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The Relationship Between K_{ATP} Channels and Energy State in Skeletal Muscle During Fatigue Development

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THE RELATIONSHIP BETWEEN K_{ATP} CHANNELS AND ENERGY STATE IN SKELETAL MUSCLE DURING FATIGUE DEVELOPMENT

By

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ABSTRACT

The objective of this study was to test the hypothesis that "During fatigue development there is greater ATP utilization in the absence than in the presence of K_{ATP} channel activity". Flexor digitorum brevis (FDB) muscle bundles were fatigued with one tetanic contraction every sec for 3 min. K_{ATP} channel activity was abolished pharmacologically using glibenclamide or genetically using Kir6.2$^{-/-}$ FDB muscle bundles; pinacidil was used to activate the channel. Abolishing K_{ATP} channel activity had no effect on the decrease in phosphocreatine (PCr), but pinacidil significantly reduced its depletion. Kir6.2$^{-/-}$ FDB showed greater and faster ATP depletion compare to wild type control, but glibenclamide did not reproduce the effect. In the presence of pinacidil ATP content remained significantly above that of control wild type. Unexpectedly Kir6.2$^{-/-}$ FDB muscles generated less lactate than those of wild type. During the first half of the fatigue period, lactate production in wild type FDB were unexpectedly in the order of pinacidil > control > glibenclamide, with the reverse order for the second half of the fatigue period.

In conclusion, not all the data supported the hypothesis because the effects of modulating K_{ATP} channel activity appeared complicated by difference between acute (pharmacological) and chronic (genetical) approaches, such as changes in ATP and lactate; as well as by time dependent effects, such as observed with lactate.

LIST OF ABBREVIATIONS

°C: degree Celsius

$[Ca^{2+}]_i$: intracellular Ca^{2+} concentration

ABC: ATP-binding cassette

ACh: acetylcholine

ADP: adenosine-5'-diphosphate

AMP: adenosine-5'-monophosphate

AMPK: adenosine monophosphate-activated kinase

ANOVA: analysis of variance

ATP: adenosine-5'-triphosphate

CK: creatine kinase

CNS: central nervous system

DHPR: Dihydropyridine receptor

DMSO: dimethyl sulfoxide

EDL: extensor digitorum longus

ER: endoplasmic reticulum

FDB: flexor digitorum brevis

GFG: glycine-phenylalanine-glycine

GLUT: glucose transporter

G-1-P: glucose-1-phosphate

G-6-P: glucose-6-phosphate

GYG: Gly-Tyr-Gly

IMP: inosine monophosphate

K_{ATP} channel: ATP-sensitive potassium channel

Kir: potassium inward rectifier

L.S.D.: least significant difference

LSGGQ: Leu-Ser-Gly-Gly-Glu

mM: millimolar

mV: millivolt

n: number of sample

NBF: Nuclear binding fold

NMJ: neuromuscular junction

PCr: phosphocreatine

PFK: phosphofructokinase

P_i: inorganic phosphate

pH_i: intracellular pH

ROS: reactive oxygen species

RKR: Arg-Lys-Arg

RYR: Ryanodine receptor

S.E.: standard error

SR: Sarcoplasmic reticulum

SUR: sulfonylurea receptor

v/v: volume/volume

VMH: ventromedial hypothalamus

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CHAPTER 1

GENERAL INTRODUCTION

During muscle exercise, energy demand increases 20- to 100- fold, depending on fiber type and activity intensity (Gibbs, 1987). In many muscular activities, energy production and utilization are matched. However, there are activities for which energy demand exceeds ATP production, leading to an energy deficit which can be deleterious to muscle integrity and function. Thus, muscles need some mechanisms to protect themselves from large and damaging energy depletion. One of the mechanisms involves the ATP-sensitive potassium channels (K_{ATP} channel). The channel was named after the discovery that binding of ATP closes the K^+ channel (Noma, 1983). More importantly, there is now evidence that mild to severe fiber damage occurs in K_{ATP} channel deficiency muscle fibers *in vivo* following swimming and treadmill running or during fatigue *in vitro* using isolated muscle fibers (Kane et al, 2004; Thabet et al, 2005; Bourassa, 2006).

The mechanism by which a deficiency in K_{ATP} channel activity leads to fiber damage is not fully understood. It has been shown that K_{ATP} channel reduces action potential amplitude (Gong et al, 2003). As a consequence of the effect on action potential, less calcium is released by sarcoplasmic reticulum (SR) (Burton and Smith, 1997; Duty and Allen, 1995) and less force is generated (Gong et al, 2003; Matar et al, 2000). Less Ca^{2+} release means that less Ca^{2+} must be pumped back in the sarcoplasmic reticulum by Ca^{2+} ATPase pumps. It also means less formation of acto-myosin links during contraction, reducing myosin ATPase activity. It has been proposed that the

reduction in Ca^{2+} ATPase and myosin ATPase activity is important to reduce ATP utilization and thus help preventing deleterious ATP depletion and fiber damage. Although the effects of K_{ATP} channels on action potential, Ca^{2+} release and force has now been demonstrated, it is still unknown how the channels affect energy metabolism. Thus the overall objective of this study is to better understand how K_{ATP} channel affects energy metabolism.

MUSCLE CONTRACTION

MUSCLE CONTRACTION CYCLE

Muscle cells are specialized to generate force and movement. There are three types of muscle tissue: cardiac, smooth and skeletal muscle. Skeletal muscle fiber contains thousands of myofibrils which in turn are made up of many sarcomeres, which are the functional units in muscle. Sarcomeres contain thin filaments and thick filaments. The thin filament primarily contains actin, tropomyosin and troponin, while the thick filament is primarily composed of myosin.

Muscle contraction can be separated into three steps. The first step involves events at the neuromuscular junction, where a chemical signal from a motorneuron, acetylcholine (ACh), initiates an action potential. After its release, ACh binds to its receptor, which is a ligand-gated channel located on the motor end plate of the neuromuscular junction (NMJ). ACh causes the opening of the channel allowing a small amount of cation (both Na^+ and K^+) to flow through. Since the Na^+ influx exceeds the K^+ efflux, the membrane depolarizes. The depolarization then activates voltage sensitive Na^+ channels, triggering an action potential on the cell membrane. An action potential

involves a Na^+ influx during the depolarization phase and a K^+ efflux during the repolarization phase.

The second step is the excitation-contraction coupling mechanism that triggers calcium release. The action potential, generated at the NMJ, propagates along the cell membrane and into transverse tubules (t-tubules) by activating neighboring voltage sensitive Na^+ channels. T-tubules, which are cell membrane invaginations, are in close proximity to SR. Two Ca^{2+} channels contribute to the calcium release from SR. One is located on the t-tubular membrane and is known as the L-type Ca^{2+} channel. It acts as a voltage sensor and is also called dihydropyridine receptor, or DHPR, because it binds a class of drugs known as dihydropyridine (Dulhunty et al, 2002). The other channel is in the sarcoplasmic reticulum and acts as the Ca^{2+} release channel; it is also known as the ryanodine receptor (RyR) (Dulhunty et al, 2002). During an action potential DHPR are activated and via a protein-protein interaction DHPR directly activates RyR, which then opens. Ca^{2+} stored in the SR then diffuses down its electrochemical gradient into the cytosol and throughout the sarcomere. Once Ca^{2+} binds to troponin, a subsequent conformational change triggers a movement of tropomyosin to shift its position on the actin filament, exposing the binding sites for myosin on actin (Dulhunty et al, 2002).

The third step is the contraction itself that involves actin and myosin. When the myosin head (also known as the crossbridge) attaches to actin, it executes a power stroke that pulls the thin filament toward the center of the sarcomere, generating a force or shortening of the sarcomere.

ENERGY UTILIZATION AND PRODUCTION DURING MUSCULAR ACTIVITY

Three major ATPases are activated during contraction. (1) The Na^+-K^+-ATPase pumps 3 Na^+ out and 2 K^+ into the cell to maintain the Na^+ and K^+ concentration gradients that are necessary for action potential generation. (2) The Ca^{2+} ATPase pumps Ca^{2+} back into the sarcoplasmic reticulum to allow muscle relaxation after cessation of action potentials. (3) The myosin ATPase converts chemical energy into mechanical work (Gibbs, 1987). Among these three ATPases, the myosin ATPase was thought to account for about 50-80% of the ATP consumption during muscle contraction (Baker et al, 1994; Szentesi et al, 2001). However, these experiments were carried out at low temperatures. One recent study conducted at the physiological temperature of 37°C showed that in mice EDL muscles the crossbridges may account for only 20% of the total ATP consumption, suggesting that the major ATP energy consuming processes involve ion pumps (Zhang, et al, 2006). All ATPase enzymes use ATP as the following chemical reaction:

$$ATP + H_2O \leftrightarrow ADP + Pi + energy.$$

To avoid ATP depletion during any activity, muscles have several mechanisms to generate and maintain ATP content. There are four major pathways supplying ATP during muscle contraction. The first source is phosphocreatine (PCr), which is a high-energy phosphate compound (Brody, 1994). During exercise, the high energy phosphate group of PCr is transferred to ADP to synthesize ATP by the enzyme creatine kinase (CK) as follows (Hochachka, 1994):

$$PCr + ADP + H^+ \rightarrow Cr + ATP$$

As there is only one enzymatic reaction involved in this energy transfer, ATP can be formed as soon as ADP is produced. However, the ATP produced from phosphocreatine is very limited, lasting only a few seconds (Sahlin et al, 1998; Neville et al, 1996).

As exercise continues, glycolysis becomes the second source of ATP synthesis. Glycolysis is believed to be activated by AMP, ADP and Pi via activation at the level of phosphofructokinase (PFK); while ATP inhibits PFK activity (Spriet, 1989). Glucose, either from glycogen or blood glucose, is the substrate of glycolysis. Glycogen is broken down into Glucose-1-P during the process of glycogenolysis; while glucose from the blood is transported into the cytoplasm by GLUT4 (Lund, et al, 1995) and phosphorylated to G-6-P. G-1-P and G-6-P then enter the glycolytic pathway, which generates ATP and two end products: pyruvic acid and NADH as follows:

$$\text{Glucose} + 2\ \text{ADP} + 2\ \text{NAD}^+ + 2\ \text{P}_i \rightarrow 2\ \text{Pyruvate} + 2\ \text{ATP} + 2\ \text{NADH} + 2\ \text{H}^+$$

Pyruvic acid has two possible fates: First, it can be converted to lactic acid using NADH. Under those conditions only 2 moles of ATP are produced per mole of glucose or 3 moles of ATP per mole of glucosyl if from glycogen. Second, in the presence of oxygen, pyruvate can enter the Kreb's cycle, which occurs in mitochondria. The NADH generated during glycolysis is also used by mitochondria to synthesize ATP. Compared to the ATP synthesis from glycolysis, the generation of ATP by the combination of glycolysis and Kreb's cycle is much higher; the net yield being either 36 moles ATP per mole of glucose or 38 moles of ATP per mole of glucosyl from glycogen. The final chemical equation for glucose being:

$$\text{Glucose} + 36\ \text{ADP} + 36\ \text{Pi} + 6\ \text{O}_2 \rightarrow 6\ \text{CO}_2 + 6\ \text{H}_2\text{O} + 36\ \text{ATP}$$

Free fatty acids (FFA) constitute the third source of energy. The process of fatty acid oxidation is termed β-oxidation since it occurs through the sequential removal of 2-carbon units by oxidation at the β-carbon position of the fatty acyl-CoA molecule. It follows the reaction:

$$FFA(n)+nATP+nCoA+nNAD+nFAD \rightarrow nAcetyl\text{-}CoA+2nP_i+nAMP+nNADH+nFADH_2$$

where n represents the numbers of carbon in the FFA divided by 2.

Each round of β-oxidation produces one mole of NADH, one mole of $FADH_2$ and one mole of acetyl-CoA. The acetyl-CoA then enters the Kreb's cycle, where it is further oxidized to CO_2 and generates more NADH and $FADH_2$. The NADH and $FADH_2$ are then oxidized by the oxidation phosphorylation pathway to produce ATP. The oxidation of fatty acids yields significantly more energy per carbon atom than does the oxidation of carbohydrates. For example, the net result of the oxidation of one mole of oleic acid (an 18-carbon fatty acid) will be 146 moles of ATP, which is much higher than ATP production from an equivalent number of glucose carbon atoms, being only 114 moles.

Finally, in some types of muscular activity, especially high intensity exercise, the rate of ATP utilization exceeds the rate of the ATP regeneration, which leads to a decrease in ATP and an increase in ADP concentration. Under those conditions, two molecules of ADP are converted to ATP and AMP by adenylate kinase (Sahlin et al, 1990). AMP is then deaminated to inosine monophosphate (IMP) by AMP deaminase (Sahlin et al, 1990). This keeps the adenylate kinase reaction toward the production of ATP. In this manner an extensive imbalance between ATP, ADP and AMP is prevented during intense exercise.

SKELETAL MUSCLE FIBER TYPE

The extent by which each metabolic pathway generates ATP depends largely on the intensity of the activity and the fiber types being recruited. Skeletal muscle fibers have been initially classified by their contraction speed as slow twitch or type I fibers and fast twitch or type II fibers (Hamalainen and Pette, 1995). Over time and with the advent of DNA cloning and sequencing, four genes encoding four different adult myosin isoforms have been found (Parry, 2001). Today, fiber types are defined according to the myosin isoforms they express; i.e., one slow twitch type I fiber and three fast twitch type IIA, IIB and IIX fibers (also known as IIC and IID) (Johnson et al, 1994).

The first major difference between fast and slow twitch fibers is the speed of contraction. Fast twitch muscles contract two-three times faster than slow twitch fibers because of the faster ATPase activity of type II isoforms compare to type I isoforms (Pellegrino et al, 2003). The fast twitch fibers split ATP more rapidly and can therefore complete the contractile cycle more rapidly than slow twitch fibers. The duration of the twitches, which is determined by how fast the SR removes Ca^{2+} from the cytosol allowing muscle fiber relax, is also faster in fast twitch fiber than slow twitch fibers (Essen et al, 1975).

The second major difference between fiber types is the metabolic characteristic. Slow twitch type I fibers contain large amount of mitochondria, myoglobin and capillary density given them a high oxidative phosphorylation capacity (Hamalainen and Pette, 1995). These fibers are usually involved in the maintenance of posture. Like type I fibers, type IIA fibers also have a very high oxidative capacity (Silverthorn, 2004), which

is important during prolonged exercise, such as running a marathon. Thus both of them work better at low levels of muscular activity where blood flow can be maintained allowing good delivery of oxygen, glucose and fatty acids (Burton et al, 1997).

Type IIB fibers, on the other hand, have the lowest aerobic capacity with the highest glycolytic capacity (Hamalainen and Pette, 1995). These muscle fibers are usually recruited during extensive and powerful exercise, such as sprinting and weight lifting. These types of exercise often stop blood flow to muscle as blood vessels are squeezed by the strong contraction. Therefore these fibers rely primarily on PCr and glycolysis as their primary source of ATP. Type IIX fibers are also glycolytic fibers; but in terms of oxidative capacity, they are intermediate between type IIA and IIB fibers (Parry, 2001).

Overall, when the production of ATP is greater or equal to ATP utilization, the muscle can work for a long period of time. However in all types of muscle activities ATP demand eventually exceeds ATP production, leading to muscle energy deficit. The time it takes to reach that point varies with the types of exercise. For low to moderate exercise, such as marathons, it can take hours because type I and IIA fibers are primarily used. For intensive exercise, such as sprinting and weight lifting, it can occur within seconds because with these types of activities the low oxidative capacity type IIB and IIX fibers are being recruited. When an energy deficit occurs, the capacity of muscle to generate force or do work diminished. This process is known as muscle fatigue.

MUSCLE FATIGUE

Muscle fatigue can be generated at the level of the central nervous system (CNS), a phenomenon known as central fatigue. In this case, a decrease in motor unit activity is

observed, resulting in less activation of skeletal muscle (Asmussen, 1979). However, an early study has shown that direct motor nerve stimulation does not improve the reduced force in human during voluntary contractions (Merton, 1954). It is then well accepted that the primary mechanism causing the decrease on force or work occurs within muscle itself and has been defined as peripheral fatigue. Associated with the development of fatigue are several changes in metabolites, the extent of the change being primarily dependent on the type of fiber being recruited.

METABOLIC CHANGES DURING FATIGUE

ATP content at rest skeletal muscle is about 27-30 mmoles/kg dry weight (Spriet 1995; Carvalho et al, 1996; Meyer et al, 1979; Matar et al, 2000) in fast twitch muscles and lower in slow twitch muscles, being about 20 mmoles/kg dry weight (Meyer et al, 1979; Matar et al, 2000). Decreases in ATP in slow twitch muscles are also slower than fast twitch muscles. When Meyer et al (1979) stimulated rat slow twitch soleus and fast twitch gastrocnemius to the same extent of fatigue, ATP decreased only 2 mmoles/kg dry weight in the former compare to about 12 mmoles/kg dry weight in the latter. The difference in ATP decrease is because slow twitch fibers contains primarily type I fibers (95% in rats), which have higher oxidative capacity and lower rate of ATP hydrolysis than fast twitch gastrocnemius that contains primarily type IIB and IIX fibers.

Overall, while force can decrease by more than 70%, most studies have reported only very small changes in ATP content. Some studies reported no change in ATP (Cady et al, 1989; Hancock et al, 2005; Jansson et al, 1987; Vollestad et al, 1988). Other studies reported about 20% decrease in muscle ATP (Bangsbo et al, 1990; Jacobs et al, 1982;

Giannesini et al, 2001; Chasiotic et al, 1987). Only rare studies showed ATP decrease exceeding 20% (Meyer et al, 1979). The lack of muscle ATP decreases can either be due to the fact that ATP generation matched ATP utilization and/or when it fails, the fatigue process is capable of decreasing ATP utilization. Here we first discuss the importance of ATP synthesis.

The resting content of PCr ranges from 70-80 mmoles/kg dry weight in human quadriceps femoris muscle (Spriet, 1995; Norman et al, 1987) and rats EDL (Carvalho et al, 1996) and can be as high as 120 mmoles/kg dry weight, such as in mice EDL muscle (Matar et al, 2000). Slow twitch soleus muscle has 74 mmoles/kg dry weight of PCr (Matar et al, 2000), which is in agreement with that fast twitch fibers contain 15-20% more PCr than slow twitch fibers (Soderlund and Hultman, 1991). PCr, being the first energy source, decreases considerably within the first 10-60 sec of short bursts intensive exercise (Sahlin et al, 1998; Neville et al, 1996; Hirvonen et al, 1987; Meyer et al, 1979). The decrease is as much as 80-90% in fast twitch gastrocnemius muscle, compare to only 60% in slow twitch soleus muscle (Meyer et al, 1979). Such a difference between fiber types may due to the higher creatine kinase activity in faster twitch skeletal muscles (Roman et al, 2002). In those high intensive exercises PCr decreases as high as 90% (Sahlin and Ren, 1989); however during prolonged exercise the decrease can be as low as 33% (Sahlin et al, 1990).

Concomitant to the decrease in PCr, creatine and Pi increases, creatine being one product of the creatine kinase reaction while Pi is released once ATP is hydrolyzed. Pi increases from 3 mmoles/kg dry weight at rest to about 25 mmoles/kg dry weight after

exercise; while Cr before and after fatigue is about 60 mmoles/kg dry weight and 130 mmoles/kg dry weight, respectively (Chasiotic et al, 1987). Pi increase has been suggested to serve as a link between PCr breakdown and glycogen utilization (Chasiotis et al, 1982).

Despite the rapid and large decreases in PCr and some decreases in ATP, increases in ADP are still small, ranging from less than 10 μM at rest to no greater than 300 μM after fatigue (Dawson et al, 1978; Tullson et al, 1990; Chasiotic et al, 1987). Although it represents more than 10-fold change in ADP, the increase is still smaller than the decrease in ATP, which is in the millimolar range. The increase in ADP during fatigue is also smaller in slow-twitch muscle than fast twitch muscle (Macdonald and Stephenson, 2006).

One reason for the lack of ADP accumulation is most likely due to the adenylate kinase and AMP deaminase reaction. The first enzyme generates 1 mole ATP and 1 mole AMP from 2 moles ADP; while the second one generates IMP and ammonia (NH_3) from AMP. AMP accumulation is also small (Chasiotic et al, 1987), which never amounted to more than 2% of the total adenine nucleotide content (TAN = ATP + ADP + AMP) (Meyer et al, 1979); IMP and NH_3 levels increase significantly to similar levels during fatigue (Sahlin and Broberg, 1990; Hellsten et al, 1991). Increases in IMP have been shown to balance the decreases in TAN, which is to about 15% after maximal exercise (Meyer et al, 1979; Sahlin et al, 1978).

Depending on the exercise intensity, IMP increase can be over 50% of the tissue's ATP content (Meyer et al, 1979; Dudley and Terjung, 1985), ranging from 0.22 mmoles/kg dry weight at rest to 15 mmoles/kg dry weight after fatigue. Such an accumulation in muscle has been found to be fiber type dependent in skeletal muscle, being about two fold

higher in fast twitch fibers than in slow twitch fibers (Winder et al, 1974; Meyer et al, 1979). It has also been shown that after exercise the IMP formed can be reaminated back to AMP by adenylosuccinate synthase and adenylosuccinase, respectively. Thereafter AMP can be deaminated to ATP, which plays a crucial role in maintaining ATP during exercise (Lowenstein, 1972). This cycle is called purine nucleotide cycle (Sjodin et al, 1991).

At rest, lactate content is very low, being about 10 mmoles/kg dry weight (Carvalho et al, 1996). With exercise, lactate is produced in large amount and can reach 60-100 mmoles/kg dry weight (Katz et al, 1986; Dudley and Terjung, 1985; Sahlin et al, 1978). As expected, lactate increases up to 25-fold in fast twitch muscles, like gastrocnemius, compare to only 3 to 4-fold in the more oxidative soleus muscle during the early stimulation period (Meyer et al, 1979). Thus production of lactate is evidence for significant activation of glycolysis during fatigue. Associated with the production of lactic acid are large decreases in intracellular pH (pH_i) as lactic acid dissociates into lactate and H^+ ions. During muscle fatigue intracellular pH decreases from approximately 7.0 (resting state) to as low as 6.2 in amphibians, 6.3 in rats and 6.4 in humans (Cady et al, 1989).

During prolonged exercise, the major energy source is glycogen. Glycogen decreases linearly with time during fatigue from about 200-300 mmoles/kg dry weight to 100 mmoles/kg dry weight (Norman et al, 1987; Sahlin et al, 1998). There is no significant difference between EDL and soleus muscle glycogen level at rest (Carvalho et al, 1996).

MECHANISMS FOR THE DECREASE IN FORCE DURING FATIGUE

For several years, investigators studied the effects of the various metabolic changes on the capacity of the sarcomere to generate force. At room temperatures, only the decrease in

pH_i and increase in Pi can depress force, but only account for one third of the total decrease in force during fatigue (Godt and Nosek, 1989). Then recent studies however have shown that at the physiological temperature of 37°C, pH_i may actually have no effect while Pi depresses force only at submaximal Ca^{2+} concentrations (Pate et al, 1995; Wiseman, et al, 1996; Debold et al, 2006). It has therefore become apparent that the primary mechanism for the decrease in force during fatigue is upstream of the sarcomere.

There is now clear evidence that the decrease in force involves a decrease in Ca^{2+} release by SR, making the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) submaximal for the activation of the sarcomere. During the early part of fatigue, tetanic $[Ca^{2+}]_i$ increases by about 50% before it significantly declines below pre-fatigue value (Westerblad and Allen, 1991). Changes in tetanic force are associated with a decrease in $[Ca^{2+}]_i$ during the final stages of fatigue (Allen et al, 2002).

It now remains to be determined if the failure of Ca^{2+} release involves a decrease membrane excitability or a failure of transmitting the signal from t-tubules to the sarcoplasmic reticulum RYR. For now, most of the researches have concentrated on a decrease in membrane excitability (Renaud, 2002).

MUSCLE FATIGUE AND MEMBRANE EXCITABILITY

At rest, membrane potential ranges between -75 to -85 mV, while action potential overshoot reaches 25-30 mV. After muscle fatigue, resting membrane potential decreases to -65 and -70 mV (Balog and Fitts, 1996), while action potential overshoot are less than 10 mV (Lännergren, 1987; Balog et al, 1994). These effects are mainly related to changes in Na^+ and K^+ concentration. A large number of action potentials lead to a net Na^+ influx and a

net K^+ efflux. When the N^+-K^+-pump is unable to return all that Na^+ out and all that K^+ back into the cell to maintain their concentration gradient, it results in large increases in intracellular Na^+ and extracellular K^+ concentration. During fatigue $[K^+]_i$ decreases by about 10-20%, while $[K^+]_e$ increases from control 4 mM (normal) up to 10 mM at exhaustion (Renaud, 2002; Vollestad et al, 1994; Hallen et al, 1994). Most studies reported $[Na^+]_i$ increases by about 1.5-2 fold (Westerblad et al, 1991), while $[Na^+]_e$ rarely changes because of a loss of water by the extracellular fluid during fatigue.

Such changes in Na^+ and K^+ concentration have profound effects on resting and action potential. Study from Cairns et al (2003) looked at the Na^+ effect on membrane excitability and force generation in unfatigued soleus muscles by mimicking the changes in Na^+ concentration gradient observed during fatigue. Decreases in the Na^+ gradient resulted in a large number of fibers becoming unexcitable fibers and a large reduction of action potential amplitude in those still capable of generating action potentials. Associated with the lost of membrane excitability and reduction in action potential amplitude were large decreases in force. These results gave strong evidence that during fatigue a decrease in Na^+ concentration gradient can contribute to the decrease in force.

For the increase in $[K^+]_e$, the direct effect is the depolarization of resting membrane potential by about 15 mV (Renaud, 2002; Renaud and Light, 1992). Such a depolarization leads to inactivation of voltage dependent Na^+ channel, which also reduces action potential amplitude, Ca^{2+} release and force (Renaud, 2002).

Some studies are also focusing on ion channels which when activated can also cause a decrease in action potential amplitude and force. One is the Ca^{2+}-activated K^+ channel

(BK_{Ca}), which can be opened in the presence of high $[Ca^{2+}]_i$. Its opening leads to an increase in membrane K^+ conductance and a decreased action potential amplitude (Fink et al, 1976), which eventually leads to less calcium release and force development. This channel may be important in preventing chronic elevation of $[Ca^{2+}]_i$, which can be detrimental in muscle fibers. See section below "K_{ATP} channel protects against Ca^{2+} overload". Another channel is the ATP-sensitive potassium channel, which is the focus of this thesis and will now be discussed.

ATP SENSITIVE POTASSIUM CHANNEL

The ATP-sensitive potassium channel was named as the binding of intracellular ATP closes the channel and because it is selective for K^+ (Noma, 1983). At the time Noma had proposed that the K_{ATP} channel is important in the regulation of cellular energy metabolism in heart, the tissue in which it was found. Thereafter, the channel was found in various tissues, including pancreas, central neurons, skeletal muscle, smooth muscle and kidney, accomplishing various functions (Ashcroft FM, 1988).

MOLECULAR STRUCTURE

The K_{ATP} channel is a hetero-octameric protein composed of two subunits: a pore-forming subunit, Kir6.x; and a regulatory subunit, sulfonylurea receptor or SUR. (Ashcroft & Gribble, 1998; Aguilar-Bryan et al, 1998; Seino, 1999). The two subunits form a functional channel in a 4:4 stochiometry (Clement et al., 1997; Inagaki et al., 1997; Shyng et al., 1997; Seino, 2003). (Fig. 1-1)

Kir subunit

Kir subunit belongs to the inward rectifier K^+ channel family, which contains seven

members (Kir1-7). Kir channels are commonly made up by either homomeric or heteromeric subunits with the same subfamily. So far, three Kir6 genes have been cloned and sequenced, including Kir6.1 and Kir6.2 in mouse, human and rat, and Kir6.3 in zebra fish. Kir6.1 and Kir6.2 have about 71% amino acid identity and four of them together form the pore of the channel. The structure includes two transmembrane regions (M1 and M2). M1 and M2 are separated by an external pore loop (H5), which is located near the pore of the channel and is believed to be part of the selectivity "filter" (Inagaki et al, 1995). Although the Gly-Tyr-Gly (GYG) motif in the H5 region is thought to be critical for the selectivity of most K^+ ion channels, the amino acid sequence for Kir6.1 and Kir6.2 was found to be Gly-Phe-Gly (GFG), suggesting a characteristic unique to the Kir6 family (Seino et al., 2003). In mammal, Kir6 also has an endoplasmic reticulum (ER) retention signal in the C-terminus, the motif being RKR (Arg-Lys-Arg). This retention signal prevents Kir6 subunits trafficking to the cell membrane in the absence of SUR subunit (Zerangue et al, 2001).

SUR subunit

The SUR subunits (receptors for the sulfonylureas, which are known as anti diabetic drugs) belong to the superfamily of ATP-binding cassette (ABC) (Higgins, 1992). The superfamily comprises large transporter proteins responsible for the movement of a wide variety of substrates, such as ions, lipids and drugs across the cell membrane. Like other ABC proteins, SUR proteins are composed of three transmembrane domains, TMD0, TMD1 and TMD2 with respectively five, six and six membrane spanning regions as well as two nucleotide binding folds (NBF-1 and NBF-2) as intracellular loops (Conti et al,

2001) (Fig. 1-1). NBF-1 is located between TMD1 and TMD2 while NBF-2 is located in the C-terminal. Each nucleotide binding fold contains Walker A and B motifs, an ABC signature motif (also called as linker sequence or LSGGQ motif) and an invariant glutamine and histidine residue (also called as the Q-loop and H-loop respectively) (Matsuo et al, 2005). These motifs play a key role for the binding of ATP and ADP. Three major isoforms, SUR1 and SUR2A and SUR2B, have extensively been studied. Two different genes encode SUR1 and SUR2, while SUR2A and SUR2B are the products of gene splicing and differ by only 42 amino acids in the C-terminus.

Assembling SUR and Kir subunits significantly increases the expression as well as the gating function of K_{ATP} channel (Shi et al, 2005). Different combinations of subunits reconstitute different types of K_{ATP} channels in various tissues, which also lead to distinct electrophysiological properties and pharmacological sensitivities. In pancreatic β-cell, K_{ATP} channel is made up of Kir6.2 and SUR1 (Inagaki et al, 1995); in cardiac and skeletal muscle, K_{ATP} channel is made up of Kir6.2 and SUR2A (Inagaki et al, 1996); in non-vascular smooth muscle, K_{ATP} channel is composed of Kir6.2 and SUR2B subunits (Yamada et al, 1997) while Kir6.1 and SUR2B constitutes the vascular smooth muscle K_{ATP} channel (Yamada et al, 1997). However, recent studies have given evidence for the expression of Kir6.1 and SUR2B in both cardiac and skeletal muscle with different locations. Kir6.1 subunits are shown to be predominantly associated with myofibril structures and are also located in mitochondria. SUR2B subunits appear strongly co-localized with t-tubules (Singh et al, 2003). Furthermore, SUR1 subunit appeared to be expressed in flexor digitorum brevis (FDB) muscles. (Tricarico et al, 2006).

Figure 1-1

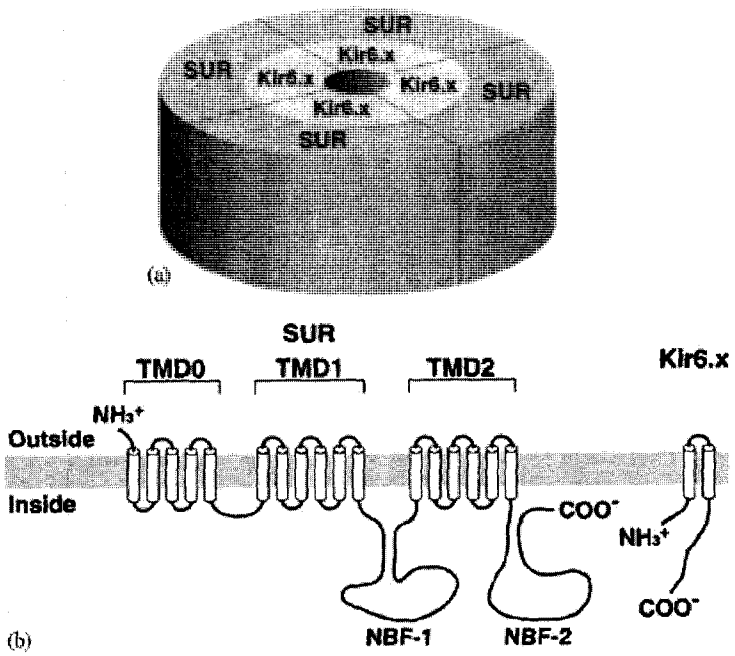


Figure 1-1. Molecular structure of K_{ATP} channel. (a) Assembly of K_{ATP} channel. The K_{ATP} channel is a hetero-octamer comprising two subunits: the pore-forming subunit Kir6.x (Kir6.1 or Kir6.2) and the regulatory subunit sulfonylurea receptor SUR (SUR1 or SUR2). (b) Membrane topology of SUR and Kir6.x. The sulfonylurea receptor has been proposed to have three transmembrane domains, TMD0, TMD1 and TMD2, each of which consists of five, six and six membrane spanning regions. Kir6.x has two transmembrane domains, while the Kir6.2 only has two membrane spanning regions (far right). (Seino, 2003)

REGULATION OF THE K_{ATP} CHANNEL ACTIVITY

K_{ATP} channel is a ligand sensitive, and voltage insensitive potassium channel. It is activated by several metabolic changes that are known to occur during fatigue, hypoxia or ischemia. The changes in metabolites include decreases in intracellular ATP, increases in intracellular ADP, lactic acid, H^+, and extracellular adenosine concentration.

Inhibition of K_{ATP} channel by ATP and ADP

The gating of a normal K_{ATP} channel involves short bursts of closing and opening state separated by long closed time. In the presence of ATP, the duration and frequency of the long closed time increases (Alekseev et al, 1997; Drain et al, 1998). The first evidence that the ATP effect was due to a binding on the Kir subunit came from a study in which the last 36 amino acids of the C-terminus were truncated to remove the ER retention signal. When expressed in a cell, it gave rise to a functional K^+ channel current, which can be inhibited by ATP (Tucker et al, 1997).

ATP binding occurs at the interface between the C-terminus of one subunit and the N-terminus of the adjacent subunits. This unique feature makes the binding of one ATP sufficient to close the channel (Markworth et al, 2000). ATP has an adenine ring and carries negative charges with its three inorganic phosphates that forms the phosphate tail (Haider et al, 2005). The phosphate tail interacts with R201 (Arg201) and K185 (Lys185) residues in the C-terminus of one subunit and with the R50 (Arg50) residue in the N-terminus of a second subunit (Antcliff, et al, 2005). The adenine ring also interacts with residues E179-I182 (Glu179-lle182) of one subunit as well as the R301 (Arg 301) of the second subunit. Mutations of any of these residues have been found to reduce ATP

sensitivity (John et al, 2003; Reimann et al, 1999; Proks et al, 1998; Antcliff et al, 2005; and Li et al, 2000). Most ATP binding sites require Mg^{2+}, such as ATPase, enzymes. One interesting characteristic of the ATP binding to Kir6.2 is that it is completely Mg^{2+} independent. Finally, while the ATP binding site is located on Kir6.2 subunit, coexpression with SUR enhances the inhibiting effect of ATP by 10-fold (Tucker et al, 1997). ADP itself can bind to Kir6.2 subunit in the absence of Mg^{2+}. It also closes the channel but has a lower affinity than ATP for the Kir6.2 subunit (Markworth et al, 2000).

Under patch clamp conditions, ATP inhibits K_{ATP} channels in the micromolar range which is far below the physiological range measured in most cells (5-10 mM). Even metabolic inhibition reduces ATP to only 1 mM (Karatzaferi et al, 2001). Although such results would suggest that the K_{ATP} channel could never be activated physiologically, there is now plenty of evidence that the channel is activated during fatigue, hypoxia and metabolic inhibition (Gramolini et al, 1997; McPherson et al, 1993). One possible reason as to how the channel is activated when ATP is still in the millimolar range is because other metabolites also activate the channel.

Activation of K_{ATP} channel by MgADP

Although ATP binding to Kir6.2 subunits is Mg^{2+}-independent, Mg^{2+}-nucleotides, such as MgATP and MgADP, interact with the SUR subunits at the NBF (Tucker et al, 1997; Gribble et al, 1998). MgATP, which can enhance the channel activity, is believed to activate the channel indirectly, after it has been hydrolyzed to MgADP at NBF-2. MgADP then attenuates the inhibition of Kir subunits by ATP, shifting the ATP dose-response curve toward higher concentrations. Therefore, increase MgADP

concentration can be an important factor activating K_{ATP} channel (Renaud, 2002).

Studies (Ueda et al, 1999a) of nucleotide binding to SUR1 have showed that when the ATP/ADP ratio decreases, ATP binds SUR1 at NBF-1 and MgADP binds at NBF-2 either directly or after the hydrolysis of MgATP to MgADP. MgADP binding at NBF-2 causes a conformational change in SUR1 which enhances the ATP-binding at NBF-1 and reduces ATP binding to Kir6.2, resulting in the opening of the K_{ATP} channel. In contrast, when the ATP/ADP ratio increases, the resultant decrease in MgADP concentration induces the dissociation of MgADP from NBF-2 and ATP from NBF-1. As ATP binds to Kir6.2, the channel closes (Ueda et al, 1999b).

In pancreatic β-cells, the changes in ATP/ADP ratio seem enough to regulate the K_{ATP} channel activity (Aguilar-Bryan and Bryan, 1999). However, in cardiac and skeletal muscle, this is not the case, since ADP changes very little during muscle fatigue as discussed in the section above "Metabolic changes during fatigue". Changes in pH, lactate and adenosine appear to be more important in activating K_{ATP} channels in cardiac and skeletal muscles.

Activation of K_{ATP} channel by lowering pH

As discussed above in the section "Metabolic changes during fatigue", pH_i can decrease from normal level of 7.0 to as low as 6.2. When such a decrease in pH_i is mimicked using NH_3Cl in unfatigued skeletal muscle, K_{ATP} channels become activated even though ATP levels are in the millimolar range (Davies et al, 1992). Xu et al (2001) also observed an activation of K_{ATP} channel by using CO_2 to acidify pH_i. Thus the change in pH_i during fatigue is one of the best candidates for the activation of the channel in

muscle.

In the absence of ATP, acidic pH has little effect on the channel activity in terms of open probability and conductance. Its effects appear to involve a decreased affinity of Kir6.2 for ATP (Davies et al, 1992). There is now evidence that the H^+ binding site is located on the Kir6 subunit rather than in the SUR (Xu et al, 2001). Three amino acids have been identified to contribute to the pH sensitivity, including Thr-71 in the N terminus, Cys-166 in the M2 region and His-175 in the C terminus. Any mutations of these residues are sufficient to completely abolish the acid-induced channel activation (Piao et al, 2001).

Activation of K_{ATP} channel by lactate and adenosine

Lactate at 20 mM, a concentration observed in plasma during fatigue, is another K_{ATP} channel activator. More importantly, it can activate the channel even when ATP levels are between 2 and 5 mM (Keung & Li, 1991; Nichols & Lederer, 1991). Adenosine, which accumulates in the extracellular space when metabolic rate increases, also activates K_{ATP} channels, via its A1 receptor in both cardiac and skeletal muscle (Olsson, 1996; Barrett-Jolley et al, 1996).

Overall, the activation of the K_{ATP} channel occurs when energy level decreases; K_{ATP} channel can sense an energy deficit either from a decrease in ATP or from the build up of metabolic end products, especially H^+, lactate and adenosine. Not only is the channel acting as an energy sensor in the cell, it also affects the electrical activity of the cell membrane. It thus links the energy states of a cell to the membrane electrical activity.

PHYSIOLOGICAL ROLES OF THE K_{ATP} CHANNEL

K_{ATP} channels play important and various functions that can be divided into two major groups: i) glucose homeostasis and ii) myoprotection in muscles.

GLUCOSE HOMEOSTASIS

The K_{ATP} channels in pancreatic β-cells are involved in glucose-induced insulin secretion. When blood glucose level increases and is transported by GLUT2 into the β-cell, glucose is metabolized via glycolysis and within mitochondria, thus leading to an increase in ATP production and a decrease in ADP level. As the ATP/ADP ratio increases, the K_{ATP} channels close allowing for a depolarization, which then activates L-type Ca^{2+} channels. The subsequent increases in Ca^{2+} influx through the Ca^{2+} channels increases $[Ca^{2+}]_i$, which triggers insulin release (Ashcroft, 2006). The K_{ATP} channels are also found in pancreatic α-cells, but their role in the secretion of glucagons is still unclear (Miki et al, 2001).

K_{ATP} channels also affect the firing pattern of glucose responsive (GR) neurons in the ventromedial hypothalamus (VMH), where the channel is made up of SUR1 and Kir6.2 subunits. These neurons then increase glucagon secretion via the autonomic neuron system under hypoglycemic conditions (Miki et al, 2001). Basal and insulin-induced glucose uptake in skeletal muscle is increased in the absence of K_{ATP} channel activity. This has been confirmed using pharmacological (Trube et al, 1986) and genetic (Chutkow et al, 2001; Miki et al, 2001; William et al, 2001) approaches with null mice for either the $Kir6.2^{-/-}$ or $SUR2^{-/-}$ gene. Pathways involved in K_{ATP} channel-associated glucose uptake still remain to be identified, but studies have already

shown that they are independent of cAMP-activated protein kinase (AMPK) and the insulin receptor substrate-1 /phosphatidylinositol 3-kinase (IRS-1/PI3K) signal (Minami et al, 2003).

MYOPROTECTION IN MUSCLE

It has been proposed that K_{ATP} channels can prevent ATP depletion and thus fiber damage by acting at the level of blood vessels and muscles themselves. At the level of blood vessels, the K_{ATP} channels in vascular smooth muscle regulate systemic blood pressure (Chutkow et al, 2001; Miki et al, 2001). They also regulate blood flow in several tissues. For example, they contribute to the vasodilatation associated with reactive hyperemia during muscle activity (Peter et al, 1996). That is, they allow for vasodilatation contributing to an increased delivery of oxygen, glucose and fatty acid, which are important energy sources during muscle activity. Within muscles themselves, K_{ATP} channel has been proposed to protect muscle fiber damage by preventing Ca^{2+} overload and energy depletion. As both cardiac and skeletal muscles are striated muscles, having a lot of similarities, we discuss this role of the K_{ATP} channel by comparing cardiac muscle and skeletal muscle.

K_{ATP} channel protects against Ca^{2+} overload

Ca^{2+} overload is defined by a large uncontrolled $[Ca^{2+}]_i$. It can be due to an increase influx through cell membrane or a failure of the various Ca^{2+} pumps. Increases in $[Ca^{2+}]_i$ is known to impair muscle function by activation of Ca^{2+}-dependent proteases (Belcastro, 1993), activation of phospholipase A_2 (Nethery, 2000) and/or an increased production of reactive oxygen species (ROS) (Nethery et al, 2000). Therefore, any mechanisms that

prevent Ca^{2+} overload contributes to myoprotection.

Cardiac muscle K_{ATP} channels are close at rest. During stress, such as treadmill running or swimming, catecholamines are released by the sympathetic neurons and adrenal glands. In cardiac cells, catecholamines increase L-type Ca^{2+} channel activity to increase force output and increase K_{ATP} channel activity to shorten action potential duration, allowing for faster heart beat. Kir6.2$^{-/-}$ mice, lacking K_{ATP} channel in cell membrane, exhibit arrhythmia and sudden death under vigorous sympathetic stimulation, such as during treadmill running and swimming. This is partly because of no reduction in action potential duration, resulting in much longer time during which Ca^{2+} comes into the cytosol during contraction. This extra Ca^{2+} influx causes a Ca^{2+} overload (Zingman et al, 2002; Kane et al, 2004). Verapamil, a Ca^{2+} channel blocker, decreases mortality during exercise in Kir6.2$^{-/-}$ mice to 17% from 73% in untreated mice, strongly suggesting that the lack of K_{ATP} channel activity increases Ca^{2+} influx through L-type Ca^{2+} channels (Zingman et al, 2002). In another study, Kir6.2$^{-/-}$ mice were compared with wild type mice following daily swimming for 28 days. Kir6.2$^{-/-}$ mice showed less augmentation in exercise capacity, lacked metabolic improvement, had damaged structure in the heart and impaired cardiac performance (Kane et al, 2004). Therefore, under physiological stress conditions, K_{ATP} channel contributes to prevent Ca^{2+} overload, arrhythmias, fiber damage and death.

In cardiac studies, the K_{ATP} channels have been studied more extensively under the condition of hypoxia or ischemia. Under these conditions, K_{ATP} channels are activated and allow for greater K^+ efflux and shortening of action potential duration (APD) (Gasser

et al, 1990). It is believed that shorter APD reduces Ca^{2+} influx and force production, reducing ATP utilization and Ca^{2+} overload. Studies have shown that K_{ATP} channels protect heart muscles from post ischemic dysfunction also by limiting Ca^{2+} influx through L-type Ca^{2+} channels (Lascano, et al., 2002). This mechanism involves a hyperpolarization of the diastolic membrane potential preventing an activation of L-type Ca^{2+} channels (Baczko et al 2004).

Skeletal muscle As observed in cardiac muscles, K_{ATP} channels are also inactive at rest in skeletal muscles (Gramolini and Renaud, 1997; Matar et al, 2000). A lack of K_{ATP} channel activity in the cell membrane of skeletal muscle *in vivo* causes significantly mild to severe fiber damage during swimming and treadmill running in hindlimb and diaphragm muscles (Thabet et al, 2005; Kane et al, 2004). Fiber damage has also been reported in FDB fibers when they are fatigued *in vitro* the K_{ATP} channel activity is abolished either pharmacologically in wild type FDB exposed to glibenclamide or genetically with $Kir6.2^{-/-}$ FDB fibers (Bourassa, 2006). Thus, as observed for cardiac muscles, K_{ATP} channels are also important for myoprotection in skeletal muscles.

Once the channel is activated under a condition such as muscle fatigue, it reduces action potential amplitude (Gong et al, 2003) but not its duration as in cardiac myocytes. As a consequence of lower action potential amplitude, less Ca^{2+} is released by sarcoplasmic reticulum (Burton et al, 1997; Duty et al, 1995) and less force is generated by the contractile component (Matar et al, 2000; Gong et al, 2003). This may be one mechanism by which a Ca^{2+} overload is prevented in skeletal muscle. In the absence of K_{ATP} channel activity, several contractile dysfunctions are observed that including: i)

large increases in resting $[Ca^{2+}]_i$ (Bourassa, 2006); ii) increases in resting tension (which occurs when muscles fail to completely relax) (Matar et al, 2000; Gong et al, 2003; Cifelli, 2006); as well as iii) an impaired capacity to recover force (probably because of fiber damage) (Matar et al, 2000; Gong et al, 2003; Cifelli, 2006). Cifelli (2006) and Bourassa (2006) both demonstrated that these contractile dysfunctions were responsible for the faster decrease in tetanic $[Ca^{2+}]_i$ and force during fatigue when there is no K_{ATP} channel activity.

Cifelli (2006) further demonstrated that lowering extracellular Ca^{2+} or exposing muscles to verapamil (L-type channel blocker) reduces the increase in resting tension and improves force recovery following fatigue. His results suggests that, as observed in heart, K_{ATP} channels are crucial in skeletal muscle to prevent Ca^{2+} overload and subsequent contractile dysfunction and fiber damage.

K_{ATP} channel protects against energy depletion

When K_{ATP} channels were first discovered (Noma, 1983), it was suggested that they affect the energy metabolism because i) they are activated by decreases in ATP; and ii) they affect membrane excitability and eventually contractility. Then as they prevent a decrease in Ca^{2+} overload, it helps preserving ATP, because lower $[Ca^{2+}]_i$ results in decreased Ca^{2+} ATPase and myosin ATPase activity (because of lower resting tension between contractions). Furthermore, the decreases in Ca^{2+} release and force production during a contraction as action potential durations are shortened in cardiac muscles or action potential amplitudes are reduced in skeletal muscle are also expected to help preserving ATP. This is crucial because any large ATP depletion during muscular

contraction affects ion pump activity and ion regulation. It can also lead to fiber damage and eventually cell death.

Cardiac muscle During ischemia, pinacidil, a K_{ATP} channel opener, reduces the extent of high energy phosphate depletion in cardiac muscle. Glibenclamide, a channel blocker, not only inhibited the pinacidil effect, it also enhances ischemic depletion of ATP (Mcpherson et al, 1993). A lack of K_{ATP} channel activity also causes greater depletion of myocardial glycogen content and higher lactate production before ischemia. Opening of the channels with diazoxide has the reverse effects (Kristiansen et al, 2005).

K_{ATP} channels have also been proposed to reduce metabolic rate, preventing the generation of excess metabolic end products (Kristiansen et al. 2005), such as reactive oxygen species (ROS), which in excess amount will lead to fiber damage (Halliwell et al, 1989 & 1992). Hydrogen peroxide or the combination of purine plus xanthine oxidase treatment resulted in a depression of myocardial contractility and significantly reduced ATP, phosphate creatine and glycogen content. However, these effects have been shown to be significantly attenuated by K_{ATP} channel opener, cromakalim (Gan et al, 1998).

Skeletal muscle Matar et al (2000) reported that in EDL muscle, neither glibenclamide, a channel blocker, nor pinacidil, a channel opener, affects the extent of the decrease in ATP and PCr when measured just before and after fatigue. In soleus muscle, on the other hand, they showed that glibenclamide caused greater decrease in ATP while pinacidil resulted in slight increase in ATP during fatigue. These effects of K_{ATP} channels were unexpected since treadmill running causes fiber damage in EDL and not in soleus muscles (Thabet et al, 2005). Furthermore, *in vitro* resting tension and force recovery is

dependent on K_{ATP} channel to a greater extent in EDL than in soleus muscles. The problem, however is that Matar et al (2000) only measured ATP and PCr just before and immediately after fatigue. Gramolini et al. (1997) measured changes in ATP and PCr during metabolic inhibition in frog muscle. The decreases in ATP and PCr during metabolic inhibition were faster in the presence than in the absence of glibenclamide. However, after 60 minutes of metabolic inhibition ATP and PCr levels were again the same in both groups.

This raises two issues: the first issue is that to understand the role of K_{ATP} channel in terms of metabolites, one cannot just measure their changes before and after fatigue. The second issue is that protective effect of K_{ATP} channels may not be limited to just the prevention of deleterious ATP depletion. It may also be important in reducing metabolic rate because similar decrease in ATP does not imply that ATP production was not greater in the absence of K_{ATP} channel activity. Moreover, there is evidence for greater glucose uptake in K_{ATP} channel deficiency muscles (Miki et al, 2001). It is then possible that greater metabolic rate results in greater generation of metabolic end products, such as reactive oxygen species (ROS) which in excess amount can be damaging.

Overall it is still unclear whether in skeletal muscle K_{ATP} channels prevent fiber damage not only by preventing Ca^{2+} overload but also by either preserving ATP or reducing metabolic rate during muscular activity. To answer this question, we need to know 1) how the K_{ATP} channel affects energy metabolism and eventually 2) whether the extent of the effect on energy metabolism is large enough to cause fiber damage.

OBJECTIVES AND HYPOTHESIS

The objective of this study was to determine how K_{ATP} channel affects energy metabolism in skeletal muscle during fatigue development (i.e. Question 1 above). The hypothesis to be tested was that "During fatigue development there is greater ATP utilization in the absence than in the presence of K_{ATP} channel activity". Two aims were pursued in the course of this study. Aim one was to determine the effects of K_{ATP} channels on the high energy phosphate reserves; i.e. PCr and ATP. Aim two was to determine the effects of K_{ATP} channels on lactate production as an index of the glycolytic flux. According to the hypothesis the expectations were that the absence of K_{ATP} channel activity would increase PCr and ATP depletion and lactate production. To test this hypothesis, the activity of K_{ATP} channel was modulated using two approaches: 1) genetic approach in which muscles from the null mice for the Kir6.2 gene ($Kir6.2^{-/-}$ mice) were used; and 2) a pharmacological approach in which wild type muscles were exposed to glibenclamide, the channel blocker and pinacidil, the channel opener to activate K_{ATP} channels.

CHAPTER 2

MATERIALS AND METHODS

ANIMALS

All experiments were carried out using two to three months old C57-Bl6 mice as wild type and null mice for the Kir6.2 gene ($Kir6.2^{-/-}$ mice) generated by Miki et al (1998). Wild type and $Kir6.2^{-/-}$ mice had been backcrossed over four generations before this study. Mice were fed ad libitum and cared according to the guidelines provided by the Canadian Council for Animal Care. All experimental procedures have been approved by the Animal Care Committee at the University of Ottawa.

MUSCLE PREPARATION

Mice were anaesthetized 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 before muscles were dissected. Experiments were carried out using either extensor digitorum longus (EDL) muscles or flexor digitorum brevis (FDB) muscle bundles. FDB muscle fibers that control the 4^{th} digit were separated from those controlling the 3^{rd} digit by cutting along the fascia that separate the fibers for 3^{rd} and 4^{th} digit, as described by Cifelli (2006).

SOLUTIONS

Muscles were kept in acrylic cuvettes measuring 1x1x4.5 cm (width, length and height) and filled with 2 ml of physiological saline solution containing (in mM): 118.5 NaCl, 4.7 KCl, 2.4 $CaCl_2$, 3.1 $MgCl_2$, 25 Na_2HCO_3, 2 NaH_2PO_4 and 5.5 D-glucose. Solution was constantly bubbled with 95% O_2 - 5% CO_2 and had a pH of 7.4.

Experiments were carried out at 37° C.

Solutions containing either 100 μM pinacidil or 10 μM glibenclamide were prepared by first dissolving the drugs in DMSO before it was added to the physiological saline solution. For these experiments, all solutions, including control solutions contained 0.1% DMSO (v/v). DMSO was not added to the physiological saline solution only when wild type and Kir6.2$^{-/-}$ muscles were compared.

FORCE MEASUREMENT

Muscles were attached with silk (Black Braided Silk Suture, 6-0, USA) at one end to a force transducer (Grass Instrument, Model FT03C, USA), and the other end to a fixed hook. The transducer was connected to a Grass Physiograph (Grass Instrument, Model 79E, USA). Peak tetanic force, defined as the maximum force generated during a provoked contraction, was calculated by measuring the difference between the maximum force during contraction and the force just before the stimulation (Fig. 2-1). Resting tension, defined as the amount of tension exerted by muscle without stimulation (i.e. between contractions when it failed to fully relax), was calculated by measuring the difference in force before fatigue and the force during fatigue (Fig. 2-1).

STIMULATION AND FATIGUE PROTOCOL

Muscles were stimulated by passing a current between parallel platinum wires located on both sides of the muscle. Stimulations were generated using a Grass S88 stimulator (USA) and Grass isolation unit (SIU5, USA). Prior to fatigue, muscles were allowed to equilibrate for 30 min in control, glibenclamide or pinacidil containing physiological solutions. In pharmacological experiments, muscles were always used in

Figure 2-1

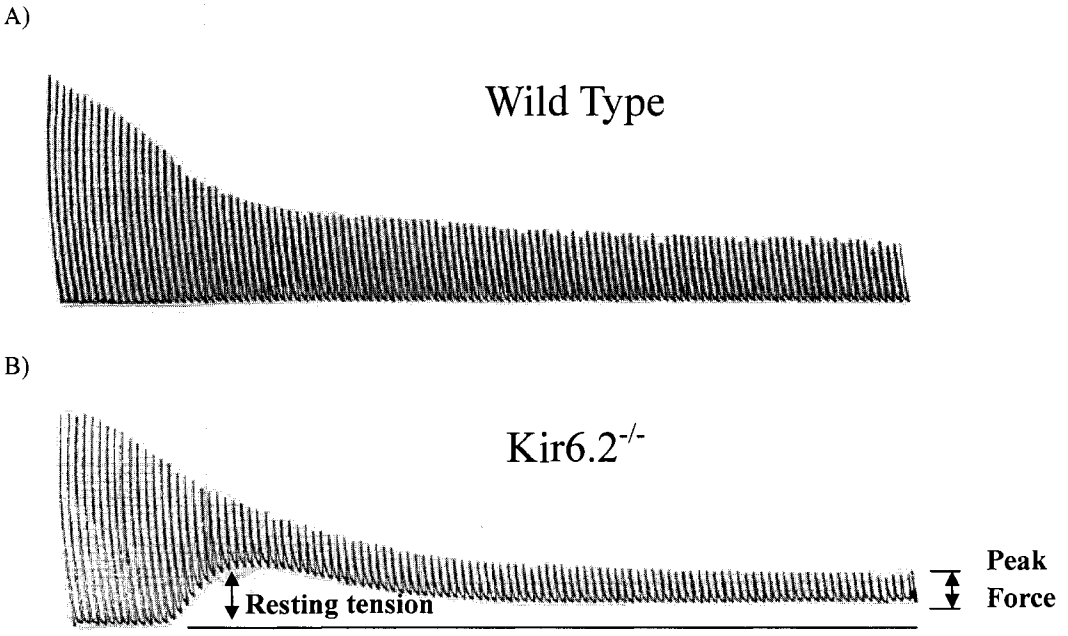


Figure 2-1. Recordings of tetanic contractions during fatigue of A) Wild Type and B) Kir6.2$^{-/-}$ FDB muscle bundles. Fatigue was elicited with one contraction every sec for 3 min. Measurements of peak tetanic force and resting tension were shown in B. Notice that resting tension barely increased during fatigue in wild type FDB compare to Kir6.2$^{-/-}$ FDB bundles.

pairs. One muscle bundle was exposed to either pinacidil or glibenclamide while the other was exposed to control solution. During that time, they were stimulated every 100 sec with 200 ms train of 0.3 ms, 8 V pulses at 200 Hz.

Previous studies in this laboratory had used a fatigue protocol elicited with one tetanic contraction every sec for 3 min. However, most of the decrease in peak tetanic force occurs within 1 min (Appendix 1A). Several fatigue protocols were tested to obtain a model for which the decreases in force were slower. When the interval between contractions was increased from 1 to 10 sec, the decrease in peak tetanic force were slower (Appendix 1A), but no resting tension was generated (Appendix 1B). However, if the time interval was reduced to one contraction every 5 sec and with 100 Hz, 100 ms trains stimulation (except for one 200 Hz, 200 ms trains every 30 sec to measure peak tetanic force), the decrease in peak tetanic force was similar to that of one every ten sec (Appendix 1A), but there was a small increase in resting tension (Appendix 1B). This was important because K_{ATP} channel opener, pinacidil, is known to abolish the resting tension and the channel deficiency muscles produce more resting tension than control. Therefore it was necessary to have a difference between the three groups of muscles. So the latter fatigue protocol with one contraction every 5 sec for 6 min was used in EDL muscles.

In the course of this study it became apparent that EDL muscle was not the best muscle preparation for studying muscle fatigue (See Discussion for further details). Furthermore, in the laboratory a new muscle preparation was characterized, the FDB muscle bundle. The remaining experiments were then carried out using this new model.

To be consistent with other studies in the laboratory, the fatigue protocol was one 200 ms tetanic contraction every second for 3 min.

METABOLITE MEASUREMENTS

At appropriate time, muscles were freeze-clamped in liquid nitrogen and stored at -80°C until subsequent analyzing. Muscles were freeze dried with a freeze drier (Freezemobile 6, Virtis, USA). To extract PCr, ATP and lactate, dried muscle fibers were separated from tendons at room temperature and weighed on an analytical balance (Mettler Toledo, XS105, USA). About 0.1-0.2 mg of dry muscles were added to 500 µl of ice cold 6% perchloric acid. After sonificated with a Microson ultrasonic cell disruptor (Heat System Ultrasonic Inc., USA) at maximum power for 15 sec, solutions were centrifuged 30 min at 20,000 g at 4° C (Beckman, Avanti J-25, USA). Supernatants were neutralized with ice cold 3 M K_2CO_3 and the K^+ salt was centrifuged (International equipment company, IEC, Micromax 3590F2282, USA) 15 min at 10,000 g and 4° C. ATP, PCr and lactate were determined enzymatically as described by Passoneau and Lowry (1983) and changes in fluorescence were measured with a Perkin Elmer fluorometer (Model LS50B, USA).

STASTISTICAL ANALYSIS

Values are given as Mean ± S.E (standard error) with the number of samples (n). Two way ANOVA (Analysis of Variance) designs were used to determine significant differences; the treatment being i) K_{ATP} channel activity and ii) time during fatigue. 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 difference (Steel and Torrie, 1980). The word "significant" refers only to a statistical difference ($P<0.05$).

CHAPTER 3

RESULTS

EFFECT OF NO K_{ATP} CHANNEL ACTIVITY IN EDL MUSCLE

When fatigue was elicited with one contraction every 5 sec in EDL muscles, peak tetanic force decreased almost linearly over time and reached about 60% of pre-fatigue value after 6 min for both wild type and Kir6.2$^{-/-}$ EDL (Fig. 3-1A). No significant difference was observed between the two groups throughout the whole fatigue period. Resting tension was greater in Kir6.2$^{-/-}$ EDL, during the second half of fatigue reaching a mean value of 2% of the pre-fatigue force compare to only 0.3% for wild type EDL (Fig. 3-1B).

Prior to fatigue, mean PCr content was 115 μmoles/g dry weight in wild type EDL (Fig. 3-2A). In Kir6.2$^{-/-}$ EDL, pre-fatigue PCr content was lower than in wild type, being only 94 μmoles/g dry weight. PCr significantly decreased during the first 2 min of fatigue to 50-52 μmoles/g dry weight in both wild type and Kir6.2$^{-/-}$ EDL. It then remained constant in both groups. Pre-fatigue ATP content in wild type EDL was 28 μmoles/g dry weight (Fig. 3-2B). No significant change in ATP content was observed during fatigue. There was also no significant difference between wild type and Kir6.2$^{-/-}$ EDL in ATP content throughout the fatigue period. Lactate content constantly increased during the 6 min fatigue period from 5 to 51 μmoles/g dry weight in wild type EDL (Fig. 3-3). The changes in lactate content in Kir6.2$^{-/-}$ EDL was similar to those observed in wild type EDL.

Figure 3-1

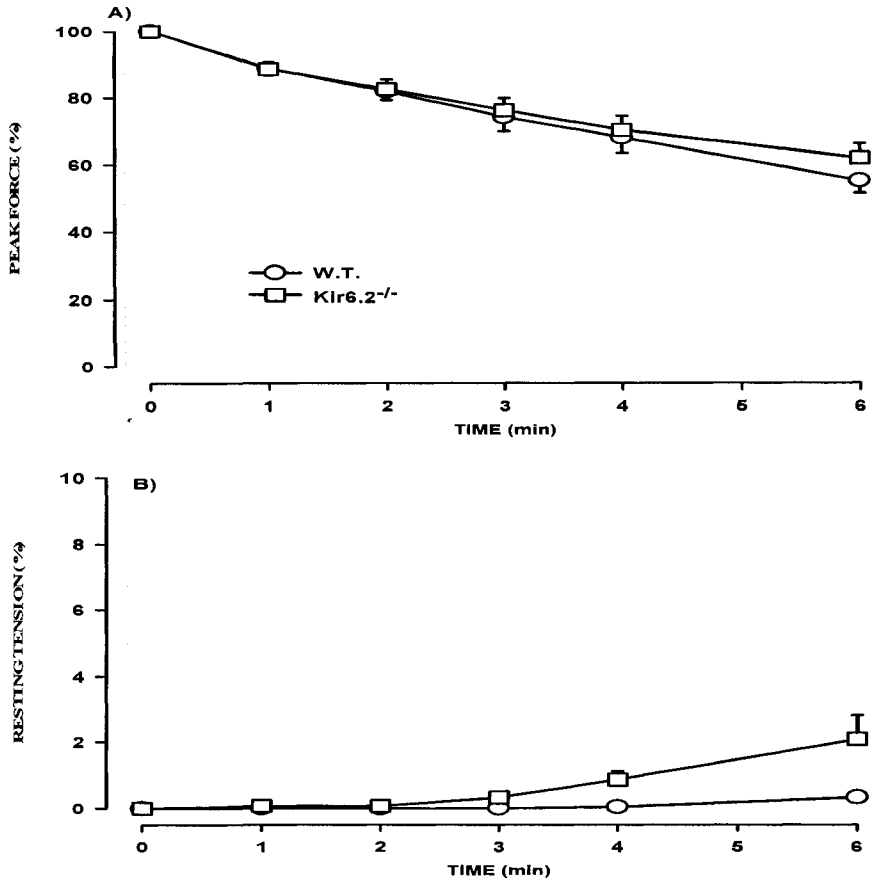


Figure 3-1. A) There was no significant difference in peak tetanic force between wild type and Kir6.2$^{-/-}$ EDL muscles while B) Kir6.2$^{-/-}$ EDL generated more resting tension. Fatigue was elicited with one tetanic contraction every 5 sec for 6 min. For clarity, data are shown at every min. Peak tetanic force is the maximum force during a contraction and resting tension is the increase in tension as muscle failed to fully relax between contractions. Both of them are expressed as a percent of the pre-fatigue peak tetanic force. Experimental temperature was 37°C. Vertical bars represent the S.E. of 5 muscles. No significant difference was observed between wild type and Kir6.2$^{-/-}$ EDL. ANOVA and L.S.D. $P > 0.05$.

Figure 3-2

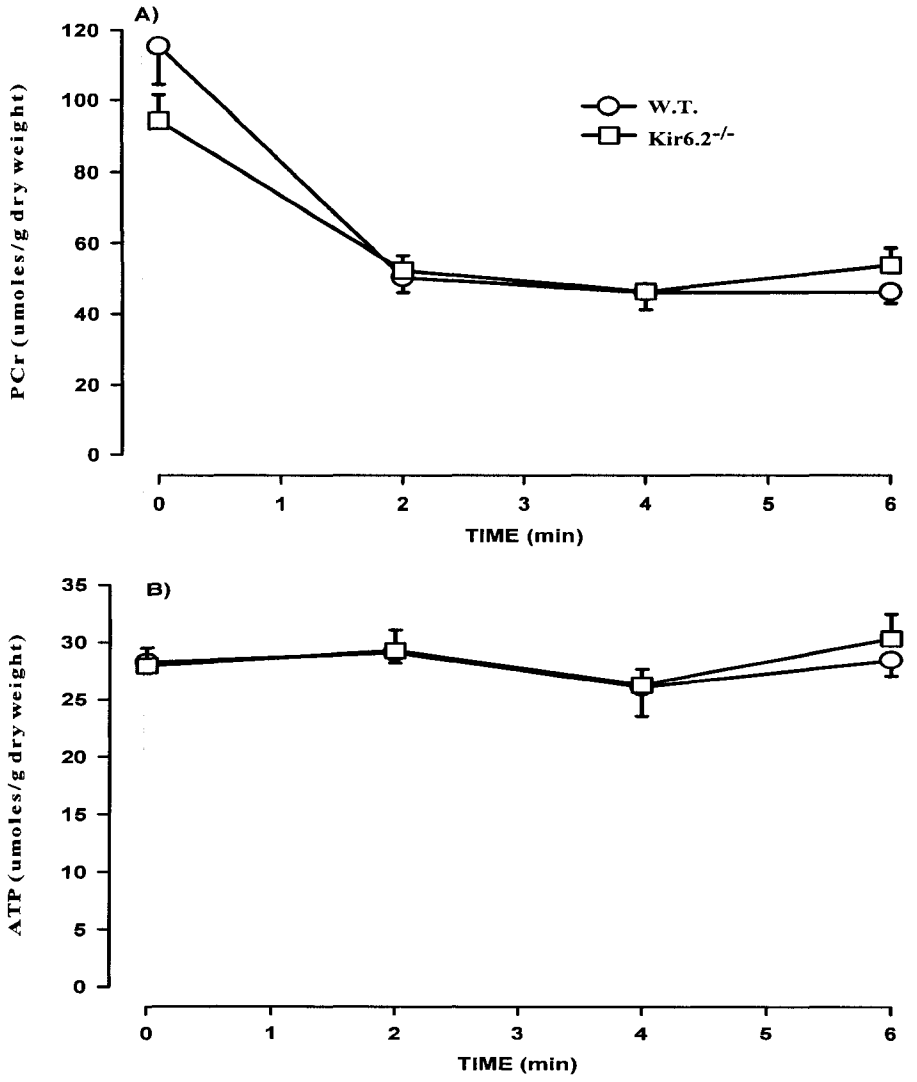


Figure 3-2. There was no significant difference in A) PCr and B) ATP content between wild type and Kir6.2$^{-/-}$ EDL muscles during fatigue. Fatigue was elicited with one tetanic contraction every 5 sec for 6 min. Experimental temperature was 37°C. Vertical bars represent the S.E. of 5 muscles.

No significant difference was observed between wild type and Kir6.2$^{-/-}$ EDL. ANOVA and L.S.D. $P > 0.05$.

Figure 3-3

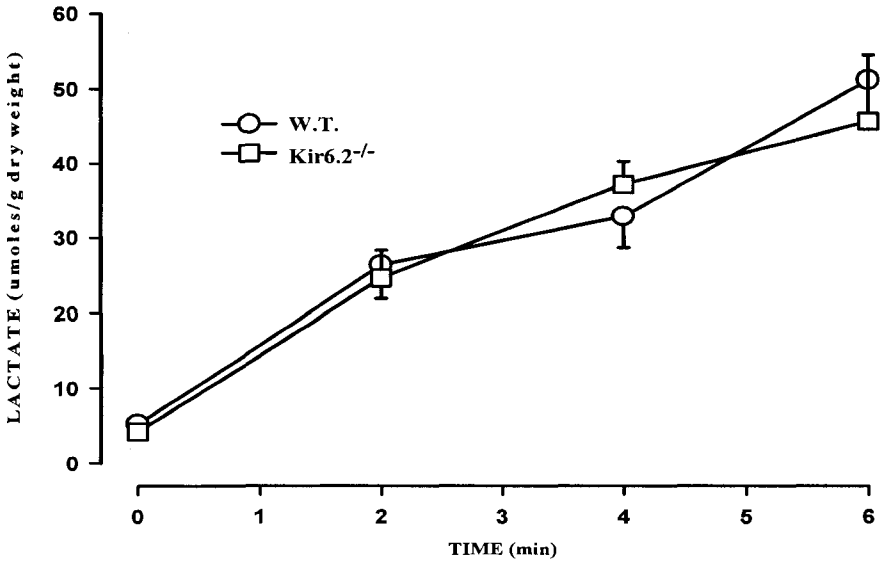


Figure 3-3. There was no significant difference in lactate content between wild type and Kir6.2$^{-/-}$ EDL muscles during fatigue. Fatigue was elicited with one contraction every 5 sec for 6 min. Experimental temperature was 37°C. Vertical bars represent the S.E. of 5 muscles.

No significant difference was observed between wild type and Kir6.2$^{-/-}$ EDL. ANOVA and L.S.D. $P > 0.05$.

EFFECT OF NO K_{ATP} CHANNEL ACTIVITY IN FDB MUSCLE

GENETIC APPROACH: WILD TYPE vs. KIR6.2$^{-/-}$ FDB

In wild type FDB, most of the decreases in peak tetanic force occurred during the first 60 sec, at which time mean peak tetanic force was 36% of pre-fatigue force; thereafter, the decrease was much smaller. At 180 sec the peak tetanic force was 24% (Fig. 3-4A). The decreases in peak tetanic force in Kir6.2$^{-/-}$ FDB were not significantly different from those of wild type FDB. Wild type FDB generated very little resting tension, which did not exceed 1% of the prefatigue force (Fig. 3-4B). However, the increase in resting tension was significantly greater in Kir6.2$^{-/-}$ than wild type FDB. It started within 20 sec and reached a maximum of 14% at 30 sec of fatigue period. It then decreased to 7% by 60 sec and was 5% at 180 sec.

Like for the pre-fatigue PCr content in EDL muscle, Kir6.2$^{-/-}$ FDB had less PCr than wild type FDB, the mean values being 64 and 51 μmoles/g dry weight in wild type and Kir6.2$^{-/-}$ FDB, respectively (Fig. 3-5A). PCr decreased significantly and rapidly during the first 5 sec of fatigue, reaching 24 μmoles/g dry weight for both wild type and Kir6.2$^{-/-}$ FDB. By 20 sec and thereafter, only very low content of about 10-15 μmoles/g dry weight was measured with no difference between wild type and Kir6.2$^{-/-}$ FDB.

ATP content in wild type FDB muscles did not decrease significantly during the first 5 sec of fatigue while it did in Kir6.2$^{-/-}$ FDB (Fig. 3-5B). During that time ATP decreased by 1 μmoles/g dry weight in wild type FDB and by 5 μmoles/g dry weight in Kir6.2$^{-/-}$ FDB, representing a 4-fold difference. ATP continued to decrease until the 20th sec. At that time, ATP content was 10 μmoles/g dry weight in wild type compare to 8 μmoles/g dry weight in

Figure 3-4

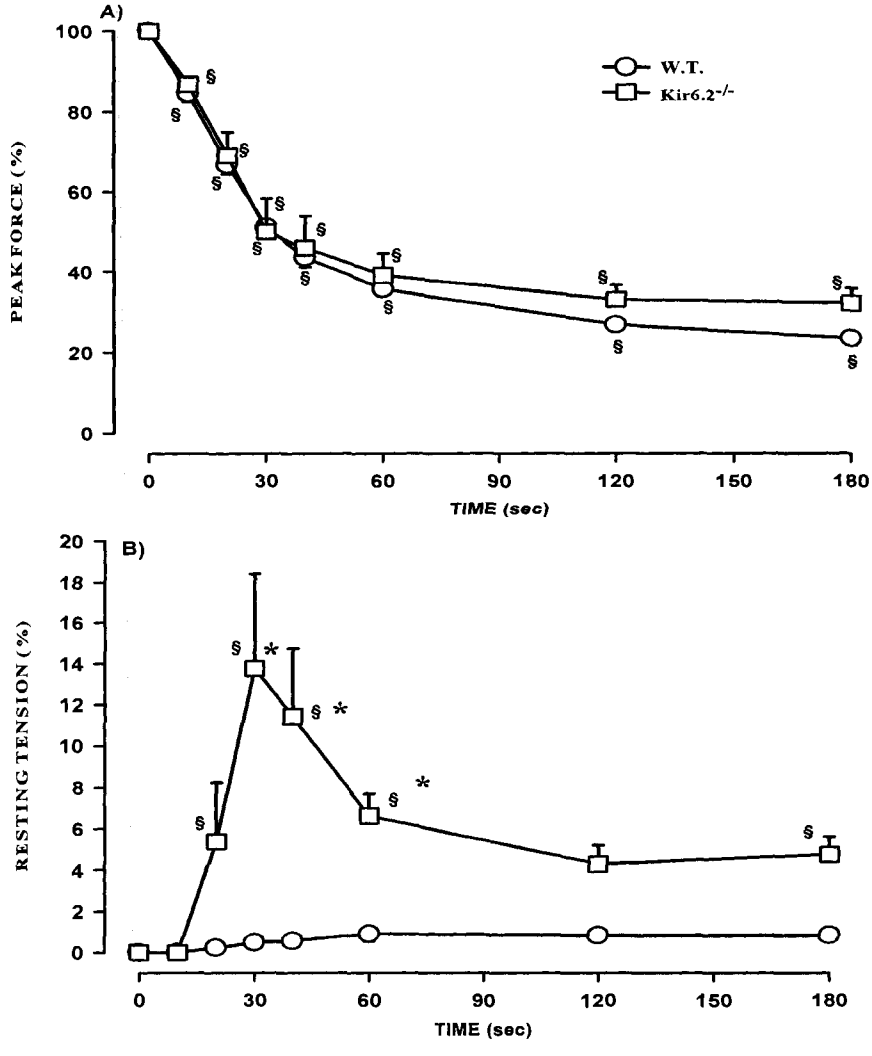


Figure 3-4. A) During fatigue the decreases in peak tetanic force were not different between wild type and Kir6.2$^{-/-}$ FDB muscles while B) Kir6.2$^{-/-}$ FDB muscle generated more resting tension. Fatigue was elicited with one contraction every sec for 3 min. Experimental temperature was 37°C. Vertical bars represent the S.E. of 8 muscles.

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

§ Significantly different from time 0 min, ANOVA and L.S.D, $P < 0.05$.

Figure 3-5

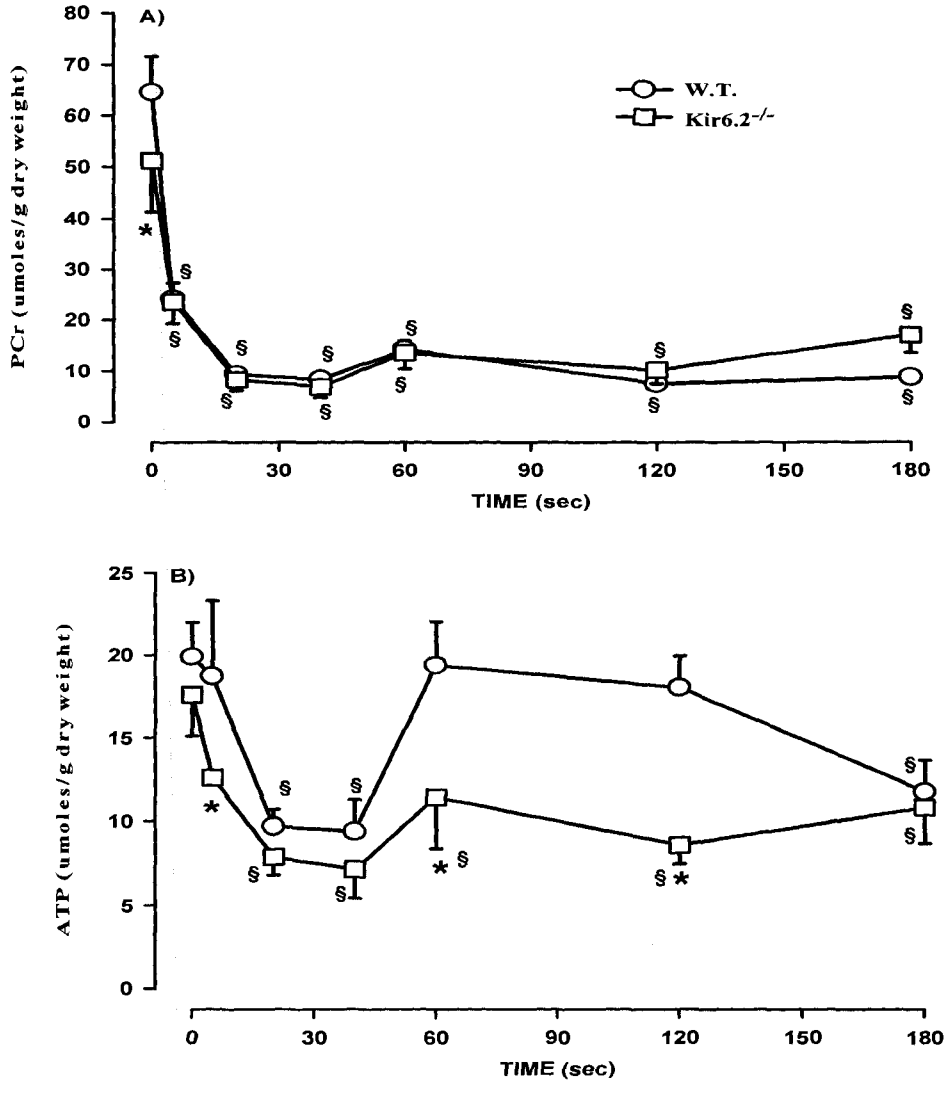


Figure 3-5. A) PCr content was lower in Kir6.2$^{-/-}$ than wild type FDB only in unfatigued muscles while B) ATP content was significantly lower in Kir6.2$^{-/-}$ FDB. Fatigue was elicited with one contraction every sec for 3 min. Experimental temperature was 37°C. Vertical bars represent the S.E. of 8 muscles.

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

§ Significantly different from time 0 min, ANOVA and L.S.D, $P < 0.05$.

Kir6.2$^{-/-}$ FDB. After remaining constant for another 20 sec, ATP content increased toward prefatigue value, reaching 19 μmoles/g dry weight by 60 sec in wild type FDB. It decreased only slightly to 12 μmoles/g dry weight by 180 sec. Compare to wild type FDB, ATP content did not re-increase as much in Kir6.2$^{-/-}$ FDB between 40 sec and 60 sec. Consequently at 60 sec and 120 sec of fatigue ATP content in Kir6.2$^{-/-}$ FDB was 9-10 μmoles/g dry weight lower than in wild type FDB. Interestingly, by 180 sec ATP levels were the same in wild type and Kir6.2$^{-/-}$ FDB.

In wild type FDB, lactate increased linearly over time from 16 to 70 μmoles/g dry weight during the first 60 sec. Thereafter it remained close to 70 μmoles/g dry weight until the end of fatigue period (Fig. 3-6). Interestingly, lactate content did not increase significantly in Kir6.2$^{-/-}$ FDB during the first 40 sec. During that time, lactate content only increased from 19 to 25 μmoles/g dry weight. Mean lactate levels became quite variable between 60 and 180 sec in Kir6.2$^{-/-}$ FDB.

PHARMACOLOGICAL APPROACH: PINACIDIL vs. GLIBENCLAMIDE

For these experiments, a new group of FDB muscle bundles were used for wild type control conditions. This is because DMSO was necessary to dissolve glibenclamide, the channel blocker, and pinacidil, the channel opener. Consequently, in these experiments, the control solution also contained DMSO whereas DMSO was not used in the experiments above, in which wild type and Kir6.2$^{-/-}$ EDL and FDB were compared. In the control group (i.e., no drug), peak tetanic force decreased rapidly to 35% of pre-fatigue peak tetanic force at 60 sec and slower thereafter, being 24% at 180 sec (Fig. 3-7A). Although glibenclamide and pinacidil had opposite effects on the K_{ATP} channel activity, they both induced a faster

Figure 3-6

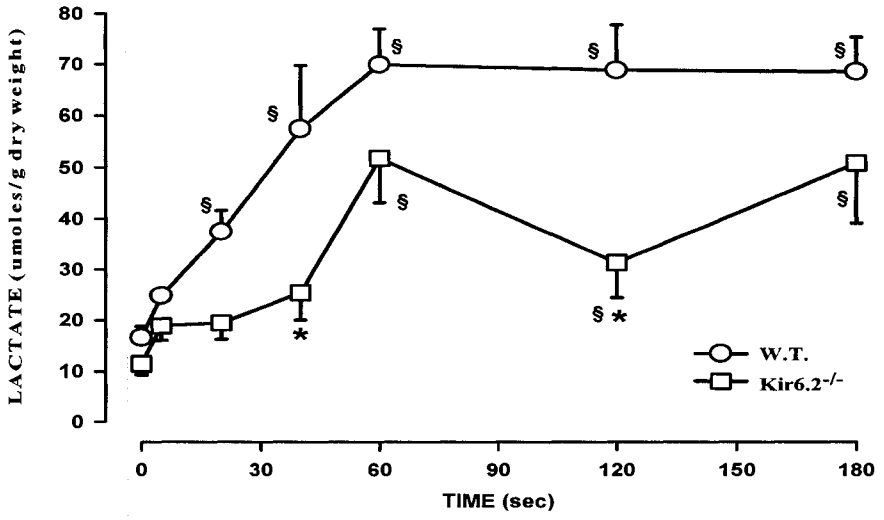


Figure 3-6. Kir6.2$^{-/-}$ FDB muscles produced significantly less lactate during fatigue than wild type FDB muscles. Fatigue was elicited with one contraction every sec for 3 min. Experimental temperature was 37°C. Vertical bars represent the S.E. of 8 muscles.

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

§ Significantly different from time 0 min, ANOVA and L.S.D, $P < 0.05$.

rate of fatigue during the first 60 sec. The decreases in peak tetanic force were 77% with glibenclamide and 80% with pinacidil compare to 65% in control condition. By 180 sec, peak tetanic force was still lower in the presence of either glibenclamide (14%) or pinacidil (19%) compare to control (24%).

In control conditions, resting tension increased to a mean value of 2.5% of the pre-fatigue force by 30 sec and then remained around 2% until the end of fatigue (Fig. 3-7B). It reached 17% in 30 sec in the presence of glibenclamide before it decreased to 11.5%. No resting tension was observed in the presence of pinacidil.

Under control condition, FDB muscle PCr content decreased rapidly during the first 40 sec from about 57 μmoles/g dry weight to 8 μmoles/g dry weight (Figure 3-8A). Thereafter PCr content remained at around 8-15 μmoles/g dry weight. As observed in Kir6.2$^{-/-}$ FDB, the glibenclamide group also had lower pre-fatigue PCr content compare to control, being 43 μmoles/g dry weight. However, from 5 sec to 180 sec of fatigue PCr content was no longer different from control FDB muscles. In the presence of pinacidil, PCr content started from a similar level as the control group. However, the decrease in PCr became slower between 5 and 60 sec. So, at 20 and 40 sec, PCr contents were respectively 35 and 25 μmoles/g dry weight, higher than in the absence of pinacidil. After 60 sec, the difference between control and pinacidil had disappeared.

In the control group, ATP decreased gradually during the first 20 sec of fatigue from 22 to 10 μmoles/g dry weight (Fig. 3-8B), which was similar to the observation in Fig 3-5B. However, unlike the observation in Fig. 3-5B, no increases in ATP content occurred between 40 and 60 sec as ATP content remained at 6-10 μmoles/g dry weight until the end

Figure 3-7

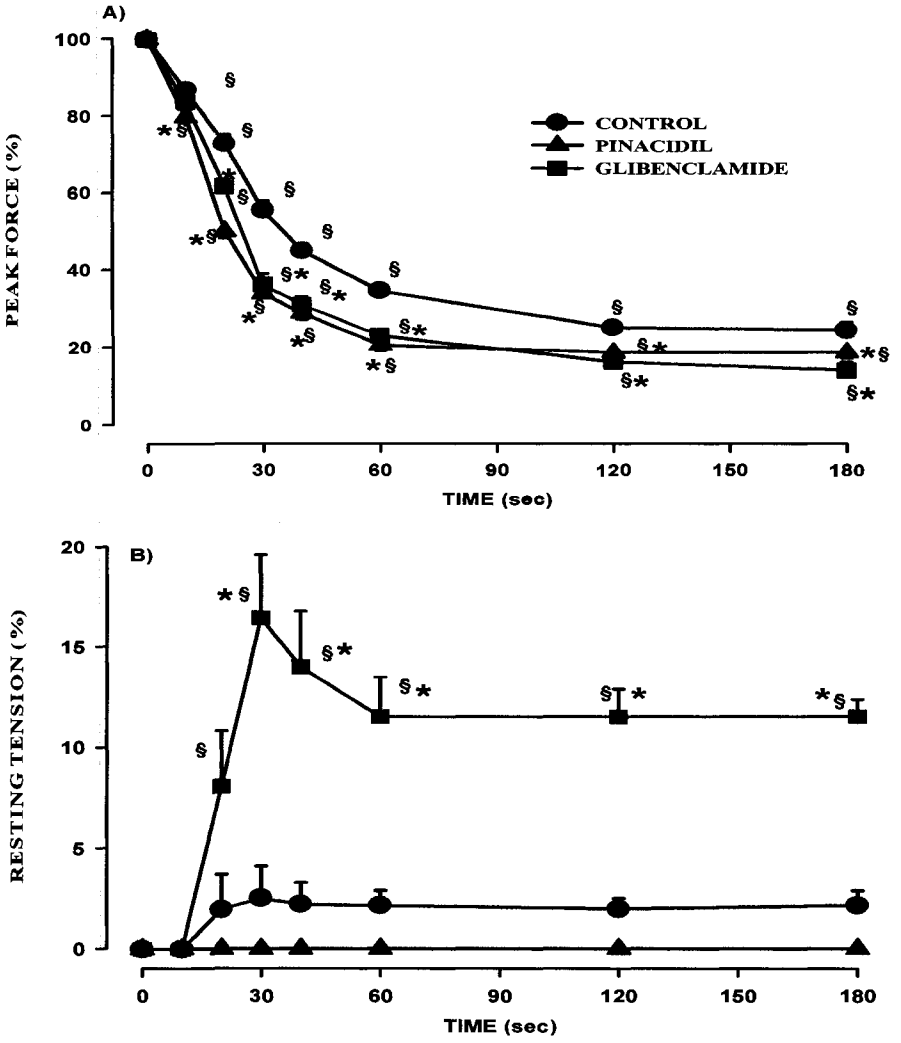


Figure 3-7. A) Glibenclamide and pinacidil increased the rate of fatigue compare to control; while B) Glibenclamide significantly increased and pinacidil completely abolished resting tension. Fatigue was elicited with one contraction every sec for 3 min. Experimental temperature was 37°C. Vertical bars represent the S.E. of 4 muscles.

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

§ Significantly different from time 0 min, ANOVA and L.S.D, $P < 0.05$.

Figure 3-8

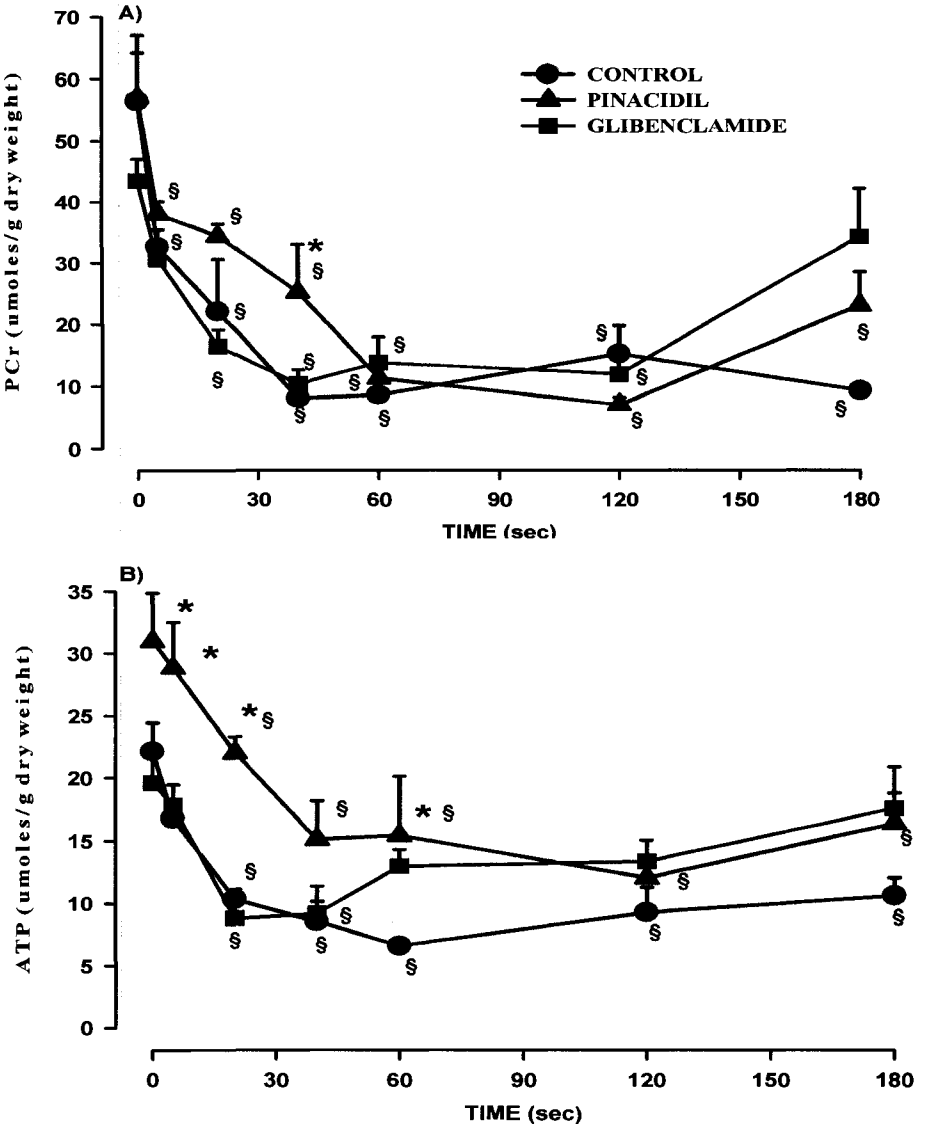


Figure 3-8. Glibenclamide had no effect on PCr level (A) and ATP level (B); while pinacidil preserved PCr (A) and ATP (B) in wild type FDB muscles. Experimental temperature was 37°C. Vertical bars represent the S.E. of 4 muscles in each group.

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

§ Significantly different from time 0 min, ANOVA and L.S.D, $P < 0.05$.

of fatigue (Fig. 3-8B). In the presence of glibenclamide, ATP content decreased from 20 to 9 μmoles/g dry weight for the first 20 sec, which was not significantly different from control FDB muscles. It then increased slightly to 13 μmoles/g dry weight at 60 sec. Pre-fatigue ATP content after 30 min in the presence of pinacidil was 31 μmoles/g dry weight, which was significantly higher compare to control FDB muscles. ATP content remained significantly higher than that of control until the 60^{th} sec.

Lactate increased during the first 40 sec of fatigue from 4 μmoles/g dry weight to 46 μmoles/g dry weight in the control group (Fig. 3-9), which is similar to the previous observation (Fig. 3-6). However, thereafter it decreased and remained at about 30 μmoles/g dry weight until the end of fatigue. For the first 40 sec, the lactate increase in the glibenclamide group was similar to control; while it was much greater in the presence of pinacidil. Thus, for the first 40 sec the increase in lactate was in the order of pinacidil > control > glibenclamide. Thereafter, lactate content in the presence of pinacidil decreased to a greater extent than in control; while in glibenclamide it remained at the level observed at 40 sec. So, by 180 sec the order for the lactate content had became the opposite of the first 40 sec, being glibenclamide > control > pinacidil.

FIGURE 3-9

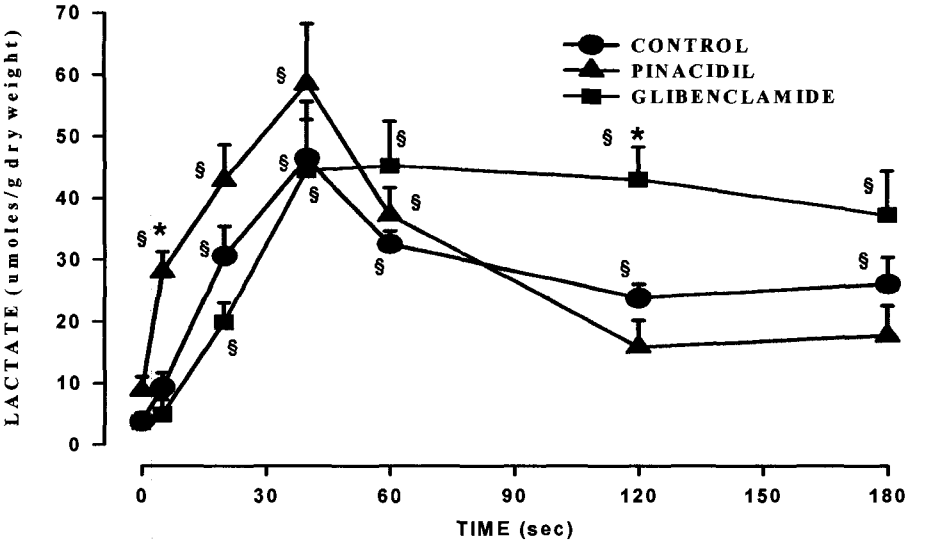


Figure 3-9: At the beginning of fatigue pinacidil increased lactate production in FDB muscles; while at the end lactate content is in an order of glibenclamide > control > pinacidil. Experimental temperature was 37°C. Vertical bars represent the S.E. of 4 muscles in each group.

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

§ Significantly different from time 0 min, ANOVA and L.S.D, $P < 0.05$.

CHAPTER 4

DISCUSSION

From rest to exercise, muscle energy utilization increases 20- to 100- fold, depending on species, muscle type and activity level (Gibbs, 1987). Although muscle itself has the ability to produce ATP from PCr, glycogen, glucose, and fatty acids, there are still conditions in which muscle energy production eventually fails to meet the energy demand, leading to a situation of energy deficit. Under such situations, fatigue is the response of muscles in which force and energy utilization are reduced to prevent deleterious energy depletion. The K_{ATP} channel is believed to be crucial in this process, acting as an energy sensor and effector.

It is considered an energy sensor because it is activated by several changes in metabolites, which occur during a metabolic stress leading to an energy deficit such as muscle fatigue. It is an effector because, being a K^+ channel, it can reduce membrane excitability (Gong et al, 2003), Ca^{2+} release (Burton and Smith, 1997; Duty and Allen, 1995) and force (Matar et al, 2000; Gong et al, 2003). The reduction in Ca^{2+} release and force is believed to be crucial in reducing ATP utilization by reducing the activity of Ca^{2+} ATPase and myosin ATPase.

To date, the effects of K_{ATP} channels on action potential, Ca^{2+} release and force have been clearly demonstrated (Burton and Smith, 1997; Matar et al, 2000; Gong et al, 2003). However, there are very few studies about the effects of the channels on energy metabolism. The objective of this study was to test the hypothesis that "During fatigue

development there is greater ATP utilization in the absence than in the presence of K_{ATP} channel activity"

STUDY WITH EDL MUSCLE

Compare to soleus, tibialis and plantaris muscles, EDL muscles in Kir6.2$^{-/-}$ mice were the most affected muscles during treadmill running; 25% of the fibers being damaged in Kir6.2$^{-/-}$ mice compare to less than 1% in wild type mice (Thabet et al, 2005). Compare to soleus muscle, it is also in EDL muscles that resting tension during fatigue and force recovery following fatigue *in vitro* are the most affected when K_{ATP} channel activity is modulated (Matar et al, 2000; Gong et al, 2003). So, if deleterious ATP depletion is the cause of fiber damage, then K_{ATP} channels should have large effects on the metabolic changes in EDL. As a first approach, the changes in ATP, PCr and lactate were measured during fatigue in EDL muscles from wild type and Kir6.2$^{-/-}$ mice. However, the decreases in PCr and increases in lactate were not significantly different between the two groups (Fig. 3-2A and 3-3), while ATP did not decrease significantly and there was again no difference between wild type and Kir6.2$^{-/-}$ EDL (Fig. 3-2B).

Although such results do not support the hypothesis, there are several possibilities as to why no difference was observed. Firstly, the decreases in peak tetanic force during fatigue were similar between wild type and Kir6.2$^{-/-}$ EDL (Fig. 3-1A), while the increase in resting tension was greater in Kir6.2$^{-/-}$ EDL (Fig. 3-1B). On a qualitative basis, these results are in agreement with those observed in previous studies (Gong et al, 2003) in which fatigue was elicited with one contraction every sec instead of every 5 sec as in this study. On a quantitative basis, Kir6.2$^{-/-}$ EDL generates a resting tension of about 10% of

pre-fatigue peak tetanic force when stimulated every one sec (Gong et al, 2003) compare to just 2% in this study. Furthermore when stimulated every sec, resting tension increased within 30 sec; while at every 5 sec the increase is only during the second half of the fatigue period, i.e. after 3 min. Thus, the fatigue protocol, used in this study, probably did not activate a sufficient number of K_{ATP} channels, perhaps because one contraction every 5 sec gave rise to a smaller metabolic stress compare to a fatigue with one contraction every sec.

Secondly, when isolated muscles are used *in vitro*, they are without a vascular perfusion. Consequently, metabolic needs have to be met by diffusion of O_2 and glucose from the surface to the middle core of the muscle. If the O_2 and glucose diffusion is too slow to meet their consumption, then an anoxic and hypoglycemic region develops in the centre of the muscle. Barclay's study (2005) showed that for mouse EDL and soleus muscle, the diffusion of O_2 at rest and 37°C is sufficient to meet the metabolic demand of the fibers located in the middle core. However, this is not the case during fatigue, where a large anoxic core develops. The importance of the anoxic core was recently demonstrated in a study by Zhang et al (2006) in which they showed that exposure of single soleus muscle fibers to N_2 causes large increases in fatigue rates compare to O_2 whereas the differences on fatigue rates between O_2 and N_2 conditions were almost inexistent in whole soleus. It then became necessary for this study to use smaller muscle preparations.

STUDY WITH FDB MUSCLE BUNDLE

In parallel of this study, Cifelli (2006) developed a new muscle preparation, in which small FDB bundles were excised from the whole FDB muscles. FDB muscle

bundles are on average 2 mg in wet weight and 8 mm long (350 fibers) compare to 10 mg wet weight and 8 mm long (more than 800 fibers) for EDL muscles. According to Barclay's model (2005), a fatigue, with a contraction duty cycle of 0.2, results in an anoxic core with a radius of 0.4 mm in EDL and just 0.1 mm in FDB bundles. Although an anoxic core still exists, FDB bundle preparation was found to be better than whole EDL (Cifelli, 2006). For example, force recovery after fatigue under control condition is 92% in FDB bundles (Cifelli, 2006) compare to only 50% and 70% in EDL and soleus muscles, respectively (Gong et al, 2003); i.e. the anoxic core results in permanent force loss (possibly due to fiber damage) in EDL and soleus, but very little in FDB. Consequently this study was continued using the same FDB bundle preparation and fatigue protocol (one contraction every sec for 3 min) as in the Cifelli's study (2006).

K_{ATP} CHANNEL EFFECTS ON PEAK TETANIC FORCE AND RESTING TENSION

When K_{ATP} channel was activated with the channel opener pinacidil, the initial rate of fatigue was significantly increased while resting tension was completely abolished (Fig. 3-7A and 3-7B). These effects are similar to those observed in previous studies using EDL and soleus muscles (Gong et al, 2000; Matar et al, 2000). It is also in agreement with the concept that K_{ATP} channel reduces action potential amplitude, leading to less Ca^{2+} release and force as discussed in the Introduction.

It is therefore expected that the absence of K_{ATP} channel activity would result in a slower rate of fatigue. However, measurements of tetanic force in FDB bundles (Cifelli, 2006) and intracellular Ca^{2+} in single FDB fibers (Bourassa, 2006) have shown that abolishing K_{ATP} channel activity actually increased the rate of fatigue and caused large

increases in resting $[Ca^{2+}]_i$ and resting tension. Furthermore, these effects were observed when the K_{ATP} channel activity was abolished either pharmacologically with glibenclamide or genetically using Kir6.2$^{-/-}$ FDB. Cifelli (2006) and Bourassa (2006) explained that the faster fatigue rates were because of severe contractile dysfunctions, including large membrane depolarization, large resting $[Ca^{2+}]_i$, increase in resting tension and fiber damage. Furthermore, Cifelli (2006) gave evidence that the large increase in resting tension was due to an uncontrolled Ca^{2+} influx through L-type Ca^{2+} channels which were activated because of the large membrane depolarization.

So the faster rates of fatigue (Fig. 3-7A) and the large resting tension (Fig. 3-7B) with glibenclamide-exposed wild type FDB bundles are in agreement with those reported by Cifelli (2006). The only difference with Cifelli's study was the lack of difference in fatigue rate between wild type and Kir6.2$^{-/-}$ FDB muscles (Fig. 3-4A). The reason for that may be related to the fact that wild type and Kir6.2$^{-/-}$ FDB muscles were from different animals and if one plots all fatigue curves from Cifelli's study, little overlap between the two groups were also observed (Cifelli and Renaud, unpublished result). It is therefore possible to observe in some cases no difference between wild type and Kir6.2$^{-/-}$ FDB. The problem between mouse variability is avoided in the pharmacological approach (i.e. with glibenclamide) because paired FDB muscle bundles are used, one as control and the other exposed to glibenclamide.

K_{ATP} CHANNEL EFFECTS ON METABOLITES

Effects on PCr

Pre-fatigue PCr content in wild type FDB muscle was 64 μmoles/g dry weight (Fig.

3-5A), which is much lower than in EDL (Fig. 3-2A; Matar et al, 2000). It is actually closer to the PCr contents in soleus muscles (Matar et al, 2000). This is interesting considering that the decreases in force during a 3 min fatigue is the same between FDB (Fig 3-4A and 3-7A) and EDL (Matar et al, 2000; Gong et al, 2003) and much faster than in soleus muscles (Matar et al, 2000; Gong et al, 2003). FDB muscles are primarily composed of type IIA (60%) and type IIX (34%) (Raymackers et al, 2000). Soleus muscles are primarily composed of type I (40%) and type IIA (60%) (Wigston et al, 1992; Thabet et al, 2005) while EDL muscles have 68% type IIB, 20% type IIX and only 12% type IIA fibers (Rosenblatt et al, 1992; Thabet et al, 2005). Thus the differences in PCr content between unfatigued EDL, FDB and soleus muscles are either because of differences among muscles or related to the amount of type IIA fibers, in which the larger the type IIA content is, the lower the PCr content.

When FDB muscles were fatigued with one contraction per sec, PCr became depleted within 30 sec in wild type FDB under control conditions (Fig. 3-5A and 3-8A). Such a rapid PCr depletion is in agreement with several other studies (Sahlin et al, 1998; Neville et al, 1996; Hirvonen et al, 1987; Meyer et al, 1979). When K_{ATP} channel activity is abolished pharmacologically or genetically, the decreases in PCr are similar to those of wild type control (Fig 3-5A and 3-8A). The lack of difference here is not surprising, as PCr is the first source of high energy phosphate that replenishes ATP as it is hydrolyzed.

The rate of PCr depletion should be relative to the energy demand at that time. For the first 10 sec of fatigue, the decreases in peak tetanic force were the same between wild type control, Kir6.2$^{-/-}$ and glibenclamide-exposed wild type FDB muscles (Fig. 3-4A and

3-7A). So during that time, energy utilization should be the same between those conditions. For the next 20 sec, the decreases in peak tetanic force was faster with glibenclamide as compared to control (Fig. 3-4A and 3-7A), but there was also a large increase in resting tension (Fig. 3-4B and 3-7B). Resting tension represents a contracture that constantly uses ATP by myosin ATPase. Thus, K_{ATP} channel deficiency muscles most likely are using more ATP than wild type control muscles during that time. The lack of a faster PCr depletion in K_{ATP} channel deficiency muscles is probably because it already reached its maximum rate with a fast decrease in peak tetanic force.

When pinacidil was used, the decrease of peak tetanic force was also faster than control FDB (Fig. 3-7A), and resting tension was completely abolished (Fig. 3-7B). The differences in force and resting tension were particularly marked between 20 and 40 sec, which corresponded to the time period when the PCr decreases were slower than in control (Fig. 3-8A). Thus, for the effects on PCr, only the pinacidil effects support the hypothesis that K_{ATP} channels reduce the rate of ATP utilization.

Effects on ATP

As observed for PCr, the pre-fatigue ATP content in wild type FDB (Fig, 3-5B and 3-8B) was comparable to those reported for soleus and lower than in EDL (Fig. 3-2B; Matar et al, 2000). Such result is expected because the creatine kinase reaction is always in equilibrium. As explained in the Result section, when wild type FDB muscle bundles were compared to Kir6.2$^{-/-}$ FDB, DMSO was not added to the solution whereas for the experiments with glibenclamide and pinacidil, DMSO was added in the control solutions. The initial decrease in ATP over the first 20 sec and the final ATP content at 180 sec of

fatigue were similar whether DMSO was present or not. However, the re-increase in ATP levels observed between the 40^{th} and 60^{th} sec in the absence of DMSO (Fig. 3-5B) was not observed in the presence of DMSO (Fig. 3-8B). It thus appears that DMSO may affect energy metabolism for unknown reasons. Here, therefore the effects of no K_{ATP} channel on metabolic contents will be discussed by comparing the effects with Kir6.2$^{-/-}$ muscles and those of the drugs with their respective controls.

It is also important to note that the re-increase in ATP level between 40^{th} and 60^{th} sec in the absence of DMSO is not an artifact. Similar decreases and re-increases in ATP during a fatigue stimulation have been reported in gastrocnemius muscle with a 4 Hz stimulation (Shoubridge et al, 1984). In that study, ATP decreased during the first 4 min of stimulation and was then resynthesized during stimulation, returning back to near pre-fatigue level. As observed in this study, the initial decrease in gastrocnemius ATP corresponded in time with most of the decrease in peak tetanic force. Perhaps when peak tetanic force reaches a steady state as during the last 2 min in Fig. 3-4A and 3-7A, the energy demand becomes much lower than the capacity of ATP synthesis, allowing for the resynthesis of ATP. The reason as to why force does not re-increase when ATP re-increases cannot be explained from the results of this study.

The major differences between wild type and Kir6.2$^{-/-}$ FDB in terms of ATP changes were: i) slightly lower pre-fatigue ATP level in Kir6.2$^{-/-}$ FDB; ii) rapid decreases in ATP within 5 sec in Kir6.2$^{-/-}$ FDB while no change was observed in wild type; iii) lower ATP by 30-40 sec in Kir6.2$^{-/-}$ FDB; and iv) the re-increase by 60 sec was smaller in Kir6.2$^{-/-}$ FDB (Fig. 3-5B). Together, these results support the hypothesis that abolishing K_{ATP}

channel activity leads to greater ATP utilization and depletion.

However, when the channel activity was abolished pharmacologically, the effects on ATP depletion were not similar to those observed in Kir6.2$^{-/-}$ FDB. When wild type FDB muscles were exposed to glibenclamide, the decreases in ATP were similar to those in control wild type FDB muscles (Fig. 3-8B). Faster ATP depletion was expected in the presence of glibenclamide considering that glibenclamide-exposed FDB muscles and Kir6.2$^{-/-}$ FDB muscles developed similar levels of resting tension. It therefore appears that an acute lack of K_{ATP} channel activity is less detrimental on ATP levels than a chronic lack of the channel activity. However, the reason for the differences cannot be explained. Furthermore, all previous studies have given evidence that abolishing K_{ATP} channel activity pharmacologically or genetically always gave rise to similar effects on fatigue rate (measured from the decrease in tetanic $[Ca^{2+}]_i$ or peak tetanic force), resting $[Ca^{2+}]_i$, resting tension, force recovery and fiber damage (Matar et al, 2000; Gong et al, 2000; Cifelli, 2006; Bourassa, 2006). Thus, this is the first time that both approaches give rise to different results.

Finally, pinacidil, the channel opener, helped in preserving ATP because its content was higher for about one min in the presence than in the absence of pinacidil (Fig. 3-8B). This result is also in agreement with the effects reported in heart muscle during ischemia (McPherson et al, 1993). Thus, overall, the pharmacological approach to activate the channel gave results that support the hypothesis that K_{ATP} channel lowers the rate of ATP utilization. Abolishing K_{ATP} channel activity with genetic approach also supported the hypothesis;. however, results from the pharmacological approach to abolish the channel

activity did not support the hypothesis.

Effects on lactate production

In wild type FDB, the pre-fatigue lactate content and increase during fatigue (Fig. 3-6 and 3-9) were comparable to other studies (Bangsbo et al, 1990; Meyer et al, 1979). Of the three metabolites studied here, lactate is the one that gave rise to the most unexpected results. The first unexpected result was with Kir6.2$^{-/-}$ FDB muscles, which generated significantly less lactate than wild type FDB muscles (Fig. 3-6). This was unexpected firstly because Kir6.2$^{-/-}$ and SUR2$^{-/-}$ muscles have greater glucose uptake at least at rest and under insulin stimulation (Miki et al 2002; Chutkow, et al. 2001). It was therefore expected that greater glucose uptake would lead to greater lactate production. Secondly, according to the hypothesis, a greater lactate production was expected in Kir6.2$^{-/-}$ FDB to support a greater ATP demand.

It is unlikely that the lower lactate production was due to a smaller ATP demand in Kir6.2$^{-/-}$ FDB muscles considering that ATP content was lower in those muscles than in wild type FDB muscles. A more likely explanation is that the chronic lack of K_{ATP} channel activity in the knockout model gives rise to some compensatory mechanisms. For example, Kir6.2$^{-/-}$ FDB muscles may transport lactate out of the fiber more effectively and/or there is greater pyruvate flux to the Kreb's cycle. If Kir6.2$^{-/-}$ oxidative capacity is higher than in wild type FDB muscles, such a compensatory mechanism would not be surprising because it would represent an adaptation to prevent ATP depletion in the absence of a protective mechanisms. If the latter case is true and assuming that wild type and Kir6.2$^{-/-}$ FDB generated the same amount of pyruvate during

fatigue, then more ATP would be generated from glucose as more pyruvate enters the Kreb's cycle in Kir6.2$^{-/-}$ FDB. For example, at 40 sec, the lactate content in wild type and Kir6.2$^{-/-}$ FDB was respectively 57 and 25 μmoles/g dry weight. If the difference is because of more oxidation of pyruvate (i.e. 32 μmoles/g dry weight), then there would be an extra 547 μmoles/g dry weight more ATP being produced in Kir6.2$^{-/-}$ than wild type FDB muscles. In that case a greater ATP production in Kir6.2$^{-/-}$ FDB would support the hypothesis that absence of K_{ATP} channel activity increases ATP demand. Further studies are therefore necessary to determine if the oxidative capacity of Kir6.2$^{-/-}$ FDB is higher than in wild type FDB.

The second unexpected result came from the pharmacological approach to abolish K_{ATP} channel activity (Fig. 3-9). During the first min, large increases in lactate content were observed in control and glibenclamide-exposed wild type FDB muscles with no difference between them, while lactate content barely increased during the first min in Kir6.2$^{-/-}$ FDB. During the last two min, lactate content became higher in glibenclamide than in control conditions, while it remained below control in Kir6.2$^{-/-}$ FDBs.

The difference between the two approaches may be explained by the fact that K_{ATP} channels are also present in mitochondria. Kir6.2$^{-/-}$ FDB muscles are expected to have normal mitochondria K_{ATP} channels activities (Suzuki et al, 2002) because there is evidence that they are mostly composed of the Kir6.1 subunits (Suzuki et al, 1997). However, glibenclamide inhibits mitochondrial K_{ATP} channels resulting in a decreased oxidative capacity (Shi et al, 2005; O'Rourke, 2004). So, as expected above, the lower lactate content in Kir6.2$^{-/-}$ FDB muscles may be an adaptation that increases oxidative

capacity, while glibenclamide in wild type FDB muscles first lowers mitochondrial activity and then forces more pyruvate to lactate. Consequently, it will not be possible to conclude how the sarcolemmal K_{ATP} channel activity affects lactate production before the effects of glibenclamide on lactate production in wild type FDB are fully understood (i.e. how it affects pyruvate flux into the Kreb's cycle) and until we confirm if Kir6.2$^{-/-}$ FDBs have greater oxidative capacity.

The last unexpected result came from the effects of pinacidil. For the last two min of fatigue, pinacidil-exposed FDB muscles had lower lactate content than control FDB muscles (Fig 3-9), which is as expected from the hypothesis. However, during the first min of fatigue the increases in lactate were higher in the presence of pinacidil, not lower than in control. Like glibenclamide, pinacidil also modulates mitochondrial K_{ATP} channel activity (O'Rourke, 2004), except that in this case pinacidil opens mitochondrial K_{ATP} channels as it does for sarcolemmal K_{ATP} channels. In isolated mitochondria, pinacidil causes mitochondrial swelling, mitochondrial membrane potential depolarization, increase respiration and decrease ATP production (Holmuhamedov et al, 1998). In the resting state, pinacidil causes a decrease in ATP content of soleus muscle, while during fatigue it causes an increase in ATP content (i.e. it prevents the usual decrease). It thus appears that the modulation of mitochondrial K_{ATP} channel activity by pinacidil results in a complex behavior of mitochondrial oxidative activity. For now it appears that at low mitochondrial activity, pinacidil may adversely affect the Kreb's cycle and oxidative phosphorylation, so that at the onset of fatigue pyruvate flux to mitochondria is reduced, resulting in greater lactate production. Then, as fatigue progresses pinacidil may become

beneficial, increasing pyruvate flux and decreasing lactate content. Thus overall, the complexity effects of the K_{ATP} channel activity, sarcolemmal and mitochondrial, is such that further experiments will be required to fully understand its effects on lactate production.

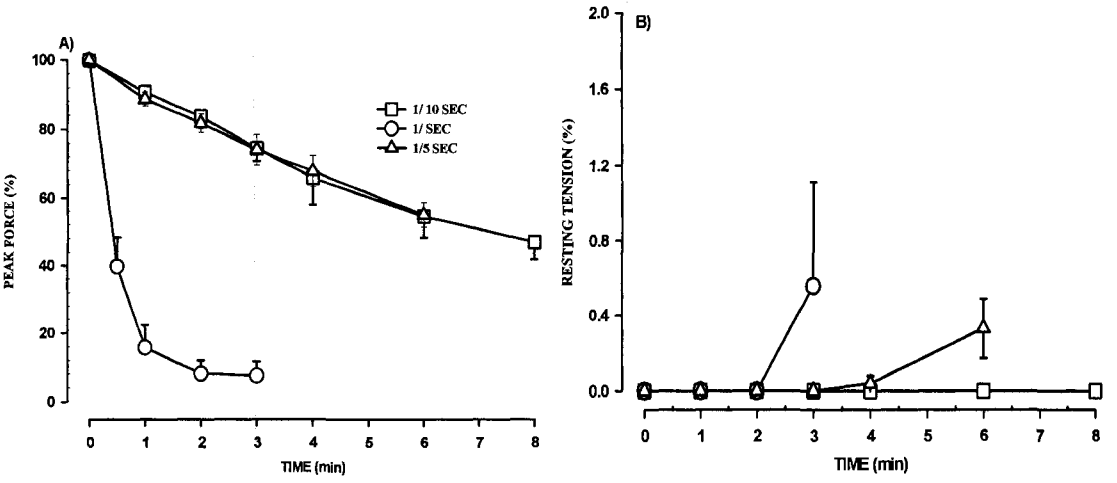
CONCLUSIONS

The objective of this study was to demonstrate that K_{ATP} channels help preserving ATP during a metabolic stress such as muscular fatigue. In regards to the measurement of PCr and ATP content, some of the data support the hypothesis: during fatigue development there was greater and faster ATP depletion in Kir6.2$^{-/-}$ than wild type FDB; and pinacidil, which activated more channels, resulted in less ATP and PCr depletion. The other results, such as the glibenclamide effects did not support the hypothesis. It is because of a lack of effect, but not because it contradicted to the hypothesis. In regards to the measurements of lactate, the analysis of the data is complicated by several factors, including: 1) the possibility of some compensatory mechanisms in Kir6.2$^{-/-}$ FDB in which there is greater oxidative capacity than wild type FDB, resulting in less lactate production in Kir6.2$^{-/-}$ FDB muscles; 2) the inhibition of mitochondrial K_{ATP} channels by glibenclamide that eventually causes greater lactate production in control conditions; and 3) a time factor in which the pinacidil affects mitochondria. Under some fatigue conditions, it reduces lactate production as expected, but under other fatigue conditions it has the reverse effects.

In this study, other K_{ATP} modulators were not used because 1) it is not always clear that they are specific for only the sarcolemmal or mitochondrial K_{ATP} channel, and 2) because so far only glibenclamide and pinacidil effects had been fully characterized for the fatigue

experiments in mouse EDL, soleus and FDB (Gong et al, 2003; Matar et al, 2000; Cifelli, 2006). It will now be necessary to use these other modulators in order to fully understand how both K_{ATP} channel affects energy metabolism.

APPENDIX 1



Appendix 1: Effects of different fatigue protocols on A) peak tetanic force and B) resting tension in wild type EDL under control conditions. All tetanic contractions were elicited with 200 ms train of pulses at 200 Hz and fatigue was elicited with one contraction every sec or every 10 sec. For another group of EDL muscles, fatigue was elicited with every 5 sec with 100 ms train of pulses at 100 Hz except at every 30 sec the train duration was increased to 200 ms and pulse frequency to 200 Hz to measure the peak tetanic force. The final fatigue protocol for the study with EDL was one contraction every sec because i) it gave rise to much slower decreases in peak tetanic force than one contraction every sec, making better time resolution for metabolites and force measurements; and ii) because it generated a bit of resting tension like one contraction every sec fatigue period. Experimental temperature was 37°C. Vertical bars represent the S.E. of 3 muscles.

CHAPTER 5

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