renaud, 1997). However, except for the studies described above, most studies fail to report an effect on the decrease in tetanic Ca^{2+} and force when KATP channel activity is abolished pharmacologically or genetically (Weselcouch *et al.*, 1993; Light *et al.*, 1994; Duty & Allen *et al.*, 1995; Comtois *et al.*, 1995; Van Lunteren *et al.*, 1998; Gong *et al.*, 2000; Matar *et al.*, 2000). The lack of an effect has raised questions as to whether the channel is indeed active or if it really affects force.

A lack of activity during fatigue is most likely not the reason because several functional impairments occur during fatigue when there is no KATP channel activity. These impairments include increased resting tension, poor force recovery (Light *et al.*, 1994; Gong *et al.*, 2000; Matar *et al.*, 2000), membrane depolarization (Gramolini & Renaud, 1997), and fiber damage (Thabet *et al.*, 2005; Bourassa & Renaud, unpublished results). More importantly, the impairments are the same in magnitude whether KATP channels are abolished pharmacologically or genetically; i.e. they are not due to any non-specific effect of glibenclamide or some other physiological compensation associated with the disruption of the Kir6.2 gene (a global knockout) in skeletal muscle itself or in other tissues.

So, on the one hand, abolishing KATP channel activity removes their effect on action potential amplitude, which should give rise to a slower fatigue rate. On the other hand, the function impairments are expected to give rise to a faster fatigue rate. It is therefore quite surprising that abolishing KATP channel activity has exactly no effect on fatigue rate. The major problem with studying muscle function *in vitro* has been the hypoxic/hypoglycemic core. It has been shown that whole skeletal muscle fatigues more

rapidly than single muscle fibers, and this effect disappears when mitochondrial respiration is eliminated in single fibers with cyanide (Zhang *et al.*, 2006). This suggests that the limited oxygen diffusion is accelerating fatigue development in skeletal muscle *in vitro*. The first objective therefore entails the use of a new muscle preparation to avoid this hypoxic core.

2-C-i-c) Is Ca^{2+} involved in fiber damage?

Ca^{2+} is an important messenger in skeletal muscle, capable of activating a wide range of processes. Ca^{2+} overload, defined as an excessive accumulation of Ca^{2+} in a cell resulting in an inability of the cell to control $[Ca^{2+}]_i$, is a serious condition involved in a wide range of situations leading to cell damage in several tissues. The Ca^{2+}-induced damage may be due to three possible mechanisms. Increases in $[Ca^{2+}]_i$ may result in activation of Ca^{2+}-activated neutral proteases (calpains) (Belcastro, 1993; Belcastro, 1998; Goll *et al.*, 2003), activation of phospholipase A_2 (Nethery *et al.*, 2000; Bruton *et al.*, 1998; Jackson *et al.*, 1984; Duncan & Jackson, 1987), and/or an increased production of reactive oxygen species (ROS) (Reid, 2001; Nethery *et al.*, 2000; Xu *et al.*, 1997; Davies *et al.*, 1982; Rajguru *et al.*, 1994). It is therefore vital for muscle cells to tightly control $[Ca^{2+}]_i$, a function performed by the combined action of the Ca^{2+}-channels and Ca^{2+}-ATPase in the cell membrane, SR, as well as the mitochondria. Any dysfunction of these channels or pumps can lead to Ca^{2+} overload.

As discussed in the section on cardiac muscle, K_{ATP} channels play an important role in preventing Ca^{2+} overload during physiological stress such as swimming and treadmill running, and during pathological stress such as ischemia. A regulation of Ca^{2+} by K_{ATP} channels has not been demonstrated in skeletal muscle. However, resting tension

and fiber damage are indirect evidence for a role of Ca^{2+}. Bourassa & Renaud (unpublished results) have recently observed large increase in resting Ca^{2+}, especially in fibers that become damaged during fatigue. It is therefore likely that the K_{ATP} channel also play a major role in the regulation of Ca^{2+} in skeletal muscle like it does in cardiac muscle. This constitutes the second objective of this thesis.

3) OBJECTIVES AND HYPOTHESES

This study has two major objectives. The first objective (Chapter 3 Part I)) is to determine how K_{ATP} channels affect force during fatigue by using smaller muscle preparations. Based on the many function impairments in the absence of K_{ATP} channel activity versus the one single effect of the action potential, it is hypothesized "that the functional impairment should override the expected slower rate of fatigue, so the rate of fatigue is faster in K_{ATP} channel deficient muscle compared to control."

The second objective (Chapter 3 Part II) is to determine if the K_{ATP} channel prevents Ca^{2+} overload, by testing the hypothesis that "the functional impairment of skeletal muscle in K_{ATP} channel deficient mice is due to an increased Ca^{2+} influx through L-type Ca^{2+} channels."

In a parallel study, Bourassa & Renaud (unpublished results) demonstrated that the decrease in peak Ca^{2+} during a second fatigue bout (FAT2) in single FDB muscle fibers under control conditions is much slower than during a first fatigue bout (FAT1) elicited 60 min before FAT2. They also showed that the increase in resting $[Ca^{2+}]_i$ during fatigue was less during FAT2 than during FAT1, especially in the presence of glibenclamide. It therefore appears that after one fatigue bout, the fatigue resistance of FDB single fibers improves while the function impairment in the absence of K_{ATP}

channel activity disappears. In their study, Bourassa & Renaud could not measure force because their single fibers had no tendon to attach them to a transducer after the collagenase treatment to disperse them. So, a third objective was added to this thesis to test the hypothesis "that the fatigue resistance as measured from tetanic force is increased and the function impairment in the absence of K_{ATP} channel disappears after one fatigue bout."

CHAPTER 2

METHODS AND MATERIALS

ANIMALS AND FDB MUSCLE BUNDLE PREPARATION

Two to three month old wild-type CD1 (from Charles River, Canada) and Kir6.2$^{-/-}$ mice all weighing 20-30 g were fed *ad libitum*, and housed according to the guidelines of the Canadian Council for Animal Care (CCAC). The Animal Care Committee of the University of Ottawa approved all experimental procedures used in this study. Mice were anesthetized with a single intraperitoneal injection of 2.5 mg ketamine, 0.5 mg xylazine, and 0.25 mg acepromazine per 10 g of body weight.

The FDB muscle is made up of three major bundles of muscle fibers that control via individual tendons the 2nd, 3rd and 4th digit of the paw (Fig. 2-1). All experiments with FDB muscle bundles were carried out by excising the bundle of fibers controlling the 4th digit, by cutting along and very close to the lateral fascia separating the muscles fibers of the 3rd and 4th digit; note that the cut was separating fibers from the 3rd digit away from the fascia, leaving the fibers of the 4th digit intact. On average, eight FDB bundles contained 350 ± 40 fibers (mean and standard error (SE)), while 42 bundles weighed 2.2 ± 0.1 mg and were 8.2 ± 0.1 mm long. Assuming a cylindrical shape and a density of 1.06 g/cm^3, the average radius was 0.31 ± 0.01 mm and cross-section area 0.30 ± 0.01 mm^2. The myosin heavy chain (MHC) isoform composition of the FDB is 4% type I, 60% type IIA, 34% type IIX, and 2% type IIB (Raymackers *et al.*, 2000). Therefore, the FDB is mainly a fast-twitch muscle, and has been used extensively for Ca^{2+} measurements (Allen *et al.*, 2002; Westerblad *et al.*, 1997). As well, the FDB was the only muscle to give viable contracting single fibers.

FIGURE 2-1

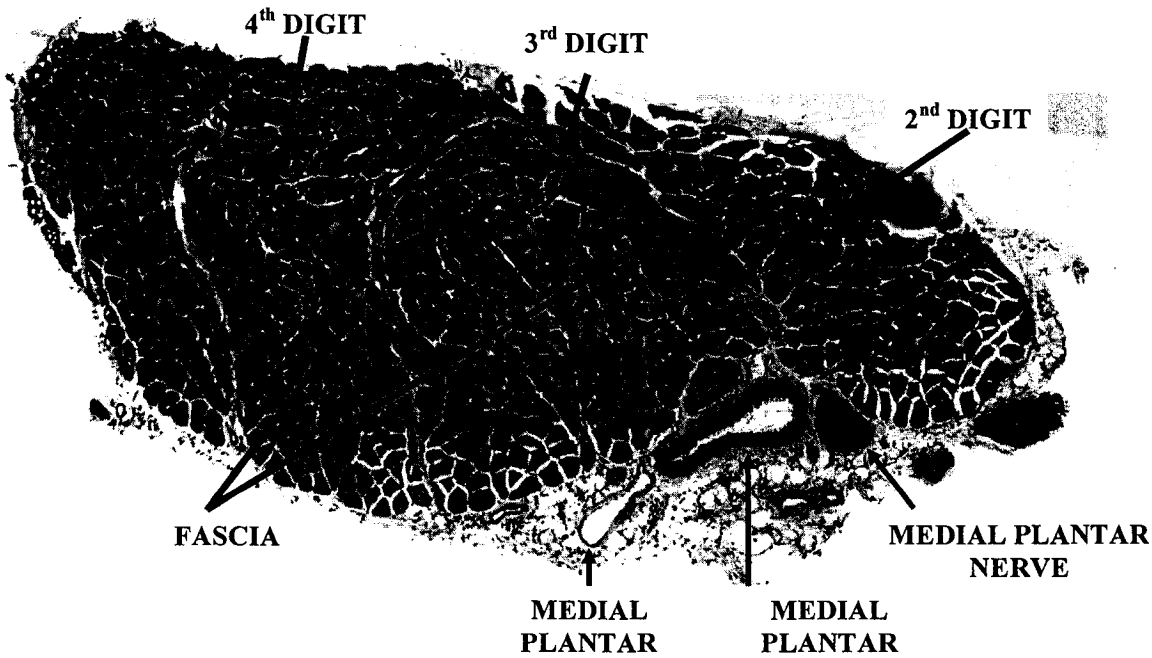

Figure 3-1. Anatomy of the left FDB muscle. The FDB was stained with hematoxylin and eosin. The FDB has three major fiber bundles controlling the movement of the 2nd, 3rd and 4th digit. The fiber bundle controlling the 4th digit was separated by cutting along the fascia on the side where muscle fibers of the 3rd digit are located. The bundle was recognized as being on the opposite side of the medial plantar artery (Banas and Renaud, unpublished results).

SOLUTIONS

During an experiment, FDB bundles were constantly immersed in physiological saline solution. The control solution contained (in mM): 118.5 NaCl, 4.7 KCl, 2.4 $CaCl_2$, 3.1 $MgCl_2$, 25 $NaHCO_3$, 2 NaH_2PO_4, and 5.5 D-glucose. All solutions were continuously bubbled with 95% O_2-5% CO_2 and had a pH of 7.4. Glibenclamide-containing solutions, used to block K_{ATP} channels, were prepared by first dissolving glibenclamide in DMSO, which was then added to the control solution. The glibenclamide concentration was 10 μM, and the DMSO concentration was 0.1% (v/v) in all solutions (including control solutions). Solution containing 0.6 and 4.8 mM Ca^{2+} (instead of 2.4 mM) had respectively 4.9 and 0.7 mM Mg^{2+} (instead of 3.1 mM) to maintain osmolarity and ionic strength. Verapamil-containing solutions, used to partially block L-type Ca^{2+} channels, were prepared by dissolving verapamil directly in the control physiological solution to a final concentration of 20 μM. All experiments were carried out at 37°C.

FORCE MEASUREMENT

Excised FDB muscle bundle was placed in a horizontal fluid-filled chamber with one tendon in a fixed position and the other tendon attached to the lever arm of the force transducer. Solution flow rate was 10 mL per minute. Muscle length was adjusted to give maximal peak tetanic force which was monitored with a Cambridge ergometer (model 300, USA) or a Kulite force transducer (Model BD100, Canada), and digitized at 5 kHz by a Keithley Metrabyte A-D board (model DAS50, USA). Peak tetanic force, defined as the maximum force, was calculated as the difference between the maximum force during contraction and the baseline 5 ms before stimulation (Gong *et al.*, 2003). Resting tension, defined as the amount of tension exerted by a muscle bundle without stimulation (as it failed to relax between contractions) was calculated as the difference in baseline 5 msec

before stimulation during the fatigue period and the baseline prior to the fatigue period (Gong *et al.*, 2003).

STIMULATION AND FATIGUE PROTOCOL

After adjusting the muscle length, FDB bundles were allowed 30 min equilibrium in the presence of control conditions, 10 μM glibenclamide, 20 μM verapamil, 0.6, or 4.8 mM Ca^{2+}. During that time, tetanic contractions were elicited every 100 s with 200 ms train of 0.3 ms, 8 V (supramaximal voltage) pulses at 200 Hz. Pulses were generated with a Grass S88 stimulator and a Grass SIU5 isolation unit (Grass Instruments, USA) and delivered using two platinum wires located on opposite sides of the bundles. Fatigue was elicited with the same tetanic contraction but every second for 3 min under the various conditions. After fatigue, muscles were stimulated 10, 20, 100, and every 100 s thereafter to measure force recovery.

STATISTICAL ANALYSIS

Analysis of Variance (ANOVA) was used to determine significant differences. Split plot designs were used because muscles were tested at all time levels. ANOVA calculations were made using the Version 9.0 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 (Steel & Torrie, 1980). The word "significant" refers only to a statistical difference ($P<0.05$).

CHAPTER 3

IMPAIRMENT OF FORCE DEVELOPMENT DURING FATIGUE IN K_{ATP} CHANNEL DEFICIENT MUSCLES INVOLVES Ca^{2+} INFLUX THROUGH L-TYPE Ca^{2+} CHANNELS

INITIAL FORCE

The peak tetanic force of 24 wild-type FDB was 53.4 N/cm^2 compared to 49.6 N/cm^2 for 14 Kir6.2$^{-/-}$ FDB. In this study, FDB muscles were always equilibrated 30 min under the different experimental conditions before fatigue was elicited. For all glibenclamide, verapamil, low and high Ca^{2+}, the decrease in force during the 30 min equilibration was always the same as those under control conditions (Table 3-1). It is therefore suggested that any effects of these treatments during fatigue are not related to a difference in peak tetanic force before fatigue.

EFFECT OF K_{ATP} CHANNEL INACTIVATION ON TETANIC FORCE

A significant difference between wild type and K_{ATP} channel deficient FDB muscle is observed during fatigue. When wild type FDB bundles are fatigued under control conditions with 1 tetanic contraction per second, the drop in peak tetanic force, defined as the difference in force between baseline (just before the contraction) and the maximum force (during the contraction), is more pronounced over the duration of fatigue in Kir6.2$^{-/-}$ FDB (Fig. 3-1). Additionally, the rise in resting tension, defined as the difference in tension between the baseline just before the contraction during fatigue and the baseline just before fatigue is elicited, is also more evident during fatigue in Kir6.2$^{-/-}$ FDB.

TABLE 3-1

	WT	Kir6.2$^{-/-}$
CON (%)	- 4.3 ± 0.21	- 6.2 ± 0.32
10 μM Glib (%)	- 5.3 ± 0.29	
0.6 mM Ca^{2+} (%)	- 8.5 ± 0.45	- 13 ± 0.82
4.8 mM Ca^{2+} (%)		- 5.8 ± 0.67
20 μM Verap (%)	- 6.3 ± 0.39	- 8.5 ± 0.43

Table 3-1. Effect of different treatments on peak tetanic force. After adjustment of muscle length to give maximum peak tetanic force, FDB muscles were equilibrated 30 min in either control, 10 μM glibenclamide (Glib), 20 μM verapamil (Verap), 0.6 or 4.8 mM Ca^{2+} (before fatigue was elicited). The force after 30 min is expressed as a percentage difference of the force before the equilibrium period. S.E. of 24 wild-type and 14 Kir6.2$^{-/-}$ FDB muscle bundles.

No significant difference was observed, ANOVA and L.S.D. $P>0.05$.

FIGURE 3-1

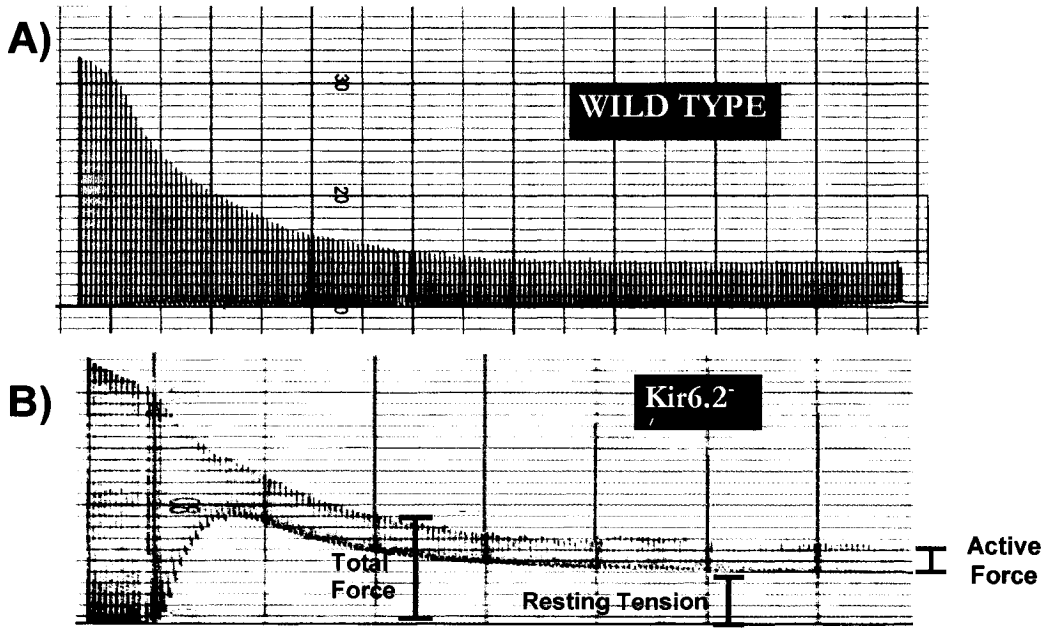

Figure 3-1. Recordings of tetanic force contractions during fatigue protocol of A) Wild-type and B) Kir6.2$^{-/-}$ mouse FDB muscle. Fatigue was elicited with one 200 ms tetanic contraction every sec for 3 min. Peak tetanic force is defined as the difference in force between baseline (just before the contraction) and the maximum force (during the contraction) as shown at the end of trace B. Resting tension is defined as the difference in tension between the baseline just before the contraction during fatigue and the baseline just before fatigue is elicited as shown in B. Notice that resting tension increased barely during fatigue in wild-type FDB and only toward the end of the fatigue period.

On an average basis, most of the decrease in peak tetanic force of wild-type FDB under control conditions occurred during the first 60 sec, the force at that time being 31% of the pre-fatigue force (Fig. 3-2A). During the last 120 sec of stimulation, peak tetanic force decreased another 10%. The decrease in peak force was significantly faster in the presence of glibenclamide. After 30 sec, peak forces in the absence and presence of glibenclamide were respectively 62% and 42%, representing a 20% difference. The difference between control and glibenclamide-exposed FDB bundles persisted until the end of fatigue period when the peak forces were respectively 21% and 11% of the pre-fatigue values.

Kir6.2$^{-/-}$ FDB bundles also fatigued faster than control wild-type ones (Fig. 3-2B). After 30 sec of stimulation the difference in peak force between wild type and Kir6.2$^{-/-}$ bundles was 23%, a difference similar to the one between control and glibenclamide-exposed wild type fibers (Fig. 3-2A). However, the difference between wild type and Kir6.2$^{-/-}$ fibers did not persist throughout the entire fatigue period as no significant difference was observed at 180 sec. Exposing Kir6.2$^{-/-}$ FDB bundles to 10 μM glibenclamide had no effect on peak tetanic force during fatigue compared to control Kir6.2$^{-/-}$ bundles (data not shown).

Resting tension, which increases when fibers fail to fully relax between contractions, increased very little in control wild type FDB bundles, reaching a maximum of 1.7% of the pre-fatigue peak tetanic force after 45 sec (Fig. 3-1A, 3-3A). Resting tension of Kir6.2$^{-/-}$ (Fig. 3-1B, 3-3B) and glibenclamide-exposed wild type bundles (Fig. 3-3A) increased sharply to 20-25% between the 15th and 30th sec of stimulation. It decreased slightly thereafter, but remained above 15% for the remainder of the fatigue

FIGURE 3-2

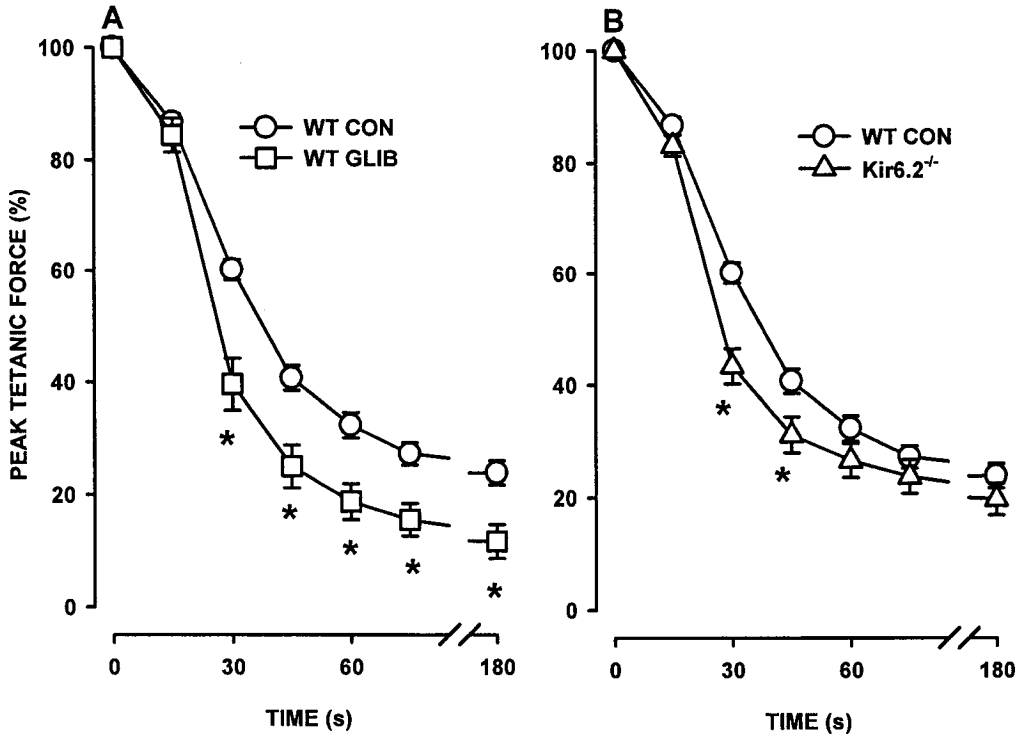

Figure 3-2. KATP channel deficient FDB muscle bundles had faster and greater decrease in tetanic force than wild type control FDB. Fatigue was elicited with one 200 ms tetanic contraction every sec for 3 min in the absence or presence of 10 μM glibenclamide (glib) for wild type (WT) FDB (A) or in the absence of glibenclamide for Kir6.2$^{-/-}$ FDB (B). Experimental temperature: 37°C. Peak tetanic force is expressed as a percent of the pre-fatigue force. Vertical bars represent the S.E. of 15 wild-type, 8 glibenclamide-exposed, and 13 Kir6.2$^{-/-}$ FDB muscles.

* Significantly different from wild type control FDB, ANOVA and L.S.D. $P < 0.05$.

FIGURE 3-3

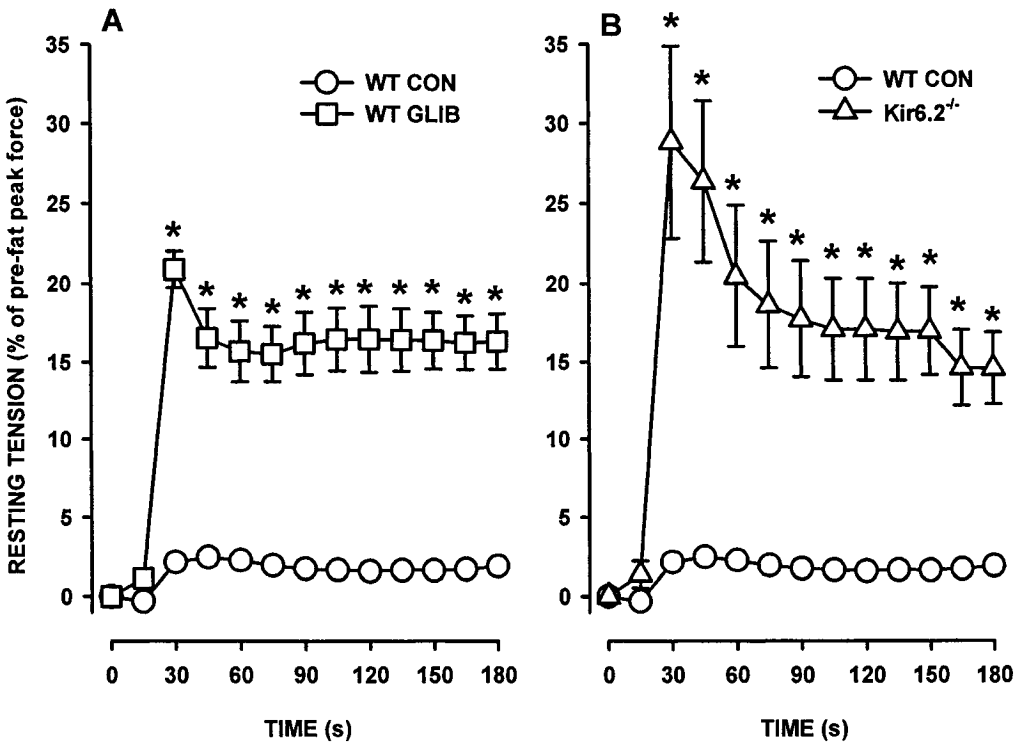

Figure 3-3. KATP channel deficient FDB muscle bundles developed more resting tension than wild type control FDB. Fatigue was elicited with one 200 ms tetanic contraction every sec for 3 min in the absence or presence of 10 μM glibenclamide (glib) for wild type (WT) FDB (A) or in the absence of glibenclamide for Kir6.2$^{-/-}$ FDB (B). Experimental temperature: 37°C. Resting tension is expressed as a percent of the pre-fatigue peak tetanic force. Vertical bars represent the S.E. of 15 wild-type, 8 glibenclamide-exposed, and 13 Kir6.2$^{-/-}$ FDB muscles.

* Significantly different from wild type control FDB, ANOVA and L.S.D. $P < 0.05$.

period. Exposing Kir6.2$^{-/-}$ FDB bundles to 10 μM glibenclamide had no effect on resting tension during fatigue compared to control Kir6.2$^{-/-}$ bundles (data not shown).

Following fatigue, peak tetanic force of control wild-type FDB recovered to 92% of the pre-fatigue value within 10 min (Fig 3-4A). The extent of force recovery was significantly less, for both Kir6.2$^{-/-}$ and glibenclamide-exposed muscle bundles, being only 73% (Fig. 3-4A, B). Exposing Kir6.2$^{-/-}$ FDB bundles to 10 μM glibenclamide had no effect on tetanic force recovery following fatigue compared to control Kir6.2$^{-/-}$ bundles (data not shown). Thus, compared to wild-type control FDB bundles, rates of fatigue were faster, resting tension greater, and force recovery less when K_{ATP} channel activity is abolished pharmacologically using glibenclamide or genetically using Kir6.2$^{-/-}$ muscles.

Taking into account the fiber damage observed in single fibers (Bourassa & Renaud, unpublished results), procion orange was added to the physiological solution to determine fiber damage in the whole muscle as discussed in Decrouy *et al* (1997). However, following *in vitro* force measurement, procion orange was only able to infiltrate a very small number of fibers in both wild-type and Kir6.2$^{-/-}$.

EFFECT OF $[Ca^{2+}]_e$ ON THE KINETICS OF FATIGUE

When the $[Ca^{2+}]_e$ was decreased from 2.4 mM (control solution) to 0.6 mM, the decrease in force during fatigue became significantly slower in Kir6.2$^{-/-}$ FDB bundles (Fig. 3-5A). For example after 30 sec, peak tetanic force was 56% at 0.6 mM compared to 38% at 2.4 mM Ca^{2+}, an 18% difference. The difference remained significant until the 75th sec. In wild type FDB, lowering $[Ca^{2+}]_e$ to 0.6 mM had no effect on how peak tetanic force decreased during fatigue (Fig. 3-5B).

FIGURE 3-4

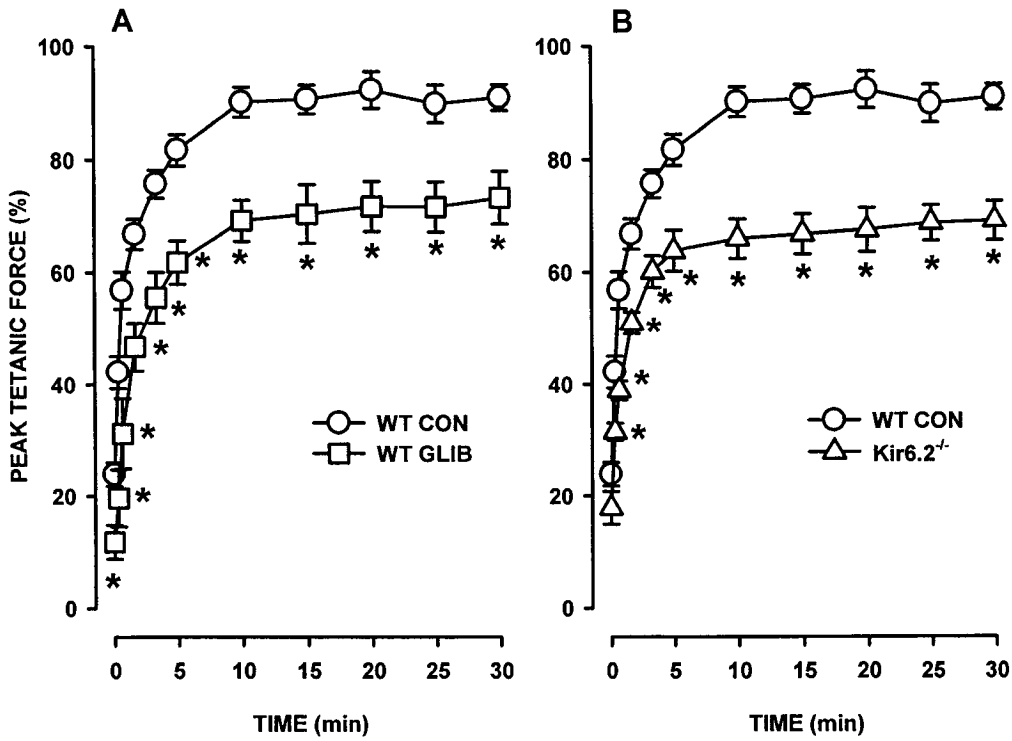

Figure 3-4. KATP channel deficient FDB muscle bundles recovered less force following fatigue than wild type control FDB. Fatigue was elicited with one 200 ms tetanic contraction every sec for 3 min in the absence or presence of 10 μM glibenclamide (glib) for wild type (WT) FDB (A) or in the absence of glibenclamide for Kir6.2$^{-/-}$ FDB (B). During recovery, tetanic contractions were elicited 10, 20, 100 sec after fatigue and then every 100 sec thereafter. Experimental temperature: 37°C. Peak tetanic force is expressed as a percent of the pre-fatigue force. Vertical bars represent the S.E. of 15 wild-type, 8 glibenclamide-exposed, and 13 Kir6.2$^{-/-}$ FDB muscles.

* Significantly different from wild type control FDB, ANOVA and L.S.D. $P < 0.05$.

FIGURE 3-5

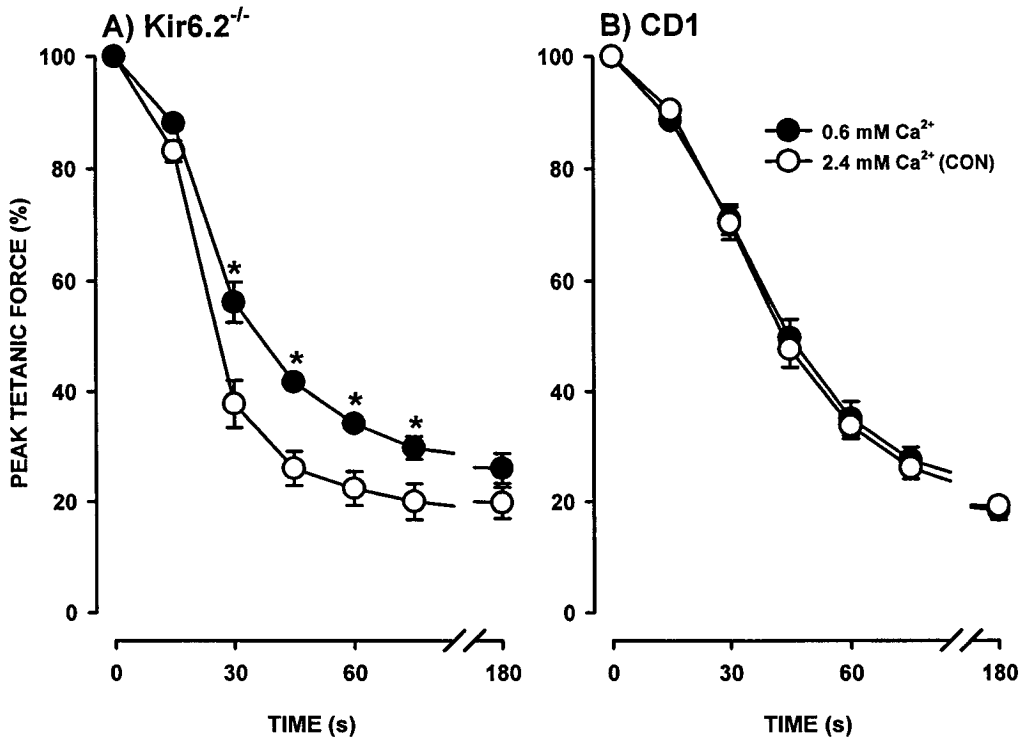

Figure 3-5. Kir6.2$^{-/-}$ FDB muscle bundles had slower decreases in tetanic force at 0.6 mM than at 2.4 mM Ca^{2+}. Fatigue was elicited with one 200 ms tetanic contraction every sec for 3 min. Experimental temperature: 37°C. Peak tetanic force is expressed as a percent of the pre-fatigue force. Vertical bars represent the S.E. of 5 wild-type and 4 Kir6.2$^{-/-}$ FDB muscles.

* Significantly different from control FDB, ANOVA and L.S.D. $P < 0.05$.

At both 0.6 and 2.4 mM Ca^{2+}, the absence of K_{ATP} channel activity increases the rate of fatigue compared to wild-type FDB. However, the differences between wild type and Kir6.2$^{-/-}$ FDB were much smaller at 0.6 mM Ca^{2+}. For example, after 60 sec, the difference in peak tetanic force between wild-type and Kir6.2$^{-/-}$ at 2.4 mM Ca^{2+} was 11%. This difference was reduced to <1% at 0.6 mM. Furthermore, the extent of fatigue (i.e., the force at 180 sec of fatigue) was not different between wild type and Kir6.2$^{-/-}$ FDB at 2.4 mM (Fig. 3-5), whereas the extent of fatigue at 0.6 mM Ca^{2+} was less for Kir6.2$^{-/-}$ than wild type FDB.

Reducing $[Ca^{2+}]_e$ to 0.6 mM reduced the development of resting tension during fatigue in Kir6.2$^{-/-}$ FDB muscle (Fig. 3-6A). While at 2.4 mM Ca^{2+}, resting tension reached a peak of 26%, it only reached 12% at 0.6 mM, a difference greater than two fold. Resting tension in Kir6.2$^{-/-}$ FDB at 2.4 mM remained above 14% for the remainder of the fatigue period, while at 0.6 mM Ca^{2+}, it decreased to 6%. A very small resting tension in wild type FDB was still observed at 0.6 mM, albeit there was a trend for lower resting tension at 0.6 than 2.4 mM Ca^{2+} between 30 and 90 sec.

Lowering $[Ca^{2+}]e$ to 0.6 mM significantly improved force recovery in Kir6.2$^{-/-}$ FDB muscle (Fig. 3-7A). For example, at 0.6 mM, peak tetanic force of Kir6.2$^{-/-}$ FDB muscle recovered to 95% of prefatigue force, which was significantly greater than at 2.4 mM Ca^{2+} at which recovery was only 70%. Interestingly, force recovery of Kir6.2$^{-/-}$ FDB at 0.6 mM Ca^{2+} was better than wild type FDB, which recovers to only 90% at both 0.6 mM and 2.4 mM Ca^{2+} (Fig 3.7B).

Thus, for Kir6.2$^{-/-}$ FDB, fatigue rates were slower, resting tension was less, and force recovery better when $[Ca^{2+}]_e$ was reduced from the control values of 2.4 mM to 0.6

FIGURE 3-6

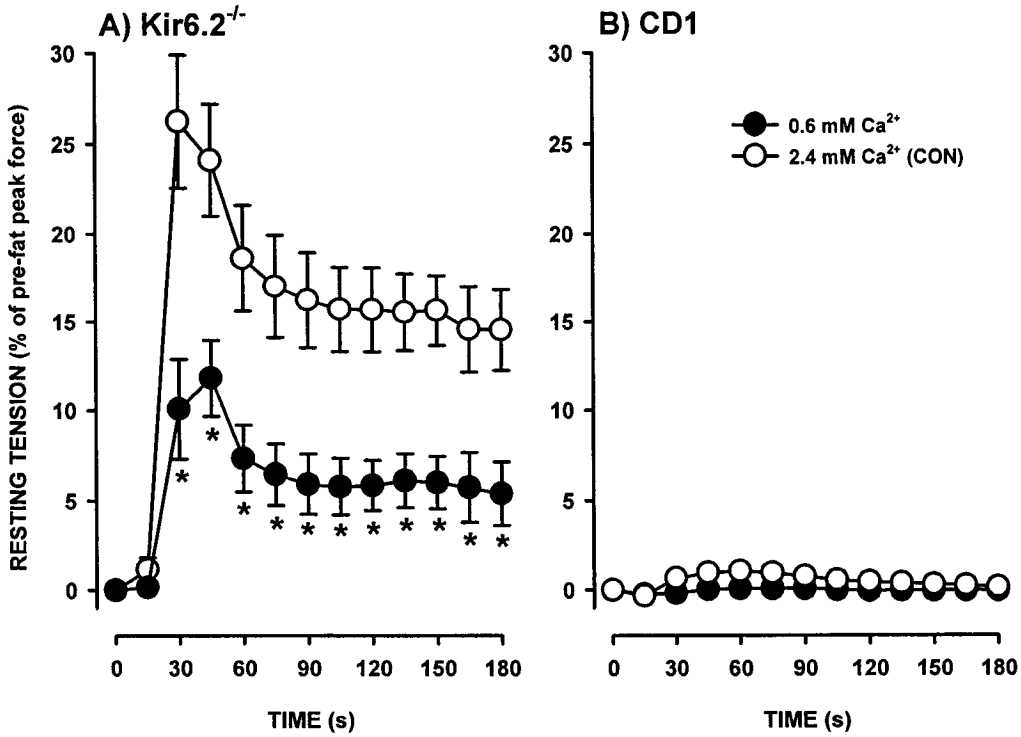

Figure 3-6. Kir6.2$^{-/-}$ FDB muscle developed less resting tension at 0.6 mM than at 2.4 mM Ca^{2+}. Fatigue was elicited with one 200 ms tetanic contraction every sec for 3 min. Experimental temperature: 37°C. Resting tension is expressed as a percent of the pre-fatigue peak tetanic force. Vertical bars represent the S.E. of 5 wild-type and 4 Kir6.2$^{-/-}$ FDB muscles.

* Significantly different from control FDB, ANOVA and L.S.D. $P < 0.05$.

FIGURE 3-7

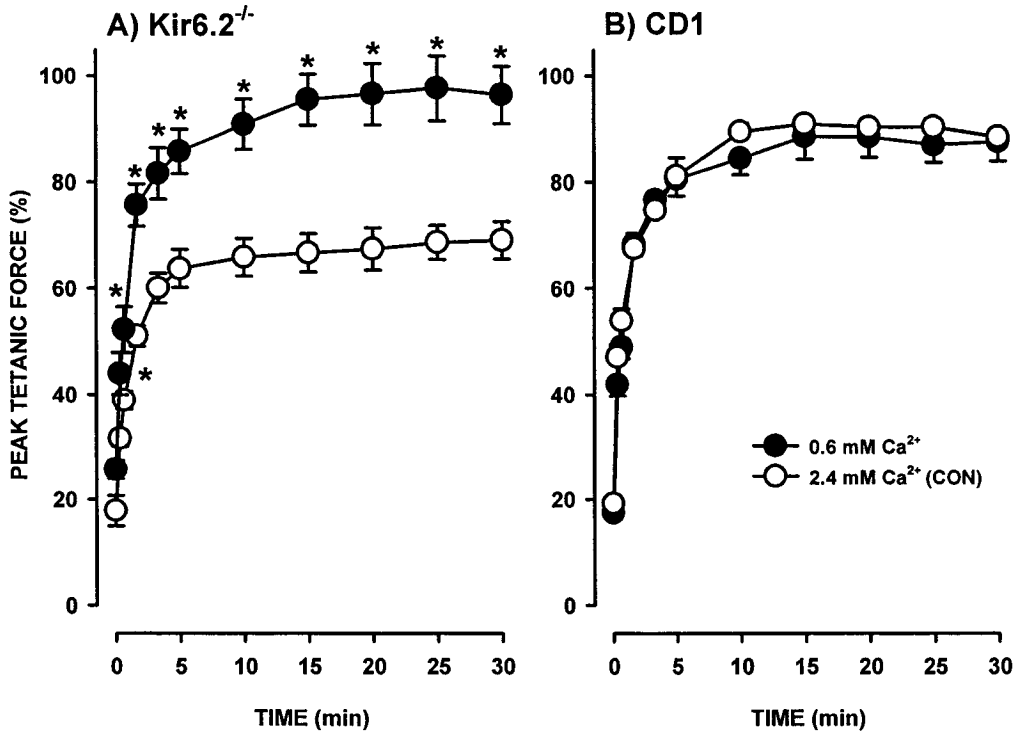

Figure 3-7. Kir6.2$^{-/-}$ FDB muscle recovered better following fatigue at 0.6 mM than at 2.4 mM Ca^{2+}. Fatigue was elicited with one 200 ms tetanic contraction every sec for 3 min. During recovery, tetanic contractions were elicited 10, 20, 100 sec after fatigue and then every 100 sec thereafter. Experimental temperature: 37°C. Peak tetanic force is expressed as a percent of the pre-fatigue force. Vertical bars represent the S.E. of 5 wild-type and 4 Kir6.2$^{-/-}$ FDB muscles.

* Significantly different from control FDB, ANOVA and L.S.D. $P < 0.05$.

mM. A similar reduction in $[Ca^{2+}]_e$ had no effect in wild-type FDB. Raising $[Ca^{2+}]_e$ from 2.4 mM to 4.8 mM had no effect on peak tetanic force and resting tension during fatigue and on force recovery in Kir6.2$^{-/-}$ FDB bundles (Fig. 3-8). Considering the lack of effect in Kir6.2$^{-/-}$ FDB, wild type FDB was not tested at 4.8 mM Ca^{2+}.

EFFECT OF VERAPAMIL ON THE KINETICS OF FATIGUE

For Kir6.2$^{-/-}$ FDB, the decrease in peak tetanic force was significantly less in the presence of 20 μM verapamil than in its absence. For example at 30 sec peak force was 60% of prefatigue value in the presence of verapamil compared to 35% in control, a 25% difference between control and verapamil conditions. The difference between control and verapamil conditions remained significant until the 60th sec. Verapamil had no effect on the rate of fatigue of wild-type FDB (Fig. 3-9B).

Resting tension increased to 28% over the first 30 sec of fatigue and plateaued at 14% for the remainder of fatigue for control Kir6.2-/- FDB. In the presence of verapamil, the increase in resting tension was basically abolished as it only reached a maximum of 1% (Fig. 3-10A). Verapamil had no effect on the resting tension of wild-type FDB (Fig. 3-10B).

Peak tetanic force recovery was significantly greater for Kir6.2$^{-/-}$ FDB muscle exposed to 20 μM verapamil. FDB muscle recovered to 90% over the 30 min following fatigue in the presence of verapamil, compared to only 75% under control conditions (Fig. 3-11A). Verapamil had no effect on the force recovery of wild-type FDB (Fig. 3-11B).

FIGURE 3-8

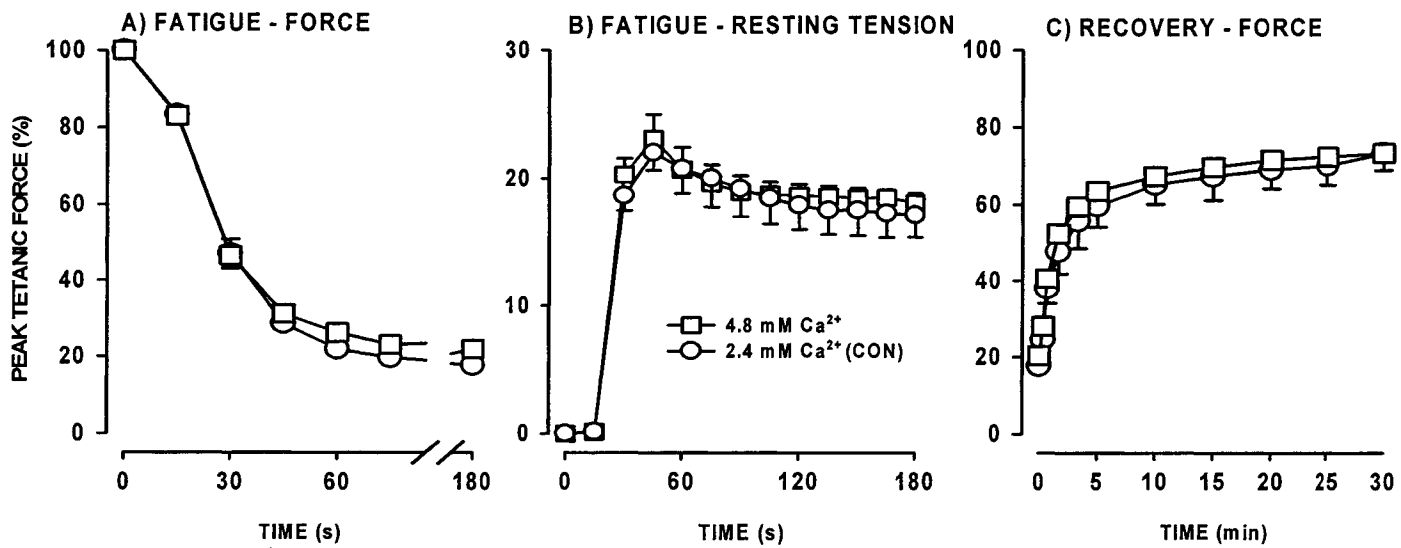

Figure 3-8. Increasing $[Ca^{2+}]e$ from 2.4 mM to 4.8 mM had no effect on the rate of fatigue (A), resting tension (B), and tetanic force recovery (C) in FDB muscles. Fatigue was elicited with one 200 ms tetanic contraction every sec for 3 min. Experimental temperature: 37°C. Peak tetanic force (A,C) is expressed as a percent of the pre-fatigue force. Resting tension (B) is expressed as a percent of the pre-fatigue peak tetanic force. Vertical bars represent the S.E. of 4 Kir6.2$^{-/-}$ FDB muscles.

No significant difference was observed between 2.4 and 4.8 mM Ca^{2+}, ANOVA and L.S.D. $P>0.05$.

FIGURE 3-9

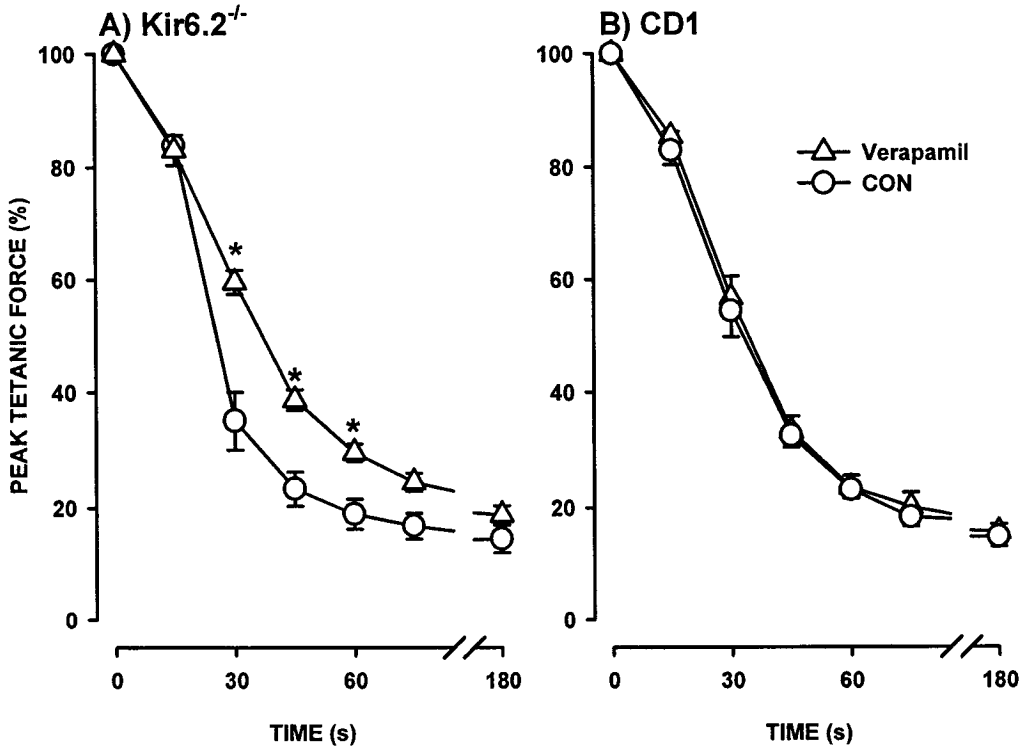

Figure 3-9. Kir6.2$^{-/-}$ FDB muscle bundles had slower decreases in tetanic force when exposed to 20 μM verapamil. Fatigue was elicited with one 200 ms tetanic contraction every sec for 3 min. Experimental temperature: 37°C. Peak tetanic force is expressed as a percent of the pre-fatigue force. Vertical bars represent the S.E. of 4 wild-type and 5 Kir6.2$^{-/-}$ FDB muscles.

* Significantly different from control FDB, ANOVA and L.S.D. $P < 0.05$.

FIGURE 3-10

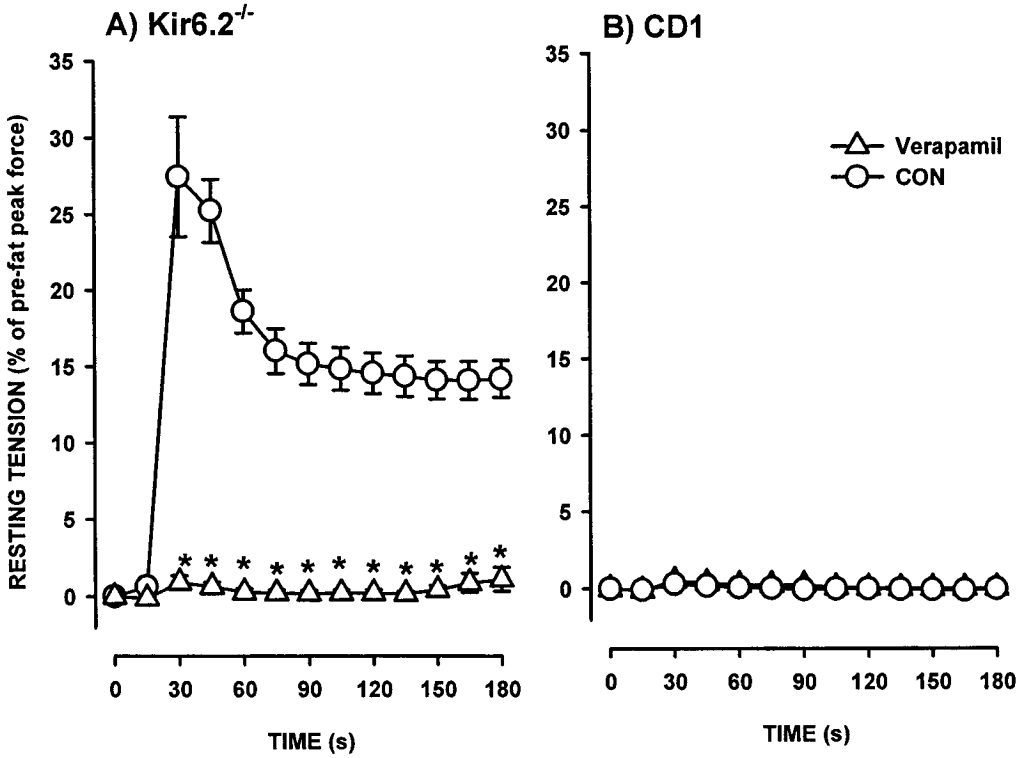

Figure 3-10. Kir6.2$^{-/-}$ FDB muscle bundles developed less resting tension when exposed to 20 μM verapamil. Fatigue was elicited with one 200 ms tetanic contraction every sec for 3 min. Experimental temperature: 37°C. Resting tension is expressed as a percent of the pre-fatigue peak tetanic force. Vertical bars represent the S.E. of 4 wild-type and 5 Kir6.2$^{-/-}$ FDB muscles.

* Significantly different from control FDB, ANOVA and L.S.D. $P < 0.05$.

FIGURE 3-11

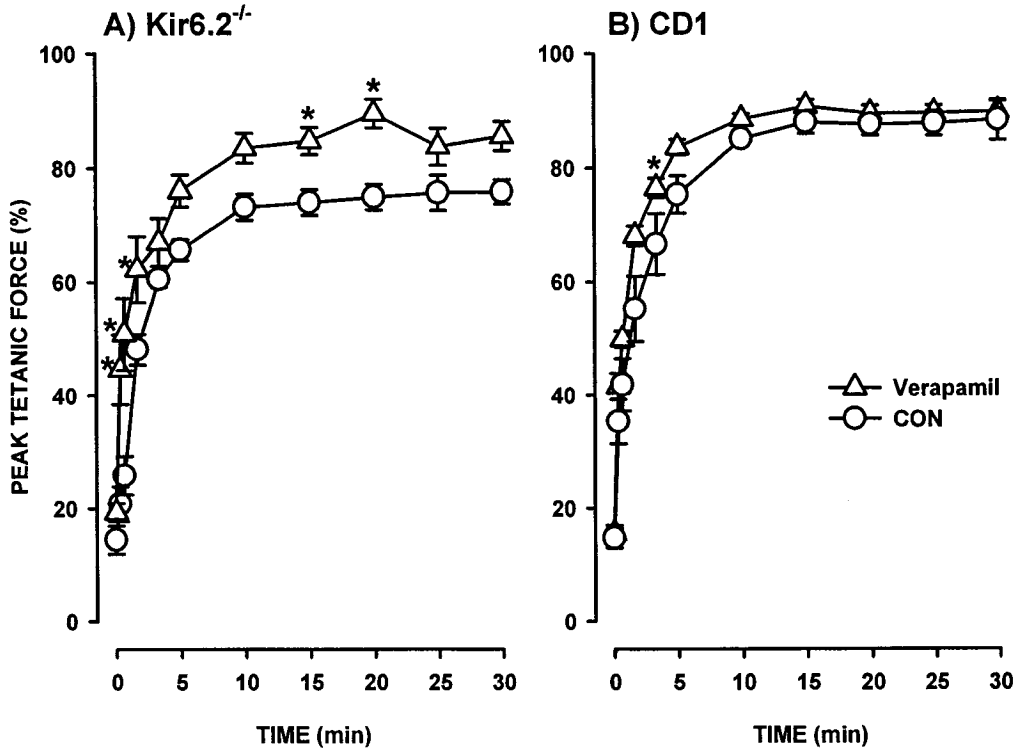

Figure 3-11. Kir6.2$^{-/-}$ FDB muscle bundles recovered better following fatigue in 20 μM verapamil. Fatigue was elicited with one 200 ms tetanic contraction every sec for 3 min. During recovery, tetanic contractions were elicited 10, 20, 100 sec after fatigue and then every 100 sec thereafter. Experimental temperature: 37°C. Peak tetanic force is expressed as a percent of the pre-fatigue force. Vertical bars represent the S.E. of 4 wild-type and 5 Kir6.2$^{-/-}$ FDB muscles.

* Significantly different from control FDB, ANOVA and L.S.D. $P < 0.05$.

When verapamil is used to partially block L-type Ca^{2+} channels of the sarcolemma and t-tubules of Kir6.2$^{-/-}$ FDB muscles, the rate of fatigue became slower, the resting tension was reduced, and force recovery was greater when compared to Kir6.2$^{-/-}$ FDB muscles fatigued in control conditions.

CHAPTER 4

FATIGUE PRE-CONDITIONING INCREASES FATIGUE RESISTANCE AND PROTECTS MUSCLE AGAINST THE IMPAIRMENTS OF NO K_{ATP} CHANNEL ACTIVITY

The experimental approach was as follows. All fatigue bouts were elicited with tetanic contractions at a rate of one contraction every second for 3 min as described in Methods and Materials, except that bundles were fatigued twice. FDB bundles were divided into three groups. The first group was used to test the effect of fatigue pre-conditioning under control conditions after the first fatigue bout (**FAT1**) (Fig. 4-1A).

FDB muscles were allowed to recover as described in Methods and Materials, and the second fatigue bout (**FAT2**) was elicited 60 min after FAT1. The second group tested the effect of blocking K_{ATP} channels with glibenclamide during FAT2. For this group, FAT1 was under control conditions while FAT2 was in the presence of 10 μM glibenclamide added 30 min prior to FAT2. This allowed for 30 min recovery from FAT1 and 30 min equilibrium in glibenclamide. The third group tested the effect of time: FDB bundles were fatigued in the absence or presence of 10 μM glibenclamide at a time when FAT2 was elicited; this fatigue was defined as delayed FAT1 (**delFAT1**) (Fig. 4-1B).

RESULTS

Most of the fatigue kinetics, decreases in peak tetanic force, resting tension and force recovery, were quite similar between FAT1 (reported in Chapter 3) and delFAT1 (reported here). Only a very few significant differences were observed. For example, under control conditions, the decrease in peak tetanic force was initially faster during delFAT1 (Fig. 4-2A) than during FAT1 (Fig. 3-2A). The differences were observed only

FIGURE 4-1

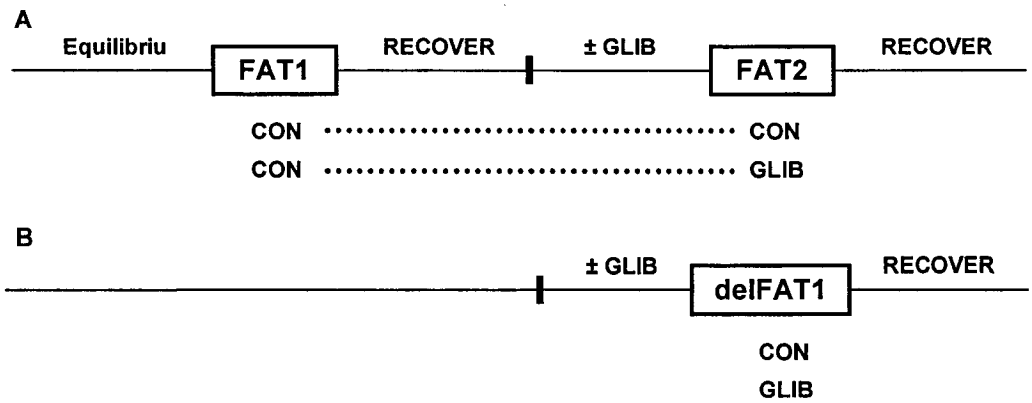

Figure 4-1. Fatigue and recovery protocol for FDB muscle bundles. All fatigue bouts were elicited with one 200 ms tetanic contraction every sec for 3 min. A) Test for fatigue pre-conditioning: the first fatigue bouts (FAT1) were all elicited under control conditions after the 30 min equilibrium and the second fatigue bouts (FAT2) were elicited after a 30 min recovery and another 30 min in the absence or presence of 10 μM glibenclamide. B) Test for the effect of time: FDB muscles were subjected to only one fatigue bout in the absence or presence of glibenclamide but at the time FAT2 was elicited, this fatigued is defined as delayed FAT1 (delFAT1).

FIGURE 4-2

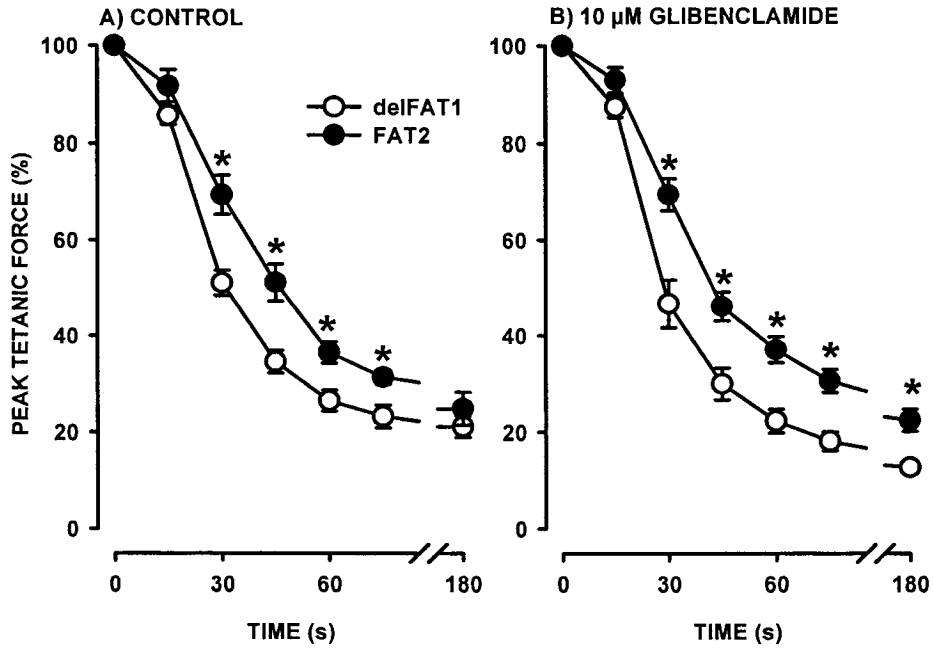

Figure 4-2. The decrease in tetanic force during FAT2 in the absence and presence of 10 µM glibenclamide is slower than during delFAT1. All fatigue bouts were elicited with 200 ms tetanic contraction every sec for 3 min. A) All fatigue bouts were elicited under control conditions: FAT1 was elicited immediately after a 30 min equilibrium, FAT2 was elicited 60 min after FAT1, and delFAT1 was elicited at the same time than FAT2 (for clarity the changes in tetanic force during FAT1 are not shown). B) FAT1 was elicited under control conditions while FAT2 and delFAT1 were elicited in the presence of 10 µM glibenclamide added 30 min prior to the fatigue bout. Peak tetanic force is expressed as a percent of the pre-fatigue force. Experimental temperature 37°C. Vertical bars represent the S.E. of 5-8 FDB bundles.

* Tetanic force significantly different from delFAT1, ANOVA, L.S.D. $P < 0.05$

between 30 and 60 sec when peak tetanic forces during delFAT1 were 5-10% lower than during FAT1. Similar to the situation observed during FAT1 (Fig. 3-3A), during delFAT1 resting tension increased little under control conditions (Fig. 4-3A) and drastically under glibenclamide conditions (Fig. 4-3B). Furthermore, under control conditions, there was no difference in force recovery after FAT1 (Fig. 3-4A) and delFAT1 (Fig. 4-4A).

Under control conditions, most of the decrease in peak tetanic force occurred within the first 75 sec when fatigue is elicited with one tetanic contraction per sec (Fig. 4-2A). During delFAT1 peak tetanic force was only 23% of the pre-fatigue force at 75 sec (Fig. 4-2A). Thereafter, peak tetanic force decreased by another 2%, being 21% at the end of the 3 min fatigue period. If two fatigue bouts are separated by 60 min, the decrease in mean peak tetanic force during the second fatigue bout (FAT2) was significantly slower than during delFAT1. For example, under control condition, mean peak tetanic force at 30 sec was 51% of the pre-fatigue values during delFAT1 compared to 69% during FAT2, an 18% difference. It is interesting to note that the decrease in peak force during FAT1 was slightly slower than during delFAT1 during the first 60 sec (data not shown); thus the longer the delay in eliciting the first fatigue the faster the decrease in force while after one fatigue bout the decrease in force becomes slower.

In the presence of 10 μM glibenclamide, the decrease in peak tetanic force was also slower during FAT2 than during delFAT1 (Fig. 4-2B). The difference at 30 sec was 23% as mean peak forces during delFAT1 and FAT2 were respectively 47% and 70%. The difference in peak force remained between delFAT1 and FAT2 remained until the end of FAT2. It is also interesting to note that the decrease in peak tetanic force during FAT2

FIGURE 4-3

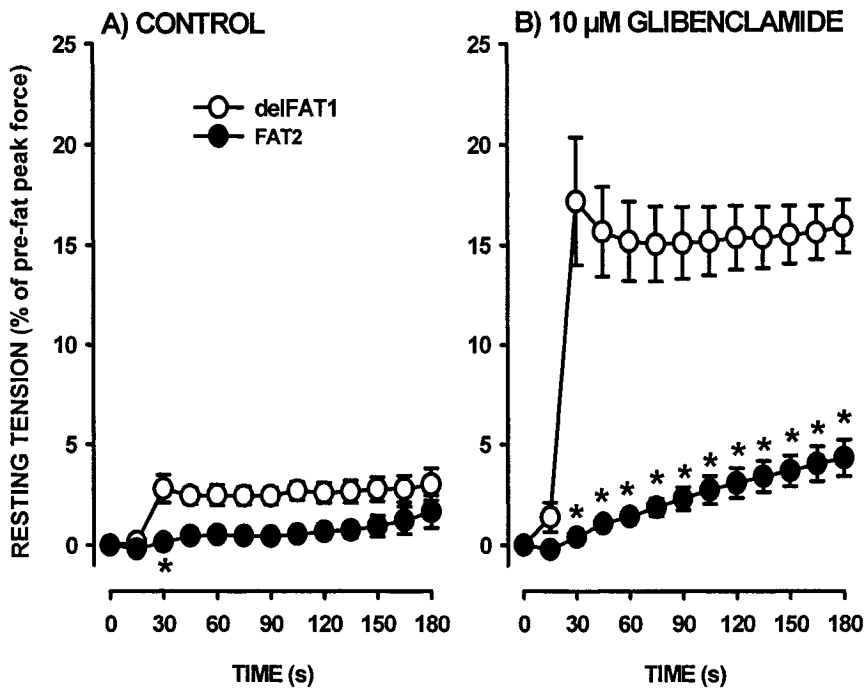

Figure 4-3. The increase in resting tension was less during FAT2 than during delFAT1, especially in the presence of glibenclamide. All fatigue bouts were elicited with 200 ms tetanic contraction every sec for 3 min. A) All fatigue bouts were elicited under control conditions: FAT1 was elicited immediately after a 30 min equilibrium, FAT2 was elicited 60 min after FAT1, and delFAT1 was elicited at the same time as FAT2 (for clarity the changes in resting tension during FAT1 are not shown). B) FAT1 was elicited under control conditions while FAT2 and delFAT1 were elicited in the presence of 10 µM glibenclamide added 30 min prior to the fatigue bout. Resting tension is expressed as a percent of the pre-fatigue tetanic force. Experimental temperature 37°C. Vertical bars represent the S.E. of 5-8 FDB bundles.

* Significantly different from delFAT1, ANOVA, L.S.D. $P < 0.05$

FIGURE 4-4

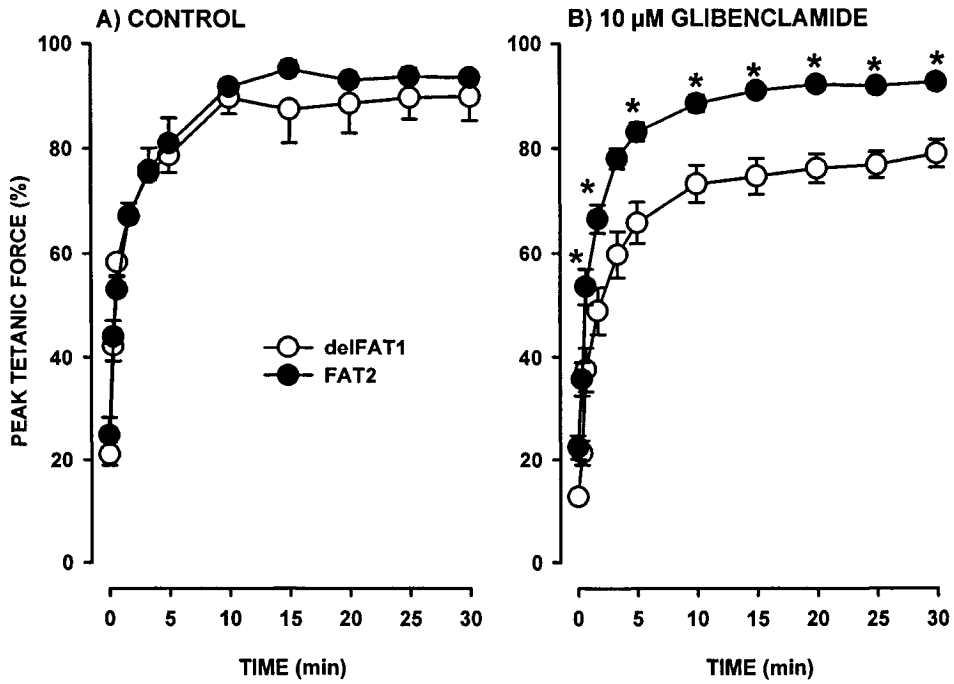

Figure 4-4. In the presence of glibenclamide, force recovery following FAT2 was significantly greater than after delFAT1. All fatigue bouts were elicited with 200 ms tetanic contraction every sec for 3 min. A) All fatigue bouts were elicited under control conditions: FAT1 was elicited immediately after a 30 min equilibrium, FAT2 was elicited 60 min after FAT1, and delFAT1 was elicited at the same time as FAT2 (for clarity the changes in tetanic force during FAT1 are not shown). B) FAT1 was elicited under control conditions while FAT2 and delFAT1 were elicited in the presence of 10 µM glibenclamide added 30 min prior to the fatigue bout and present during the recovery period. Peak tetanic force is expressed as a percent of the pre-fatigue force. Experimental temperature 37°C. Vertical bars represent the S.E. of 5-8 FDB bundles.

* Significantly different from delFAT1, ANOVA, L.S.D. $P < 0.05$

was not significantly different between control conditions (Fig. 4-2A) and in the presence of glibenclamide (Fig. 4-2B). This is in sharp contrast with Fig. 3-2, which shows that during one fatigue bout (equivalent to FAT1) the decrease in peak force was significantly faster and greater in FDB fibers that have no K_{ATP} channel activity. It thus appears that following one fatigue bout under control conditions the impairment of abolishing K_{ATP} channel activity is no longer observed in a subsequent second fatigue bout.

The disappearance of the function impairment of no K_{ATP} channel activity was especially evident for the resting tension as the increase in resting tension during FAT2 was less than during delFAT1 (Fig. 4-3). Under control conditions, the differences were small and only one significant difference was observed at 30 sec (Fig. 4-3A). In the presence of glibenclamide, resting tension increased to 17% of the pre-fatigue peak tetanic force and remained above 15% for the remaining fatigue period during delFAT1 while during FAT2 resting tension increased gradually to 4% at 180 sec (Fig. 4-3B).

Under control conditions, peak tetanic force recovered to 90% and 94% after delFAT1 and FAT2, respectively (Fig. 4-4A). In the presence of glibenclamide, on the other hand, force recovery was less following delFAT1, reaching a maximum of 79%. This extent of recovery following delFAT1 was significantly less than the recovery of 93% after FAT2 also in the presence of glibenclamide (Fig. 4-4B). So, after one 3 min fatigue bout, the decreases in tetanic force are slower, the increases in resting tension are less and force recovery improved during a second fatigue bout, especially when K_{ATP} channels are blocked with glibenclamide.

CHAPTER 5

DISCUSSION

Muscle activity increases metabolic rate 20- to 100-fold depending on fiber type and activity intensity (Gibbs, 1987). Although muscles are capable of increased ATP production, most muscular activity leads to an energy deficit. It is believed that muscle fatigue is a physiological response that decreases force development to prevent ATP rundown and irreversible fiber damage (Lascano *et al*, 2002). For such a mechanism to work, it must first be able to have components that sense an energy deficit. The K_{ATP} channel can be such a sensor. Its activity first depends on the ATP:ADP ratio for which a decrease in ratio activates the channel. It is therefore a channel that can be activated during any metabolic stress. In skeletal and cardiac muscle the channel can also be activated by a decrease in pH, an increase in lactate and adenosine which are produced during fatigue.

It is currently accepted that the decline in force that characterizes fatigue in skeletal muscle is primarily due to a failure of the SR to release Ca^{2+}, which results in submaximal $[Ca^{2+}]_i$, that no longer fully activates the sarcomere (Allen, 1989; Westerblad, 1991; Baker, 1993). This preserves ATP as less ATP is consumed by the myosin- and Ca^{2+}-ATPases, which together account for 99% of ATP consumption during activity (Gibbs, 1987). The cause for lower release of SR Ca^{2+} is still unknown, but is most likely a multifactorial event (Fitts, 1994). Some of the mechanisms involve the DHP and RyR and others a change in membrane excitability (Westerblad, 1991). In the latter case, reduction in action potential amplitude reduces Ca^{2+} release and force production

(Matar, 2000; Gong, 2003). Recent evidence has shown that KATP channels contribute to a decrease in action potential amplitude by increasing K^+ conductance and by decreasing the Na^+ depolarization effect (Gong *et* al., 2000). Thus, the KATP channel is not only an energy sensor, but is a candidate contributing to the phenomenon of fatigue.

Given that activation of KATP channels increases the rate at which tetanic force decreases during fatigue, blocking KATP channel activity should have the reverse effect, i.e. slow down the rate of fatigue. However, as argued in the Introduction, there are several function impairments during fatigue in the absence of KATP channels. These impairments include larger resting tension, poor recovery, large membrane depolarization (Gramolini & Renaud, 1997), and fiber damage (Bourassa & Renaud, unpublished results). These impairments are expected to increase the rate at which force decreases during fatigue, overriding the expected slower decrease in force via an effect on action potential amplitude. It is therefore interesting that most studies reported absolutely no effect on the rate of fatigue when there is no KATP channel activity (Weselcouch *et al.*, 1993; Duty & Allen, 1995; Light *et al.*, 1994; Comtois *et al.*, 1995; Van Lunteren *et al.*, 1998; Matar *et al.*, 2000; Gong *et al.*, 2000; Gong *et al.*, 2003). Although this has raised questions as to whether KATP channels really affect force development, it was argued in the Introduction that the apparent lack of an effect of no KATP channel activity on fatigue rate may have been related to the fact that most studies use EDL and soleus muscles; two muscles that are quite large to be used at 37°C.

Therefore, one objective of this study was to use FDB muscle bundles, which are much smaller than EDL and soleus muscles, to test the hypothesis that the "functional

impairment should override the expected slower rate of fatigue, so the rate of fatigue is faster in KATP channel deficient muscle compared to control."

THE FDB: A BETTER MUSCLE MODEL TO STUDY THE EFFECT OF NO KATP CHANNEL

EDL muscles contain approximately 750 fibers (Thabet *et al.*, 2005), weigh 9 mg and are 7 mm long (James *et al.*, 2004). In comparison, FDB muscle bundles contain 350 fibers, weigh 2.2 mg and were 8.2 mm long. Assuming a cylindrical shape and a density of 1.06 g/cm^3, the average radius for FDB was 0.31 mm and has a cross-sectional area of 0.3 mm^2. These values are much smaller than in EDL with a radius of 0.5 mm and a cross-sectional area of 0.8 mm^2, while the muscles have similar fatigue kinetics. The larger size of the EDL muscle hinders a bulk of the central fibers from receiving the oxygen and glucose necessary to sustain several contractions over a short period of time because of slow diffusion. It is therefore expected that FDB has a smaller hypoxic/hypoglycemic core than the EDL. At 37°C, it has been shown that such a hypoxic/hypoglycemic core can mask the effect of some experimental manipulations (Zhang *et al.*, 2006).

Some investigators resolved this issue by using lower experimental temperatures. In resting mouse, the subcutaneous temperature close to the FDB is 30°C (Bruton *et al.*, 1998). The author of that study argued that muscle fatigue should therefore be performed at 30°C. However, during exercise the core body temperature in rat increases 3-4°C, while the temperature of the skin, which does not generate heat but receives warmed blood from active muscles, increases from 30°C to 36°C (Fuller *et al.*, 1998; Gonzales-Alonso *et al.*, 1999). It is therefore more than likely that during exercise the temperature of active skeletal muscles reaches or exceeds 37°C. Furthermore, Duty and Allen (1995)

did not report any fiber damage with glibenclamide at 22°C, whereas at 37°C extensive fiber damage occurred in the absence of K_{ATP} channel activity (Bourassa & Renaud, unpublished results) as reported for swimming (Kane *et al.*, 2004) and treadmill running (Thabet *et al.*, 2005). We therefore suggest that 37°C is representative of normal physiological conditions.

When EDL muscles are fatigued at 37°C, the maximum recovery of tetanic force under control conditions is 50% (Matar *et al.*, 2000; Gong *et al.*, 2003). The incomplete recovery is most likely linked to the hypoxic/hypoglycemic core, is a known cause for fiber damage (Fredsted *et al.*, 2005). Once damaged, the fibers no longer contribute to the development of force resulting in a net loss of force during recovery. Although EDL under control conditions recovers poorly, it was useful to test the effects of activating K_{ATP} channels using pinacidil, which improved force recovery after fatigue to 80 – 85% of its prefatigue force. Such findings support the purported cytoprotective role of the K_{ATP} channel. However if 50% of the fibers are lost during fatigue, it then becomes difficult to study the effect of no K_{ATP} channel activity.

Following fatigue, the FDB has a much better recovery compared to the EDL as force returned to 90 – 92% of prefatigue force (Fig. 3-4). The better force recovery in FDB muscles compared to EDL is not evidence that a hypoxic core does not develop during fatigue in the FDB, but that it is at least not as lethal as in the EDL. It is thus suggested that FDB muscle bundles represent a better preparation not only for the study of K_{ATP} channels, but also for the mechanisms of fatigue in general, because, as proposed in the Introduction, if fatigue is a cytoprotective mechanism, then one expects full recovery of force following fatigue.

Previous studies, using EDL and soleus, have reported greater increase in resting tension during fatigue and slightly lower capacity to recover force following fatigue in KATP channel deficient fibers compared to normal muscles (Gong *et al.*, 2000). Thus, the greater resting tension during fatigue (Fig. 3-4) and the lower capacity to recover force (Fig. 3-5) following fatigue in KATP channel deficient FDB muscles are in agreement with the findings of previous studies using EDL and soleus (Gong *et al.*, 2000; Light *et al.*, 1994; Matar *et al.*, 2000). It should be noted though that the differences between control and KATP channel deficient muscles are greater for the FDB than for EDL or soleus. For example, Kir6.2$^{-/-}$ FDB muscle resting tension increased to a maximum of 29%, while Kir6.2$^{-/-}$ EDL and soleus respectively increased to a maximum of 8% and 13% (Gong *et al.*, 2000). Resting tension is considered an impairment as wild-type muscles can completely or almost completely relax upon cessation of the fatiguing stimulus. As well, in Ca^{2+}-imaging studies, resting Ca^{2+} was shown to be elevated in FDB abolished of KATP activity compared to wild-type. The difference in force recovery between control and KATP channel deficient muscle were, on the other hand, quite similar between FDB and EDL, for which the differences were 3%. The differences are however greater when compared to those of the soleus where the difference was 15%. So, in regard to impairment in resting tension and force recovery, the effects of no KATP channel activity are the same between FDB, EDL, and soleus, but greater differences in magnitude are observed in FDB bundles.

ABOLISHING KATP CHANNEL ACTIVITY INCREASES THE RATE OF FATIGUE

Abolishing KATP channel activity in FDB muscle, either pharmacologically using glibenclamide or genetically using Kir6.2$^{-/-}$ FDB muscles resulted in faster decreases in

tetanic force (Fig. 3-2). In a parallel study, Bourassa & Renaud (unpublished results) also observed faster decreases in tetanic $[Ca^{2+}]_i$ during fatigue in KATP channel deficient single muscle fibers compared to control fibers. It thus appears that, as proposed in the first hypothesis, the lack of KATP channel activity gives rise to faster rate of fatigue and such an effect can only be observed with small muscle bundles or single muscle fibers at 37°C. This faster rate of fatigue also agrees with the matter that severe function impairment prevents slower fatigue rate as expected if the channel no longer affects the action potential.

The question now is how to demonstrate that the depressing effects of the function impairment are indeed greater than the expected slower decrease from no effect on action potential. If the incomplete force recovery is due to a loss of fibers as they become damaged, then one can calculate what the force should be during fatigue if there was no damage. In single fibers, damage occurred during the first 60 sec of fatigue (Bourassa & Renaud, unpublished results). At 60 sec, tetanic force is 30% of pre-fatigue force in wild-type FDB. For these FDB, recovery was 90%. If for now we assume that the 10% loss of force represents 10% of damaged fibers, then if there was no damage, force at the 60^{th} sec of fatigue should be 34% (Fig. 5-1). Such a difference is very small. However for KATP channel deficient muscle, force at 60 sec is 20% and recovery is only 70%. Repeating the same calculations means that in the absence of damage, force at 60 sec of fatigue would be 27% instead of 20%. Although this shows the possible impact of fiber damage in KATP channel deficient FDB, such a correction stills shows a force at 60 sec of fatigue that is less in Kir6.$2^{-/-}$ than wild-type FDB muscle.

FIGURE 5-1

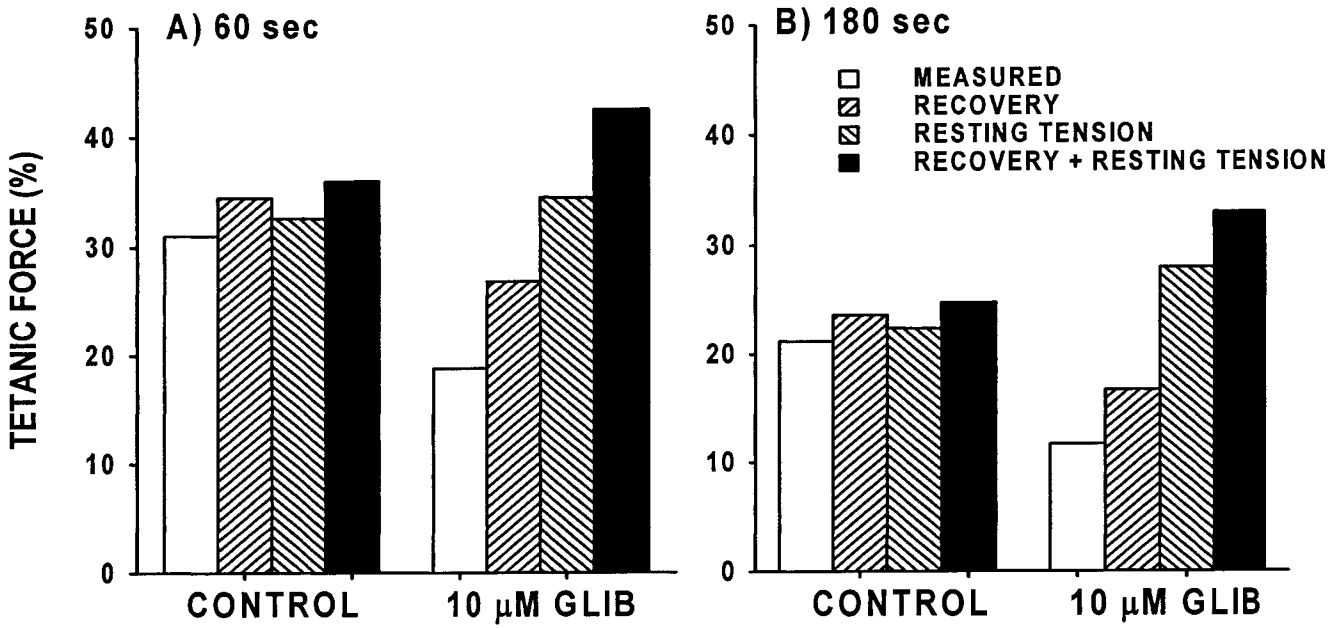

Figure 5-1. Effect of functional impairment on the development of tetanic force during fatigue. Measures are the values given at A) 60 sec, and B) 180 sec in Fig. 3-2A for wild-type and Fig. 3-2B for Kir6.2-/- under control conditions (i.e. no glibenclamide). The expected force during fatigue if there was no fiber damage (using the net loss of force after recovery) and resting tension are also shown. See text for how the calculations were made.

As discussed in the Introduction, the capacity of the FDB to generate force during fatigue becomes limited because there is less Ca^{2+} release resulting in sub-maximal activation of the sarcomere. Then, other metabolites, such as P_i can further limit force generation by decreasing Ca^{2+} activation of the sarcomere. In this study we measure the active force which is the increase in force upon stimulation. For KATP channel deficient FDB muscle, the active force may have been only 18%, but the resting tension at the same time was 16%. So in reality, the total force generating capacity of the FDB at 60 sec was in fact 34%, which is the sum of the active force and the resting tension. When this calculation is made, it then becomes obvious that the maximum capacity of KATP channel deficient FDB to generate force at 60 sec is the same as the one for control wild-type FDB. Finally, when the correction for fiber damage and resting tension are simultaneously taken into account, then at 60 sec the force in KATP channel deficient FDB would be 43% which is greater than the 35% for wild-type FDB. A calculation for the force at the end of the fatigue period shows a similar pattern of difference between $Kir6.2^{-/-}$ and wild-type FDB (Fig. 5-1B).

The calculations above are not meant to obtain what the exact force should have been if a lack of KATP channel activity did not increase resting tension and cause fiber damage. For example, a 10% or 30% loss of force recovery does not directly translate into 10% and 30% in the number of fibers being damaged, because not all fibers are of the same size and force is a function of cross-sectional area. Furthermore, if some of the damage involves the cell membrane, an uncontrollable Ca^{2+} influx can result in a contraction that can contribute to resting tension. Instead the calculations were used to illustrate how these two function impairments can easily mask the expected slower

decrease in force when the lack of KATP channel activity no longer reduces action potential amplitude. It is also important to realize that there is at least one function impairment that has not been considered. The cell membrane does not depolarize during metabolic inhibition unless KATP channels are blocked with glibenclamide (Gramolini & Renaud, 1997). The depolarization can be as large as 30 mV. Such a large depolarization is known to increase the inactivation of Na^+ channels, which also contributes to the depression of force. Although similar depolarization has not been shown during fatigue, it undoubtedly occurs as the lack of KATP channel activity reduces K^+ conductance. Consequently, the 43% value estimated for Kir6.2$^{-/-}$ FDB at 60 sec may even be higher if the membrane depolarization is taken into consideration.

It is therefore proposed, based on previous studies with channel openers and this study, that i) KATP channels are active during fatigue, ii) that they contribute to the decrease in force during fatigue and, iii) that a slower rate of fatigue does not occur when KATP channel activity is abolished because of severe function impairments that reduce the capacity of muscles to generate force.

INCREASED INFLUX OF EXTRACELLULAR Ca^{2+} VIA L-TYPE Ca^{2+} CHANNELS CAUSES FUNCTION IMPAIRMENT OF KATP CHANNEL DEFICIENT SKELETAL MUSCLE

It has been shown that function impairment and fiber damage in cardiac muscles depend in part from an influx of Ca^{2+} through the L-type Ca^{2+} channels that are located in t-tubules. So, the second objective of this thesis was to test the hypothesis that "the functional impairment of skeletal muscle in KATP channel deficient mice is due to an increased Ca^{2+} influx through L-type Ca^{2+} channels." Two experimental approaches were used to test this hypothesis. The first approach was a reduction in $[Ca^{2+}]_e$ from 2.4 to 0.6

mM and the other approach involved a partial block of L-type Ca^{2+} channels with 20 μM verapamil.

When the $[Ca^{2+}]_e$ was lowered from 2.4 to 0.6 mM, at that concentration, the peak force was unaffected before fatigue as previously reported for skeletal muscle (Vires *et al.*, 1988). This was an important issue because any decrease in force would indicate decreases in tetanic $[Ca^{2+}]_i$. Then, the rate of ATP utilization by Ca^{2+}-ATPase and myosin-ATPase would be lower at the start of fatigue. The lack of force loss at 0.6 mM Ca^{2+} suggests that the Ca^{2+}-ATPases and myosin-ATPase, are working at similar capacities at 0.6 and 2.4 mM Ca^{2+}. Interestingly, at 0.6 mM Ca^{2+} the rate of fatigue measured from the decrease in force was slower than at 2.4 mM Ca^{2+} in Kir6.2$^{-/-}$ FDB, while in wild-type FDB lowered $[Ca^{2+}]_e$ had no effect. This is therefore strong evidence that somehow K_{ATP} channels reduce Ca^{2+} influx through the cell membrane during fatigue, reducing the impairment of force development. This notion is further supported by the fact that less resting tension was developed and more force was recovered after fatigue (i.e. less fiber damage at 0.6 than 2.4 mM Ca^{2+}).

It is also interesting to point out that if tetanic force decreases at a slower rate at 0.6 mM Ca^{2+}, then the energy demand is also greater during each contraction through the fatigue period compared to the situation at 2.4 mM Ca^{2+}. However, the lower resting tension during fatigue at 0.6 mM $[Ca^{2+}]_e$ (Fig. 3-6) probably contributes to lower ATP consumption by the Ca^{2+}-ATPase and myosin-ATPase especially that active force was generally for only 200 msec each second, while resting tension last the full second between contraction cycles. This may also be another reason why at low $[Ca^{2+}]_e$ fatigue rates were slower. Thus, overall, lowered $[Ca^{2+}]_e$ diminished function impairment that

occurs during fatigue in KATP channel deficient FDB. Since the electrochemical gradient favours Ca^{2+} influx, it is suggested that KATP channel effects reduce Ca^{2+} influx. The next question is the mechanism of action for this Ca^{2+} influx.

In cardiac muscle, there are two sources of Ca^{2+} for the activation of the sarcomeres. One pool being in the t-tubules entering via the L-type Ca^{2+} channel and the other being in the SR. When there is a lack of KATP channel activity and action potential shortening does not occur, more Ca^{2+} enters from the t-tubules leading to a Ca^{2+} overload. In skeletal muscle however, DHP receptors or L-type Ca^{2+} channels, interact directly with the RyR. A Ca^{2+} influx is not necessary to trigger contractions (Carafoli, 1987). So while the presence of extracellular Ca^{2+} is necessary for contraction in heart muscle, none is necessary in skeletal muscle. In KATP deficient cardiac muscle, it is known that Ca^{2+} influx occurs through the DHP, or L-type Ca^{2+} channel (Lascano *et al.*, 2002).

In this study verapamil was used to block the L-type Ca^{2+} channels because it is a very effective and specific blocker of the L-type Ca^{2+} channel in skeletal muscle (Soza *et al.*, 1986; Vazquez *et al.*, 1998; Morgan *et al.*, 1997; Morelli *et al.*, 2001). As discussed for low Ca^{2+}, it was important that the initial stress imposed at the onset of fatiguing stimulation be the same in the presence of verapamil as in its absence. At 50 μM, verapamil depresses tetanic force (Jones *et al.*, 1984). From dose-response studies, we found that 20 μM has no effect on force prior to fatigue (data not shown).

Binding and IV studies have shown partial block of L-type Ca^{2+} channels occurs even at 10 μM (Vires *et al.*, 1988). So at 20 μM, verapamil only partially blocks L-type Ca^{2+} channels. As in 0.6 mM Ca^{2+}, the rate of fatigue (Fig. 3-9), resting tension (Fig. 3-10), and extent of recovery (Fig. 3-11) were improved when Kir6.2$^{-/-}$ FDB were exposed

to 20 μM verapamil. This is to be expected if the influx of Ca^{2+} is through the L-type Ca^{2+} channels. However, verapamil had a better effect on resting tension, which was almost eliminated in Kir6.2$^{-/-}$ muscles. As mentioned earlier, the electrochemical gradient is reduced in 0.6 mM Ca^{2+}, however this still favours an influx of Ca^{2+}, and therefore may still result in Ca^{2+} overload, albeit less than that at 2.4 mM Ca^{2+}. A partial block of the L-type Ca^{2+} channels maintains the electrochemical gradient, but appears sufficient to block any excess Ca^{2+}influx.

Our observation on control skeletal muscle agrees with the role of extracellular Ca^{2+}, which does not affect force, resting tension, and recovery when $[Ca^{2+}]_e$ is lowered (Figs. 3-5B, 3-6B, 3-7B) or when L-type Ca^{2+} channels are blocked (Figs. 3-9B, 3-10B, 3-11B). However in Kir6.2$^{-/-}$ FDB muscle, lowering Ca^{2+} influx by either lowering $[Ca^{2+}]_e$ or adding verapamil slows down the fatigue rate (Figs. 3-5A, 3-9A), reduces resting tension (Figs. 3-6A, 3-10A), and improves force recovery (Figs. 3-7A, 3-11A). These finding suggest that K_{ATP} channels help prevent a damaging Ca^{2+} overload by reducing Ca^{2+} influx through L-type Ca^{2+} channels.

MECHANISMS OF ACTION FOR THE K_{ATP} CHANNEL

The mechanism by which the K_{ATP} channel protects muscle against fiber damage is not fully understood, but taking into account previous studies, thus far two functions are emerging. The first function is important during contraction as illustrated using channel openers (Fig. 5-2A). In this case, the increased K^+ conductance associated with the activation of K_{ATP} channels reduces action potential amplitude (Gong *et al.*, 2003) because the K^+ efflux reduces the extent of the Na^+ depolarization. As a result of lower action potential amplitude, less Ca^{2+} is released by sarcoplasmic reticulum

FIGURE 5-2

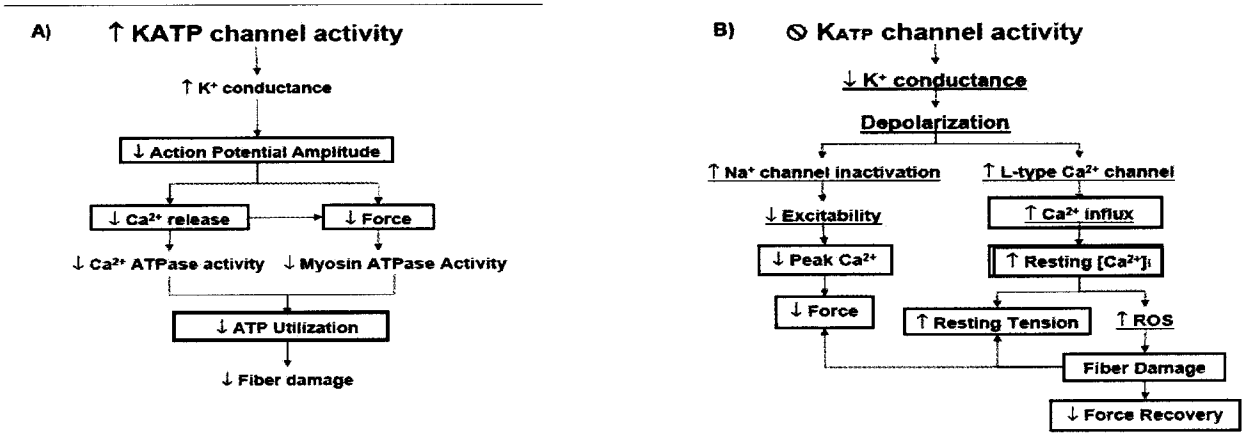

Figure 5-2. Proposed physiological functions for the KATP channel during exercise and fatigue in skeletal muscle. A) Function of the channel during contraction starting with action potentials; the model is based on evidence obtained from channel openers. B) Function of the channel between contractions starting with the resting membrane potential; the model is based on evidence obtained from KATP channel deficient muscles. Texts in black boxes: are effects known to be associated with modulating KATP channel activity; texts in red boxes have been shown in this study; underlined texts are effects known from other studies but which have not yet been shown to be associated with KATP channel; other texts are effects that are proposed to be associated with the channel, but for which there is no evidence. See text for all references.

(Burton & Smith, 1997; Duty & Allen, 1995), increasing the rate at which force decreases (Gong *et al.*, 2003; Matar *et al.*, 2000). Studies from this laboratory have now given evidence that this process may be important to reduce Ca^{2+}- and myosin-ATPase activity to preserve ATP (Li & Renaud, unpublished results).

The second function of the KATP channel is important between contractions and becomes evident from studies in which the channel activity is abolished (Fig. 5-2B). The cell membrane does not depolarize during metabolic inhibition unless the channels are blocked; the extent of the depolarization being 30-50 mV (Gramolini & Renaud, 1997). Similar depolarizations have yet to be shown during fatigue. However, lower K^+ conductance in the absence of KATP channel activity undoubtedly results in resting membrane depolarization, which can then affect Na^+ and Ca^{2+} channels. For Na^+ channels, the depolarization will increase the degree of inactivation reducing membrane excitability (Hodgkin & Huxley, 1952); i.e., it can be one reason for the faster fatigue rate in KATP channel deficient muscle compared to normal muscle as shown in Fig. 3-2. This study now gives evidence that Ca^{2+} channels are more active in KATP channel deficient muscle. In this case the depolarization may activate them. As Ca^{2+} influx increases it contributes to the increase in resting tension. The greater $[Ca^{2+}]i$ then leads to fiber damage by activating Ca^{2+}-sensitive proteases or phospholipase-A or by increasing the production of reactive oxygen species (Jackson *et al.*, 1984; Neerunjun & Dubowitz, 1979; Supinski *et al.*, 1999). The lower capacity to recover force following fatigue in KATP channel deficient muscle (Fig. 3-4) may then be related to the damaged fibers which no longer generate force. The loss of fibers may also contribute to the faster rate of fatigue.

FATIGUE PRECONDITIONING

This study reports a novel phenomenon where the fatigue kinetics in FDB fibers are significantly slower during a second fatigue bout (FAT2) elicited 60 min after a first fatigue bout (FAT1); the slower fatigue kinetics being characterized by slower decrease in tetanic force (Fig. 4-2), and slower increases in resting tension (Fig. 4-3). The slower kinetics during FAT2 were especially marked when K_{ATP} channels were blocked with glibenclamide. As shown in Fig. 4-2, during a fatigue bout equivalent to FAT1, the lack of K_{ATP} channel seriously impairs muscle function as K_{ATP} channel deficient FDB fibers had much faster fatigue kinetics compared to control FDB fibers. None of these impairments were observed during FAT2 following FAT1 under control conditions. In fact, the fatigue kinetics during FAT2 and the subsequent recovery were no longer different between control and glibenclamide-exposed FDB muscles. These findings are in agreement with those of Bourassa and Renaud (unpublished results), who were the first to notice this new phenomenon as after FAT1, the decrease in tetanic Ca^{2+} was slower than during FAT2 whether or not glibenclamide was present.

The slower fatigue kinetics during FAT2 is not a temporal phenomenon. The fatigue kinetics during delFAT1 (Fig. 4-2) (initiated at the same time as FAT2) are actually slightly faster than those reported during FAT1 (Fig. 3-2). Thus, the longer the delay before a first fatigue bout is elicited at 37°C the lower the fatigue resistance is under control conditions. This time effect is opposite to the increase in fatigue resistance after one fatigue bout. We define this novel phenomenon as fatigue pre-conditioning.

One important characteristic of fatigue pre-conditioning is its temperature dependency. At 22°C, three fatigue bouts have similar decreases in peak Ca^{2+} and tetanic

force in mouse single FDB fibers (Chin & Allen, 1997). An increase in fatigue resistance is not even apparent when FAT2 is at 37°C after FAT1 at 22°C (Moopanar & Allen, 2005). Only when both fatigue bouts are at 37°C does the fatigue resistance becomes greater during the second fatigue bout.

It is not possible from our results to determine the mechanism(s) of fatigue pre-conditioning. It is unlikely to involve mechanisms such as those that occur during chronic muscular activity or training because the latter occurs over a period of weeks and requires important changes in protein content (Kane *et al.*, 2004; Nielsen *et al.*, 2004), whereas fatigue pre-conditioning is observed within 60 min. Instead, fatigue pre-conditioning most likely involves some intracellular signaling pathways, which may be similar to those of ischemic pre-conditioning. Ischemia occurs when blood flow to a tissue ceases. If the ischemic period is too long, it eventually leads to function impairment and fiber damage. In cardiac muscle a damaging ischemia occurs within 30 min. When cardiac muscles are exposed to short bouts of non-damaging ischemic periods, their resistance to function impairment and fiber damage during the 30 min ischemia is greater. Ischemic pre-conditioning has also been observed in skeletal muscle (Kohin *et al.*, 2001; Pang *et al.*, 1995).

Both fatigue (Gibbs, 1987) and ischemia (McPherson *et al.*, 1993) involve a metabolic stress. In the case of fatigue, the metabolic stress starts with an increase in energy demand that tends to deplete ATP (Matar *et al.*, 2000). During ischemia, blood flow is cut and even if there is no activity, the basal metabolic rate also causes an ATP depletion (Pang *et al.*, 1995). Thus, for both fatigue and ischemia, an ATP depletion may incur reactions that increase resistance to fatigue, function impairment, and fiber damage

(Andersen & Clausen, 1993; Garlid *et al.*, 1997). Finally, the protective effects of the pre-conditioning are observed within 30-60 min for both cardiac and skeletal muscle. It will therefore be interesting to determine in future studies if the factors involved in ischemic pre-conditioning, such as adenosine (Andersen & Clausen, 1993; Gürke *et al.*, 2000), mitochondrial K_{ATP} channel (Garlid *et al.*, 1997; Oldenburg *et al.*, 2002) and protein kinase C (Hopper *et al.*, 2000), are also involved in fatigue pre-conditioning.

Another important point to consider is the target of those signaling pathways. The major mechanism leading to the decrease in force during fatigue appears to be related to a failure of the sarcoplasmic reticulum to release adequate amount of Ca^{2+} to activate the contractile components. As a consequence of lower Ca^{2+} release, the activity of the Ca^{2+}-ATPase pump and myosin ATPase decreases protecting muscle against deleterious ATP depletion. If fatigue is a protective mechanism to prevent damaging ATP depletion, then a link between the Ca^{2+} release mechanism and the energy status of the fiber must exist. In fact, evidence for such a link exists. For example, glycogen depletion results in a decrease in peak $[Ca^{2+}]_i$ and tetanic force much sooner than under normal glycogen content (Chin & Allen, 1997)

One possible target for any signaling pathways involved in fatigue pre-conditioning must affect ATP production, ATP utilization or both. There is already evidence for greater glucose and free fatty acid transports following a period of muscle activity, which can be one mechanism for greater ATP production. Decreases in ATP utilization during prolonged contractions has also been reported in mammalian muscles. Greater production and/or lower utilization of ATP can then better sustain the activity of two important pumps: i) the Na^+K^+ pump, which maintains the necessary Na^+ and K^+

concentration gradients for action potential and which also contribute to the resting membrane potential, and ii) the Ca^{2+} ATPase pump to prevent increases in $[Ca^{2+}]_i$ and resting tension.

An effect on energy metabolism cannot however be the only mechanism in fatigue pre-conditioning. First, the decreases in tetanic force are not prevented but delayed, thus an energy deficit still occurs. One function of the K_{ATP} channel is to prevent function impairment and fiber damage by preventing damaging ATP depletion and intracellular Ca^{2+} accumulation. This K_{ATP} channel function is evident during a first fatigue bout, but not in subsequent fatigue bouts. Consequently, if an energy deficit still occurs during fatigue after fatigue pre-conditioning then another protective mechanism has to be activated to replace the K_{ATP} channels. Perhaps, the BK_{Ca} channels become more sensitive to increases in $[Ca^{2+}]_i$ during FAT2. Being a K^+ channel it can then have the same effects as those of the K_{ATP} channel.

CONCLUSION

This study began with two objectives. The first objective was to employ smaller muscle preparations to determine how K_{ATP} channels affect the kinetics of force during fatigue. It was found that although activation of K_{ATP} channels contributes to the decrease in force (i.e. increase the rate of fatigue), fatigue also occurs earlier when K_{ATP} channel activity is abolished. However, it was also demonstrated that in the latter case, the faster fatigue is the result of severe function impairment that adversely affects force production. It was then shown that the functional impairment was linked to an increase in Ca^{2+} influx via the L-type Ca^{2+} channel. Thus, one major function for the K_{ATP} channel is to reduce Ca^{2+} influx to prevent Ca^{2+} overload. In performing these experiments, a phenomenon

was observed in which one fatigue bout at 37°C improves fatigue resistance within 60 min. The initial fatigue bout reduced the dependency of muscle on K_{ATP} channels to protect them against function impairment and fiber damage in subsequent fatigue bouts. This phenomenon is referred to as 'fatigue pre-conditioning'.

CHAPTER 6

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