# The effect of modulating ATP-sensitive potassium channels in frog skeletal muscle, *in vitro*, during fatigue and metabolic inhibition

by

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# ABSTRACT

The ATP-sensitive potassium ($K^+_{(ATP)}$) channel is a $K^+$ channel which is activated as the energy state of a muscle decreases. It has been hypothesized that once activated, $K^+_{(ATP)}$ channels decrease the excitability of the cell and cause decreased contractility, such as during fatigue, in order to prevent energy levels from falling to dangerously low levels. The purpose of this study was to test this hypothesis and to determine under which conditions $K^+_{(ATP)}$ channels can contribute to a decrease in force during a metabolic stress in the sartorius muscle of the frog, *Rana pipiens*.

In the first series of experiments, sartorius muscle fibres were fatigued with 100 msec long tetanic contractions every second for three minutes, a condition known to activate ATP-sensitive potassium channels. So if $K^+_{(ATP)}$ channels contribute to a decrease in force during fatigue, an activation of $K^+_{(ATP)}$ channels with channel openers should further decrease membrane excitability and contractility. However, pinacidil at 200 $\mu mole \cdot L^{-1}$ or levcromakalim at 100 $\mu mole \cdot L^{-1}$ (two known $K^+_{(ATP)}$ channel openers) did not affect the depolarization of the cell membrane normally observed during fatigue. They also fail to affect the action potential and the rate of fatigue as measured from the decrease in tetanic force. They also failed to affect the recovery of the resting potential, action potential repolarization phase and the tetanic force. These results are therefore not consistent with the hypothesis that $K^+_{(ATP)}$ channels contribute to the decrease in force during fatigue.

Abstract

In a second series of experiments, muscles were subjected to metabolic inhibition which is known to activate a large number of $K^+_{(ATP)}$ channels in order to better understand the relationship between $K^+_{(ATP)}$ channel activity, the bioenergetic state, and force. The goal was to determine if $K^+_{(ATP)}$ channels can contribute to a decrease in force under a bioenergetic state that is within physiological limits. A metabolic inhibition was induced with 2 mmole·L$^{-1}$ cyanide (CN) and 1 mmole·L$^{-1}$ iodoacetate (IOA). One hundred μmole·L$^{-1}$ glibenclamide was used to block, while 100 μmole·L$^{-1}$ pinacidil was used to further activate $K^+_{(ATP)}$ channels. During the metabolic inhibition, muscles were stimulated to elicit a tetanic contraction every 10 minutes. Within 10 minutes of metabolic inhibition, the membrane conductance of control muscles increased by 15%. After 30 minutes of metabolic inhibition the increase reached 64%. Glibenclamide reduced the increase in membrane conductance by 2.8 fold, while pinacidil had no effect. After the first 30 minutes of metabolic inhibition, the membrane conductance of control muscles suddenly increased to greater than 200% and changed no further until the 60th minute. During that time, neither glibenclamide nor pinacidil affected the membrane conductance.

During a metabolic inhibition, the decrease in tetanic force became significant after 20 minutes and reached zero force in 60 minutes. While pinacidil had no effect, glibenclamide had a significant effect on the tetanic force during the metabolic inhibition. When added 60 minutes prior to metabolic inhibition, glibenclamide caused a faster decrease in tetanic force than in control muscles. Furthermore, glibenclamide exposure caused an earlier and greater increase in resting tension. However, when glibenclamide was added 8 minutes into metabolic inhibition, the tetanic force increased during the next 12

minutes. Thereafter, the decrease in tetanic force became significantly greater than in control as the tetanic force reached zero at 50 minutes of metabolic inhibition; that is 10 earlier than control muscles. When glibenclamide was added 18 minutes into metabolic inhibition, glibenclamide had no significant effect on the tetanic force compared to controls. The addition of glibenclamide 8 and 18 minutes into metabolic inhibition caused significant increases in resting tension similar to the increase observed when glibenclamide was added 60 minutes prior to metabolic inhibition.

During metabolic inhibition, the resting potential did not change significantly. In the presence of glibenclamide, added either 60 minutes prior to, 8 or 18 minutes after the onset of metabolic inhibition, large depolarizations were observed. The lowest resting potential was -48 mV after 40 minutes of metabolic inhibition. Additionally, the action potential half-repolarization time of control muscle was significantly reduced within 10 minutes of metabolic inhibition. This shortening was blocked by glibenclamide. Pinacidil was without effects on the resting potential and the half-repolarization time during a metabolic inhibition.

Levels of phosphocreatine in control muscles declined over time during the metabolic inhibition from 65.6 ± 6.2 μmole/g dwt to 11.6 ± 3.0 μmole/g dwt. In the presence of glibenclamide, phosphocreatine levels decreased at a significantly faster rate, from 62.4 ± 3.8 μmole/g dwt to 1.9 ± 1.2 μmole/g dwt. Prior to metabolic inhibition, ATP concentrations were not different between control and glibenclamide-treated muscles: 15.6 ± 1.3 and 18.5 ± 1.3 μmoles/g dwt, respectively. ATP levels declined only slightly for

control muscles during 50 minutes of metabolic inhibition decreasing from control values to 15.1 ± 0.8 μmole/g dwt, while in the presence of 100 μM glibenclamide the ATP concentrations decreased to 4.1 μmoles/g dwt. ADP concentrations in control muscle increased from 1.4 ± 0.3 to 3.8 ± 0.6 μmole/g dwt, while in the presence of glibenclamide, the increase was from 1.5 ± 0.1 μmole/g dwt to 8.2 ± 2.0 μmole/g dwt at 60 minutes of metabolic inhibition, and the difference compared to control was significant.

From the study on metabolic inhibition, it is proposed that $K^+_{(ATP)}$ channels can contribute to a decrease in force under physiological conditions. However, when $K^+_{(ATP)}$ channels are blocked it eventually causes a greater increase in resting tension, greater depolarization, a greater decrease in tetanic force and a greater decrease in the bioenergetic state of muscle fibers than in control muscles suggesting that $K^+_{(ATP)}$ channels play an important myoprotective role in skeletal muscle. Finally, it is also suggested that the lack of an effect of the two ATP-sensitive potassium channel openers, pinacidil and levcromakalim, is probably because they did not activate a sufficient number of $K^+_{(ATP)}$ channels above what is already activated during fatigue so they did not significantly increase the rate of fatigue.

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| $\lambda$ | length constant |
| µl | microlitre |
| µmole·L$^{-1}$ | micromolar |
| ADP | adenosine-5'-diphosphate |
| AMP | adenosine-5'-monophosphate |
| ANOVA | analysis of variance |
| ATP | adenosine-5'-triphosphate |
| $Ca^{2+}$ | calcium ion |
| $Cl^-$ | chloride ion |
| $CO_2$ | carbon dioxide |
| Cr | creatine |
| DMSO | dimethyl sulfoxide |
| EDL | extensor digitorum longus muscle |
| g dwt | gram dry weight |
| $G_m$ | specific membrane conductivity |
| $H^+$ | hydrogen ion |
| $HCO_3^-$ | bicarbonate ion |
| HPLC | high-performance liquid chromatography |
| $I_o$ | amount of current injected |
| IOA | iodoacetic acid |
| $K^+$ | potassium ion |

| | |
|---|---|
| $K^+_{Ca2+}$ | calcium-dependent $K^+$ channel |
| $K^+_{(ATP)}$ | ATP-sensitive $K^+$ channel |
| $K_i$ | concentration to close half of the channels |
| $K_d$ | concentration to open half of the channels |
| L | Litres |
| M | molar |
| MCT | duncan multiple comparison test |
| $Mg^{2+}$ | magnesium ion |
| ml | millilitres |
| $mmole \cdot L^{-1}$ | millimolar |
| $Na^+$ | sodium ion |
| NaCN | sodium cyanide |
| $O_2$ | oxygen |
| PCA | perchloric acid |
| PCr | phosphocreatine |
| $pH_i$ | intracellular pH |
| $P_i$ | inorganic phosphate |
| $PO_4^{2-}$ | phosphate ion |
| radius | radius of the muscle fiber |
| $R_{in}$ | input resistance |
| $r_m$ | membrane resistance |
| $R_m$ | specific membrane resistance |
| S.E.M. | standard error of the mean |

| | |
|---|---|
| $SO_4^{2-}$ | sulfate ion |
| $V_o$ | membrane voltage change at site of current injection |
| $V_x$ | membrane voltage change at recording electrode of distance x |
| x | distance between stimulating and recording electrodes |
| °C | Celsius |

# CHAPTER 1: STATEMENT OF PURPOSE

Muscle fatigue, defined as a decreased force generating capacity, develops gradually during exercise and is considered a protective mechanism against deleterious depletion of energy and against impairment of muscle function. This implies that a muscle must have a mechanism which is sensitive to the bioenergetic state and which can cause a decrease in contractility to reduce the rate of energy utilization. For years, many studies were carried out to determine how the changes in intracellular metabolites affect the contractility of the sarcomere (for review, see Fitts, 1994). During fatigue the decrease in force is at least 70% (Fitts, 1994; Westerblad, 1992; Renaud, 1989) and the change in metabolites account for only 40% of the decrease in force (Godt & Nosek, 1989). The $K^+_{(ATP)}$ channel actually provides a much better protective mechanism to explain the decrease in force during fatigue because it links the bioenergetic state of the cell to electrical activity of the cell membrane.

The $K^+_{(ATP)}$ channel, present in skeletal, cardiac, and smooth muscle, as well as the brain and pancreatic cells (Hong & Chang, 1991; Hussain *et al.*, 1994; Noma, 1983; Gasser & Vaughan-Jones, 1990; Haddad & Jiang, 1994; Aizawa *et al.*, 1994), is a $K^+$ channel that is regulated by ATP. These $K^+_{(ATP)}$ channels are time- and voltage-insensitive with a unitary conductance varying between 15 and 135 pS depending on the $K^+$ concentrations in the patch pipette and the bath solution (Ashcroft, 1988). The channel is open in the absence of ATP and closes when ATP binds to the intracellular side of the channel, with a $K_i$ value of 20 - 140 $\mu mole \cdot L^{-1}$ (Ashcroft, 1988; Nichols & Lederer, 1991). With such a low $K_i$ value for ATP and the normal cellular [ATP] range in the $mmole \cdot L^{-1}$ range, the physiological

relevance of the channel can be questioned. However, these channels are also activated by several other metabolites. Increases in $[ADP]_i$, intracellular $H^+$, as well as lactic acid all have been shown to activate the channel (Ashcroft, 1988; Edwards & Weston, 1993). Since muscle fatigue exhibits all of the changes in the metabolic factors required for the activation of $K^+_{(ATP)}$ channels, several investigators have hypothesized that ATP-sensitive $K^+$ channels are activated and contribute to the decrease in force during fatigue or other metabolic stresses by affecting membrane excitability (Standen, 1992).

There is evidence that $K^+_{(ATP)}$ channels are activated when frog sartorius muscles are fatigued with one 200 msec tetanic contraction every second for 180 seconds (Light *et al.*, 1994; Comtois *et al.*, 1995). If $K^+_{(ATP)}$ channels contribute to the decrease in force during fatigue, then blocking these channels should decrease the rate of fatigue with respect to tetanic force development. However, Light *et al.*, 1994, and Comtois *et al.*, 1995, have shown that blocking $K^+_{(ATP)}$ channels in frog skeletal muscle, with two different specific channel antagonists, tolbutamide and glibenclamide, does not affect the decrease in force during fatigue. These results suggest that either the channels are not involved in fatigue, or not enough $K^+_{(ATP)}$ channels are activated during fatigue, at least in their fatigue model. It was also observed, however, that the capacity of muscle to recover tetanic force following fatigue is irreversibly reduced when $K^+_{(ATP)}$ channels are blocked during fatigue (Light *et al.*, 1994; Comtois *et al.*, 1995). This reduced capacity of a muscle to recover its force suggests that blocking the channel is deleterious, which is consistent with Noma's notion that $K^+_{(ATP)}$ channels serve as a protective mechanism preventing irreversible impairment of muscle function (Noma, 1983). More importantly, if blocking $K^+_{(ATP)}$ channels is deleterious to the

muscle then the deleterious effects may be counteracting the expected slower decrease in tetanic force during fatigue in the presence of glibenclamide and tolbutamide. So it is still not clear whether $K^+_{(ATP)}$ channels contribute to a decrease in force during fatigue.

## A. Objectives of study

The goal of this study was to further test the hypothesis that ATP-sensitive $K^+$ channels contribute to the decrease in force during fatigue. Two approaches were used to test this hypothesis. First, since blocking the channel is deleterious to the muscle, we examined the effect of channel agonists during fatigue, with the expected result being a faster decrease in force. Furthermore, if blockage of the channels reduced the capacity of the muscle to recover, then the ability to recover should be improved in the presence of channel openers. Second, muscles were subjected to a metabolic inhibition in the presence and absence of $K^+_{(ATP)}$ channel modulators in order to i) determine the relationship among bioenergetic state, force and $K^+_{(ATP)}$ channel activity, and ii) to determine whether or not $K^+_{(ATP)}$ channels can be activated and affect force following a change in the bioenergetic state which is within physiological limits.

# CHAPTER 2: INTRODUCTION

## A. General Background

The cell membrane has a phospholipid bilayer structure in which proteins are inserted at intervals (Singer & Nicholson, 1972). This lipid structure of the cell membrane is very impermeable to charged molecules, resulting in ions crossing the membrane by means of membrane-spanning transport proteins. These are of two types: carriers and channels. Carrier proteins undergo a conformational change for each ion (or a few ions) transported, so their transport rate is relatively slow ($10^4$ ions/sec), but with appropriate energy input they can transport ions up an electrochemical gradient (Standen, 1992). Carrier proteins include the Na-K pump and several other active transport mechanisms (Noble *et al.*, 1992; Radowski, 1991). In contrast, channels transport ions only down an electrochemical gradient.

Ionic channels are transmembrane proteins sitting in the lipid bilayer of the cell membrane. These channels have the ability to conduct ions across the cell membrane at extremely rapid rates (up to $1.0 \times 10^8$ ions/second), thus providing a large flow of ionic current (Kandel *et al.*, 1991). This current flow allows for the rapid changes in membrane potential required for neuronal signalling. The current flow is obtained not from a single type of channel, but rather from a combination of various channels. The major channels found within excitable tissues are $Na^+$, $Ca^{++}$, $Cl^-$, and $K^+$ channels. The $Na^+$ channel is activated when the membrane is depolarized, and once opened allows sodium ions to

rapidly enter into the cell and to cause further cellular depolarization. One important function of the sodium channel is to initiate the depolarizing phase of the action potential. $Ca^{++}$ influx is governed largely by $Ca^{++}$ channels. The main role of the calcium channel is to regulate $Ca^{++}$-dependent intracellular events such as contractions, gating of other channels, and various secretions. Voltage-sensitive $Ca^{++}$ channels normally are activated upon depolarization, while other $Ca^{++}$ channels can be regulated by G-proteins (Kandel *et al.*, 1991). When $Cl^-$ channels are opened they inhibit the cellular excitability by moving $E_m$ towards $E_{Cl}$ (Kandel *et al.*, 1991).

The most impressive diversification of ion channels is observed among $K^+$ channels (Rudy, 1988). $K^+$ channels exist in as many as 20 or more separate classes, the pharmacological and therapeutic significance only now being understood (Triggle, 1990). It is because of this large diversity that the role of the $K^+$ channels is incredibly varied. However, amidst all of this diversity, all of the $K^+$ channels are very selective at conducting only $K^+$ ions and few other ions. Among the best known $K^+$ channels, are the A channel, the M channel, the delayed rectifier, calcium-dependent channel, and the inward rectifier. Open $K^+$ channels stabilize the membrane by drawing the membrane potential nearer to the $K^+$ equilibrium potential and further from the firing threshold (Hille, 1992). Among the voltage-sensitive $K^+$ channels, the delayed rectifier opens only after the membrane is depolarized, while others such as the inward rectifier are further activated only after an hyperpolarization. Other $K^+$ channels, such as the $Ca^{2+}$-activated $K^+$ channel, are

modulated not only by changes in membrane potential but also by intracellular ligands, such as $Ca^{2+}$.

Recent evidence has also brought attention to a class of $K^+$ channels that is sensitive to adenosine triphosphate (ATP). This $K^+_{(ATP)}$ channel has been suggested to provide a link between the bioenergetic state of the cell and the electrical excitability of the cellular membrane, and prevents serious energy depletion and/or impairment of the cellular metabolism (Noma, 1983). Decreases in ATP, increases in ADP, decreases in $pH_i$, and increases in lactate have all been shown to activate $K^+_{(ATP)}$ channels in cardiac and/or skeletal muscle. The following three sections will deal with the channels distinctive attributes with regards to 1) voltage and metabolic characteristics, 2) pharmacology, and 3) proposed function.

## B. $K^+_{(ATP)}$ Channel Voltage and Metabolic Characteristics

### *1. ATP Sensitivity*

$K^+_{(ATP)}$ channels are inhibited by the binding of ATP at the intracellular side of the membrane: extracellular ATP is without effect on channel activity (Ashcroft, 1988). $K^+_{(ATP)}$ channels in patch-clamp experiments are inhibited by $[ATP]_i$, with half maximal closures ($K_i$) ranging between 20 and 140 $\mu mole \cdot L^{-1}$ (Standen *et al.*, 1992; Spruce *et al.*, 1987; Woll *et al.*, 1989; Ashcroft, 1988; Nichols & Lederer, 1991). It is suspected that there are at least two

binding sites within the channel: one for ATP and another for MgATP (Noma, 1983). Although free ATP is required for channel inhibition, millimolar concentrations of the Mg-bound form (i.e., MgATP) are slightly more effective in causing the channel to close (Nichols & Lederer, 1991). Most studies show that ATP binding increases the channel closed time and reduces the channel open time, with no effect on the current amplitude (Woll *et al.*, 1[illegible] Spruce *et al.*, 1987).

Since intracellular ATP concentrations never reach values below the millimolar range even in conditions such as severe ischemia or metabolic inhibition (Weselcouch *et al.*, 1993; Yamada *et al.*, 1993; Deutsch *et al.*, 1991), the relevance of these channels in physiological conditions is questioned because the $K_i$ value for ATP is in the micromolar range. However, $K^+_{(ATP)}$ channels have been found to be active in rabbit ventricular myocytes even though the ATP levels dropped only 25 percent, from 5.5 mmole·L$^{-1}$ to 4.2 mmole·L$^{-1}$ (Deutsch *et al.*, 1991). A number of mechanisms have been proposed to explain the activation of $K^+_{(ATP)}$ channels under these conditions. First, there are differences in channel behaviour in excised patch clamp studies and intact fibre studies. For example, the $K_i$ for glibenclamide, a $K^+_{(ATP)}$ channel blocker, in excised cardiac membrane patches is 3 μmole·L$^{-1}$ while it is 30 μmole·L$^{-1}$ for intact ventricular cells. Furthermore, it is virtually impossible to duplicate all of the cellular conditions in a bathing solution. It has been suggested that channel activation in intact fibres is more likely to occur under conditions which are more physiological (Deutsch *et al.*, 1991).

Second, the density of the $K^+_{(ATP)}$ channel (at least in skeletal muscle) has been found to be equal to or greater than 2 channels/$\mu m^2$, which is greater than that of the delayed rectifier (Spruce *et al.*, 1985; Spruce *et al.*, 1987; Hussain *et al.*, 1994). It has been calculated that less than 1% of the available $K^+_{(ATP)}$ conductance of 200-1000 nS/cell is sufficient to shorten the action potential by 50% in cardiac muscle (Findlay & Faivre, 1991; Yao *et al.*, 1993). Thus, a small drop in [ATP] may activate sufficient numbers of $K^+_{(ATP)}$ channels because of the large number available (Yao *et al.*, 1993). However, in order for this mechanism to work, it has also been proposed that variations of membrane ATP consumption may cause intracellular ATP levels, or the ATP near the membrane, to be heterogeneous. For example, if 10% of the cellular membrane area is facing a low ATP concentration, and the remaining 90% has normal levels, the bulk ATP level may change little, but the increase in $K^+_{(ATP)}$ conductance is calculated to be in the range of 20-100 nS/cell, and this would completely abolish the cellular membrane excitability (Findlay & Faivre, 1991; Yao *et al.*, 1993; Nichols & Lederer, 1991; Deutsch *et al.*, 1991).

Third, it is possible that the cell membrane senses a different pool of ATP than the rest of the cytoplasm and that near-membrane [ATP] may fall more quickly than the bulk [ATP] in energy deprivation (Post & Jones, 1991). Thus, ATP synthesis and availability may depend on metabolic pathways selective for intracellular or membrane-related functions. Weiss and Lamp (1987) lowered ATP content to similar levels by two separate methods, by inhibiting aerobic metabolism or by inhibiting glycolytic metabolism. They demonstrated that the ATP produced from glycolytic pathways is more effective than the ATP from

oxidative metabolism in suppressing $K^+_{(ATP)}$ channel activity in cardiac muscle (Post & Jones, 1991). The preferential inhibition may be due to the presence of key glycolytic enzymes located near the membrane or adjacent cytoskeleton near the channels (Weiss & Lamp, 1987). Furthermore, processes closely associated with the membrane, such as ATPases, are likely to be involved in regulation of the channel activity (Faivre & Findlay, 1990; Weiss & Lamp, 1989; Weiss & Lamp, 1987). For example, repeated action potential generation will lead to an increased activity of the $Na^+$-$K^+$ pump. This will lead to the utilization of ATP resulting in a regional depletion of ATP which could in turn affect $K^+_{(ATP)}$ channels.

While some mechanisms have been suggested for an activation of $K^+_{(ATP)}$ channels when the ATP concentration decreases, it was also found that other metabolites modulate the activity of the channel and displace the ATP inhibition to higher $K_i$ values. These are nucleotides diphosphates, $H^+$, lactate, and phosphorylation processes (Davies *et al.*, 1992; Standen *et al.*, 1992; Kandel *et al.*, 1991; Allard & Ladzunski, 1992; Stanfield *et al.*, 1994).

### 2. *Nucleotide Diphosphates*

Muscle fatigue or any other form of metabolic stress results in an increase in the amount of ADP, from approximately 30 to over 200 $\mu mole \cdot L^{-1}$ (Fitts, 1994; Westerblad *et al.*, 1991). ADP, like ATP, is an inhibitor of the channel. However, ADP is a weaker inhibitor as its $K_i$ value is in the range of 0.5 to 3.1 $mmole \cdot L^{-1}$ in frog skeletal muscle which is 10 fold higher than for ATP (Forestier & Vivaudou, 1993). Since ADP activation may

alleviate ATP inhibition by competitive binding, this combination may allow for channel activation because of an increase in the $K_i$ value for ATP. For example, under conditions of ATP in the micromolar range, the addition of 1 mmole·$L^{-1}$ ADP causes a reduction of the inhibition of the channel (Allard & Ladzunski, 1992). On the other hand, in the presence of millimolar concentrations of ATP, ADP is without effect, most likely because the higher concentration of ATP is sufficient to completely block the channel (Allard & Ladzunski, 1992).

A large amount of the ATP and ADP are bound to $Mg^{2+}$ within the cell. It is possible that ADP affects the amount of intracellular *free* ATP, as opposed to magnesium-bound ATP, by acting as a competitor for the $Mg^{2+}$ (Vivaudou *et al.*, 1991). As MgATP is a stronger inhibitor of the channel than ATP, any reduction in the levels of MgATP lead to a weaker inhibition of the channel. Furthermore, it is suspected that $Mg^{2+}$ has an important role in determining how ADP modulates the channel. However, under low concentrations (in the micromolar range), MgADP acts as a channel activator whereas free ADP acts as a channel inhibitor.

Experimentally, other NDP's such as GDP, UDP, and CDP exhibit the same characteristics: alone they inhibit the channel, but with $Mg^{2+}$ present they open the channel (Allard & Ladzunski, 1992; Tung & Kurachi, 1991; Terzic *et al.*, 1994). The free nucleotides are able to inhibit channel activity by possibly binding to the ATP binding site, and acting as competitive inhibitors. As they are less specific in their ability to block the channel, the

degree of inhibition is less for the NDP's. The role of Mg-bound nucleotides is more difficult to discern. As these studies were performed on excised membrane patches, it is very likely that at these low concentrations of the nucleotides the activation is due to channel phosphorylation (discussed in Chapter 2, section B.4).

### *3. $H^+$ and Lactic Acid*

During fatigue or a metabolic stress, there is an increase in the intracellular amount of lactic acid as well as $[H^+]$. Both of these metabolites have been demonstrated to affect the $K^+_{(ATP)}$ channel in cardiac and/or skeletal muscle (Keung & Li, 1991; Cuevas *et al.*, 1991; Davies *et al.*, 1992).

Lactate rises from 4 to 35 mmole·L$^{-1}$ during sustained ischemia (Keung & Li, 1991). Lactate at concentrations in the range of 20 to 40 mmole·L$^{-1}$ activates the $K^+_{(ATP)}$ channel in cardiac myocytes, even in the presence of 2 to 5 mmole·L$^{-1}$ ATP (Keung & Li, 1991; Han *et al.*, 1993). This activation was blocked by 100 μmole·L$^{-1}$ glibenclamide (Keung & Li, 1991; Han *et al.*, 1993). Furthermore, when 20 mmole·L$^{-1}$ lactate is present in the bathing solution, the action potential of intact ventricular myocytes is significantly shortened within 15 minutes of lactate exposure, whereas action potentials of control myocytes (ie, no lactate) do not shorten within this time period (Keung & Li, 1991). Lactate is believed to act via reducing the ATP inhibition or by acting as a channel opener. Therefore, lactate and not the decrease in ATP may play a significant physiological role in activating cardiac $K^+_{(ATP)}$

channels (Keung & Li, 1991; McKillen *et al.*, 1994; Han *et al.*, 1993). The role of lactate on regulating $K^+_{(ATP)}$ channels in skeletal muscle is unknown because i) to date there has been no study which has addressed this issue; and ii) cardiac and skeletal muscle $K^+_{(ATP)}$ channels have different characteristics as shown below for the effect of hydrogen ion.

Studies in cardiac myocytes have revealed a minor pH dependency. Cuevas *et al* (1991) found that by lowering $pH_i$ from 7.4 to 6.5, in the presence of 0.2 mmole·$L^{-1}$ ATP, there was an increase in the mean open time of the channel. The data indicate that pH modulates cardiac $K^+_{(ATP)}$ channel properties only when ATP levels are near or at the $K_i$ values. These results indicate that pH has no effect when the ATP concentration is within the physiological range. Thus, unlike lactic acid, $H^+$ may not be important in activating $K^+_{(ATP)}$ channels during ischemia in cardiac muscle.

This is in contrast to skeletal muscle $K^+_{(ATP)}$ channels where, under resting levels of ATP, intracellular $H^+$ significantly affects the channel. A decrease in intracellular pH from 7.2 to 6.3 increases the mean open time of the channel and increases the $K_i$ value for ATP (Davies, 1990). The $K_i$ value at pH 7.2 is 17 μmole·$L^{-1}$ and increases to 260 μmole·$L^{-1}$ when the pH is lowered to 6.3 (Davies *et al.*, 1992). Davies *et al.* (1992) proposed a model that two $H^+$ ions competitively inhibit by binding to channel proteins and prevent the binding of, or closure of the channel, by ATP. In intact frog sartorius muscle fibers, a decrease in intracellular pH from 7.2 to 6.3 caused an increase in outward current which was blocked by glibenclamide, a $K^+_{(ATP)}$ channel blocker (Standen *et al.*, 1992). Considering

that during muscle fatigue, the intracellular pH of intact muscle fiber ranges between 6.3 and 6.5 (Renaud, 1989), it was proposed by Davies *et al.* that changes in intracellular pH during fatigue may be important in the regulation of $K^+_{(ATP)}$ channel activity of skeletal muscle.

Thus, changes in both ADP and ATP during most metabolic stresses appeared too small to activate the $K^+_{(ATP)}$ channel of both cardiac and skeletal muscle. In cardiac tissue, lactate appears to be a major determinant of the channel's activation, while in skeletal muscle $pH_i$ is a major determinant. It has been proposed that ATP binds to the channel to set a very low background probability of being open, while changes in pHi and probably other factors in skeletal muscle, and lactate in cardiac muscle, act to change the probability of the channel being open ($P_{open}$) by modulating the degree of inhibition by ATP (Standen, 1992).

*4. Phosphorylation*

One special feature of the $K^+_{(ATP)}$ channel is the so called 'run-down' characteristic (Kamouchi & Kitamura, 1994; Terzic *et al.*, 1994; Furukawa *et al.*, 1994). In excised patch clamp studies, the channel is characterized by a decrease in activity over time, referred to as 'run-down', when it is exposed to ATP-free solutions. It has been proposed that $Ca^{2+}$ in the cytoplasmic solution promotes run-down in cardiac muscle by activating $Ca^{2+}$-dependent phosphatases (Furukawa *et al.*, 1993; Deutsch & Weiss, 1993). This decreased activity can be antagonized by the application of ATP in the presence but not in the absence of $Mg^{2+}$ (Tung & Kurachi, 1991; Kamouchi & Kitamura, 1994). Other nucleotide diphosphates have shown similar effects (Tung & Kurachi, 1991). In all these conditions, the reactivation of the channel depends critically on $Mg^{2+}$ (Tung & Kurachi, 1991; Kamouchi & Kitamura, 1994). As $Mg^{2+}$ is required for hexokinase activity, and thus the ability of phosphoryl groups to be transferred from ATP to an acceptor, it appears that phosphorylation is involved. Furthermore, as nonhydrolyzable analogues of ATP do not substitute for ATP in reactivation from rundown, phosphorylation of the channel appears to be a mechanism by which run-down is prevented (Nichols & Lederer, 1991).

The ability to be reactivated would suggest that the channel, when excised, undergoes some kind of modification, possibly a dephosphorylation reaction which would deactivate the channel by locking it into a closed state (Deutsch & Weiss, 1994). Furthermore, the addition of trypsin to channel patches allows for the stabilization of the channel making it

resistant to the effects of dephosphorylation (Deutsch & Weiss, 1994). The mechanism of action of trypsin may involve a proteolytic digestion of lysine residues located in the cytosolic regions of the channel protein which are involved in the phosphorylation/dephosphorylation process (Furukawa *et al.*, 1993). Tryptic proteolysis did not affect the other characteristics of the channels including gating kinetics, unitary conductance, or the sensitivity to ATP (Furukawa *et al.*, 1993). It would seem likely that the channel when excised is modified at the structural level which the application of agents such as trypsin is able to modulate.

There are several other plausible mechanisms proposed for the run-down of the $K^+_{(ATP)}$ channel. One of these is that the channel run-down is mediated by $Mg^{2+}$ ions and perhaps other divalent ions, binding to the channel resulting in an allosteric change and in a long-lived closed state (Furukawa *et al.*, 1993). Although many possible explanations for the mechanism of channel run-down have been postulated, an exact mechanism is still largely unknown.

## C. $K^+_{(ATP)}$ Channel Pharmacology

As a pharmacological approach is necessary to investigate the channels' role in skeletal muscle of the frog, this section deals with the pharmacology of various $K^+_{(ATP)}$ channel modulators. In recent years, numerous openers and blockers have been developed which specifically act on the $K^+_{(ATP)}$ channel. The most commonly used openers in the study

of $K^+_{(ATP)}$ channels are nicorandil, pinacidil, cromakalim, SR44866, and levcromakalim--the active enantiomer of cromakalim. The most common $K^+_{(ATP)}$ channel blockers are glibenclamide, tolbutamide, and chlorpromazine.

*1. Channel openers*

The $K^+_{(ATP)}$ channel openers are a diverse group of agents originally characterized by their ability to open smooth muscle K-channels. The opening of smooth muscle $K^+_{(ATP)}$ channels results in an hyperpolarization-induced vasorelaxation (Ashcroft, 1988). However, it is known that the these agents exert their opening effect in a wide variety of tissue types including the pancreatic $\beta$-cell, neurons, skeletal muscle, and cardiac muscle (Ashcroft, 1988). The channel openers appear to decrease the channel sensitivity to ATP, shifting the $K_i$ for channel inhibition to higher ATP levels (Nichols & Lederer, 1991).

One interesting characteristic of these channel openers is their dependency on the intracellular ATP concentration. Pinacidil (N-cyano-4-pyridyl-N-1,2,2, trimethylpropyl-guanidine monohydrate) activates $K^+_{(ATP)}$ channels better at low ATP concentrations than at high ATP concentrations: pinacidil at 1 mmole·$L^{-1}$ ATP has a $K_d$ of 70 µmole·$L^{-1}$, whereas at 10 mmole·$L^{-1}$ ATP the $K_d$ is greater than 158 µmole·$L^{-1}$. Furthermore, the maximal current measured under pinacidil activation decreases as the concentration of ATP is increased (Fan *et al.*, 1990). Similarly, nicorandil (2-Nicotinamiodethyl nitrate) exerts

greater channel opening at 2 mmole·$L^{-1}$ ATP with a $K_d$ of 0.5 mmole·$L^{-1}$, whereas at 10 mmole·$L^{-1}$ ATP the $K_d$ is 2.9 mmole·$L^{-1}$ (Nakayama *et al.*, 1991).

Pinacidil (0.2 to 1.0 μmole·$L^{-1}$), and 0.3 - 1.0 mmole·$L^{-1}$ nicorandil (lower doses are for pancreatic tissues and the higher doses are for cardiac tissue), increase the open probability of the channel without changing the unitary current amplitude (Fan *et al.*, 1990; Nakayama *et al.*, 1990). It is hypothesized that pinacidil and nicorandil have either their own binding site or a receptor complex that interacts with the ATP-binding site inhibiting the ATP effects (Hiraoka *et al.*, 1993; Fan *et al.*, 1990).

Pinacidil, at higher concentrations (mmole·$L^{-1}$) may also produce closure of channel activity in addition to its activation effects at low concentrations (μmole·$L^{-1}$) (Fan *et al.*, 1990). Further, pinacidil exerts a voltage-dependent blockage of channel activity by decreasing the mean open time and increasing the mean closed time at positive potentials (Fan *et al.*, 1990). Due to this dual role of pinacidil, it is suspected that there are multiple binding sites for pinacidil: one for activation and another one for blockage.

Levcromakalim (BRL 38227) is a new orally active anti-hypertensive agent. It is the biologically active 3S, 4R enantiomer of cromakalim, another $K^+_{(ATP)}$ channel opener (Hamilton *et al.*, 1993). The opening of $K^+_{(ATP)}$ channels by 100 μmole·$L^{-1}$ levcromakalim increases the outward current in mouse skeletal muscle fiber patches which can be blocked by 10 μmole·$L^{-1}$ glibenclamide, a $K^+_{(ATP)}$ channel blocker (Hussain *et al*, 1994).

Furthermore, in the presence of 100 $\mu mole \cdot L^{-1}$ levcromakalim, frog skeletal muscle has been shown to have up to a 50 % reduction in the membrane resistance which is blocked by tolbutamide, another $K^+_{(ATP)}$ channel blocker, indicative of an opening of $K^+_{(ATP)}$ channels (Hong & Chang, 1991; Benton & Haylett, 1992). Finally, an increase in $K^+$ efflux and hyperpolarization of 6.6 mV in frog skeletal muscle has been observed which is consistent with the opening of $K^+_{(ATP)}$ channels shifting the $E_m$ towards $E_K$ (Benton & Haylett, 1992).

Smooth muscle and cardiac muscle are sensitive to the effects of all channel activators. However, skeletal muscle appears to be less sensitive to these channel activators as a 10- to 100- fold higher concentration of these drugs is required to affect channel activity. The openers which have been found to be relatively effective in skeletal muscle are: 1 $mmole \cdot L^{-1}$ cromakalim (mammalian), 200 $\mu mole \cdot L^{-1}$ nicorandil (mammalian), 1 $mmole \cdot L^{-1}$ pinacidil (mammalian), 100 $\mu mole \cdot L^{-1}$ SR44866 (frog) and 100 $\mu mole \cdot L^{-1}$ levcromakalim (frog) (for review see Edwards & Weston, 1993). In this study, levcromakalim will be used at it has been shown that 100 $\mu mole \cdot L^{-1}$ levcromakalim reduces the membrane resistance in frog skeletal muscle (Hong & Chang, 1991; Benton & Haylett, 1992). SR44866 will not be used due to its non-specific effects on the sodium channel (Sauviat *et al.*, 1991). As pinacidil has been demonstrated to be highly effective in cardiac tissue in affecting the action potential and the membrane current (for review see Edwards & Weston, 1993), pinacidil will also be used in this study to determine its effects in skeletal muscle.

## 2. *Channel blockers*

Agents that restrict the movement of $K^+$ ions through the $K^+_{(ATP)}$ channel have been variously described as blockers, antagonists or inhibitors. $K^+_{(ATP)}$ channel blockers include the non-specific $K^+$ channel blockers tetraethylammonium (TEA), 4-aminopyridine, and various cations such $Ba^{2+}$, $Mg^{2+}$, and $Cs^{2+}$ (Cook & Quast, 1989.). These blockers are not only nonspecific, but often will not completely block the $K^+_{(ATP)}$ channel. Blockers specific for the $K^+_{(ATP)}$ channel include the phenothiazines and the sulfonylureas.

Chlorpromazine and related phenothiazines are used clinically to treat a number of psychiatric disorders and are known to interact with dopamine receptors at low concentrations. However, at higher concentrations (mmole·$L^{-1}$), these drugs interact with a variety of other ionic channels such as the $Ca^{++}$-sensitive $K^+$ channel. At low concentrations (below 50 μmole·$L^{-1}$), these compounds interact with the pancreatic B-cell $K^+_{(ATP)}$ channel and may inhibit the activity of the channel almost as effectively as the sulfonylureas (Muller *et al.*, 1991). However, chlorpromazine does not affect $K^+_{(ATP)}$ channels in frog skeletal muscle (Benton & Haylett, 1992).

The best known sulfonylurea compounds are tolbutamide and glibenclamide. The concentration of these drugs used to block $K^+_{(ATP)}$ channels in muscle varies depending on experimental conditions and tissues. Under excised patch clamp conditions tolbutamide inhibits channel activity at concentrations of 0.5 - 1.0 mmole·$L^{-1}$ for pancreatic tissues, 1.0

to 1.5 mmole·$L^{-1}$ for cardiac muscle, and 1.0 to 2.0 mmole·$L^{-1}$ for skeletal muscle. For glibenclamide, the ranges of concentrations are 0.2 to 0.3 μmole·$L^{-1}$ for pancreatic cells, 1 to 3 μmole·$L^{-1}$ for cardiac muscle, and greater than 3 μmole·$L^{-1}$ for skeletal muscle. Another interesting aspect is that 10-fold higher concentrations of tolbutamide or glibenclamide are required to block $K^+_{(ATP)}$ channels in intact fibres in comparison to those in excised patch clamp (Nakayama *et al.*, 1990; Nakaya *et al.*, 1991; Gasser & Vaughan-Jones, 1990; Sauviat *et al.*, 1991; Castle & Haylett, 1987; Woll *et al.*, 1989). The reason for the difference in efficiency between the different preparations is unknown.

Both tolbutamide and glibenclamide have been used extensively to study the function of $K^+_{(ATP)}$ channels because of their specificity. Tolbutamide (up to 2 mmole·$L^{-1}$) has no effect on the $K^+$ inward rectifier or the $K^+$ delayed rectifier, the resting potential, action potential, or membrane conductance in cardiac tissue (Gasser & Vaughan-Jones, 1990; Nichols & Lederer, 1991). However, Comtois *et al.*, 1993, have shown that tolbutamide is less specific in frog skeletal muscle than in cardiac tissue (Comtois *et al.*, 1993). Tolbutamide at acidic extracellular pH depolarizes the sarcolemma, decreases the number of excitable muscle fibres, and decreases tetanic force. The mechanism of action is unknown, but the effect could not be explained by an effect on the $K^+_{(ATP)}$ channel. Tolbutamide also increases lactate production by increasing anaerobic metabolism mediated by activation of phosphofructokinase and phosphorylase (Kramer *et al.*, 1983). For these reasons, tolbutamide will not be used in this study.

Glibenclamide (up to 200 $\mu mole \cdot L^{-1}$) has no effect on the mammalian cardiac $K^+$ inward rectifier channel and $K^+$ delayed rectifier (Gasser & Vaughan-Jones, 1990; Escande & Cavero, 1992). At doses up to 100 $\mu mole \cdot L^{-1}$, glibenclamide has no effect on the delayed rectifier channel, the inward rectifier, or the calcium-activated $K^+$ channel in rat skeletal muscle (Light & French, 1994). In frog sartorius muscle, glibenclamide (up to 50 $\mu mole \cdot L^{-1}$) has no effect on the inward rectifier (Davies *et al.*, 1992), and has no effect on the $Na^+$ channel or the delayed rectifier as indicated by a lack of effect on the action potential (Light *et al.*, 1994). While glibenclamide is quite specific for the $K^+_{(ATP)}$ channel, some non-specific effects have also been found. It has been shown to open an unknown channel current (Quastoff *et al.*, 1990). However, this channel current has not been found in skeletal muscle so this non-specific effect is not relevant in muscle. In the liver, glibenclamide also inhibits A-kinase that regulates the enzyme fructose-6-phosphate-2-kinase and such an effect would shift the glycolytic pathway in the direction of pyruvate production (Caro, 1990). The subsequent net synthesis of ATP could result in an inhibition of $K^+_{(ATP)}$ and could account for some of the effects of this agent on $K^+_{(ATP)}$ channels in the liver (Caro, 1990). The result of the altered metabolism is an inhibition of the $K^+_{(ATP)}$ channel. However, if pyruvate production is increased, channel activation may occur as pyruvate activates the $K^+_{(ATP)}$ channel in cardiac muscle (McKillen et al., 1994). These effects of glibenclamide on cellular metabolism have not been demonstrated in skeletal muscle.

## D. Proposed Function of the $K^+_{(ATP)}$ Channel

$K^+_{(ATP)}$ channels may provide a link between the bioenergetic state of a muscle fiber and the electrical activity of the cell membrane. When Noma (1983) first discovered the $K^+_{(ATP)}$ channel, he suggested that the $K^+_{(ATP)}$ channel prevents further depletion of ATP and protects the cell from irreversible impairment of its energy metabolism. This can be achieved if $K^+_{(ATP)}$ channels reduce the muscle contractility during any metabolic stress (Standen *et al.*, 1992).

Two mechanisms can be proposed by which the $K^+_{(ATP)}$ channel can contribute to a decrease in force during a metabolic stress in both cardiac and skeletal muscle. First, an increase in the channel activity provides an increased outward current which then reduces the duration of the action potential. As the repolarization phase of the action potential is shorter, less $Ca^{2+}$ is released by the sarcoplasmic reticulum into the intracellular space, leading to a reduction in force development.

A second mechanism by which $K^+_{(ATP)}$ channels can contribute to a decrease in force during fatigue is by increasing the extracellular concentration of $K^+$. During fatigue or other forms of metabolic stress, there is a large net efflux of $K^+$ ions which leads to an accumulation of $K^+$ in the t-tubule network and interstitium (Sjogaard *et al.*, 1985). This $K^+$ accumulation then causes a depolarization of the cell membrane leading to the inactivation of $Na^+$ channels which blocks action potential generation (Bigland-Ritchie *et*

*al.*, 1979; Fitts, 1994). Recent results have shown that when the extracellular [$K^+$] reaches 10 mmole·$L^{-1}$, muscle fibres rapidly become inexcitable (Renaud & Light, 1992). Furthermore, if there is also a decrease in extracellular $Na^+$ (which may occur in t-tubules during fatigue), then the [$K^+$] at which the muscle fiber become inexcitable is less than 10 mM (Bouclin *et al.*, 1995).

Both mechanisms might lead to a decrease in membrane excitability resulting in less calcium release which in turn leads to a decrease in force production. The main advantage of such mechanisms is that they reduce the activity of the major ATPases within the muscle: namely: myosin ATPase, calcium ATPase, and the Na-K ATPase. During contractions, the energy utilization is 3 orders of magnitude above resting conditions (Baker *et al.*, 1994). Contractile processes, i.e. myosin ATPase, utilize 57% of this energy, whereas the remaining 43% is used by non-contractile processes, i.e. calcium ATPase and Na-K ATPase (Baker *et al.*, 1994). Since such a tremendous amount of energy is utilized during contractions (a 1000-fold in comparison to resting conditions), any reduction in contractility would drastically reduce the energy expenditure when the bioenergetic state decreases to protect the cell from serious damage (Noma, 1983).

In cardiac muscle, activation of $K^+_{(ATP)}$ channels in conditions of ischemia contributes to the $K^+$ efflux, action potential shortening, and decrease in force. The $K^+_{(ATP)}$ specific blocker, glibenclamide, counters all of these effects (Weiss *et al.*, 1992; Nakaya *et al.*, 1991). $K^+_{(ATP)}$ channel activators such as nicorandil and pinacidil mimic these ischemic

conditions in resting control cardiac muscle cells (Satoh, 1993; Yao & Gross, 1994). In addition, in ischemic conditions the presence of $K^+_{(ATP)}$ channel openers leads to a faster rate of recovery following these periods, indicating a cardioprotective role of the channel. Thus, in cardiac muscle, there is excellent evidence that activation of $K^+_{(ATP)}$ channels during ischemia alters the electrophysiological and contractile properties of the muscle and that activation of $K^+_{(ATP)}$ channels is an important endogenous mechanism to prevent energy depletion (Auchampach *et al.*, 1992; Yao *et al.*, 1993).

In skeletal muscle, $K^+_{(ATP)}$ channels contribute to the $K^+$ efflux during a metabolic stress. The activity of $K^+_{(ATP)}$ channels in skeletal muscle has been measured in metabolically exhausted fibres, where there is a large increase in $K^+$ efflux and a very large increase in membrane conductance, which is blocked by the presence of glibenclamide (Castle & Haylett, 1987; Light *et al.*, 1994). This would strongly suggest that in exhausted fibres, $K^+_{(ATP)}$ channels are active in large numbers and contribute to the $K^+$ efflux. However, complete metabolical exhaustion does not represent a true fatigue condition because muscles are inexcitable and do not recover, whereas fatigued muscle are still excitable and are capable of fully recovering force. During fatigue, or other metabolic stresses, there is an increase in the venous concentration of $K^+$ (Fitts, 1994; Westerblad *et al.*, 1991). This increase in the $K^+$ concentration in the venous return of diaphragm and gastrocnemius muscles is reduced when dogs are injected with either tolbutamide or glibenclamide (Comtois *et al.*, 1994; Paterson *et al.*, 1992). These results suggest that $K^+_{(ATP)}$ channels are activated during fatigue.

There is evidence that activation of $K^+_{(ATP)}$ channels shortens the action potential in skeletal muscle. Sauviat *et al.* (1994) demonstrated this is in unfatigued frog skeletal muscle with the channel opener SR44866. Light *et al.* (1994) demonstrated that glibenclamide or tolbutamide prolonged the action potential duration in fatigued muscle fibers (Light *et al.*, 1994; Comtois *et al.*, 1995). These results suggest that $K^+_{(ATP)}$ channels are active during fatigue and contribute to the action potential repolarization phase.

Despite the fact that $K^+_{(ATP)}$ channels contribute to the $K^+$ efflux and the action potential repolarization phase in skeletal muscle as in the heart, the role of $K^+_{(ATP)}$ channels with respect to the decrease in force is less defined. Evidence that $K^+_{(ATP)}$ channels contribute to a decrease in force comes from a study on the effect of the channel opener, SR44866 on frog skeletal muscle by measuring twitch forces (Sauviat *et al.*, 1991). SR44866 increases the activity of $K^+_{(ATP)}$ channels and as it decreases the action potential duration it also reduces the twitch force in resting unfatigued muscle (Sauviat *et al.*, 1991). SR44866 has similar effects on skeletal muscle as nicorandil and pinacidil have on cardiac muscle. However, even though an effect was found on the action potential and the twitch force, it must be pointed out that SR44866 also affects the sodium current, so it is not possible to determine if the shortening of action potential and the decrease in twitch force are due to an effect on the $Na^+$ channels, $K^+_{(ATP)}$ channels or both.

Weselcouch *et al.* (1993) demonstrated that activation of $K^+_{(ATP)}$ channels with 300 μM cromakalim, a $K^+_{(ATP)}$ channel opener, does not affect the rate of fatigue development

when rat extensor digitorum longus (EDL) muscles are stimulated to generate twitches at a frequency of 0.2 Hz under normoxic conditions. However, under conditions of anoxia, cromakalim significantly increases the rate of fatigue (Weselcouch *et al.*, 1993). In that study, the activity of $K^+_{(ATP)}$ channels was not measured, but these results suggest that a critical number of channels is required to be opened before the capacity of the muscle to generate force is reduced. At a frequency of 0.2 Hz, under normoxic conditions, the change in the metabolites is probably too small to activate a sufficient number of channels to affect the twitch force, while, under anoxic conditions the change in metabolites is large enough so that the addition of cromakalim causes a further decrease in force.

Using a higher frequency of stimulation and a fatigue model for which there was evidence for an activation of $K^+_{(ATP)}$ channels, Light *et al.* (1994) and Comtois *et al.* (1995) tested the effect of blocking $K^+_{(ATP)}$ channels on the rate of fatigue as measured by the decrease in tetanic force. If $K^+_{(ATP)}$ channels contribute to the decrease in force during fatigue, then blocking these channels should decrease the tetanic force development. However, neither tolbutamide nor glibenclamide, two specific $K^+_{(ATP)}$ channel blockers, affected the decrease in force during fatigue in frog sartorius muscle. Their results suggest the channels do not contribute to the decrease in force during fatigue, or that not enough $K^+_{(ATP)}$ channels were activated in their fatigue model.

In their studies, however, Light *et al.* (1994) and Comtois *et al.* (1995) also observed that the capacity of frog sartorius muscle to recover tetanic force from fatigue is irreversibly

impaired when $K^+_{(ATP)}$ channels are blocked during fatigue. This impairment of force recovery suggests that blocking the channel is deleterious, which is consistent with Noma's notion (1983) that $K^+_{(ATP)}$ channels serve as a protective mechanism preventing irreversible impairment of muscle function. It is also consistent with the cardioprotective effect observed in cardiac muscle. More importantly, if blocking $K^+_{(ATP)}$ channels is deleterious then the deleterious effects may have counteracted the expected slower decrease in tetanic force during fatigue in the presence of glibenclamide and tolbutamide. It therefore remains to be determined if $K^+_{(ATP)}$ channels contribute to the decrease in force in skeletal muscle during fatigue and under what magnitude of metabolic stress.

The goal of this study was to further test the hypothesis that $K^+_{(ATP)}$ channels contribute to the decrease in force during fatigue. Two approaches were used to test this hypothesis. First, to determine if channel activation contributes to the decrease in force during fatigue, one must examine the effect of a channel agonist during fatigue, with the expected result being a faster decrease in force. Furthermore, if blockage of the channels reduced the capacity of the muscle to recover, then the ability to recover should be enhanced in the presence of channel openers. Second, to better understand under what metabolic state $K^+_{(ATP)}$ channels contribute to the decrease in force and whether or not such a metabolic state is within normal physiological condition, muscles were subjected to metabolic poisoning in the presence and absence of $K^+_{(ATP)}$ channel modulators since it is known that under these conditions a large number of $K^+_{(ATP)}$ channels are activated.

# CHAPTER 3: MATERIALS AND METHODS

## A. Animals and Muscle

### *1. Animals*

Frogs, *Rana Pipiens*, with weights in the range of 25 to 40 grams, were purchased from Anilab, Québec, Canada and maintained as described by Light *et al.* (1994). Briefly, frogs were kept in 90 x 40 x 55 cm teflon tanks containing water (20 cm deep) at 20 - 22 °C; three platforms were provided above the water to provide dry areas. Groups of 15 animals or less were kept in each tank. The photoperiod was 12 : 12 hours (light : dark), and the frogs were provided with mealworms supplemented with vitamins (Centrum brand) and $Ca^{2+}$ (Caltrate 600) three times a week for at least one month prior to an experiment. Frogs were maintained according to the guidelines of the CCAC (Canadian Council of Animal Care) and protocols were approved by the Animal Care Committee of the University of Ottawa.

### *2. Sartorius Muscle Preparation*

Frogs were killed by double pithing and sartorius muscles were carefully dissected out with a piece of the pelvic bone and a 4.0 surgical silk thread tied around the distal tendon. The pelvic bone was held in place by a clamp in a muscle chamber (10 ml volume), while the distal tendon end was attached by the silk thread to a light weight wire attached

to a force transducer (unless otherwise stated). The force transducer was mounted on a vernier in order to adjust the muscle length.

## B. Solutions

The muscles were superfused with solutions at all times during an experiment. The control solution, buffered at pH 7.2 contained (in mmole·$L^{-1}$): 120 $Na^+$, 3 $K^+$, 1.8 $Ca^{2+}$, 1 $Mg^{2+}$, 113.6 $Cl^-$, 1 $SO_4^{2-}$, 2 $PO_4^{2-}$, and 7 $HCO_3^-$. All solutions were continuously gassed with 99% $O_2$ and 1% $CO_2$. The flow of solution through the muscle chamber (total volume of 10 ml) was maintained at 10 - 15 ml/min at all times during an experiment. Experimental temperature was 20 °C. All solutions containing metabolic poisons (Chapter 5) were recirculated with a Harvard Apparatus peristaltic pump. The total volume of the recirculating solutions was 300 ml. Solutions for fatigue experiments were not recirculated (Chapter 4).

Metabolic inhibition was induced by exposure to solutions containing 2 mmole·$L^{-1}$ sodium cyanide (to block oxidative phosphorylation) and 1 mmole·$L^{-1}$ iodoacetate (to block glycolytic phosphorylation) (Lydrup *et al.*, 1994; Yamada *et al.*, 1993). The solutions were prepared by adding proper amounts of each compound to the control solution. Solutions containing glibenclamide (Sigma, USA) were obtained by adding the proper volume of a stock solution of 20 mmole·$L^{-1}$ glibenclamide in 100% DMSO (Sigma, dimethyl sulfoxide). Solutions of pinacidil (a gift from Eli Lilly and Company, Lilly Corporate Center, Indianapolis, USA), or levcromakalim (a gift from SmithKline Beecham Pharmaceutical)

were obtained by adding the proper volume of a stock solution of 50 mmole·$L^{-1}$ in 100 % DMSO The final concentration of DMSO for all experimental solutions was less than 0.20 % v/v. This concentration of DMSO had no significant effect on resting skeletai muscle action potentials or tetanic force (Appendix I). Furthermore, there was no effect of DMSO on the kinetics of fatigue and recovery (Appendix II).

## C. Stimulation

The muscles were stimulated by passing a current between parallel platinum wires placed above and below the muscle. Stimulations were given by a Grass S88 (USA) stimulator via an isolation unit (Grass SIU5, USA). Tetanic contractions were elicited by 200 msec trains of 0.5 msec rectangular pulses at a frequency of 140 Hz with a supramaximal strength of 6 V (threshold values for the muscle were in the range of 0.5 to 0.9 V).

## D. Measurement of Tetanic Force

The pelvic bone was held in place by a clamp in the muscle chamber, while the distal tendon was hooked to a lightweight wire attached to a Kulite BG-100 force transducer. The force transducer was connected to a Kipp and Zonen chart recorder (BD 100) to measure the resting tension--defined as the tension in the absence of stimulation. The force transducer was also connected to a DAS-50 (Keithley, Metrabyte Corporation; USA) analogue-digital converter to digitize tetanic contractions at a sampling rate of 5 KHz. The

following parameters were later obtained by analyzing the waveforms on a microcomputer. Tetanic force, defined as the maximum force developed during a contraction, was calculated as the difference between the baseline and maximum force. The tetanic force was calculated in units of $N/cm^2$, assuming a cylindrical shape of the muscle with a uniform density of 1.0 mg/cm. Force-200, an appraisal of the ability of the muscle to maintain a constant force during the plateau phase, was the force measured at the end of the 200 msec train of stimuli and expressed as a percentage of the maximum tetanic force. The first derivative of the tetanic contraction was calculated from the slope of every ten data points (time interval 2 msec) of the digitized tetanic contraction. The maximum rate of relaxation (-dF/dT) was then defined as the difference between the baseline and the lowest peak during the relaxation phase.

## E. Measurement of Resting and Action Potentials

Membrane potentials were measured using conventional micro-electrodes. The reference electrode, measuring the potential of the bathing solution, was made from a 3.0 cm long porous glass rod and soaked in 3 M KCl between experiments. Resistance of the reference electrode was 1 MΩ. Recording micro-electrodes were made from 1.5 mm o.d. (outer diameter) borosilicate glass (1B150F-4; World Precision Instruments, Inc, USA), pulled daily on a Sutter Flaming/Brown Micropipette Puller (Model P-87; USA) and back-filled with 3 M KCl. Resistances ranged from 5 to 16 MΩ, and tip potentials were less than 5 mV. Ag-AgCl wires were used as the reversible half-cells for both reference and micro-electrodes. A silver sheet (1 cm x 1 cm) in the muscle chamber was used as a ground

electrode. Micro-electrodes and reference electrodes were connected to a World Precision Instruments Inc (model S-7071A: USA) amplifier system which allowed for capacitance compensation. The amplifier system was then connected to a Kipp and Zonen chart recorder (for resting potential measurement upon penetration of the muscle fiber) and to the DAS-50 analogue digital converter. Action potentials (APs) were elicited with a single 0.5 msec rectangular pulse. The stimuli were applied only at the surface of the muscle using fine platinum electrodes and stimulus strength was adjusted to 0.4 - 0.8 V to stimulate a small number of fibres to minimize movement artifacts. Action potentials were digitized as described for tetanic force except that the sampling rate was 200 KHz. The following variables were later analyzed: resting membrane potential, overshoot, and the half repolarization time (time interval between the peak of the action potential and the time repolarization was half complete). For a given experimental condition, action potentials were measured in a minimum of three fibers chosen at random. The variability between these fibers was less than the variability between animals. For each variable of the action potential an average value was calculated for each muscle and the mean values reported in this study were calculated from these muscle averages, not from individual measurements. Thus, the sample size (n) represents the number of muscles, and not the number of muscle fibers tested.

## F. Measurement of Membrane Conductance

Membrane conductance was measured from the low frequency cable properties using three micro-electrodes. One micro-electrode (filled with 2 M potassium acetate) was used

to inject a current (between -95 nA and 50 nA) while the other two micro-electrodes (filled with 3 M KCl) were used to measure the change in membrane potential at two different distances from the injection site. Distances were measured using a micrometer in the ocular lens. The first electrode was placed approximately 50 μm from the injecting micro-electrode while the second electrode was placed 1200 - 2300 μm from the injecting micro-electrode. The first distance was chosen to be as close as possible to the current injecting site to have a good estimate of $V_o$, the change in membrane potential at the injecting site. The second electrode was positioned near the expected length constant, $\lambda$, to better estimate the decay of the membrane potential along the muscle fiber. Membrane potential changes were measured on the Kipp and Zonen chart recorder described above for the resting potential. This technique was chosen over the one in which a voltage measuring microelectrode is moved 3-4 times along the muscle fiber for two reasons. One, the cell membrane became unstable during metabolic inhibition (results not shown). Two, this technique allows constant measurements while changing solutions.

Calculations of the membrane conductance from the change in membrane potential were as described by Renaud & Mainwood (1985). Briefly, a linear regression of the natural log of the membrane potential changes versus the microelectrode distances was used to estimate by extrapolating to the y-intercept the change in membrane potential at the injection site ($V_o$), and the length constant, $\lambda$, from the reciprocal of the slope. From these two values the input resistance, $R_{in}$, membrane resistance, $r_m$, and conductance, $G_m$, were calculated as follows (Renaud & Mainwood, 1985):

1) input resistance: $R_{in} = V_c/I_c$, where $I_c$ is current injected

2) membrane resistance: $r_m = 2 \cdot \lambda \cdot R_{in}$

3) specific membrane resistance $R_m = r_m \cdot 2 \cdot \pi \cdot \text{radius}$

4) specific membrane conductance: $G_m = 1 / R_m$

## G. Biochemical Assays

### *1. Tissue preparation*

Muscle samples were freeze clamped in liquid nitrogen and stored at -80°C. Prior to analysis, muscle were lyophilized in a vacuum freeze-drier kept at -45°C overnight. Samples were weighed and then immediately pulverized with a glass rod before 1.0 ml 6% w/v perchloric acid (PCA) was added to the muscle tissues. The tissue samples were kept on ice and vortexed every five minutes for twenty minutes. The suspension was centrifuged for 10 minutes at 12 000 RPM and 800 µl of the resulting supernatant transferred to a fresh 1.5 ml eppendorf test tube on ice. 100 µl of 2.0 M $K_2CO_3$ was added to neutralize PCA. The $K^+$ precipitate was centrifuged for 10 minutes at 12 000 RPM. The resulting supernatant was aspirated off and stored at -80 °C until analysis for the nucleotides.

## II. High Performance Liquid Chromatography Determination and Analysis

Skeletal muscle high-energy phosphates were determined on an anion-exchange Partisil SAX-10 (0.4 x 25 cm, particle size 10 μm; Cat # 4226-001; Whatman, Maidstone, Great Britain) column with the aid of a Waters Intelligent Sample Processor (W.I.S.P.) 712 automatic refrigerated injector (Millipore). The method was slightly modified from the method described in the Journal of Chromatography (Harmsen *et al.*, 1982). Briefly, the sample injection volume was 20 μl, and absorbances were measured at a wavelength of 214 nm. Samples were read and then compared to standards of nucleotides containing in $\mu mole \cdot L^{-1}$: ATP, 750; ADP, 500; AMP, 100; PCr, 1000; Cr, 1000. Due to large differences in polarity between AMP and ATP, a salt gradient elution was necessary. The two buffers were prepared fresh every three days and filtered through a 0.45 μm filter (Millipore). The column was eluted with buffer A at a flow rate of 1.5 ml/min. Buffer A consists of 10 $mmole \cdot L^{-1}$ $NH_2PO_4$, pH adjusted with 10 $mmole \cdot L^{-1}$ $H_3PO_4$ and $NH_3$ to a pH of 2.85; buffer B consists of 0.75 M $NH_2PO_4$, pH of approximately 4.0 to 4.4. Within 30 minutes AMP, IMP, ADP, ATP, and PCr can be separated and quantified in the tissue extract. After the separation, an additional 30 minutes were necessary for the restoration of the starting conditions (15 min) and column equilibration (15 min). Peaks of ATP, PCr, ADP, and AMP, were identified by comparing retention times with standards and were quantified against the standard curve.

## I. Statistics

All data are reported as mean ± S.E.M. (standard error of the mean). Analysis of variance (ANOVA) was used to test the significance of the different treatments. A split plot design was used when a muscle was used for all levels of a given treatment. All calculations were made using the GLM procedures (General Linear Model procedures) of the SAS statistical software (SAS Institute, Cary, NC, USA). When a main effect or an interaction was significant, a Duncan multiple comparison test (MCT) was used to determine the significant differences. When necessary, the data were transformed to obtain homoscedasticity. In this study, the word "significant" refers only to a statistical difference ($P < 0.05$).

# CHAPTER 4: Do $K^+_{(ATP)}$ channels contribute to the decrease in force during fatigue?

## A. INTRODUCTION:

It is well known that cardiac muscle exposed to a metabolic stress such as ischemia or hypoxia has a shorter action potential duration, a higher $K^+$ efflux and a reduced capacity to develop force. Several studies have shown that $K^+_{(ATP)}$ channels are activated under these metabolic stresses and contribute to these changes (Duncker *et al.*, 1993; Downey, 1993; Weiss *et al.*, 1992; Faivre & Findlay, 1990; Gasser & Vaughan-Jones, 1990). Additionally, there is also evidence for a cardioprotective effect of $K^+_{(ATP)}$ channels in cardiac muscle as the capacity of the muscle to recover from these metabolic stresses is reduced when the channel is blocked (Nakaya *et al.*, 1991) or enhanced when the channel is further activated with channel openers (Cole, 1993; Faivre & Findlay, 1990). The role of $K^+_{(ATP)}$ channels in skeletal muscle is much less understood as there are fewer studies. It has been postulated that $K^+$(ATP) channels are activated and contribute to the decrease in force during fatigue (Standen, 1992).

Although there is evidence for an activation of $K^+_{(ATP)}$ channels during fatigue, the rate of fatigue was not affected when the channels were blocked with either tolbutamide or glibenclamide (Comtois *et al.*, 1995; Light *et al.*, 1994). However, it has been proposed that blocking $K^+_{(ATP)}$ channels is deleterious and the deleterious effect may be counteracting the expected slower rate of fatigue when $K^+_{(ATP)}$ channels are blocked. The objective here was

therefore to determine if a further activation of $K^+_{(ATP)}$ channels will increase the rate of fatigue.

## B. EXPERIMENTAL PROTOCOL:

### *1. Dose response curve of pinacidil on the action potential, resting potential and membrane conductance of unfatigued muscle fibers*

Two agonists were used in this study: levcromakalim and pinacidil. 100 $\mu mole \cdot L^{-1}$ levcromakalim has been demonstrated to activate $K^+_{(ATP)}$ channels in frog skeletal muscle. The presence of 100 $\mu mole \cdot L^{-1}$ levcromakalim reduces the membrane resistance and increases $K^+$ efflux by 40% (Hong & Chang, 1991; Benton & Haylett, 1992). Pinacidil activates mammalian cardiac and skeletal muscle $K^+_{(ATP)}$ channels (McPherson *et al.*, 1993; Fan *et al.*, 1990; Nakayama *et al.*, 1990), but has not been tested in frog muscle. Therefore, a dose-response curve for pinacidil was carried out by exposing sartorius muscle to concentrations of 0, 100, 250, and 500 $\mu M$ pinacidil in that order. After 30 minutes at each concentration, the resting and action potentials were measured as described in Chapter 3. A 30 minute time period was used to ensure that fibers at the surface of the sartorius muscle were in a steady state condition. After measurements at 500 $\mu M$ and while still in the presence of 500 $\mu M$ pinacidil, 100 $\mu M$ glibenclamide was added to determine if the pinacidil effect can be reversed. After these last measurements, pinacidil and glibenclamide were washed out to determine the reversibility of these effects.

In separate experiments, the membrane conductance was measured from the low frequency cable properties (Section F, Chapter 3). The effect of 100 and 500 $\mu mole \cdot L^{-1}$ pinacidil was tested on two different groups of muscles. The conductance was measured in a muscle fiber prior to the addition of pinacidil and the same muscle fiber was again penetrated after 30 and 60 minutes of exposure to pinacidil. 100 $\mu mole \cdot L^{-1}$ glibenclamide was then added (while pinacidil was still present) for a period of thirty minutes, after which a measurement of the membrane conductance was again taken in the same muscle fiber.

2. *Effect of Pinacidil and Levcromakalim on the Kinetics of Fatigue and Recovery*

After the 30 minute equilibration period in control solution, muscles were exposed to either 100 $\mu mole \cdot L^{-1}$ levcromakalim or 200 $\mu mole \cdot L^{-1}$ pinacidil for a 60 minute period prior to, during fatigue, and during recovery. A 60 minute drug incubation was used instead of 30 minutes as above because for the dose-response curve measurements were made from surface fibers only, while in these experiments the tetanic force from the whole muscle was measured. In order that all muscles were fatigued at the same time, control muscles (no agonists) were superfused another 60 minutes with control solution prior to fatigue. One hundred $\mu mole \cdot L^{-1}$ levcromakalim was used based on the study of Benton & Haylett (1992). Two hundred $\mu mole \cdot L^{-1}$ pinacidil was used because it was the highest concentration that could be used without any non-specific effects (see Discussion).

Fatigue was elicited with 100 msec tetanic contractions every second for three minutes. One hundred msec train duration was used for the fatigue protocol as it has been

found that continuous 200 msec trains damage the muscle and the fibres do not recover well (Renaud & Mainwood, 1985). This fatigue model was also chosen because there is evidence for $K^+_{(ATP)}$ channel activation (Standen *et al.*, 1992; Light *et al.*, 1993; Comtois *et al.*, 1995). Every 30 contractions the duration was increased back to 200 msec to measure tetanic force and Force-200. Following fatigue, muscles were allowed to recover while being stimulated with 200 msec long tetanic contractions every 5 minutes for 100 minutes. In these experiments, action potentials were measured between tetanic contractions as described in Chapter 3.

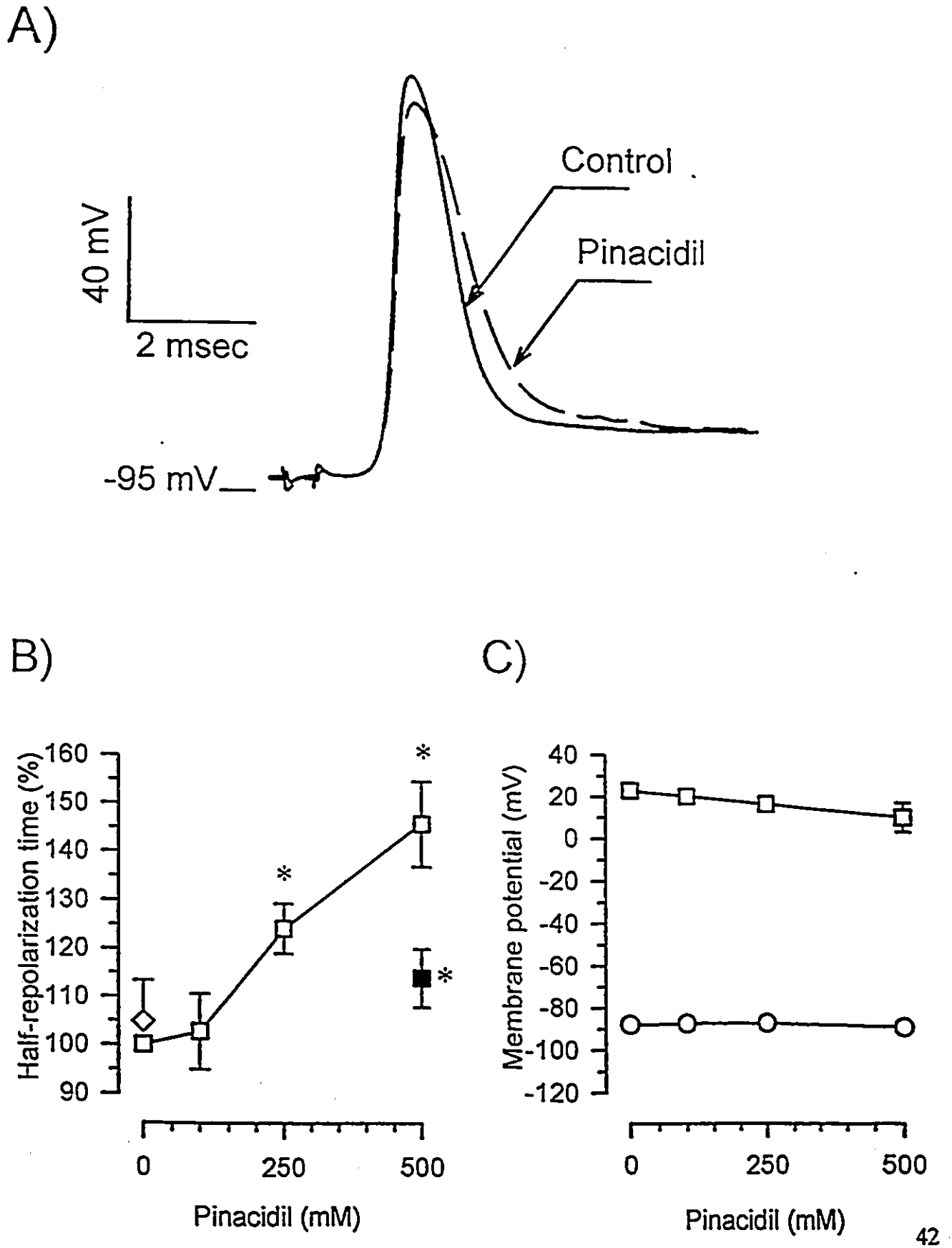
## C. RESULTS

### *1. Dose response curve for pinacidil in unfatigued muscle fibers*

At 500 μmole·L$^{-1}$, pinacidil caused a small decrease in the overshoot and increased the duration of the action potential (Figure 1A). The increased duration is due to a slower repolarization phase. The initial mean half-repolarization time was 0.70 ± 0.04 msec and did not significantly change in the presence of 100 μmole·L$^{-1}$ pinacidil, while it significantly increased by 26% and 44% in the presence of 250 and 500 μmole·L$^{-1}$ pinacidil, respectively (Figure 1B). Addition of 100 μmole·L$^{-1}$ glibenclamide in the presence of 500 μmole·L$^{-1}$ pinacidil largely reduced the duration of the half repolarization time but it was still 14% above control values (absence of pinacidil). A complete washout of pinacidil and glibenclamide restored the half-repolarization time back to the control values.

The initial mean action potential overshoot was 23.4 mV and did not change with 100 μmole·L$^{-1}$ pinacidil (Figure 1C). At 250 and 500 μmole·L$^{-1}$ pinacidil, the mean overshoot decreased by 6.5 and 12.8 mV, respectively, but these decreases were not significant. Addition of 100 μmole·L$^{-1}$ glibenclamide in the presence of 500 μmole·L$^{-1}$ pinacidil restored the mean overshoot to 20.5 ± 10.1 mV (results not shown). The mean resting membrane potential was not affected by the presence of pinacidil up to a concentration of 500 μmole·L$^{-1}$. The control resting membrane potential is -87.7 ± 2.1 mV, whereas the resting potential with 500 μM pinacidil is -88.4 ± 3.0 mV.

Figure 1: The effect of pinacidil on the action potential of frog sartorius muscle. A) Action potential in the presence and absence of 500 $\mu mole \cdot L^{-1}$ pinacidil. B) Dose response curve of pinacidil on the half-repolarization time. The half-repolarization time is the time interval between the time to peak and the time when the membrane has repolarized by 50%. Symbols: (□), pinacidil; (■) 100 $\mu mole \cdot L^{-1}$ glibenclamide and 500 $\mu mole \cdot L^{-1}$ pinacidil; (◇) washout of both drugs. C) Dose response curve of pinacidil on the resting potential (O) and overshoot (□). Vertical bars represent the SEM of 5 muscles. * mean is significantly different from the mean at 0 μM pinacidil ($p<0.05$, students paired t-test).


A)
Control
Pinacidil
40 mV
2 msec
-95 mV
B)
Half-repolarization time (%)
160
150
140
130
120
110
100
90
0
250
500
Pinacidil (mM)
C)
Membrane potential (mV)
40
20
0
-20
-40
-60
-80
-100
-120
0
250
500
Pinacidil (mM)

*2. The effect of pinacidil on membrane conductance in unfatigued muscle fibres*

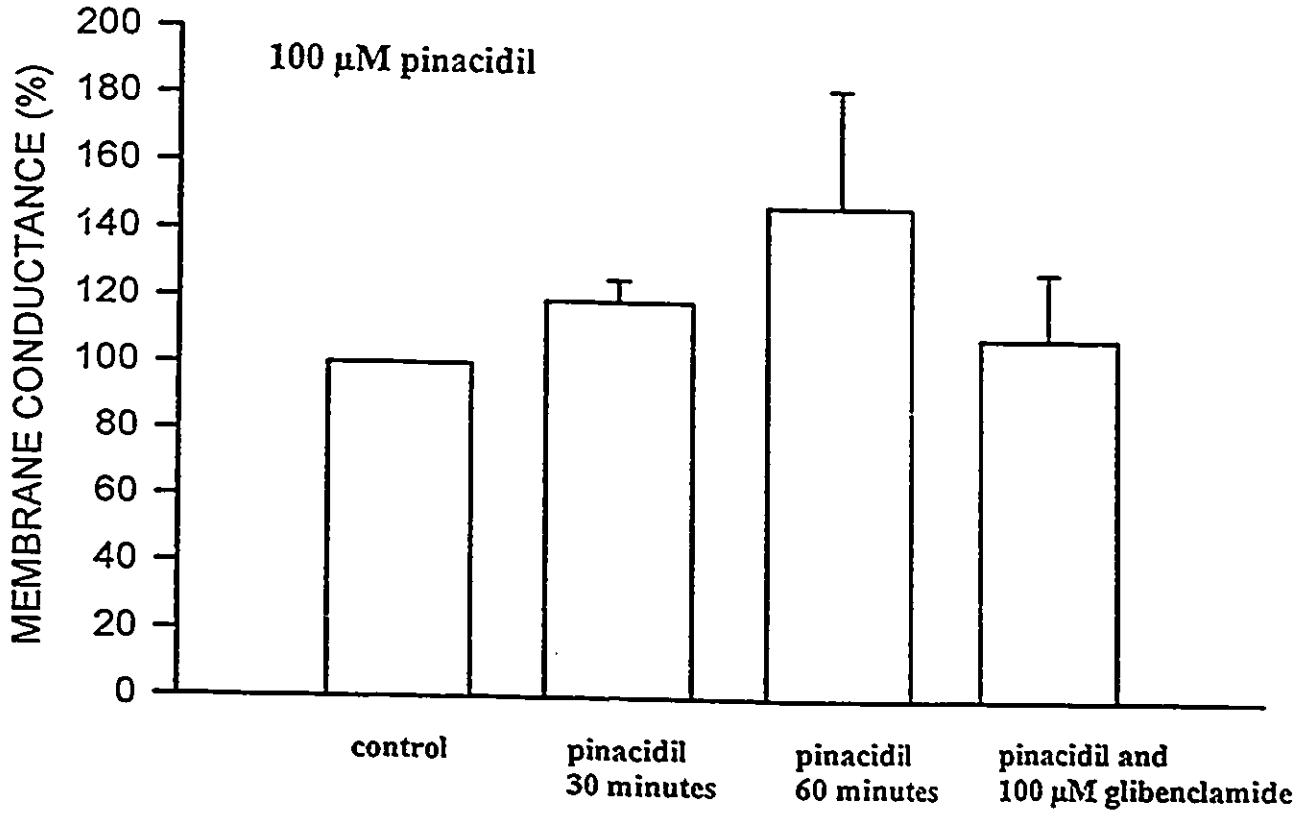
In the absence of pinacidil, the initial membrane conductance, as measured from the low frequency cable properties using a current of -95 nA was 385 ± 48 $\mu S \cdot cm^2$, while the length constant was 1.71 ± 0.07 mm. The effect of adding 100 $\mu mole \cdot L^{-1}$ pinacidil was quite variable; some fibers showed a large increase in membrane conductance, whereas others showed no change. However, no fibers showed a decrease in conductance. Overall, the mean membrane conductance did not significantly change in the presence of 100 $\mu mole \cdot L^{-1}$ pinacidil (Figure 2A).

In a separate series of experiments the effect of 500 $\mu mole \cdot L^{-1}$ pinacidil on the membrane conductance was examined. In these experiments the initial membrane conductance was 450 ± 35 $\mu S \cdot cm^2$ (n=7) and the length constant was 1.75 ± 0.11 mm. Both values are within the range of the first experiment. In comparison to the 100 $\mu mole \cdot L^{-1}$ pinacidil condition, the muscle fibers did not display as much variability. The membrane conductance did not change significantly in the presence of 500 $\mu mole \cdot L^{-1}$ pinacidil or in the presence of 500 $\mu mole \cdot L^{-1}$ pinacidil and 100 $\mu mole \cdot L^{-1}$ glibenclamide (Figure 2B).

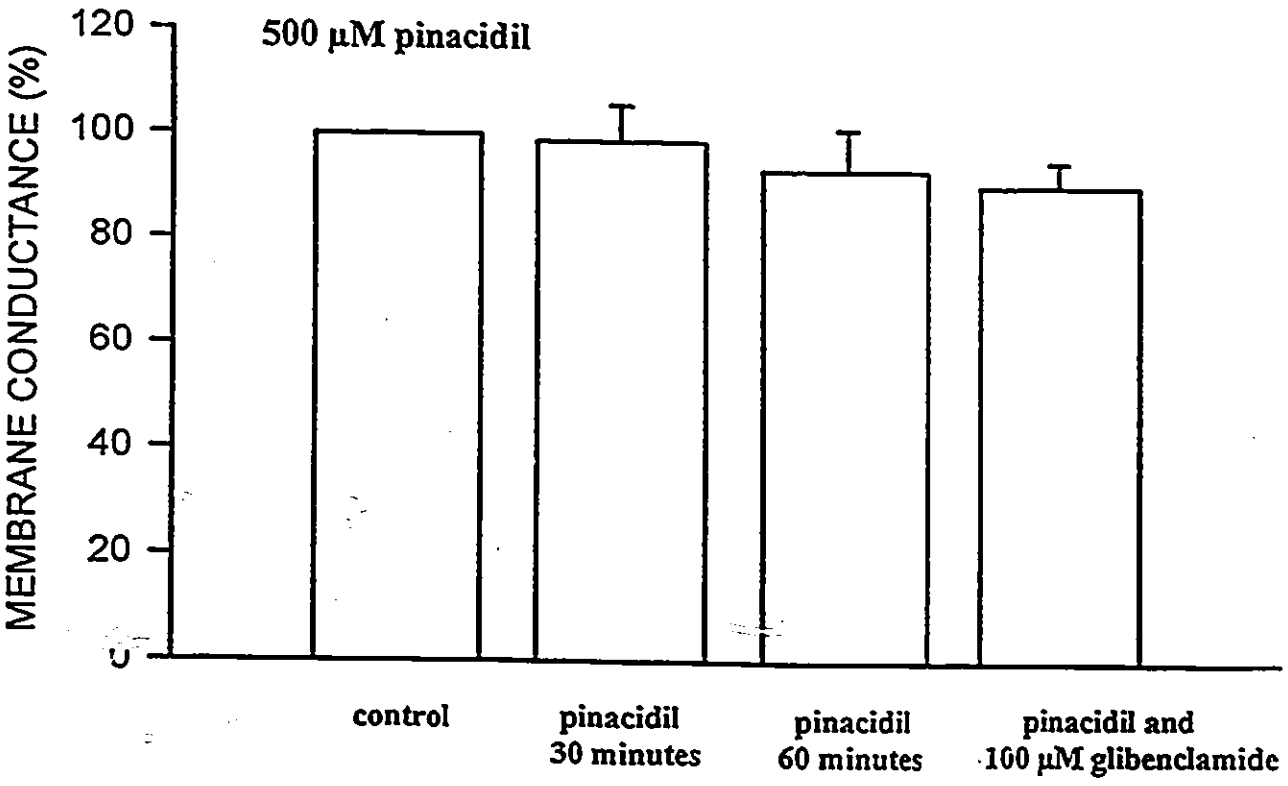
Similar results were obtained when the current injection was between -50 nA to +10 nA, however, the degree of variability was larger (results not shown).

Figure 2: The effect of A) 100 $\mu mole \cdot L^{-1}$ and B) 500 $\mu mole \cdot L^{-1}$ pinacidil on the membrane conductance of frog sartorius muscle. Membrane conductance was measured from the low frequency cable properties. Membrane conductance was measured in the same muscle fiber for all pinacidil and glibenclamide concentrations so that membrane conductance is expressed as a percent change, taking the conductance in the absence of pinacidil as 100%. Results are shown with a current injection of -95 nA. Measurements at 100 (n=5) and 500 $\mu mole \cdot L^{-1}$ (n=7) were done in separate experiments. No significant differences were observed, $p>0.05$, Students paired t-test.

A)


100 µM pinacidil
MEMBRANE CONDUCTANCE (%)
200
180
160
140
120
100
80
60
40
20
0
control
pinacidil 30 minutes
pinacidil 60 minutes
pinacidil and 100 µM glibenclamide

B)


500 µM pinacidil
MEMBRANE CONDUCTANCE (%)
120
100
80
60
40
20
0
control
pinacidil 30 minutes
pinacidil 60 minutes
pinacidil and 100 µM glibenclamide

*3. The effect of pinacidil and levcromakalim on the resting and action potential during fatigue and recovery*

During the 60 minutes preceding the fatigue, the resting potential did not change in control and pinacidil-exposed muscle, while in the presence of levcromakalim muscle fibers hyperpolarized from -80.3 mV ± 1.7 to -86.8 ± 2.0 mV (results not shown). The mean resting potential of controls significantly decreased by 9.0 mV following fatigue (Figure 3). The decrease in the resting potential was similar in the presence of pinacidil, 7.1 mV, and slightly greater in the presence of levcromakalim, 15.5 mV. The resting potential of control muscle was no longer significantly different from the pre-fatigue values after 30 minutes of recovery. Neither pinacidil nor levcromakalim significantly affected the rate of recovery of the resting membrane potential.

Prior to fatigue, levcromakalim and pinacidil had no effect on the action potential half-repolarization time. The half-repolarization time increased significantly during fatigue from 0.70 ± 0.04 sec to 1.47 ± 0.08 sec for control muscles. The increase in the mean half-repolarization time was not significantly affected by either levcromakalim or pinacidil. In the presence of pinacidil and levcromakalim the half-repolarization time increased to approximately the same extent: 0.72 ± 0.05 sec to 1.51 ± 0.16 sec while in the presence of pinacidil, and from 0.69 ± 0.04 sec to 1.70 ± 0.16 sec in the presence of levcromakalim

Figure 3: The effect of pinacidil and levcromakalim on the resting potential. Pinacidil and levcromakalim were added 60 minutes prior to fatigue and were present for the remainder of the experiment. Vertical open bar labelled F: muscles were fatigued with 100 msec long tetani every second for 180 seconds. Resting potentials were measured in a minimum of three muscle fibers and these values were averaged for each muscle. These averages were then used to obtain the mean. Thus, sample size represents the number of muscles, not individual fibers. Vertical bars represent the SEM, n=5. * mean is significantly different than before fatigue (ANOVA, Duncan Multiple Comparison Test [DMCT], $p<0.05$). Neither pinacidil nor levcromakalim significantly affected the resting potential (ANOVA, $p>0.05$).

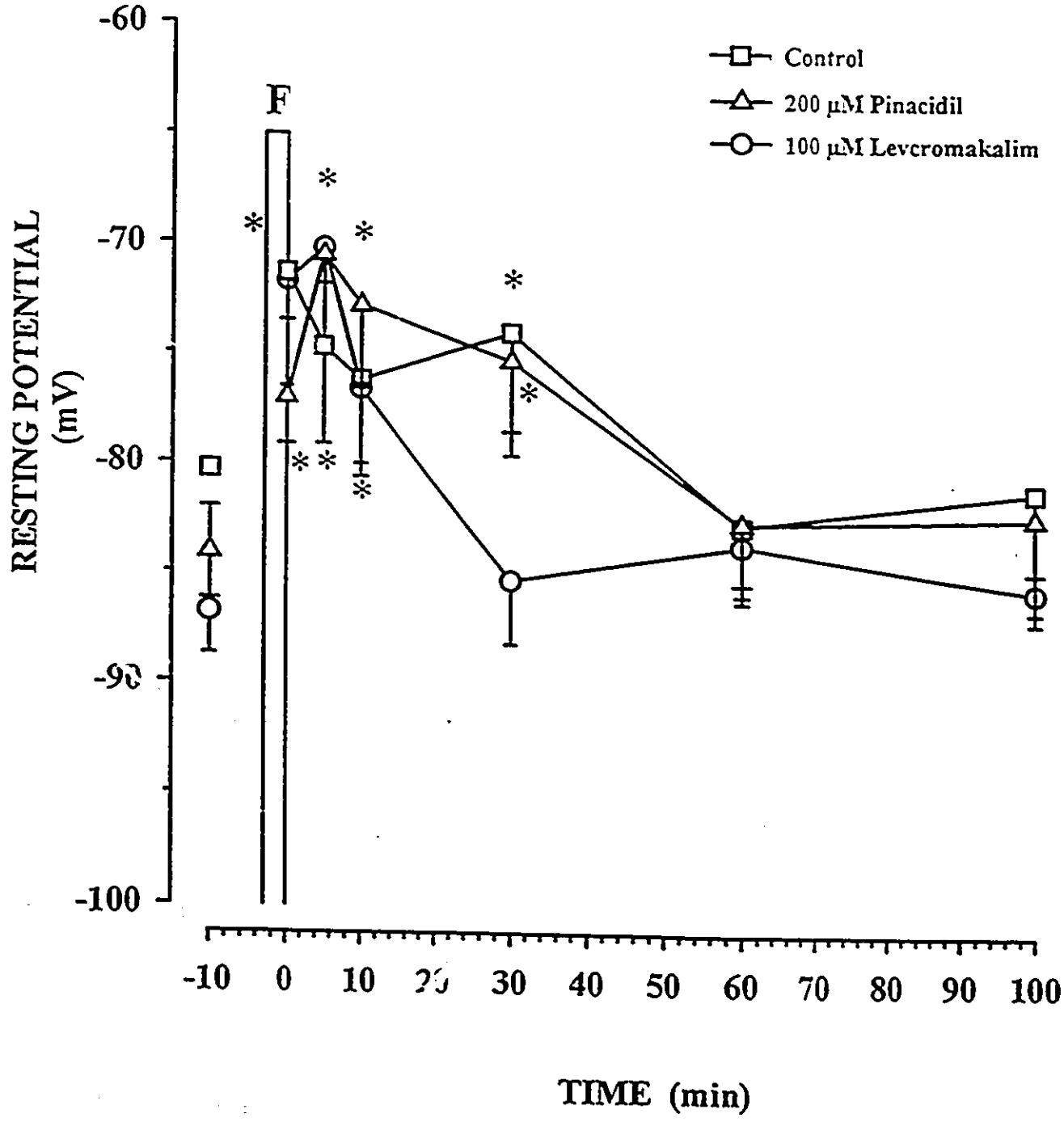
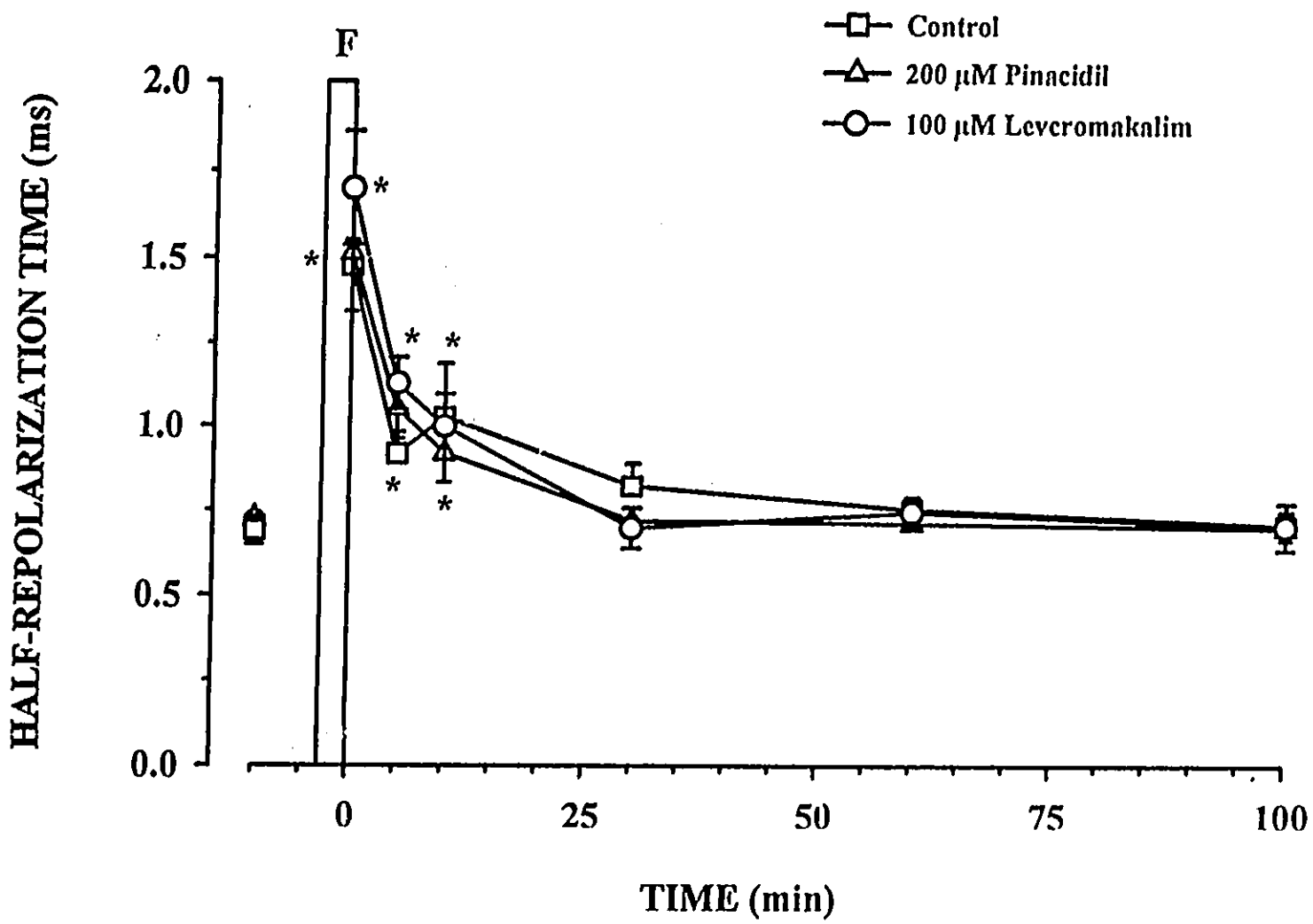

-60
-70
-80
-90
-100
RESTING POTENTIAL (mV)
F
Control
200 μM Pinacidil
100 μM Levcromakalim
-10
0
10
30
40
50
60
70
80
90
100
TIME (min)

Figure 4: The effect of pinacidil and levcromakalim on the action potential half-repolarization time. Pinacidil and levcromakalim were added 60 minutes prior to fatigue. Vertical open bar labelled F: muscles were fatigued with 100 msec long tetani every second for 180 seconds. Action potentials were measured in a minimum of three muscle fibers and these values were averaged for each muscle. These averages were then used to obtain the mean. Thus, sample size represents the number of muscles, not individual fibers. Vertical bars represent the SEM, n=5. Pinacidil and levcromakalim were not significantly different from control, (ANOVA,$p > 0.05$). * mean is significantly difference from before fatigue. Neither pinacidil nor levcromakalim significantly affected the half-repolarization time (ANOVA,$p > 0.05$).


F
Control
200 μM Pinacidil
100 μM Levcromakalim
HALF-REPOLARIZATION TIME (ms)
2.0
1.5
1.0
0.5
0.0
0
25
50
75
100
TIME (min)

(Figure 4). Following fatigue, the half-repolarization time of control muscles decreased back toward pre-fatigue levels and was no longer significantly different from the pre-fatigue values after 30 minutes. Neither levcromakalim nor pinacidil affected the recovery of the half repolarization time.

Neither compound had an effect on the overshoot of the action potential during the 60 minute drug-exposure time. The overshoot of the action potential was not significantly affected during fatigue and recovery (Table I).

*4. The effect of $K^+_{(ATP)}$ channel activation on the tetanic force during fatigue and recovery*

The mean tetanic force before fatigue was 23.7 ± 1.0 N/cm$^2$ for control. The tetanic force of muscles exposed to pinacidil or levcromakalim for 60 minutes prior to fatigue was not significantly different from controls; the tetanic force was 19.6 ± 1.9 N/cm$^2$ in the presence of pinacidil, and 22.2 ± 1.1 N/cm$^2$ in the presence of levcromakalim. Tetanic force for control muscle declined from 100% at the onset of fatigue to 7.0 % at the end of the fatigue protocol (Figure 5A). The decrease in tetanic force during fatigue was not significantly different in the presence of either pinacidil or levcromakalim (Figure 5A). In the absence of pinacidil or levcromakalim, muscles recovered 87.8 % of their pre-fatigue tetanic force after 80 minutes (Figure 5B). Both pinacidil- and levcromakalim-exposed muscles had a slightly slower rate of recovery, but this was not significant. After 100 minutes of recovery,

Table I: The effect of the $K^+_{(ATP)}$ channel activators, pinacidil and levcromakalim, on the recovery of the action potential overshoot.

| TIME (min) | CONTROL (mV) | 200 µmole·L$^{-1}$ PINACIDIL (mV) | 100 µmole·L$^{-1}$ LEVCROMAKALIM (mV) |
|---|---|---|---|
| -5 | 18.1 ± 7.2 | 24.8 ± 3.1 | 28.4 ± 2.1 |
| 0 | 20.9 ± 5.7 | 24.3 ± 3.1 | 29.0 ± 4.2 |
| 5 | 18.7 ± 6.7 | 25.4 ± 3.5 | 29.1 ± 3.4 |
| 10 | 18.5 ± 7.8 | 29.0 ± 2.3 | 29.7 ± 1.9 |
| 30 | 11.1 ± 6.7 | 21.9 ± 4.2 | 26.4 ± 5.6 |
| 60 | 16.3 ± 8.2 | 16.6 ± 2.9 | 22.6 ± 1.5 |
| 100 | 25.7 ± 9.0 | 31.1 ± 6.7 | 23.4 ± 3.8 |

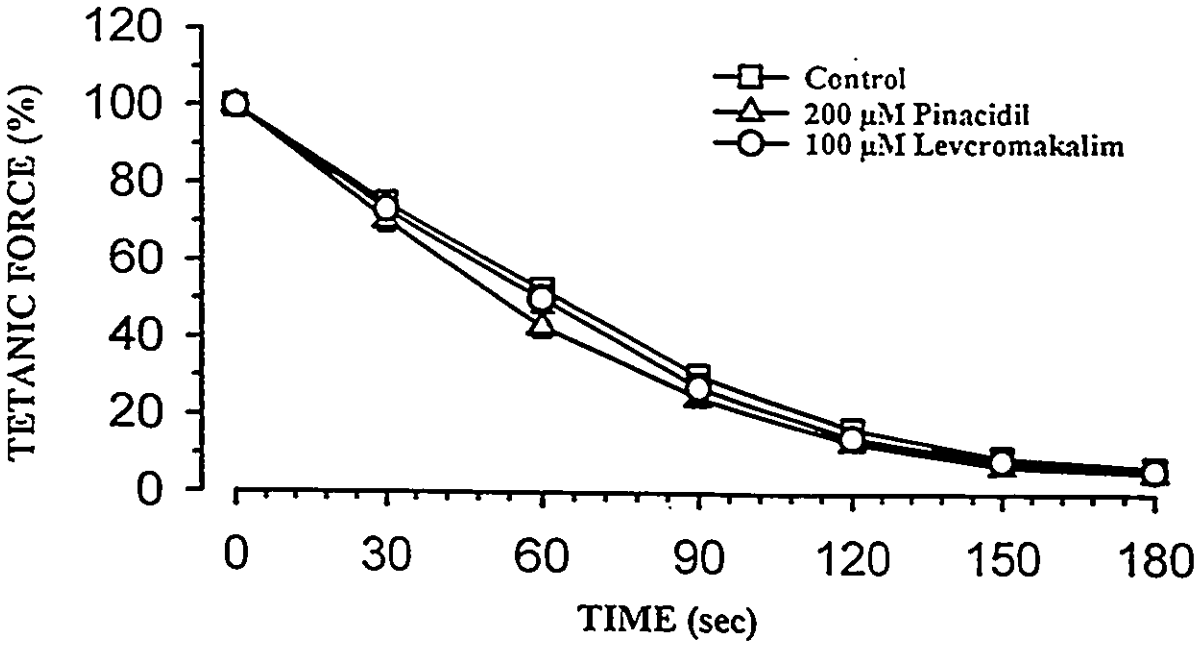
**Fatigue was induced with a 100 msec train of pulses every sec for 180 sec. Time represents the time during the recovery. Time -5 represents values measured 5 minutes before fatigue. Data are given as the mean ± S.E.M., n = 5. No significant differences were observed between the groups, ANOVA, $p > 0.05$.**

the tetanic forces in the presence of levcromakalim and pinacidil were similar to those of control muscle, being 87.1%, and 87.8% of pre-fatigue values, respectively.

In contrast to the tetanic force, the plateau phase of the tetanic contraction was affected by both pinacidil and levcromakalim. As shown in Figure 6, frog muscles were unable to maintain a constant force during the plateau phase of a tetanus after 5 minutes of recovery. After 30 minutes of recovery, the plateau phase of control muscle was back

Figure 5: The effect of pinacidil and levcromakalim on the tetanic force during A) fatigue and B) recovery. Tetanic force is the maximum force developed during the tetanus and is expressed as a percent of pre-fatigue values: Time 0 in A, time -10 in B. Every 30 seconds the train duration was increased to 200 msec to measure the tetanic force. In B, the fatigue period is shown by a vertical open bar labelled 'F'. During the recovery period muscles were stimulated once every 5 minutes. Vertical bars represent the SEM, n=5. The decrease in force during fatigue was significant (ANOVA, $p<0.05$), but neither pinacidil nor levcromakalim had a significant effect compared to control (ANOVA, $p>0.05$).

A)


120
100
80
60
40
20
0
TETANIC FORCE (%)
Control
200 µM Pinacidil
100 µM Levcromakalim
0
30
60
90
120
150
180
TIME (sec)

B)

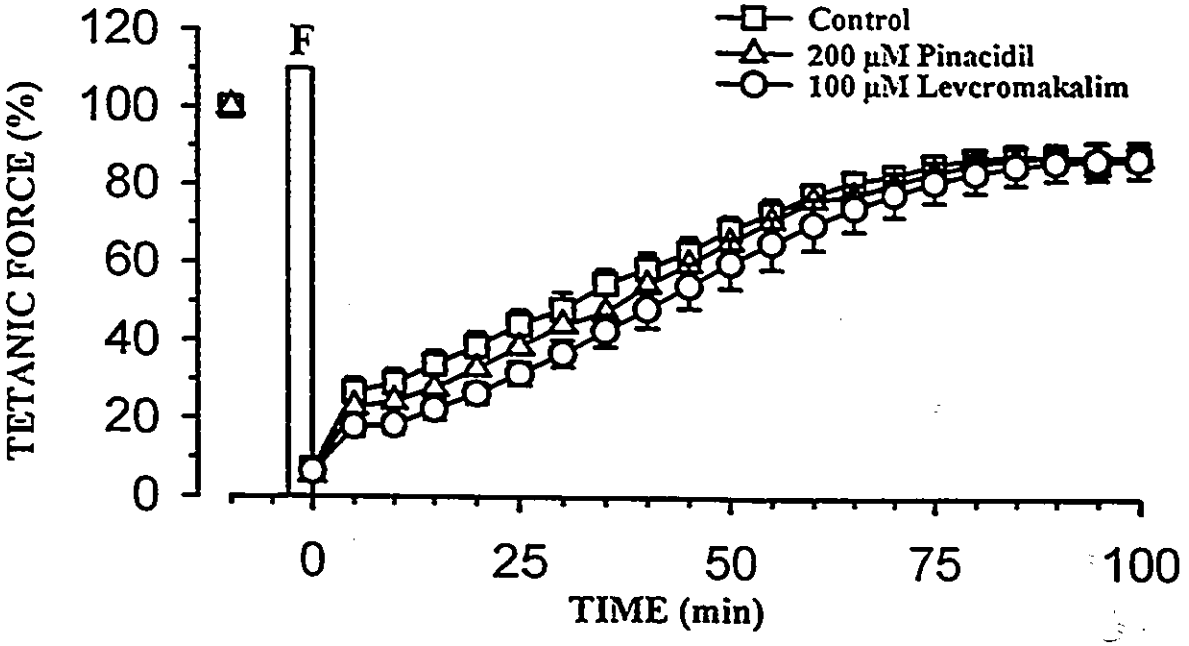
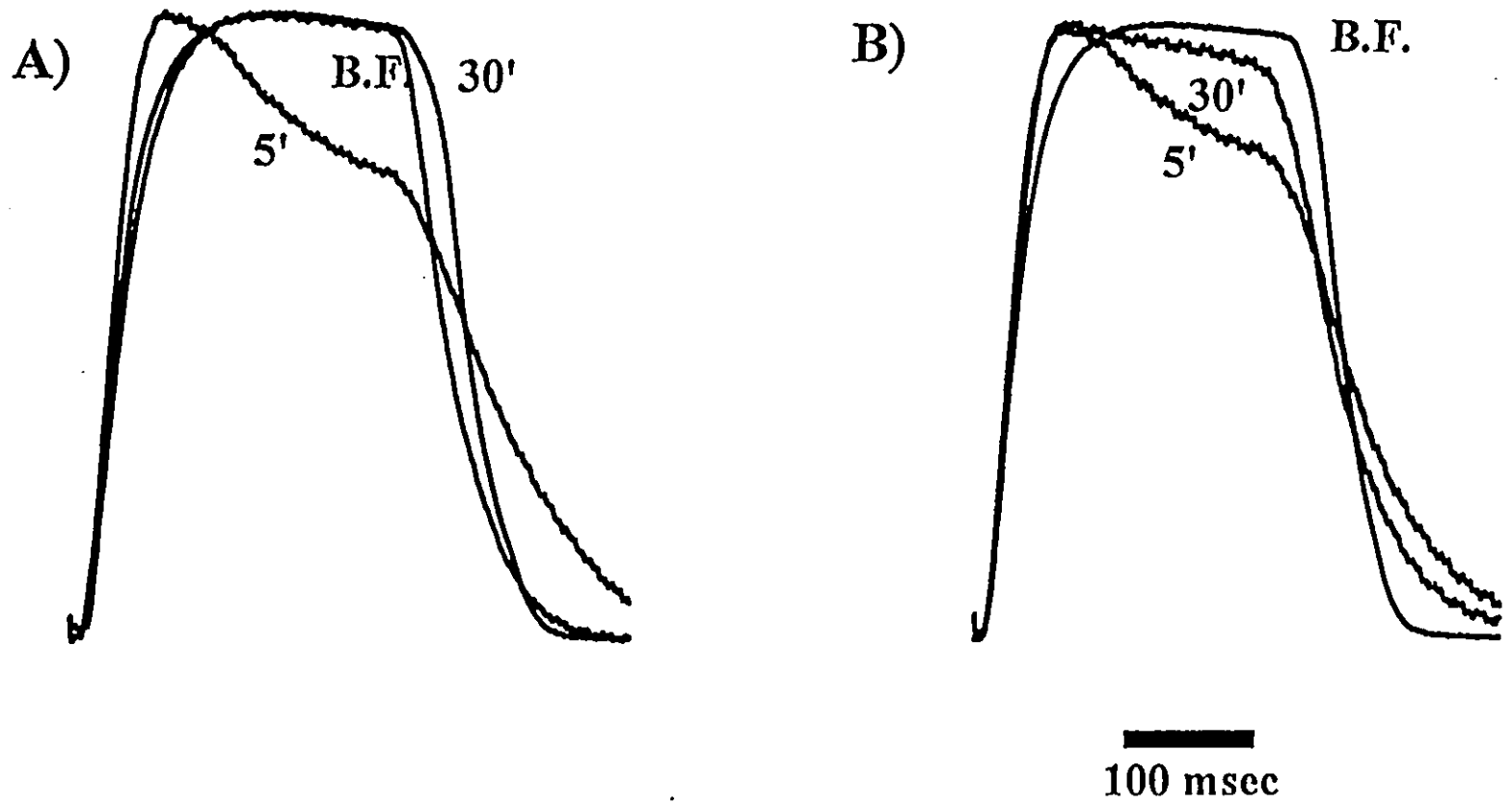

120
100
80
60
40
20
0
TETANIC FORCE (%)
F
Control
200 µM Pinacidil
100 µM Levcromakalim
0
25
50
75
100
TIME (min)

Figure 6: The effect of fatigue on the plateau phase of the tetanic contraction in A) the absence and B) the presence of 100 $\mu$mole·L$^{-1}$ levcromakalim. Each tetanic contraction is plotted as a percent of the maximum force of each contraction. B.F.: before fatigue. The times 5' and 30' represent the time in minutes during the recovery period.

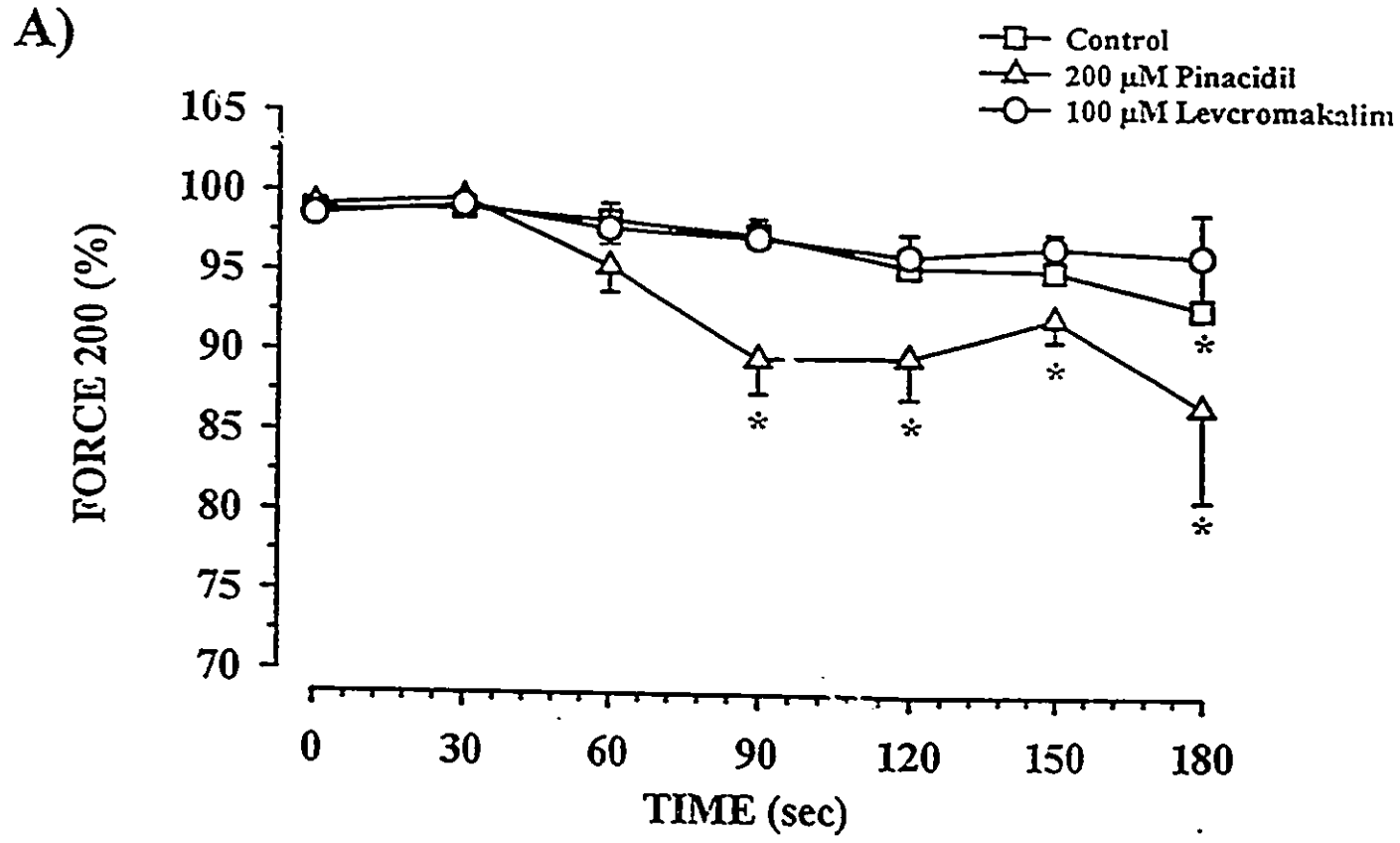

A)
B.F.
30'
5'
B)
B.F.
30'
5'
100 msec

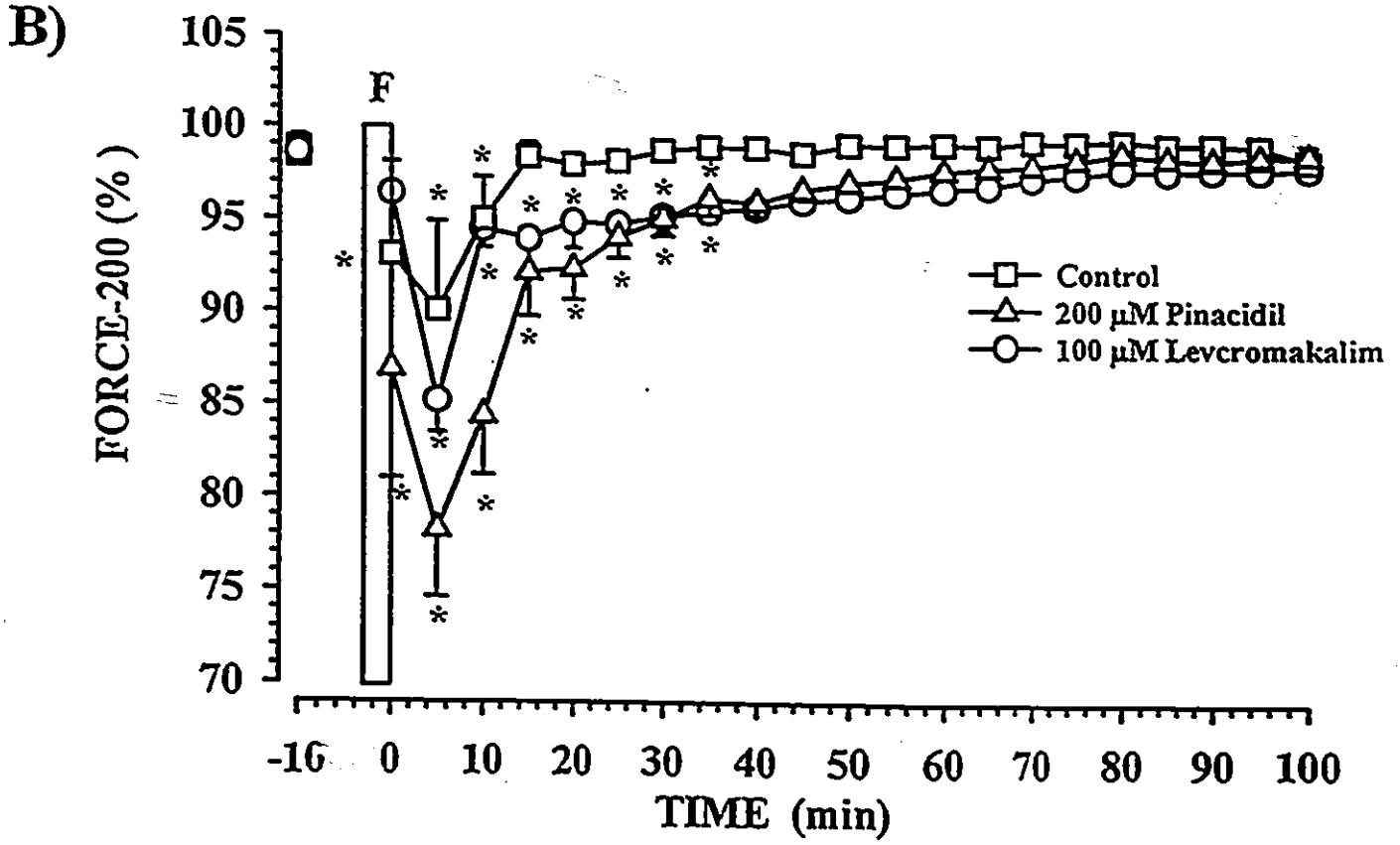
to normal, while in levcromakalim-exposed muscles there was still a noticeable decrease in force during the plateau phase.

To estimate the decrease in force during the plateau phase, the Force-200 was measured. The Force-200 is defined as the force at the end of the 200 msec stimulation and expressed as a percentage of peak tetanic force. Before fatigue, Force-200 of control muscles was greater than 98% in control muscle (Figure 7A). This means that after reaching the maximum force, force decreased by less than 2 % during the plateau phase of the tetanus. Neither pinacidil nor levcromakalim affected the Force-200 prior to fatigue (Figure 7A). Force-200 of control muscles decreased during fatigue, but became significantly different from the pre-fatigue values only at the end of the fatigue period where it reached 93.1 ± 5.0%. In the presence of pinacidil, a significant difference during fatigue was noticed sooner than control as after 90 sec of stimulation Force-200 was 89.7 ± 2.2 % (Figure 7A). Levcromakalim had no effect on the rate of Force-200 during fatigue, as the Force-200 decreased only to 96.4 ± 0.4 %. During the first five minutes of recovery Force-200 decreased significantly for all three experimental groups before it started to recover back towards pre-fatigue levels (Figure 7B). In control muscles, Force-200 returned to pre-fatigue levels within 15 minutes (Figure 7B). The recovery of Force-200 was significantly slower in the presence than in the absence of both levcromakalim and pinacidil.

Figure 7: The effect of pinacidil and levcromakalim on the Force-200 during A) fatigue and B) recovery. Force-200, an appraisal of the ability of the muscle to maintain a constant force during the plateau phase, was the force measured at the end of the 200 msec train and expressed as a percentage of maximum tetanic force on the same contraction. In B, the fatigue period is shown by a vertical open bar labelled 'F'. Vertical bars represent the SEM, n=5.

* mean is significantly different than pre-fatigue values (ANOVA, $p<0.05$).


A)
Control
200 µM Pinacidil
100 µM Levcromakalim
FORCE 200 (%)
105
100
95
90
85
80
75
70
0
30
60
90
120
150
180
TIME (sec)


B)
F
FORCE-200 (% )
105
100
95
90
85
80
75
70
Control
200 µM Pinacidil
100 µM Levcromakalim
-10
0
10
20
30
40
50
60
70
80
90
100
TIME (min)

## D. DISCUSSION

The major findings are that in the presence of 200 μmole·L-1 pinacidil or 100 μmole·L-1 levcromakalim 1) the action potential duration was not significantly reduced before and after fatigue, 2) the rate of fatigue, as measured from the decrease in tetanic force, was not significantly affected, 3) the rate of tetanic force recovery was also not affected while 4) the recovery of the Force-200 was significantly reduced.

### *1. Effect of pinacidil during fatigue and recovery on the resting and action potential*

Pinacidil appears ineffective in activating $K^+_{(ATP)}$ channels as there is no significant increase in membrane conductance observed at concentrations up to 500 μmole·$L^{-1}$. Thus, contrary to the activation of $K^+_{(ATP)}$ channels by 30 - 100 μmole·$L^{-1}$ pinacidil in mammalian muscle (Weik & Neumke, 1990), and 100 μmole·$L^{-1}$ levcromakalim in frog muscle (Benton & Haylett, 1992), pinacidil appears ineffective in activating $K^+_{(ATP)}$ channels in intact frog sartorius muscle fibers.

The lack of an effect of pinacidil on membrane conductance is consistent with the lack of a pinacidil effect on the action potential at a concentration of 200 μmole·$L^{-1}$ or less. However, pinacidil significantly prolonged the action potential repolarization phase at 250 and 500 μmole·$L^{-1}$ in unfatigued muscle. This prolongation was observed while pinacidil had no significant effect on the resting potential and overshoot of the action potential.

Therefore, the prolongation of the repolarization phase must involve an effect of pinacidil on an ionic channel which contributes to the repolarization phase. Since the prolongation of the action potential is blocked by glibenclamide, it suggests that the pinacidil effect must involve $K^+_{(ATP)}$ channels. However, the prolongation is not consistent with an increase in $K^+$ conductance, so the pinacidil effect can not involve an increased $K^+_{(ATP)}$ channel activity. Although at high concentrations pinacidil has been shown to block $K^+_{(ATP)}$ channels (Hehl & Neumcke, 1994), it is unlikely that such an effect could explain the prolongation of the action potential because $K^+_{(ATP)}$ channels are inactive in unfatigued muscle (Standen *et al.*, 1992; Comtois *et al.*, 1995). It would thus appear that the prolongation of the action potential repolarization phase is either due to a decreased activity of $K^+$ delayed rectifiers, the $K^+$ channel normally responsible for the repolarization phase, or a decrease in the inactivation kinetics of $Na^+$ channels. Thus, like SR44866, another $K^+_{(ATP)}$ channel opener, it would appear that pinacidil at high concentrations (similar to 250 $\mu mole \cdot L^{-1}$) lacks specificity, at least in frog sartorius muscle.

2. *Effect of levcromakalim during fatigue and recovery on the resting and action potential*

One hundred $\mu mole \cdot L^{-1}$ levcromakalim is known to activate $K^+_{(ATP)}$ channels in frog sartorius muscle, but also fails to shorten the action potential in both unfatigued and fatigued frog sartorius muscle fibers. These results are therefore not consistent with the study of Sauviat *et al.* (1991). In that study, they demonstrated in frog skeletal muscle a shortening of the action potential in the presence of SR44866, a $K^+_{(ATP)}$ channel opener. However, they also demonstrated that SR44866 reduces the sodium current. It is therefore

possible that the shorter action potential in the presence of SR44866 is due to a combined decrease in $Na^+$ and an increase in $K^+$ current, and that an activation of $K^+_{(ATP)}$ channels alone with levcromakalim has little effect on the action potential repolarization phase in frog sartorius muscle.

The lack of an effect of levcromakalim in frog sartorius muscle is also in sharp contrast with the situation observed in cardiac muscle. In cardiac tissue, $K^+_{(ATP)}$ channel openers are highly effective in decreasing the action potential duration. This difference between cardiac and skeletal tissues may be related to the different kinetics of the action potential. In cardiac muscle, the activation of the $K^+$ delayed rectifier occurs at least 200 msec after the $Na^+$-induced depolarization phase (Hille, 1992). When the voltage insensitive $K^+_{(ATP)}$ channels are active they provide an early repolarization before the delayed rectifiers are fully activated. Thus, in cardiac muscle, the $K^+_{(ATP)}$ channel becomes the dominant channel contributing to the outward current that is required to repolarize the cell. The situation is different in skeletal muscle as the delayed rectifiers are activated within 0.5 - 1.0 msec of the depolarization phase. When $K^+_{(ATP)}$ channels are active in skeletal muscle they provide an additional outward current to that of the delayed rectifiers. It would appear that the activation of $K^+_{(ATP)}$ channels by levcromakalim does not cause a large enough increase in the outward current to significantly shorten the action potential duration in skeletal muscle. It is possible that to shorten the action potential duration in skeletal muscle a much higher degree of $K^+_{(ATP)}$ channel activation is necessary compared to skeletal muscle.

*3. Effect of $K^+_{(ATP)}$ channels during fatigue and recovery on the tetanic force*

According to the hypothesis that $K^+_{(ATP)}$ channels contribute to the decrease in force during fatigue (Standen, 1992), a further activation of $K^+_{(ATP)}$ channels was expected to increase the rate of fatigue; that is, the decrease in tetanic force was expected to be faster in the presence than in the absence of levcromakalim and pinacidil. However, pinacidil at a concentration of 200 $\mu mole \cdot L^{-1}$, and levcromakalim at a concentration of 100 $\mu mole \cdot L^{-1}$, did not significantly affect the tetanic force before, during, or following fatigue. Regarding pinacidil this was not surprising since it fails to activate $K^+_{(ATP)}$ channels. It is also not surprising for levcromakalim if we consider that it did not affect the action potential which is one mechanism by which $K^+_{(ATP)}$ channels are expected to cause a decrease in force development (Chapter 2, section D). The lack of an effect of levcromakalim on the rate of fatigue is, however, surprising because levcromakalim is effective in increasing the $K^+$ efflux rate in frog skeletal muscle, and an accumulation of $K^+$ in the extracelluiar space is a factor expected to contribute to the decrease in force (Fitts, 1994). This suggests that either the $K^+$ efflux through the delayed rectifiers is already quite high and that the contribution of the ATP-sensitive channel is relatively small or that $K^+$ is not an important factor contributing to the decrease in force in this fatigue model as it has recently been argued by Renaud and Comtois (1994) and Bouclin *et al.* (1995). Therefore, these results are not consistent with the hypothesis that $K^+_{(ATP)}$ channels contribute to a decrease in force during fatigue.

Although the tetanic force was not significantly affected, both pinacidil and

levcromakalim significantly affected the plateau phase during the recovery phase. Considering i) that pinacidil is ineffective in activating $K^+_{(ATP)}$ channels; ii) that neither pinacidil nor levcromakalim have an effect on the tetanic force; and iii) that both pinacidil and levcromakalim have similar effects on Force-200, it is unlikely that this latter effect is due to an activation of $K^+_{(ATP)}$ channels. A possible mechanism for the lack of an effect of force may be an effect on $Ca^{++}$ levels, as there is evidence that some $K^+_{(ATP)}$ channel openers affect $Ca^{++}$ channels (Fischmeister & Hartzell, 1985). It may be that in fatigued muscle fibers, pinacidil and levcromakalim affect the calcium release mechanism to a greater extent by affecting either the calcium channel in the t-tubule or in the sarcoplasmic reticulum causing a depression of the calcium levels during the plateau phase.

In summary, both pinacidil (200 $\mu mole \cdot L^{-1}$) and levcromakalim (100 $\mu mole \cdot L^{-1}$), two $K^+_{(ATP)}$ channel openers, fail to affect the recovery of the action potential and to increase the rate of fatigue as measured by the decrease in tetanic force. In the case of pinacidil, it appears to be due to a lack of $K^+_{(ATP)}$ channel activation. Thus, contrary to the situation with mammalian muscles, pinacidil appears ineffective in activating $K^+_{(ATP)}$ channels in unfatigued and fatigued frog muscle fibers. Levcromakalim, on the other hand, has been shown to be effective in frog muscle but still failed to affect the action potential duration and the rate of fatigue. Such results are not consistent with the hypothesis that $K^+_{(ATP)}$ channels contribute to the decrease in force as proposed by Standen (1992). Thus either the $K^+_{(ATP)}$ channel do not affect force in skeletal muscle during fatigue, or in this fatigue model there is still not a sufficient number of active channels.

# CHAPTER 5: What level of $K^+_{(ATP)}$ channel activity is necessary to affect force development during metabolic inhibition?

## A. INTRODUCTION:

Although there is evidence for an activation of $K^+_{(ATP)}$ channels during fatigue under normoxic conditions, a significant effect on the rate of fatigue is not observed in the presence of the channel blockers glibenclamide and tolbutamide (Light *et al.*, 1994; Comtois *et al.*, 1995), or in the presence of the channel openers levcromakalim (Chapter 4) and cromakalim (Weselcouch *et al.*, 1993). However, since cromakalim was shown to increase the rate of fatigue in rat EDL under anoxic conditions, it suggests that $K^+_{(ATP)}$ channels can contribute to a decrease in force. It also suggests that a larger metabolic stress than a fatigue under normoxic conditions is required to activate a critical number of channels to cause a decrease in force. To better understand the relationship among $K^+_{(ATP)}$ channels and force development, it becomes necessary to understand the relationship among $K^+_{(ATP)}$ channel activity, the bioenergetic state and force in order to determine if there is a bioenergetic state within physiological limits in which these channels can contribute to a decrease in force.

To obtain different bioenergetic states, metabolic inhibition was induced by exposure to 2 mM cyanide and 1 mM iodoacetate, known to activate a large number of $K^+_{(ATP)}$ channels (Castle & Haylett, 1987; Light *et al.*, 1994).

## B. EXPERIMENTAL PROTOCOL:

(NOTE: The experiments described in this chapter were the first series of experiments that were carried out at the beginning of this research project. At the time, levcromakalim was not available. Pinacidil was used based on the knowledge that it becomes more effective in activating $K^+_{(ATP)}$ channels when the ATP concentration decreases (Fan *et al.*, 1990; Nakayama *et al.*, 1990), and that a greater decrease in ATP was expected during a metabolic inhibition than during fatigue. When levcromakalim became available (as a gift from SmithKline Beecham) there was just enough to carry out one series of experiments. The effects of levcromakalim were tested under a fatigue condition instead of metabolic inhibition since the former represents a physiological condition.)

### *1. Experiment I: Effect of pinacidil and glibenclamide on the tetanic force and action potential during metabolic poisoning.*

For these experiments, 100 $\mu mole \cdot L^{-1}$ glibenclamide or 100 $\mu mole \cdot L^{-1}$ pinacidil was used. Pinacidil and glibenclamide were added 60 minutes prior to the metabolic inhibition. During that time, control muscles were superfused with normal control solution. In frog sartorius the $K_i$ for glibenclamide is 3 $\mu mole \cdot L^{-1}$, but 50 $\mu mole \cdot L^{-1}$ glibenclamide blocks only 95% of the channels (Standen *et al.*, 1992). Therefore, 100 $\mu mole \cdot L^{-1}$ glibenclamide was used in this study in an attempt to block all the channels. One hundred $\mu mole \cdot L^{-1}$ pinacidil was used to activate the channels while avoiding non-specific effects (see Chapter 4). The 60 minute drug-exposure period was to ensure that a steady state condition was

achieved and to make sure that all compartments of the muscle had been exposed to either compound. Following the 60 minute exposure in the absence or presence of a $K^+_{(ATP)}$ channel modulator, metabolic poisoning was induced in the absence and presence of either pinacidil and or glibenclamide with 2 mmole·$L^{-1}$ sodium cyanide, to block oxidative phosphorylation, and 1 mmole·$L^{-1}$ iodoacetic acid, to block glycolytic metabolism.

During the metabolic poisoning, muscles were stimulated to generate a 200 msec long tetanus once every 10 minutes. Action potentials were measured in three fibers, every 10 minutes following the contraction (as described in Chapter 3).

2. *Experiment II: Effect of blocking $K^+_{(ATP)}$ channels with glibenclamide during metabolic inhibition.*

The protocol was the same as Experiment I except that glibenclamide was added not before but 8 or 18 minutes after the addition of cyanide and iodoacetate. These experiments were carried out to determine if the changes when glibenclamide was added 60 minutes prior to metabolic inhibition could also be observed when muscles were exposed to glibenclamide during the metabolic inhibition, and that the effects were not due to a metabolic change that occurred when $K^+_{(ATP)}$ channels are blocked at the onset of metabolic inhibition.

3. *Experiment III: Effect of pinacidil and glibenclamide on the membrane conductance during metabolic inhibition.*

The protocol was the same as for Experiment I and the membrane conductance was measured (as described in Chapter 3) every 10 minutes during the metabolic inhibition prior to a tetanic contraction. Measurements of membrane conductance were first done in a few fibers prior to metabolic inhibition. Then at different times during the metabolic inhibition, these fibers were used for another measurement in order to calculate the percent change in membrane conductance. A muscle fiber was never used more than once during the metabolic inhibition. Measurements were completed within 5 minutes. This protocol was chosen because of the large variability of the specific membrane conductance due to underestimation of the t-tubular surface area (Hodgkin & Nakajima, 1972). Intermittent measurement of membrane conductance was also chosen over a continuous recording since the latter technique gave rise to a very large increase in membrane conductance (Appendix III) possibly due to some damage to the cell membrane over time.

4. *Experiment IV: Effect of glibenclamide on intracellular metabolites during metabolic inhibition.*

The protocol was the same as for Experiment I, except that intracellular metabolites (ATP, ADP, AMP, PCr) were measured by HPLC. Muscles were frozen in liquid nitrogen prior to the 200 msec tetanic contraction.

Control and glibenclamide-exposed muscle (60 minute pre-metabolic poisoning) were used for the metabolite determinations. Pinacidil exposed muscles were not used because no significant effects were seen on the action potential, the tetanic force, nor the membrane conductance of these muscles.

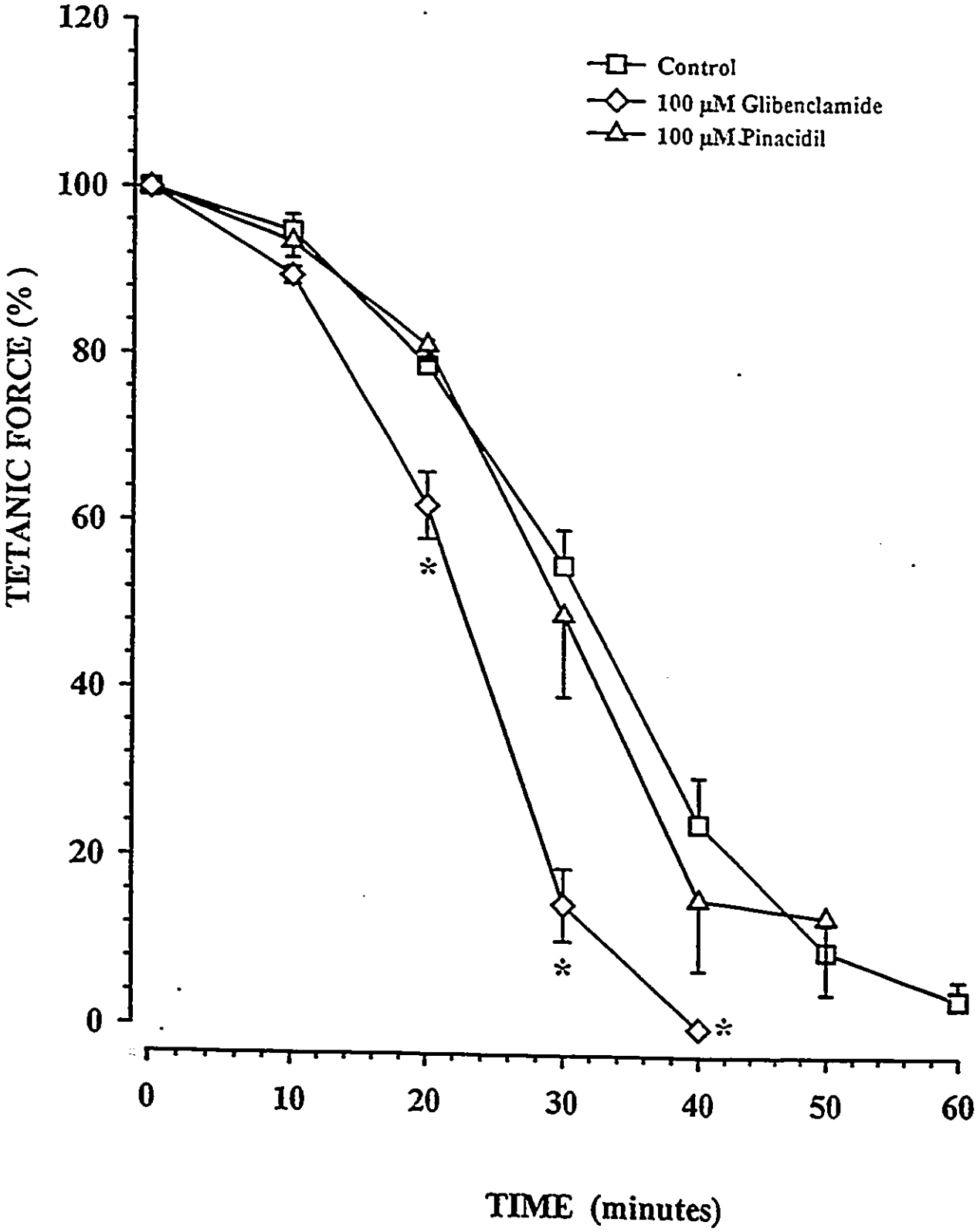
## C. RESULTS

### *1. The effect of modulating $K^+_{(ATP)}$ channel activity on the tetanic force during metabolic inhibition*

During the 60 minutes preceding the metabolic inhibition, the tetanic force of control muscle declined by less than 4%. Neither glibenclamide nor pinacidil caused a greater change than 4% in the decrease in tetanic force. The mean tetanic force just before metabolic inhibition was 19.6 ± 0.8, 21.8 ± 2.8, and 17.3 ± 1.9 N/cm$^2$ for control, pinacidil-exposed, and glibenclamide-exposed muscles, respectively.

The peak tetanic force of control muscle decreased by 20% during the first twenty minutes (Figure 8). Thereafter, the tetanic force decreased at a much faster rate as it decreased another 58% during the next 20 minute of metabolic inhibition. After 60 minutes of cyanide and iodoacetate exposure, the tetanic force was 3.6% of the tetanic force prior to metabolic inhibition. Pinacidil had no significant effect when compared to control muscles. However, when muscle fibres were exposed to 100 μmole·L$^{-1}$ glibenclamide 60 minutes prior to metabolic inhibition, the decrease in tetanic force became significantly faster than control muscles following 10 minutes of metabolic poisoning (Figure 8). The greatest difference between control and glibenclamide-exposed muscle was observed after 30 minutes when the tetanic force was 17% in the presence of glibenclamide compared to 57% for control muscle. Furthermore, tetanic contractions could not be elicited past 40 minutes in the presence of glibenclamide compared to more than 60 minutes in control muscles.

Figure 8: The effect of pinacidil and glibenclamide on the tetanic force during metabolic inhibition. Tetanic force is defined as the maximum amount of force during a tetanus and is expressed as a percentage of force at time 0. Metabolic inhibition was induced by adding 1 mM IOA and 2 mM CN at time 0. Pinacidil and glibenclamide were added 60 minutes prior to the onset of metabolic inhibition. A 200 msec long tetanus was elicited by field stimulation every 10 minutes. Vertical bars represent the SEM, n=5. * mean tetanic force is significantly different from control values at that time point, ANOVA, $p<0.05$.


120
100
80
60
40
20
0
TETANIC FORCE (%)
Control
100 µM Glibenclamide
100 µM Pinacidil
*
*
*
0
10
20
30
40
50
60
TIME (minutes)

When glibenclamide was added after 8 minutes into metabolic inhibition, the mean tetanic force increased during the next 12 minutes. So after 20 minute of metabolic inhibition the tetanic force was still not significantly different from the pre-inhibition values, but it was significantly greater when compared to control muscles, as well as muscles exposed to glibenclamide 60 minutes prior to metabolic inhibition (Figure 9). This slower decrease in tetanic force when glibenclamide was added after 8 minutes of metabolic inhibition, however, lasted only for those 12 minutes: from the 20th to 30th minute of metabolic inhibition the tetanic force suddenly decreased by 44% compared to 22% for control muscle and 49% for muscle exposed to glibenclamide 60 minute prior to metabolic inhibition. The decrease in tetanic force in the presence of glibenclamide added after 8 minutes of metabolic inhibition had zero peak tetanic force after 50 minutes of metabolic inhibition, compared to more than 60 minutes for control muscles. When glibenclamide was added after 18 minutes of metabolic inhibition, the decrease in tetanic force was not significantly different from control muscle, except for the fact that after 60 minutes of metabolic inhibition, none of these muscles generated force while the mean value for control muscles was 3.6%.

During fatigue, many muscles fail to maintain a constant force during the plateau phase of the tetanus (Renaud, 1989, Chapter 4). Force-200 is an appraisal of the muscle's ability to maintain a constant force during the plateau phase. The Force-200 of control muscles did not significantly change during the metabolic inhibition (Figure 10). The presence of either 100 $\mu mole \cdot L^{-1}$ pinacidil or glibenclamide added 60 minutes prior to

Figure 9: The effect of adding glibenclamide during metabolic inhibition on the tetanic force. Metabolic inhibition was induced by adding 1 mM IOA and 2 mM CN at time 0. In these experiments, glibenclamide was added either 60 min prior to metabolic inhibition, or 8 min or 18 min after metabolic inhibition was started. Note that the data for control and glibenclamide (60 minutes prior to metabolic inhibition) are the same as in Figure 8. Vertical bars represent the SEM, n=4. * mean is significantly different than control values at that time, ANOVA, $p<0.05$. ** mean is significantly different from the mean when glibenclamide was added 60 minutes prior to metabolic inhibition, ANOVA, $p<0.05$.

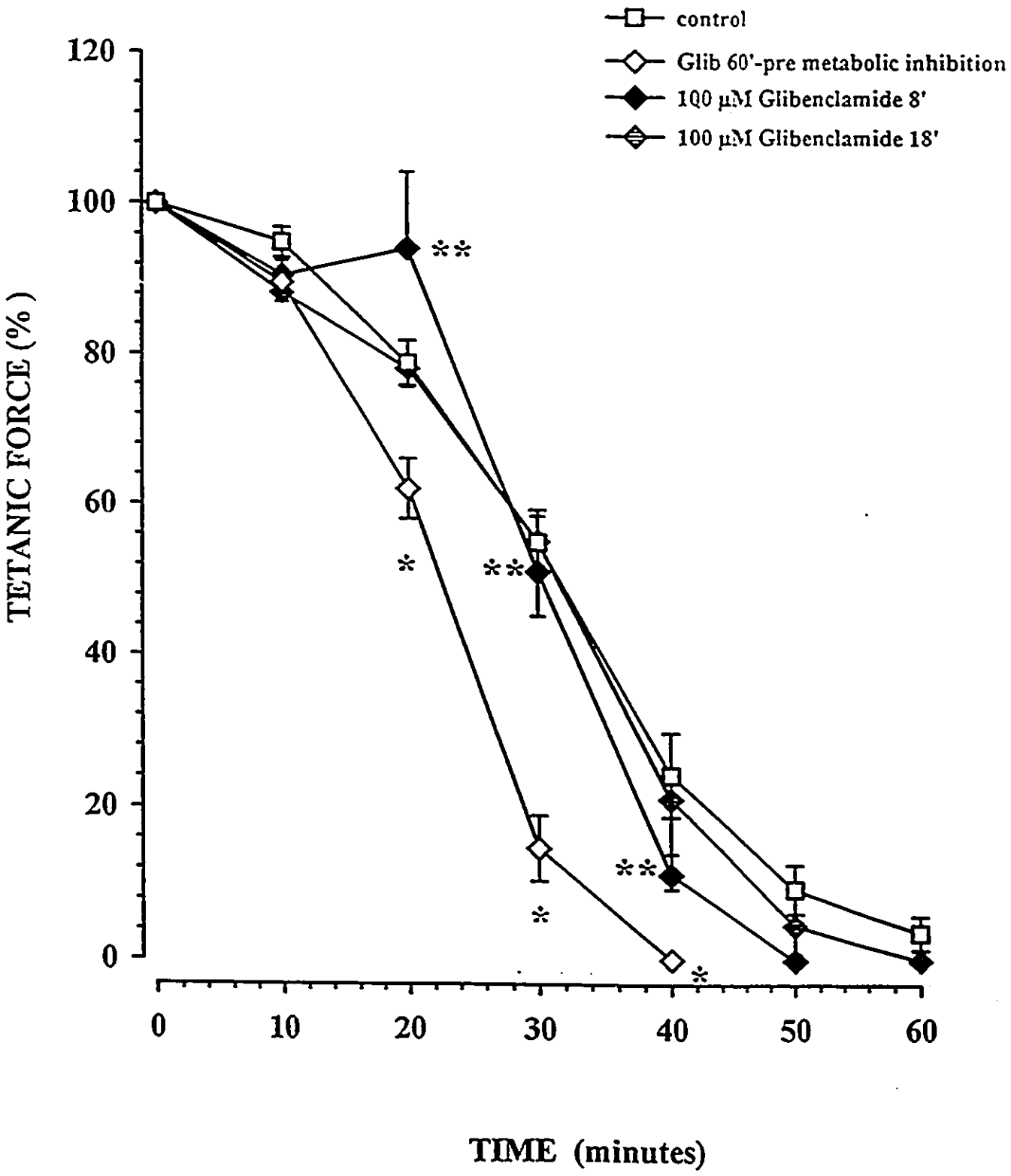
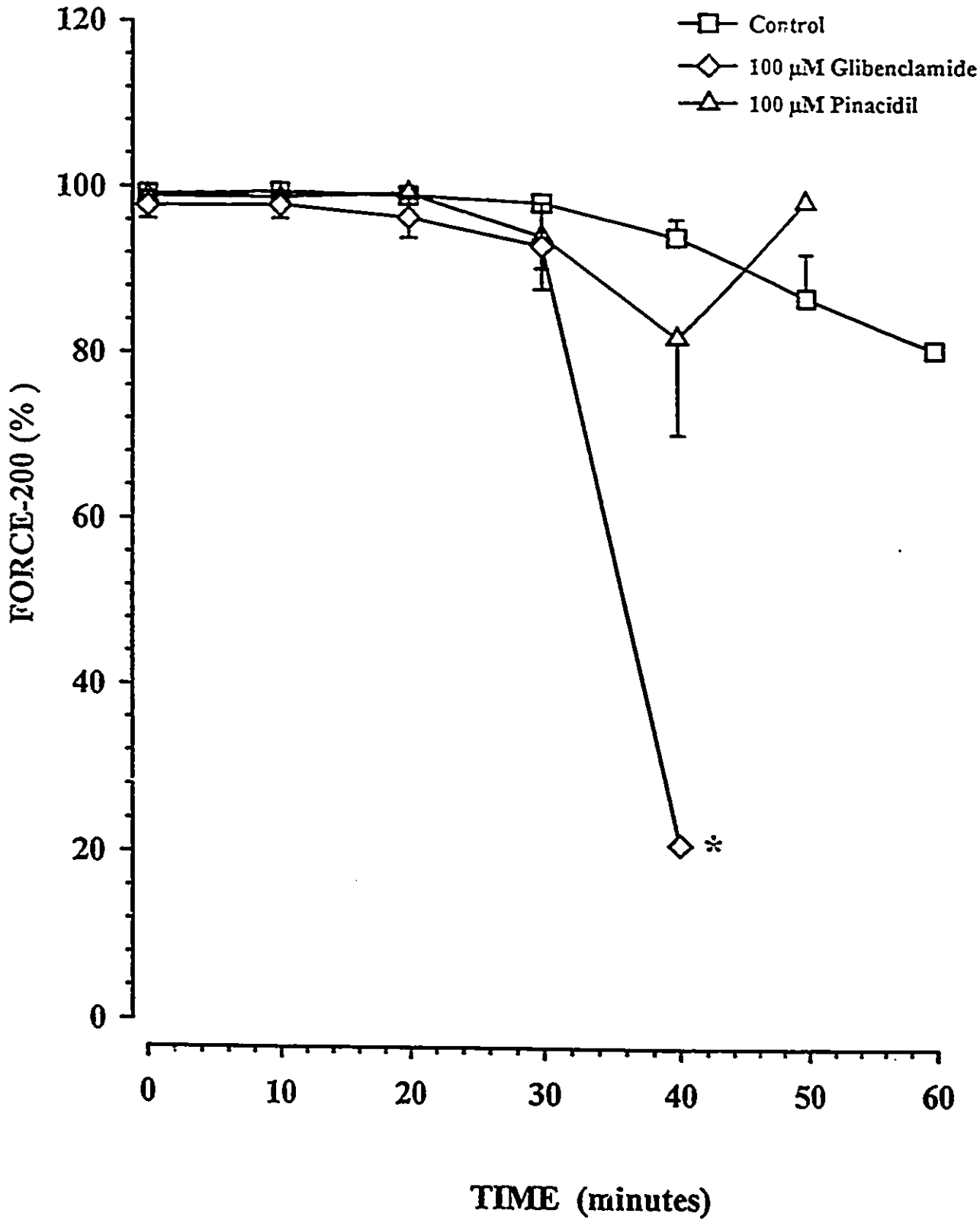

control
Glib 60'-pre metabolic inhibition
100 µM Glibenclamide 8'
100 µM Glibenclamide 18'
TETANIC FORCE (%)
120
100
80
60
40
20
0
0
10
20
30
40
50
60
TIME (minutes)

Figure 10: The effect of pinacidil and glibenclamide on the Force-200 during metabolic inhibition. Force-200, an appraisal of the ability of the muscle to maintain a constant force during the plateau phase, was the force measured at the end of the 200 msec train and expressed as a percentage of maximum tetanic force on the same contraction. Vertical bars represent the SEM, n=5. * mean is significantly different than control values at that time, ANOVA, $p<0.05$.


Control
100 µM Glibenclamide
100 µM Pinacidil
120
100
80
60
40
20
0
FORCE-200 (%)
0
10
20
30
40
50
60
*
TIME (minutes)

metabolic inhibition did not affect the Force-200 through 30 minutes. Glibenclamide-treated muscle had an effect only after 40 minutes of metabolic poisoning. However, this was measured in only one muscle since most were not capable of generating a tetanus; and for the one that did, the peak tetanic force was less than 1 $N/cm^2$.

The resting tension of control muscles started to increase after 24 minutes of metabolic poisoning and reached a plateau of 2.87 $N/cm^2$ after 45 minutes (Figure 11). The increase in resting tension in the presence of pinacidil had a similar time course and reached a maximum of 3.34 $N/cm^2$, a value not significantly different from control muscles. There was, however, a significantly earlier onset of resting tension, in the presence of glibenclamide. Resting tension started to increase after 10 minutes of metabolic inhibition and became significantly greater than control muscles after 12 minutes. The time at which resting tension started to increase significantly in glibenclamide exposed muscles also corresponded to the time when the largest decrease in tetanic force was observed. The resting tension of glibenclamide exposed muscle reached a steady state after 38 minutes which was much earlier than in control muscles. Finally, the resting tension of glibenclamide exposed muscle was 6.21 $N/cm^2$ which was 1.9-fold greater than in control muscle.

Although the resting tension increased within 12 minutes when glibenclamide was added 60 minutes prior to metabolic inhibition, adding glibenclamide 8 minutes after the start of metabolic inhibition resulted in an increase in resting tension 22 minutes after the glibenclamide addition. Consequently the increase in resting tension was after the increase

Figure 11: The effect of pinacidil and glibenclamide on the resting tension during metabolic inhibition. Resting tension is the tension measured in the absence of stimulation. Resting tension was measured every two minutes directly from a chart recorder. Vertical bars represent the SEM, n=5. * mean is significantly different than control values at that time, ANOVA, $p<0.05$.

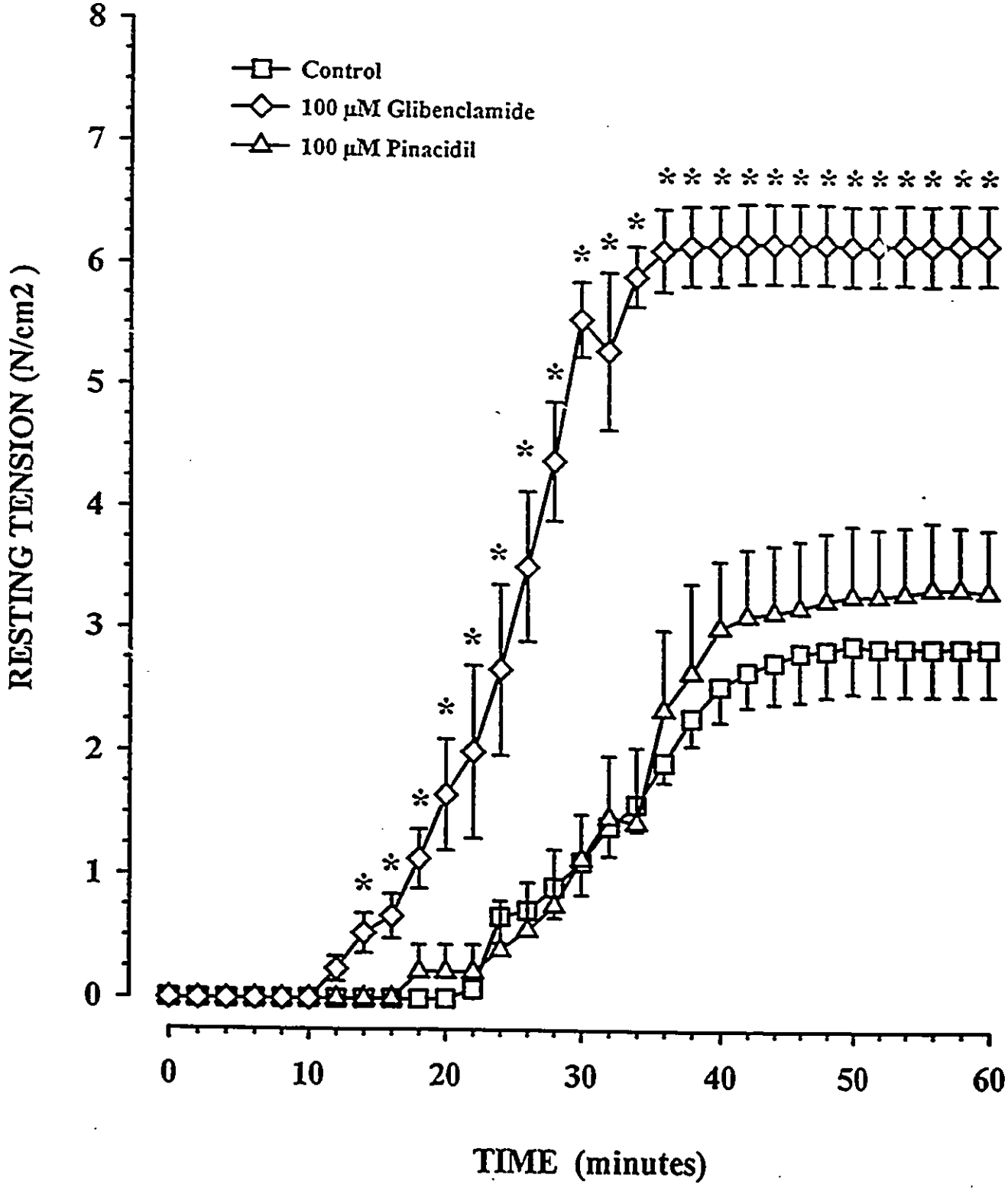
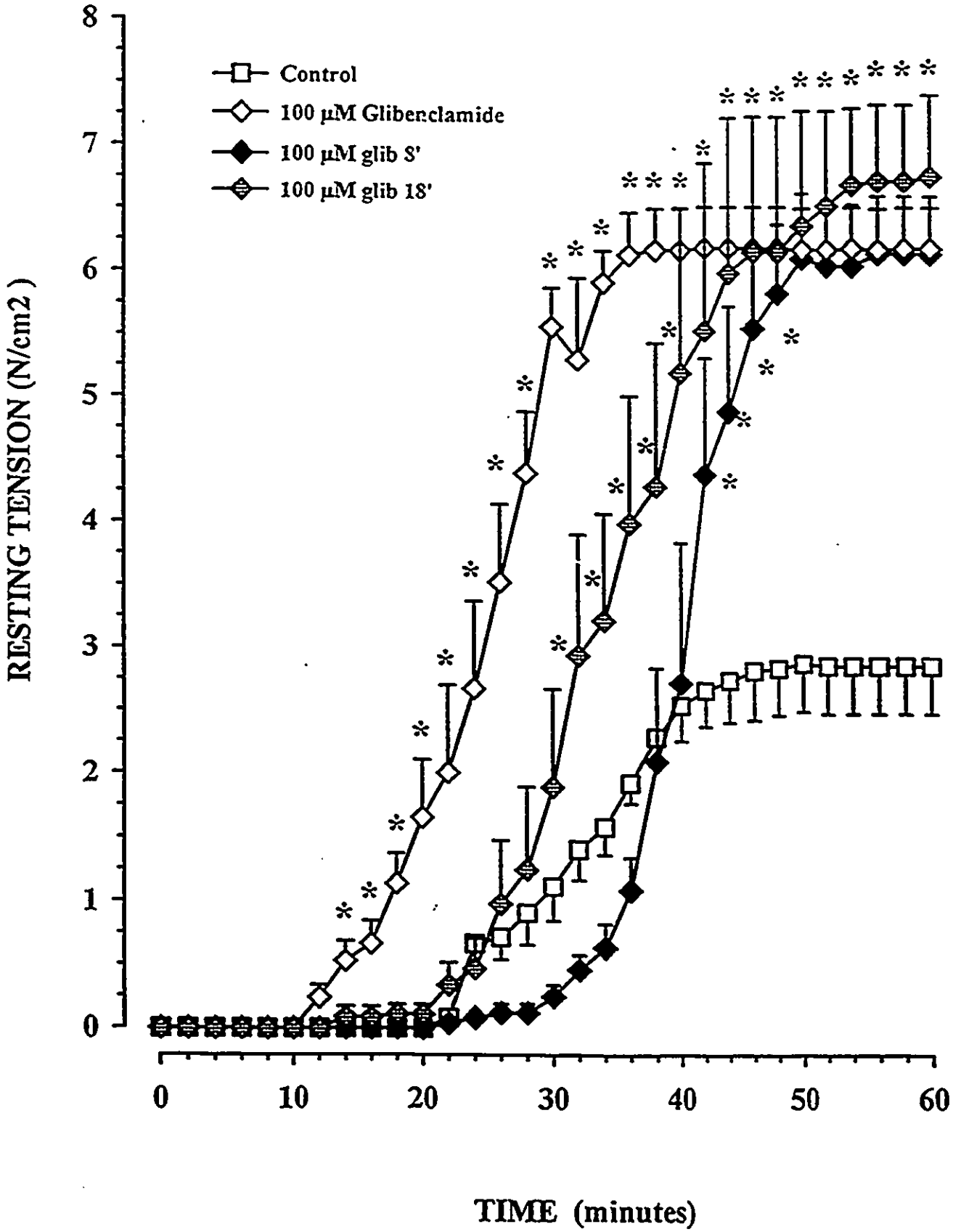

Control
100 µM Glibenclamide
100 µM Pinacidil
RESTING TENSION (N/cm2 )
TIME (minutes)
0
1
2
3
4
5
6
7
8
0
10
20
30
40
50
60

Figure 12: The effect of adding glibenclamide during metabolic inhibition on the resting tension. Resting tension is the tension in the absence of stimulation, measured from the chart recorder every two minutes. In these experiments, glibenclamide was added either 60 min prior to metabolic inhibition, or 8 min or 18 min after metabolic inhibition was started. Muscles were stimulated with 200 msec long tetanic contractions every 10 minutes. Note that the data for control and glibenclamide (60 minutes prior to metabolic inhibition) are the same as in Figure 11. Vertical bars represent the SEM, n=4. * mean is significantly different from control values at that time, ANOVA, $p<0.05$.

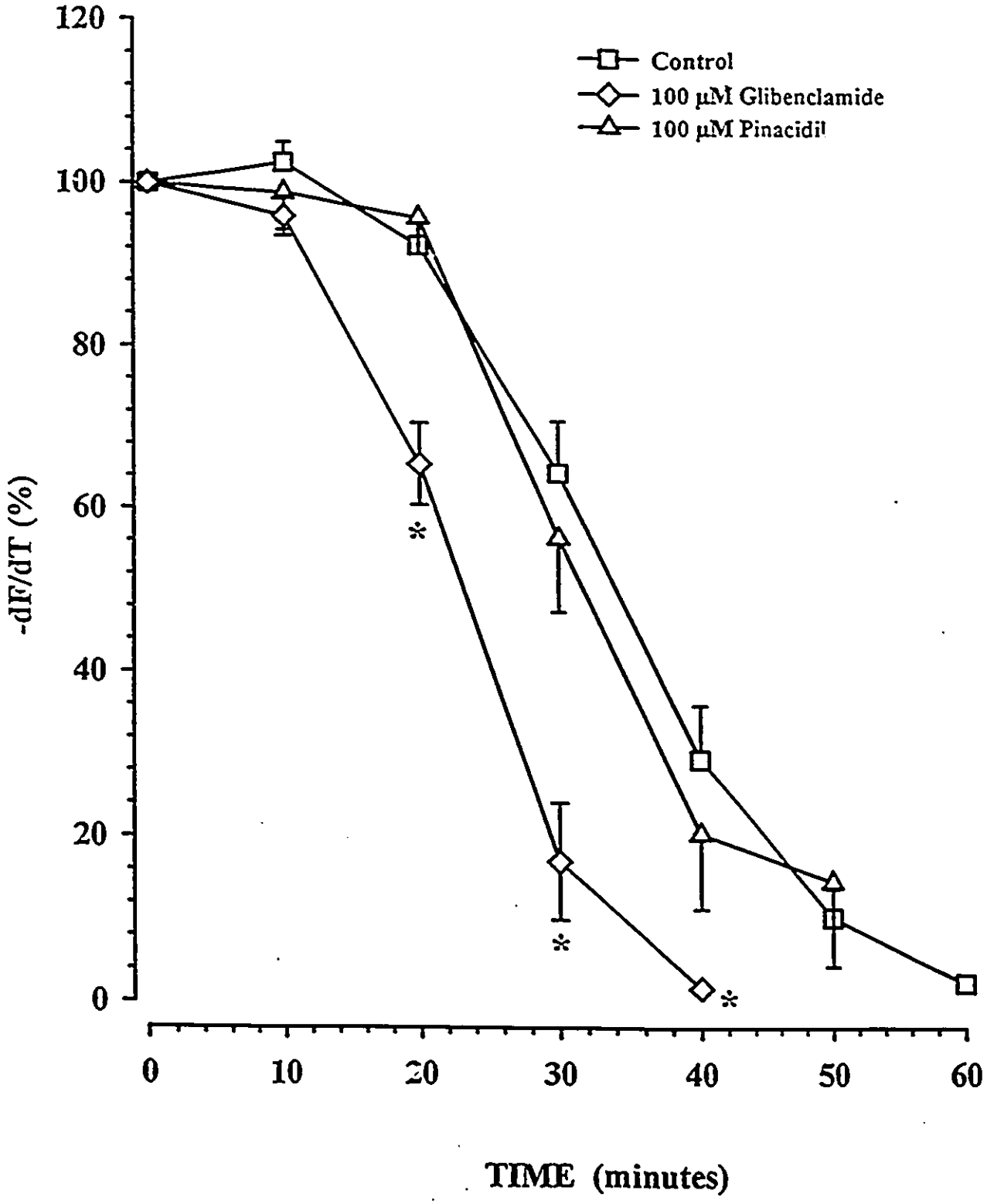

Control
100 µM Glibenclamide
100 µM glib 5'
100 µM glib 18'
RESTING TENSION (N/cm2 )
TIME (minutes)
0
1
2
3
4
5
6
7
8
0
10
20
30
40
50
60

observed in control muscles and 10 minutes after the tetanic force suddenly decreased in the presence of glibenclamide (Figure 12). However, adding glibenclamide 18 minutes after the start of metabolic inhibition caused within 4 minutes an increase in resting tension which was much faster than when glibenclamide was added after 8 minutes (Figure 12). The final increase in resting tension in the presence of glibenclamide was the same regardless of the time glibenclamide was added: the resting tension was 6.2 and 6.7 $N/cm^2$ when glibenclamide was added after 8 and 18 minutes of metabolic inhibition, respectively.

The maximum rate of relaxation of control and pinacidil muscles changed very little during the first 20 minutes of metabolic inhibition as the mean values of the -dF/dT were 92.5 % and 95.9 %, respectively (Figure 13). This is in sharp contrast with the tetanic force which decreased by 20 % during the same time period (Figure 8). This suggests that the relaxation phase became shorter than pre-metabolic inhibition values during the first 20 minutes. After 30 minutes and until the 60th minute of metabolic inhibition, the decrease in the maximal rate of relaxation was similar to that of the tetanic force, suggesting that during this time the time course of the relaxation phase was similar to the time course before metabolic inhibition and that the decrease in maximal rate of relaxation was related to the decrease in tetanic force.

The maximum rate of relaxation decreased at a significantly faster rate in glibenclamide-exposed muscle than in control muscle. However, contrary to control muscle, the decrease in the maximal rate of relaxation in glibenclamide-exposed muscle was similar

Figure 13: The effect of pinacidil and glibenclamide on the -dF/dT during metabolic inhibition. -dF/dT is defined as the maximum rate of relaxation of force during a tetanus and is expressed as a percentage of -dF/dT at time 0. Vertical bars represent the SEM, n=5. * mean tetanic force is significantly different from control values at that time point, ANOVA, $p<0.05$.


120
100
80
60
40
20
0
-dF/dT (%)
0
10
20
30
40
50
60
TIME (minutes)
Control
100 µM Glibenclamide
100 µM Pinacidil
*
*
*

to the decrease in tetanic force throughout the 40 minutes of metabolic inhibition needed to abolish contractility. Thus, in glibenclamide-exposed muscle the change in maximal rate of relaxation was primarily related to the decrease in tetanic force.

### 2. *The effect of $K^+_{(ATP)}$ channel modulators on membrane characteristics: resting potential, action potential, and membrane conductance*

Prior to metabolic inhibition, the resting potential was not significantly affected by the presence of either glibenclamide or pinacidil (Figure 14). The resting potential of control muscles did not show a significant depolarization during metabolic inhibition. The resting potential of control muscles after 60 minutes of metabolic inhibition was -84.4 ± 0.7 mV compared to -88.3 ± 0.7 mV before metabolic inhibition. The lack of a depolarization during a metabolic exhaustion is in agreement with the results of Fink and Luttgau (1976). The resting potential in the presence of pinacidil was not significantly different from controls. In the presence of glibenclamide, the resting potential was quite variable. However, there was a definite trend as the membrane began to depolarize once cyanide and iodoacetate was added, but became significantly different from controls after 30 minutes of metabolic inhibition. The membrane potential after 30 minutes of metabolic inhibition in the presence of 100 $\mu mole \cdot L^{-1}$ glibenclamide was -68.9 ± 4.2 mV compared to -86.1 ± 1.3 mV in control muscles. The mean resting potential continued to decrease until 50 minutes of metabolic inhibition; being -62.7 ± 3.8 mV at that time.

Figure 14: The effect of pinacidil and glibenclamide on the resting potential during metabolic inhibition. Pinacidil and glibenclamide were added 60 minutes prior to the onset of metabolic inhibition. Resting potentials were measured prior to a contraction in a minimum of three muscle fibers and the mean values were calculated as described in Chapter 3. Vertical bars represent the SEM. n=5. * mean is significantly different than control (ANOVA, DMCT, $p<0.05$).

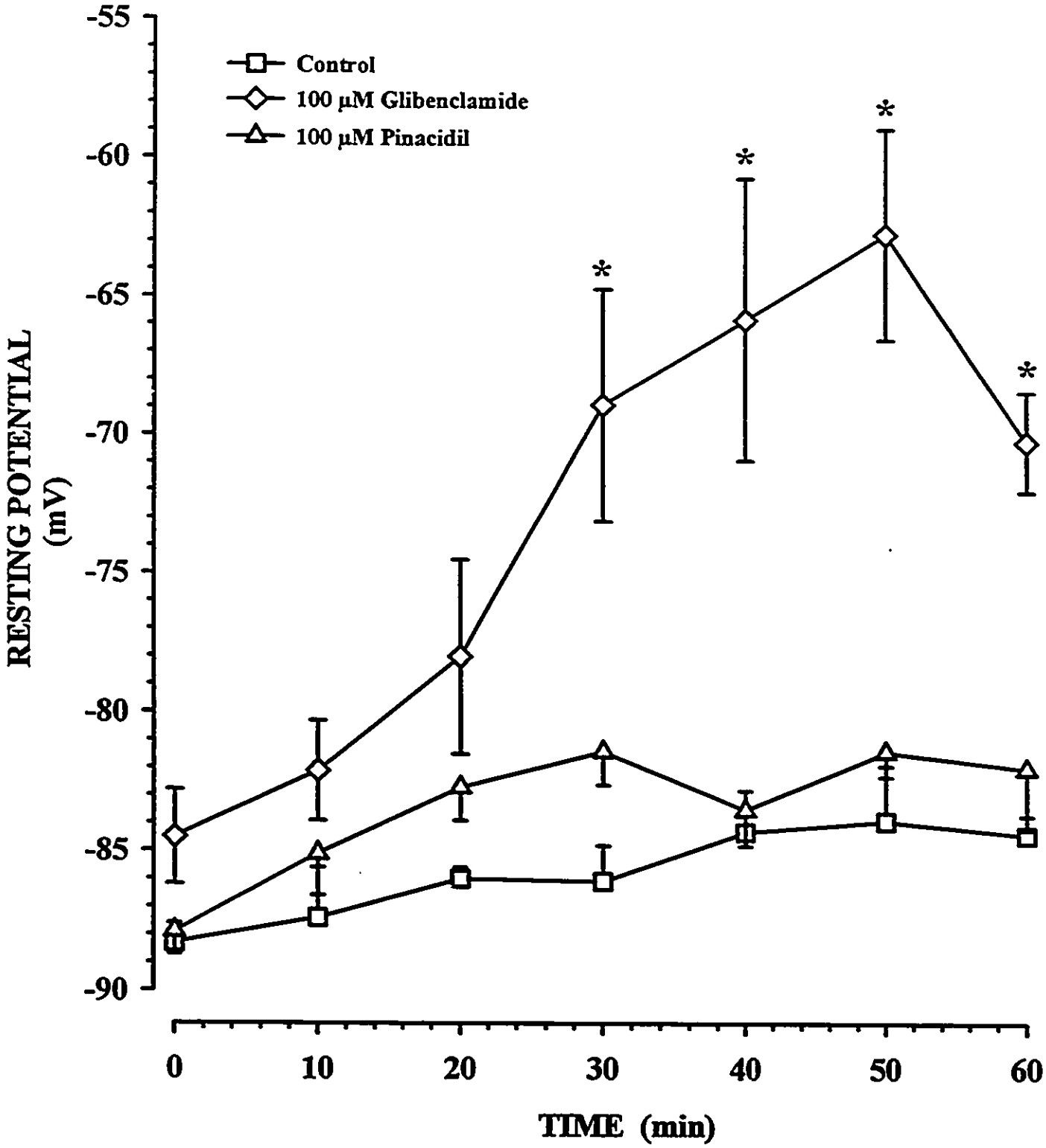
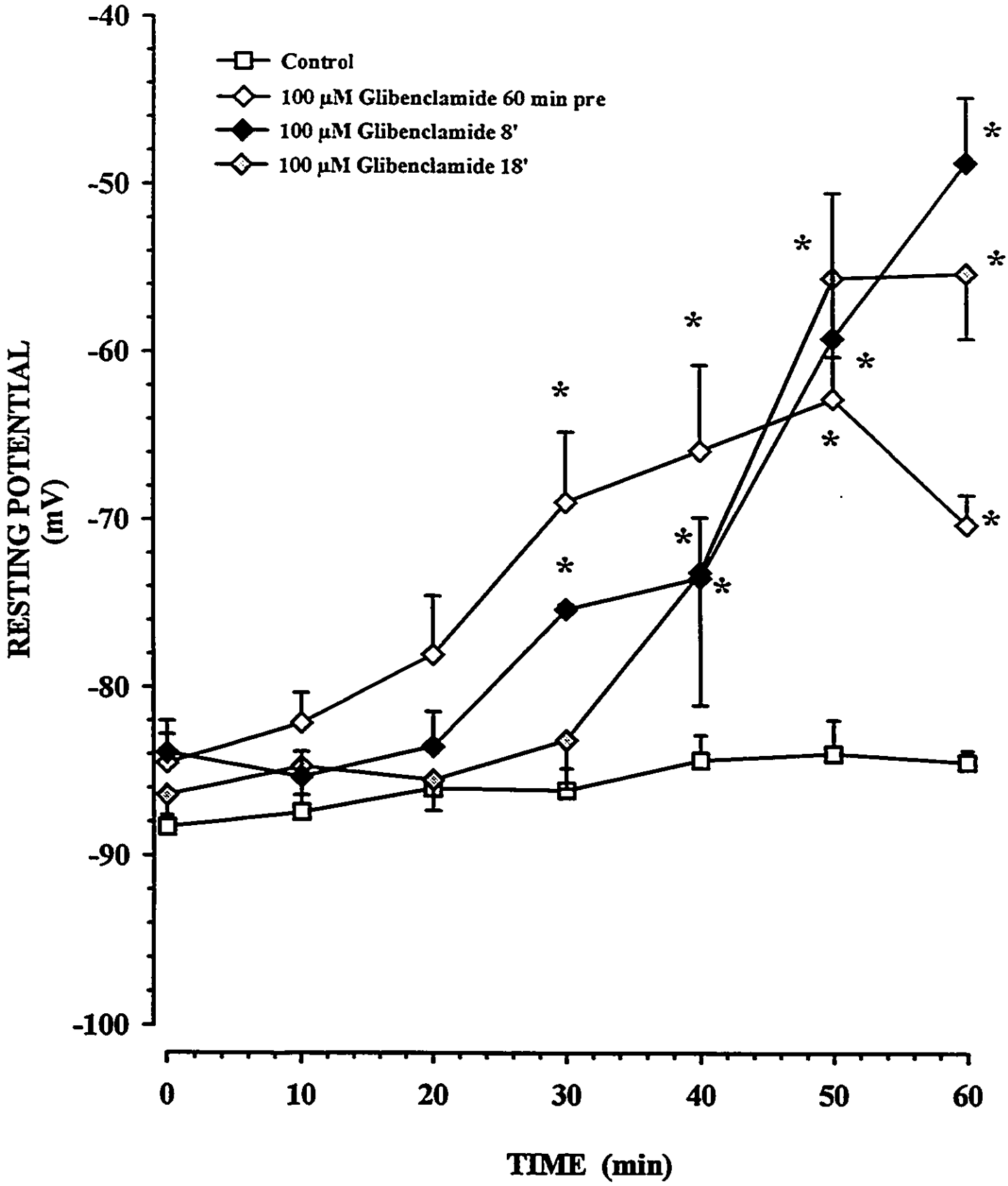

Control
100 µM Glibenclamide
100 µM Pinacidil
RESTING POTENTIAL (mV)
-55
-60
-65
-70
-75
-80
-85
-90
0
10
20
30
40
50
60
TIME (min)
*
*
*
*

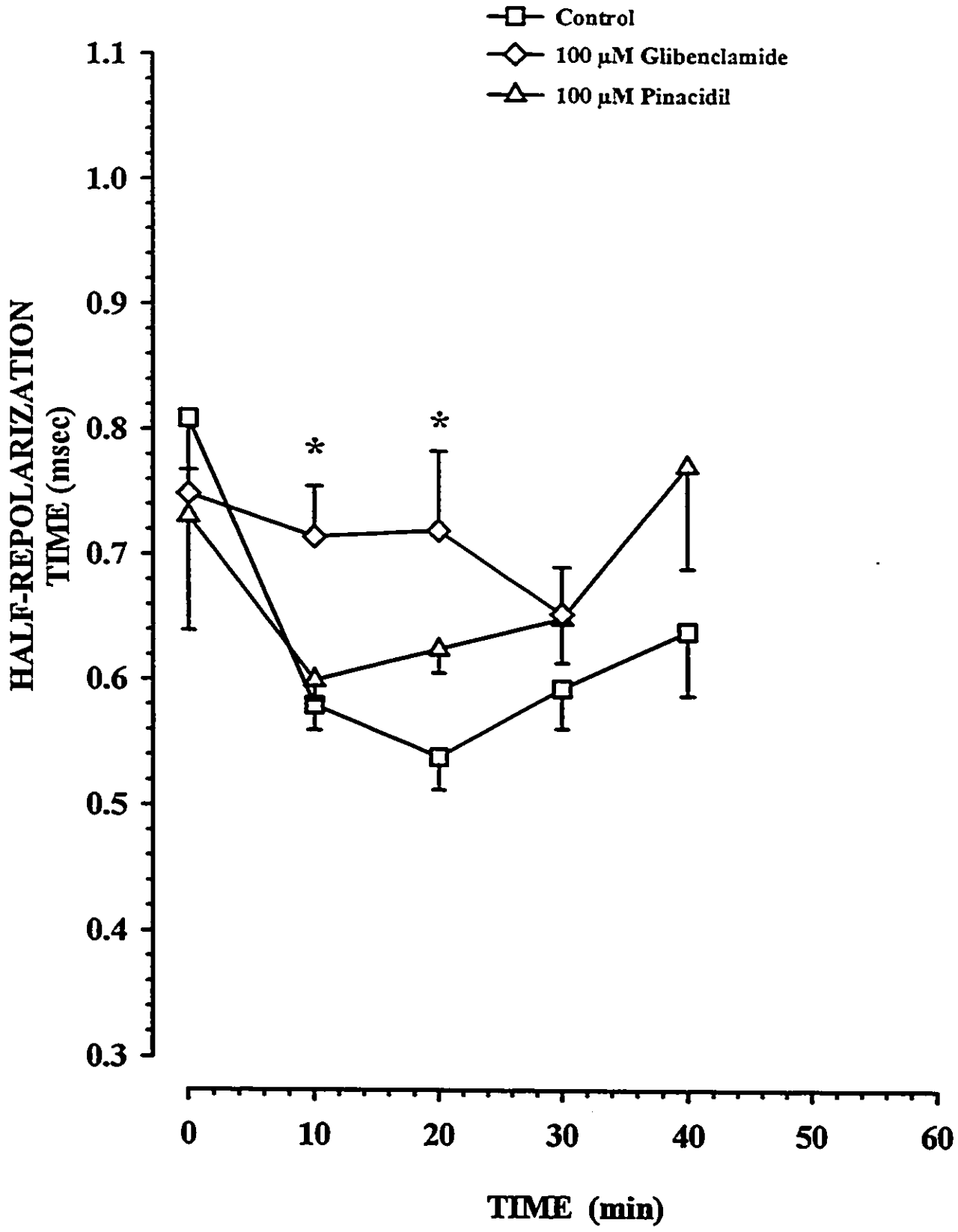
Figure 15: The effect of adding glibenclamide during metabolic inhibition on the resting potential. Metabolic inhibition was induced by adding 1 mM IOA and 2 mM CN at time 0. In these experiments, glibenclamide was added either 60 min prior to metabolic inhibition, or 8 min or 18 min after metabolic inhibition was started. Note that the data for control and glibenclamide (60 minutes prior to metabolic inhibition) are the same as in Figure 14. Vertical bars represent the SEM, n=4. * mean is significantly different from control values at that time, ANOVA, $p<0.05$.


Control
100 µM Glibenclamide 60 min pre
100 µM Glibenclamide 8'
100 µM Glibenclamide 18'
RESTING POTENTIAL (mV)
-40
-50
-60
-70
-80
-90
-100
0
10
20
30
40
50
60
TIME (min)

When glibenclamide was added 8 minutes after the addition of the metabolic inhibition, the membrane potential became significantly depolarized after 30 minutes of cyanide and iodoacetate exposure (Figure 15). Glibenclamide added 18 minutes after the addition of cyanide and iodoacetate caused the resting potential to be significantly different than controls after 40 minutes. Both groups continued to depolarize over the next 20 minutes. The mean resting potential after 60 minutes of metabolic inhibition was -55 mV when glibenclamide was added 8 minutes into CN and IOA exposure compared to -48 mV when glibenclamide was added after 18 minutes (Figure 15). The effect of glibenclamide on the resting potential appeared to require approximately 15 minutes to see an effect, and a maximal effect was observed after 40 minutes.

In the presence of cyanide and iodoacetate alone (control muscles), there was a shortening of the repolarization phase of the action potential within 10 minutes from .81 ± 0.06 to .58 ± 0.08 msec (Figure 16). This significant shortening was observed until 40 minutes of metabolic inhibition. No data are shown for 50 and 60 minutes as it was very difficult to obtain action potentials as the tetanic force was less than 10 % of pre-metabolic inhibition values. Pinacidil-exposed muscles were not significantly different from controls. The shortening of the action potential repolarization phase was not observed in the presence of glibenclamide added 60 minutes prior to metabolic inhibition. At time 0, the mean half repolarization time was 0.75 msec and after 10 minutes of metabolic inhibition it was 0.72 msec. Consequently, the half-repolarization time of glibenclamide-exposed muscles became significantly different from controls within 10 minutes and until the 20th

Figure 16: The effect of pinacidil and glibenclamide on the action potential half-repolarization time during metabolic inhibition. Vertical bars represent the SEM, n=5. * mean is significantly difference from control (ANOVA, DMCT, $p<0.05$).


Control
100 µM Glibenclamide
100 µM Pinacidil
HALF-REPOLARIZATION TIME (msec)
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
*
*
0
10
20
30
40
50
60
TIME (min)

minute of metabolic inhibition. When glibenclamide was added at 8 or 18 minutes of metabolic inhibition, the action potential half repolarization did not change significantly (Table II).

**Table II: The effect of glibenclamide exposure on the action potential half-repolarization time during metabolic inhibition.**

| TIME (min) | Glibenclamide 8' (msec) | Glibenclamide 18' (msec) |
|---|---|---|
| 0 | 0.64 ± .04 | 0.73 ± .09 |
| 10 | 0.59 ± .03 | 0.58 ± .09 |
| 20 | 0.68 ± .08 | 0.63 ± .03 |
| 30 | 0.57 ± .07 | 0.64 ± .06 |
| 40 | -- | -- |
| 50 | -- | -- |

**Metabolic inhibition was induced by adding 1 mM IOA and 2 mM CN at time 0. Muscles were stimulated with 200 msec long tetanic contractions every 10 minutes. In these experiments, glibenclamide was added either 8 min or 18 min after metabolic inhibition was started. Action potentials were measured in a minimum of three muscle fibers and these values were averaged for each muscle. These averages were then used to obtain the mean. Thus, sample size represents the number of muscles, not individual fibers. Data are given as the mean ± S.E.M., n = 4 muscles.**

The repolarization phase can be affected by changes in $K^+$ current as well as by a slower depolarization phase giving rise to a slower activation of $K^+$ delayed rectifiers. To determine if the changes observed during the repolarization phase can be due to changes in the depolarization phase, the effect of pinacidil and glibenclamide during a metabolic inhibition were measured on the dV/dT, the maximum rate of depolarization (Table III), and the overshoot (Figure 17). The dV/dT of control muscle did not significantly change through 40 minutes of metabolic inhibition being 290.8 ± 65.1 V/sec compared to 370.9 ± 63.6 V/sec prior to metabolic inhibition. Pinacidil was without effect. However in the presence of glibenclamide added 60 minutes prior to metabolic inhibition, dV/dT became significantly less than the pre-inhibition values after 20 minutes of metabolic inhibition.

In control muscle, the initial overshoot of the action potential was 24.3 ± 3.8 mV and did not change significantly during the 40 minutes of metabolic inhibition (Figure 17), while the repolarization phase was significantly shortened (Figure 16). In the presence of pinacidil, the overshoot decreased, but not significantly. However, in the presence of glibenclamide added 60 minutes prior to metabolic inhibition, the overshoot was significantly lower than in control muscles at 20 minutes of metabolic inhibition.

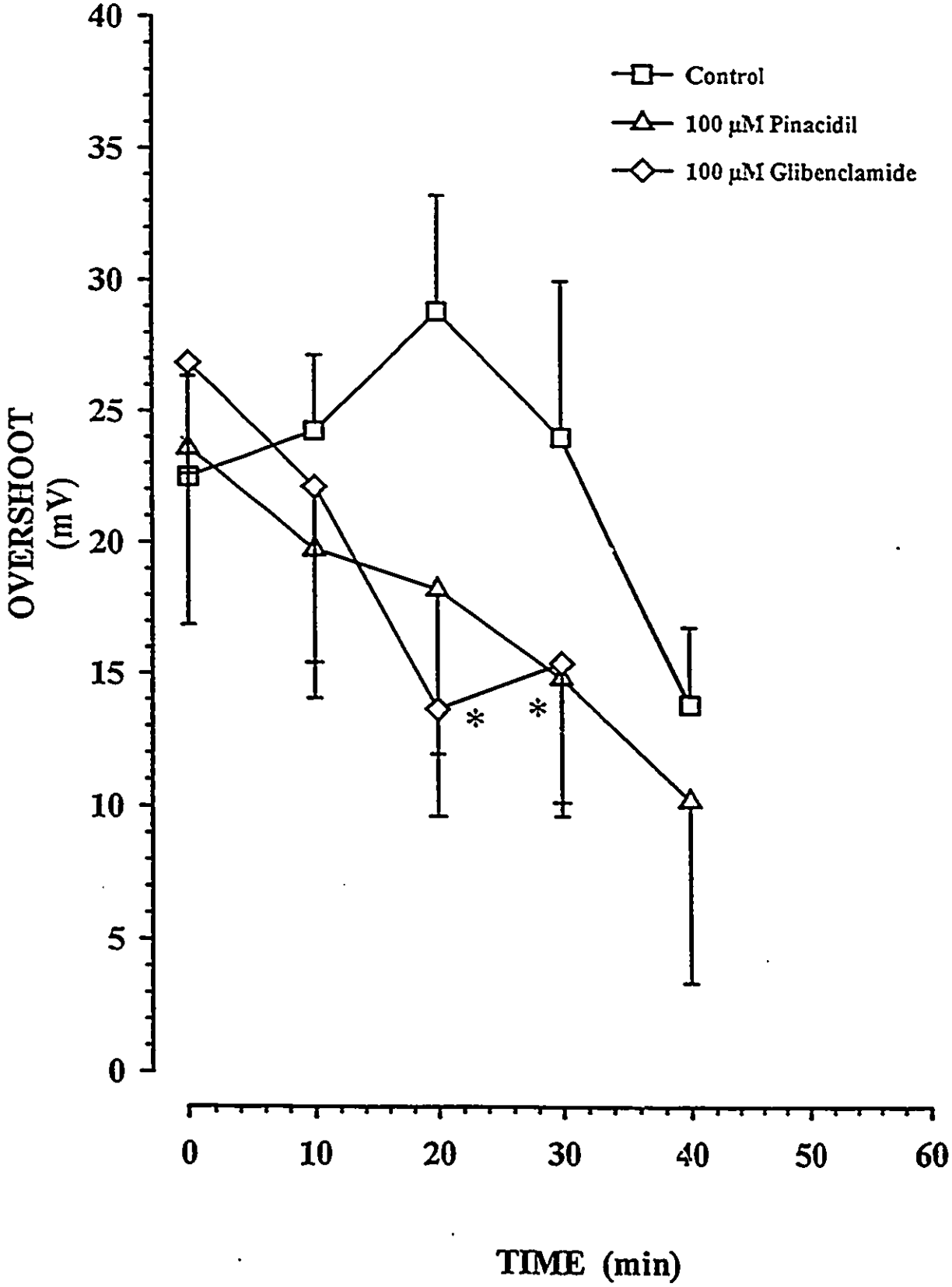
In control conditions, the specific membrane conductance measured from the low frequency cable properties was 476 ± 51 $\mu S \cdot cm^2$ (n=8) with a length constant ($\lambda$) of 1.66 ± 0.16 mm (n=8), which is similar to values reported previously in control frog skeletal

Table III: The effect of cyanide and iodoacetate exposure on the action potential dV/dT in the presence and absence of $K^+_{(ATP)}$ channel modulators.

| TIME (min) | Control (V/sec) | Pinacidil (V/sec) | Glibenclamide (V/sec) |
|---|---|---|---|
| 0 | 370.9 ± 63.6 | 367.8 ± 123.0 | 417.2 ± 71.0 |
| 10 | 381.5 ± 29.3 | 281.0 ± 73.5 | 335.1 ± 46.1 |
| 20 | 404.6 ± 20.4 | 309.0 ± 92.7 | 241.8 ± 28.4 * |
| 30 | 394.3 ± 112.6 | 254.4 ± 87.2 | 234.7 ± 31.0 * |
| 40 | 290.8 ± 65.1 | 208.2 ± 82.2 | -- |
| 50 | -- | -- | -- |
| 60 | -- | -- | -- |

**Pinacidil and glibenclamide were added 60 minutes prior to the onset of metabolic inhibition. Metabolic inhibition was induced by adding 1 mM IOA and 2 mM CN at time 0. Muscles were stimulated with 200 msec long tetanic contractions every 10 minutes. Action potentials were measured prior to a contraction in a minimum of three muscle fibers and the mean values were calculated as described in Chapter 3. Time represents the time during the metabolic poisoning. Data are given as the mean ± S.E.M., n = 5. * mean is significantly different from controls at that time, ANOVA, $p < 0.05$.**

Figure 17: The effect of pinacidil and glibenclamide on the action potential overshoot during metabolic inhibition. Action potential overshoot is the peak of the action potential. Vertical bars represent the SEM, n=5. * mean is significantly difference from control (ANOVA, DMCT, $p<0.05$).


Control
100 µM Pinacidil
100 µM Glibenclamide
OVERSHOOT (mV)
40
35
30
25
20
15
10
5
0
0
10
20
30
40
50
60
*
*
TIME (min)

muscle (Hodgkin & Nakajima, 1972, and Chapter 4). During metabolic inhibition, the membrane conductance increased to 115.3 % of pre-metabolic inhibition values, within the first 10 minutes of cyanide and iodoacetate exposure, with no difference in the presence of glibenclamide, 118.0 ± 8.8 %, or pinacidil, 116.2 ± 15.5% (Figure 18). After 20 minutes of metabolic inhibition the membrane conductance of control muscles had increased to 164.0 ± 18.5 %. However, in the presence of 100 μM glibenclamide, the increase in membrane conductance was less; being 123.1 ± 7.3 %. After 40 minutes of metabolic inhibition the membrane conductance increased and differences between control and glibenclamide-exposed muscles could not be observed at 40 minutes and 60 minutes of metabolic inhibition. Furthermore, during that time the variability was also very large in control and glibenclamide-exposed muscle: some muscles had very large increases while smaller increases were observed in other muscles. A similar variability was observed by Fink & Luttgau (1976). Pinacidil was ineffective at increasing the level of membrane conductivity above that of control muscle.

### 3. *The effect of $K^+_{(ATP)}$ channel modulators on intracellular metabolite concentrations*

Prior to metabolic inhibition, ATP concentrations were not different between controls and glibenclamide-treated muscles as the mean ATP levels were 15.6 ± 1.3 and 18.5 ± 1.3 μmoles/g dwt, respectively (Figure 20). ATP levels declined only slightly for control muscles during 50 minutes of metabolic inhibition being 15.1 ± 0.8 μmole/g dwt after 50 minutes.

Figure 18: The effect of pinacidil and glibenclamide on the membrane conductance during the first 30 minutes of metabolic inhibition. Membrane conductance was measured prior to a contraction from the low frequency cable properties. Results are shown with a current injection of -95 nA. Pinacidil and glibenclamide were added 60 minutes prior to the onset of metabolic inhibition. Vertical bars represent the SEM, n=6.

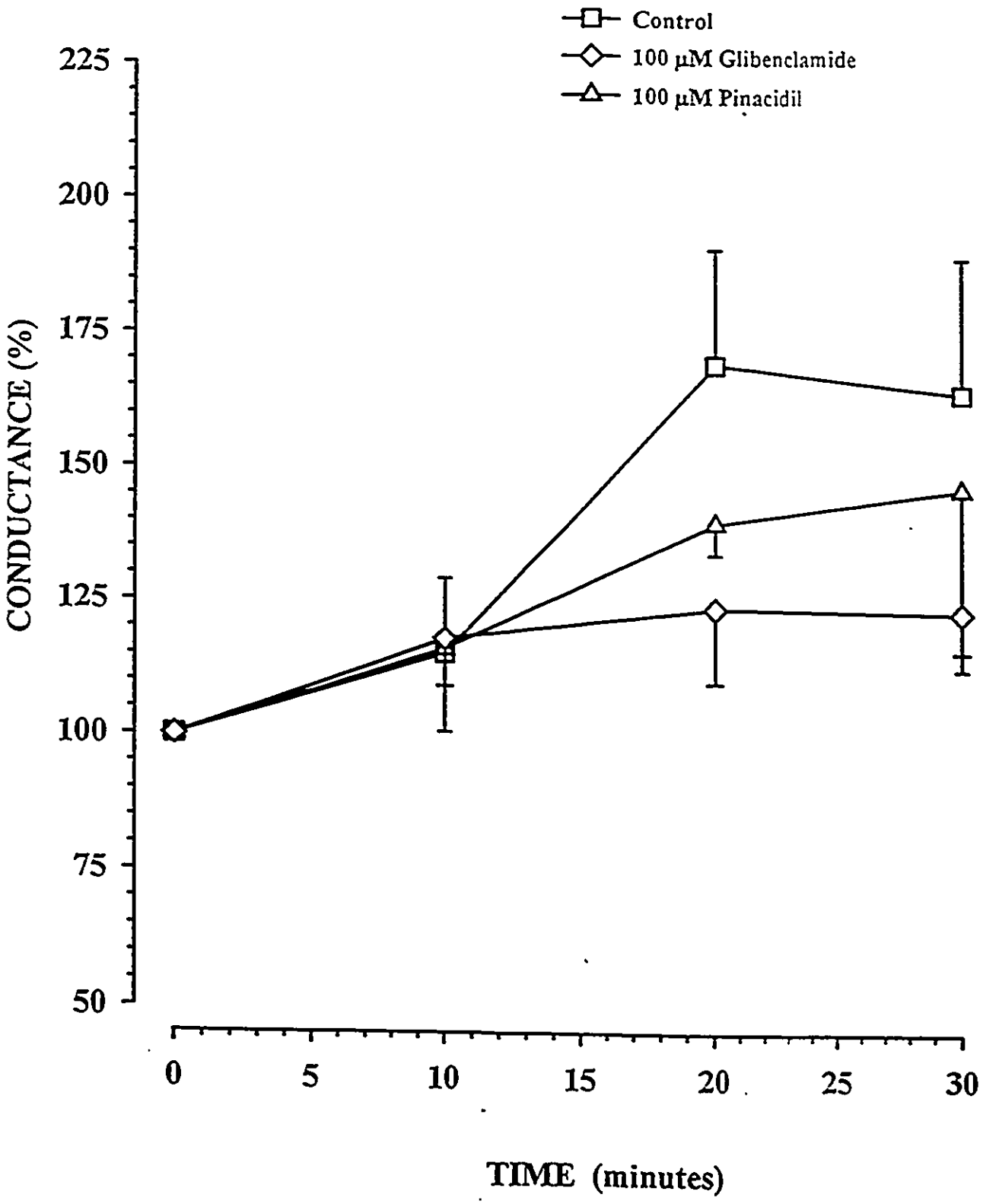
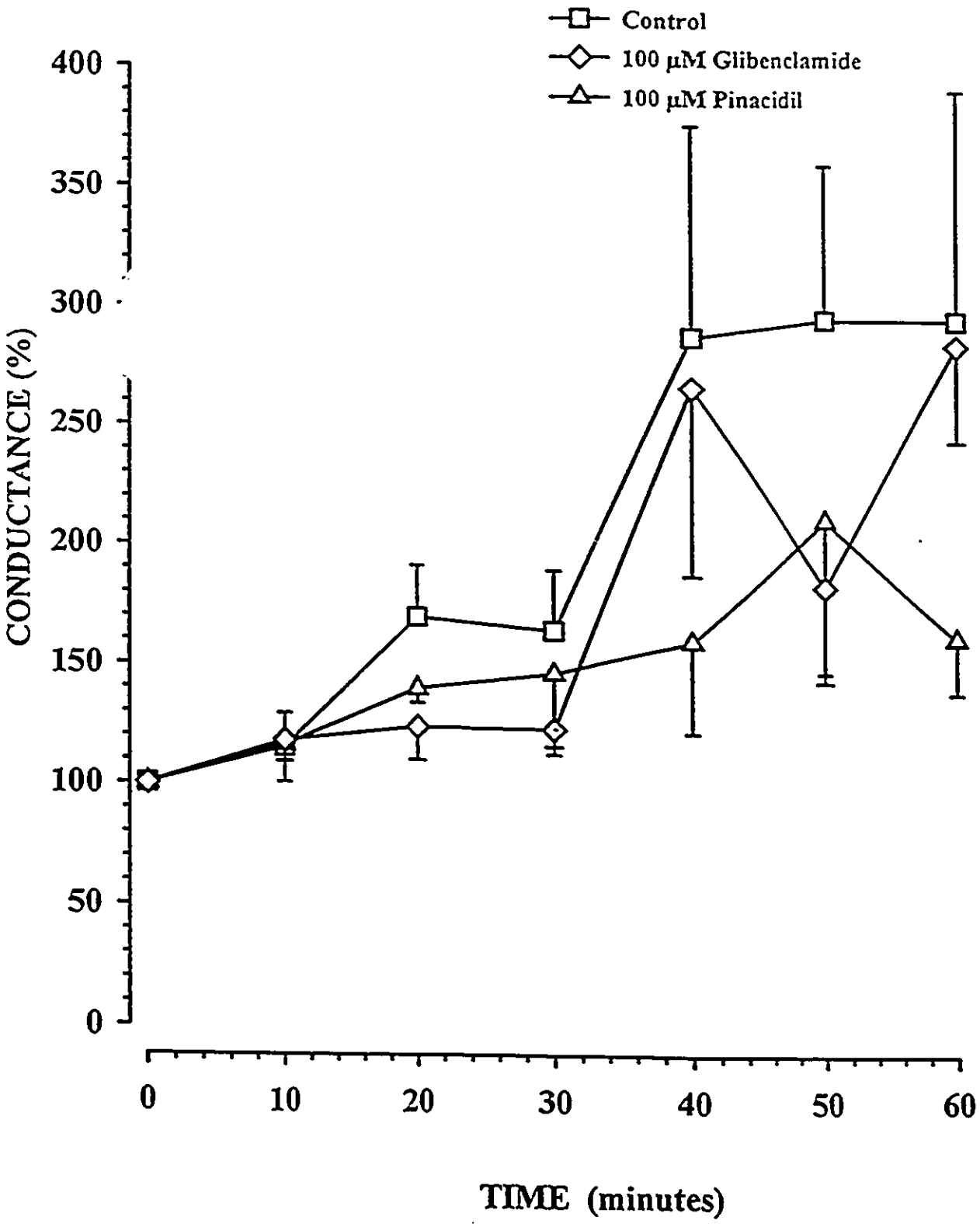

Control
100 µM Glibenclamide
100 µM Pinacidil
225
200
175
150
125
100
75
50
CONDUCTANCE (%)
0
5
10
15
20
25
30
TIME (minutes)

Figure 19: The effect of pinacidil and glibenclamide on the membrane conductance during the full sixty minutes of metabolic inhibition. Membrane conductance was measured prior to a contraction from the low frequency cable properties. Results are shown with a current injection of -95 nA. Vertical bars represent the SEM. n=6. No significant differences from control muscles (ANOVA.$p>0.05$).


Control
100 µM Glibenclamide
100 µM Pinacidil
CONDUCTANCE (%)
400
350
300
250
200
150
100
50
0
0
10
20
30
40
50
60
TIME (minutes)

A large decrease in ATP was observed only for the last 10 minutes of metabolic inhibition in control muscles as ATP levels dropped to 2.1 μmoles/g dwt. In the presence of 100 μM glibenclamide, ATP levels were not significantly different from control muscles for the first 40 minutes of metabolic inhibition. The sudden decrease in ATP concentrations in the presence of glibenclamide preceded the decrease in control muscles by 10 minutes. After 50 minutes of metabolic inhibition in the presence of glibenclamide the ATP levels were 4.1 ± 1.4 μmoles/g dwt compared to 15.1 ± 0.8 μmoles/g dwt in control muscle.

ADP concentrations in control muscle increased from 1.4 ± 0.1 to 3.8 ± 0.8 μmole/g dwt during the 60 minutes of metabolic inhibition (Figure 21). In the presence of glibenclamide, the increase in ADP concentration was significantly greater: increasing from 1.5 μmole/g dwt to 8.2 ± 2.1 μmole/g dwt at sixty minutes of metabolic inhibition.

It has been suggested that the ATP/ADP ratio is a more important determinant of $K^+_{(ATP)}$ channel activity than either ATP or ADP alone. As such, the ATP/ADP ratio was examined in this experiment (Figure 22). In control muscle at time zero, the ATP/ADP ratio was 11.5 ± 0.8 and decreased constantly during the 60 minutes of metabolic inhibition to 0.8 ± 0.9. In the presence of glibenclamide, the ratio is initially higher (15.9 ± 1.0) than control values. This is due to the slightly higher levels of ATP measured at time zero for the glibenclamide exposed muscle (Figure 20). The ATP/ADP ratios of glibenclamide-treated muscles were significantly lower than in control muscles after 10 minutes and until the 50 th minute of metabolic inhibition.

Levels of phosphocreatine of control muscles declined over time during metabolic inhibition from 65.6 ± 6.3 μmole/g dwt at time zero to 6.4 ± 3.0 μmole/g dwt at 60 minutes of metabolic inhibition (Figure 23). In the presence of glibenclamide, phosphocreatine levels decreased at a significantly faster rate. Prior to metabolic inhibition, glibenclamide-exposed muscles had phosphocreatine at a concentration of 62.4 ± 3.8 μmole/g dwt which declined to 1.9 ± 1.2 μmole/g dwt at 60 minutes in the presence of glibenclamide.

At time zero, both control muscles and glibenclamide-exposed muscles had levels of AMP which were not measurable (Table IV). However, the levels of AMP in glibenclamide-exposed muscles were significantly greater than controls after 30 minutes of metabolic inhibition.

Figure 20: The effect of glibenclamide on the ATP concentration during metabolic inhibition. Muscles were frozen in liquid nitrogen prior to a contraction. ATP concentrations were determined by HPLC (as described in Chapter 3). Vertical bars represent the SEM, n=5. * mean is significantly different from controls at that time (ANOVA, $p<0.05$).

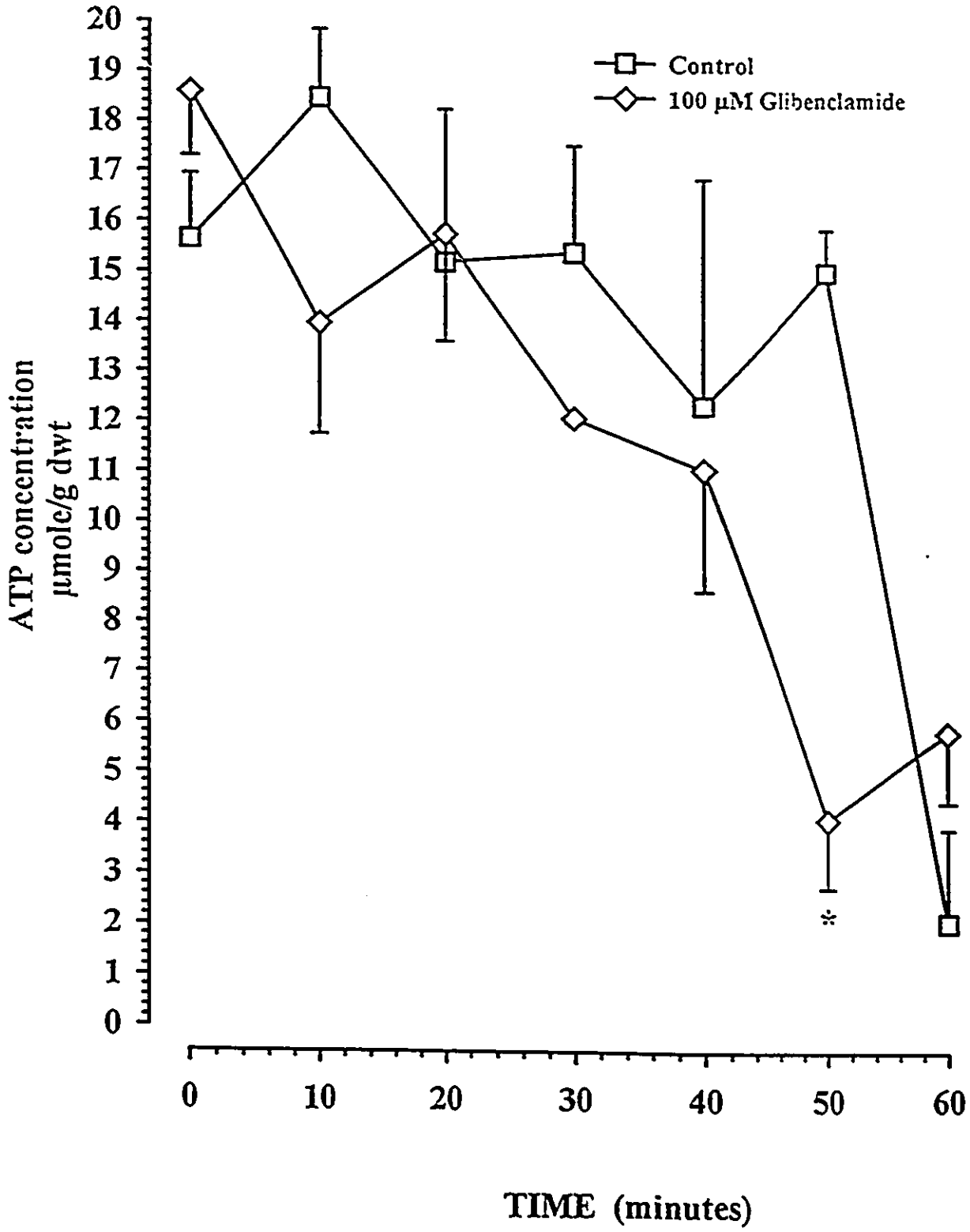

Control
100 µM Glibenclamide
ATP concentration
µmole/g dwt
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
0
10
20
30
40
50
60
*
TIME (minutes)

Figure 21: The effect of glibenclamide on the ADP concentration during metabolic inhibition. ADP concentrations were determined by HPLC (as described in Chapter 3). Vertical bars represent the SEM, n=5. * ADP concentrations of glibenclamide-exposed muscles are significantly different than controls, ANOVA main effect, $p<0.05$.

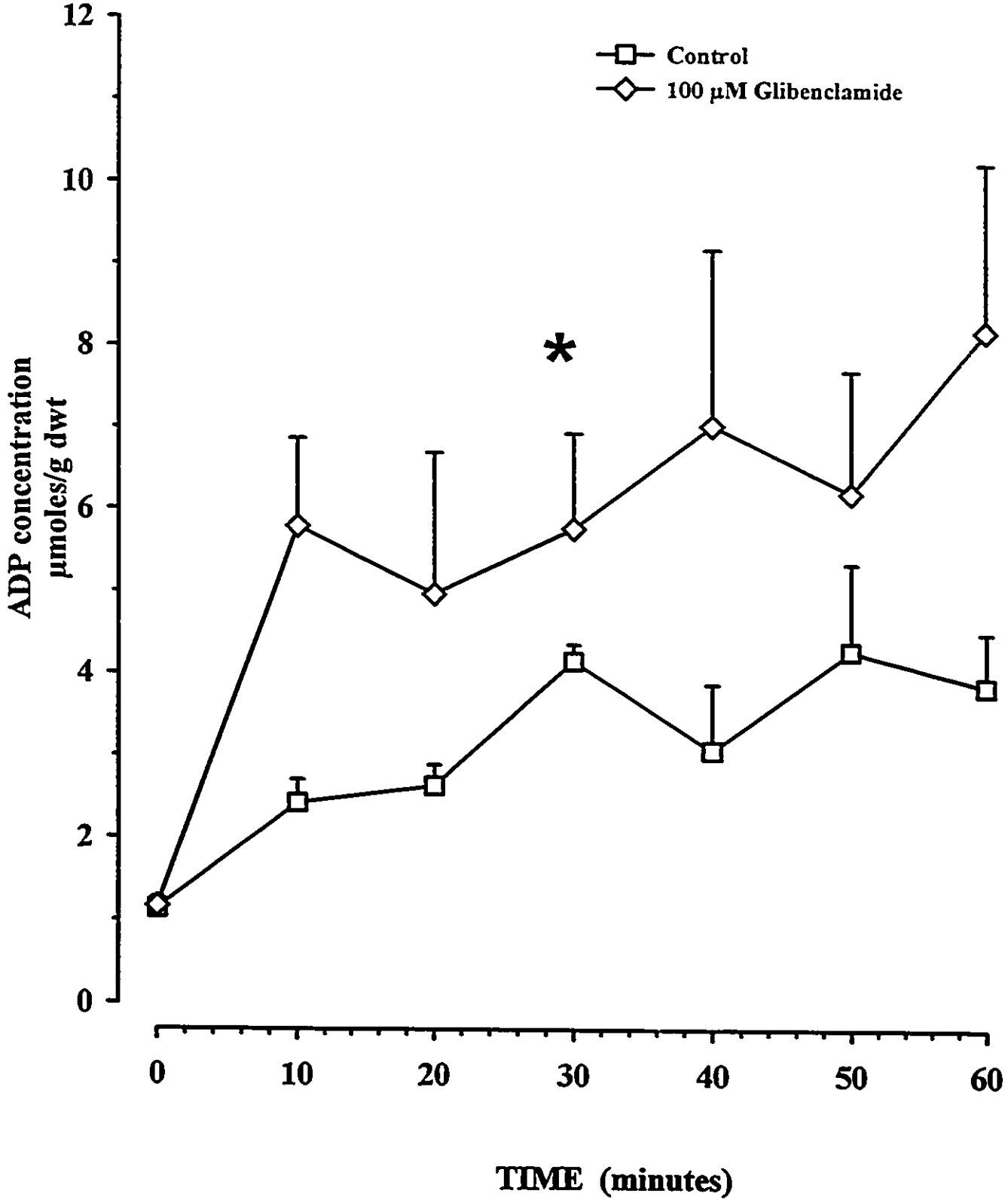

Control
100 µM Glibenclamide
ADP concentration
µmoles/g dwt
12
10
8
6
4
2
0
*
0
10
20
30
40
50
60
TIME (minutes)

Figure 22: The effect of glibenclamide on the ATP/ADP ratio during metabolic inhibition. ATP and ADP concentrations were determined by HPLC (as described in Chapter 3). Vertical bars represent the SEM, n=5.

* mean is significantly different from controls, ANOVA, $p<0.05$.

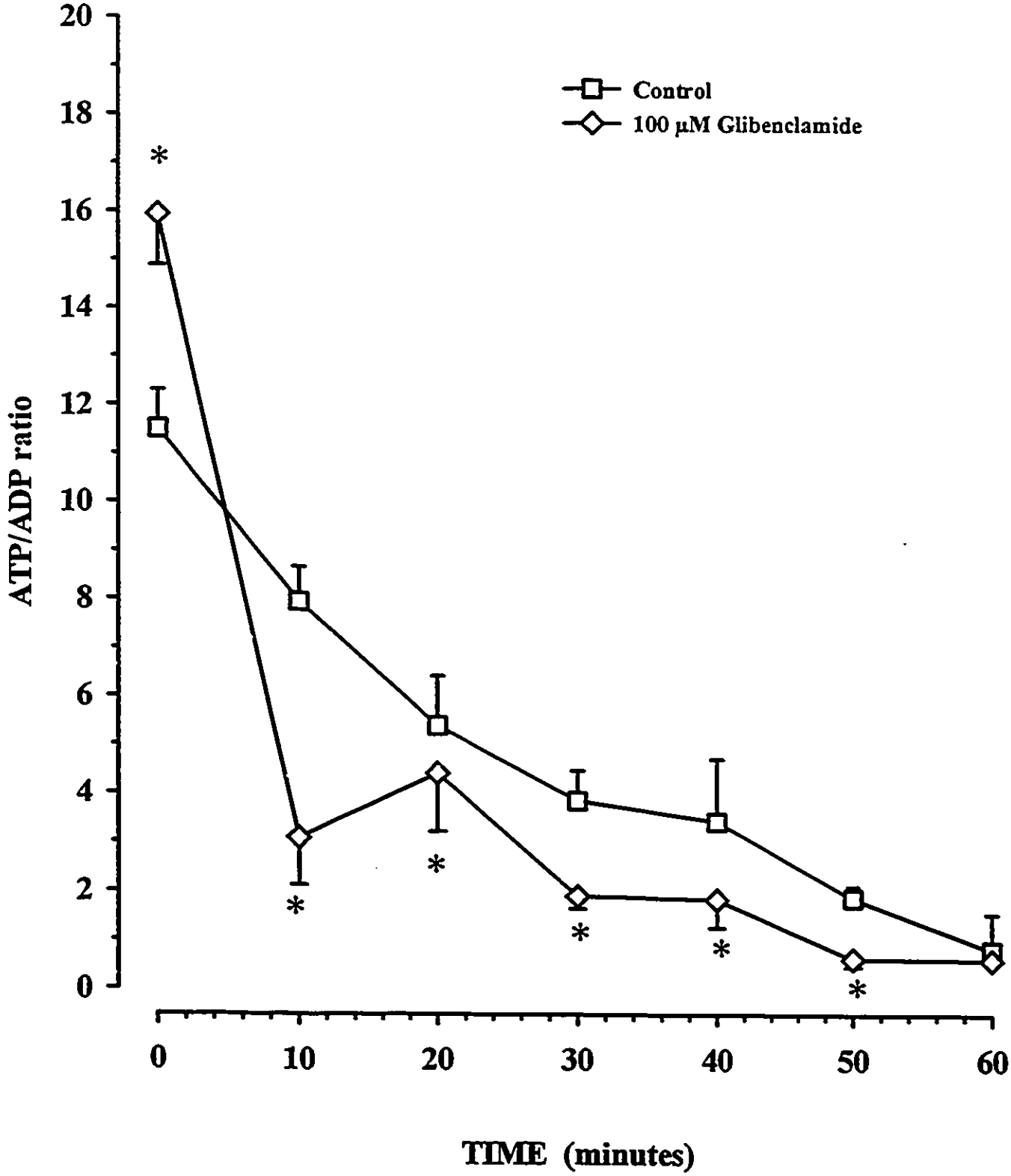

Control
100 µM Glibenclamide
ATP/ADP ratio
TIME (minutes)
0
2
4
6
8
10
12
14
16
18
20
0
10
20
30
40
50
60

Figure 23: The effect of glibenclamide on the PCr concentration during metabolic inhibition. PCr concentrations were determined by HPLC (as described in Chapter 3). Vertical bars represent the SEM, n=5. * PCr concentrations of glibenclamide-exposed muscles are significantly different than controls. ANOVA main effect, $p<0.05$.

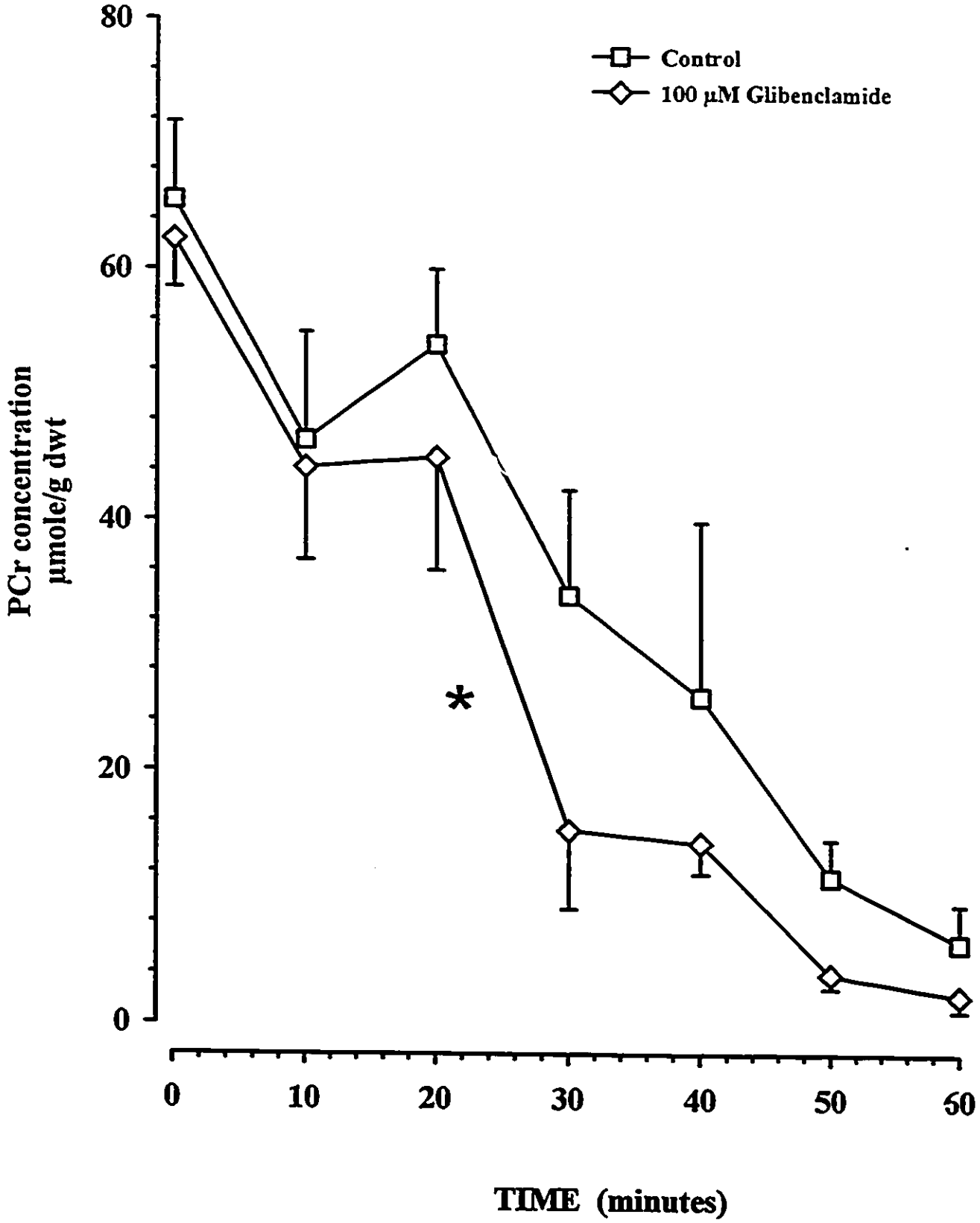

80
60
40
20
0
PCr concentration
µmole/g dwt
Control
100 µM Glibenclamide
*
0
10
20
30
40
50
60
TIME (minutes)

Table IV: The effect of CN and IOA exposure on the concentration of AMP, adenine 5'-monophosphate, in the presence and absence of the $K^+_{(ATP)}$ channel antagonist, glibenclamide.

| TIME (min) | Control [AMP] (μmole/g dwt) | Glibenclamide [AMP] (μmole/g dwt) |
|---|---|---|
| 0 | n.m. | n.m. |
| 10 | 0.9 ± 0.2 | 0.5 ± 0.3 |
| 20 | 1.0 ± 0.5 | 0.3 ± 0.3 |
| 30 | 1.5 ± 0.1 | 3.9 ± 3.1 |
| 40 | 1.2 ± 0.5 | 10.3 ± 4.1 * |
| 50 | 0.9 ± 0.1 | 13.3 ± 2.8 * |

**Metabolic inhibition was induced by adding 1 mM IOA and 2 mM CN at time 0. Muscles were stimulated with 200 msec long tetanic contractions every 10 minutes. In these experiments, glibenclamide was added either 60 min prior to metabolic inhibition, or 8 min or 18 min after metabolic inhibition was started. Metabolite determinations were done prior to the 200 msec contraction. Data are given as the mean ± SEM, n = 5. Metabolite concentrations were measured by HPLC. n.m. (not measurable). Values represent the mean ± S.E.M., n = 5. * mean is significantly different from controls at that time period (ANOVA, $p<0.05$).**

## D. DISCUSSION:

The major findings in this chapter when frog sartorius muscles are subjected to a metabolic inhibition with CN and IOA are: 1) pinacidil was without effects on the tetanic force, resting tension, resting potential and action potential; 2) the cell membrane of glibenclamide-exposed muscle was significantly depolarized while no depolarization was observed in control muscles; 3) the half-repolarization time of control muscles became significantly shorter within 10 minutes of metabolic inhibition and this effect was completely blocked in the presence of glibenclamide when added 60 minutes prior to metabolic inhibition; 4) the membrane conductance of control muscle increased. This was partially blocked by glibenclamide during the first thirty minutes; 5) when glibenclamide was added 60 minutes prior to metabolic inhibition, the decrease in tetanic force and the half-relaxation time were greater than control; 6) when glibenclamide was added 8 minutes into metabolic inhibition, the tetanic force initially increased for 12 minutes before it decreased at a faster rate than in control muscles; while adding glibenclamide after 18 minutes had no effect compared to control; 7) the increase in resting tension in the presence of glibenclamide was significantly greater than in control muscles, the onset of the increase being the earliest when glibenclamide was added 60 minutes prior to metabolic inhibition and latest when added after 8 minutes of metabolic inhibition; and 8) greater energy depletion was observed in the presence than in the absence of glibenclamide.

## 1. *Effect of $K^+_{(ATP)}$ channel modulation during metabolic inhibition on the membrane conductance*

Metabolically exhausted fibers have been shown to have a large membrane conductance (Fink & Luttgau, 1976). Recent studies have now given evidence that this increased conductance is in large part due to activation of $K^+_{(ATP)}$ channels as the addition of glibenclamide caused large reductions in membrane conductance (Light *et al.*, 1994) and $K^+$ efflux (Castle & Haylett, 1987). The initial increase in membrane conductance, during the first ten minutes, was not blocked by glibenclamide, suggesting that $K^+_{(ATP)}$ channels were not activated during that time and that the increase in conductance is either an increase in $K^+$ conductance from another $K^+$ channel or an increase in chloride conductance as chloride channels also contribute to the increased conductance during a metabolic inhibition (Fink & Luttgau, 1976).

Between 10 - 30 minutes of metabolic inhibition, the increase in membrane conductance was greater in the absence than in the presence of glibenclamide. The difference was not significant because of the large variability which has also been reported by Fink & Luttgau (1976). However, after 20 minutes of metabolic inhibition, the mean increase in conductance in the absence of glibenclamide (i.e., control muscles) was 2.8-fold greater than in the presence of glibenclamide. A similar difference was observed after 30 minutes. Since glibenclamide has no effect on the inward rectifier in frog skeletal muscle (Standen *et al.*, 1992) and does not affect the delayed rectifier and $K_{Ca2+}$ in mammalian muscle (Light & French, 1994) it is therefore suggested that $K^+_{(ATP)}$ channels are activated

between the 10 - 30 minutes of metabolic inhibition and these channels contribute 64% of the increase in membrane conductance. This activation of $K^+_{(ATP)}$ channels is observed while the bioenergetic state is still sufficiently high as ATP levels are unchanged, while the decrease in PCr is less than 15% from the pre-inhibition value. As discussed in the Introduction (Chapter 2), studies suggest that a change in $pH_i$ is the most important activator of $K^+_{(ATP)}$ channels as the $K_i$ value for ATP is too low, being in the micromolar range. However, under the present metabolic inhibition a decrease in $pH_i$ is not expected since glycolysis was blocked. This is further supported by the fact that the decrease in the rate of relaxation of the tetanus is due to a decrease in tetanic force and not to a slower time course as would be expected if there was a decrease in $pH_i$ (Renaud & Stevens, 1981). Thus, this study is indicating that in intact sartorius muscle fibers, $pH_i$ is not the only important activator of $K^+_{(ATP)}$ channels and that a large activation can occur with small decreases in ATP and PCr together with small increases in ADP. It is not possible, however, from this study to determine the exact mechanism by which these changes in metabolites affected the $K^+_{(ATP)}$ channel activity.

After the first thirty minutes of metabolic inhibition there is a large and sudden increase in membrane conductance and there was no difference between control and glibenclamide exposed muscle. There are two possibilities for the lack of a glibenclamide effect at that time. First, glibenclamide becomes ineffective as the metabolic inhibition progresses as has been shown in cardiac tissue (Findlay, 1992). However, this is unlikely in this muscle preparation since Light *et al.* (1994) reported a large decrease in membrane conductance upon exposure of fully metabolically exhausted muscle fibers to glibenclamide.

Second, there is an opening of another channel. The most likely candidate is the calcium-dependent $K^+$ ($K_{Ca++}$) channel. In the presence of glibenclamide, the increase in resting tension and the depolarization of the cell membrane during metabolic inhibition is significantly greater than in control muscles. Considering i) that the increase in resting tension most likely reflects an increase in intracellular calcium levels, ii) that there is evidence for an activation of $K_{Ca++}$ channels in metabolically exhausted fibers (Fink *et al.*, 1983), and iii) that both an increase in calcium and a depolarization activate $K_{Ca++}$ channels (Moczydlowski & Latorre, 1983), it is suggested that in the presence of glibenclamide, there is a greater activation of $K_{Ca++}$ channels than in control muscles. It is therefore suggested that $K^+_{(ATP)}$ channels are still active during the last 30 minutes of metabolic inhibition, but in the presence of glibenclamide there is a greater $K_{Ca++}$ activation so that the membrane conductance of control and glibenclamide-exposed muscle is no longer different.

2. *Effect of $K^+_{(ATP)}$ channel modulation during metabolic inhibition on the action and resting potential.*

During metabolic inhibition the cell membrane of control muscle did not depolarize, which is in agreement with the findings of Fink & Luttgau (1976). In the presence of glibenclamide, however, large depolarizations were observed whether glibenclamide was added prior to or during the metabolic inhibition. Thus, the results suggest that the $K^+_{(ATP)}$ channel is an important channel responsible for maintaining the resting potential in control muscle during a metabolic inhibition as it gives a high $K^+$ conductance and allows the membrane to remain near $E_K$.

A shortening of the action potential was observed within the first ten minutes of metabolic inhibition. This shortening was not observed when $K^+_{(ATP)}$ channels were blocked with glibenclamide whether it was added before or after the onset of metabolic inhibition. Since glibenclamide has no effect on the delayed rectifier of mammalian muscle or on the repolarization phase of frog muscle action potentials prior to metabolic inhibition (this study, and Comtois *et al.*, 1993), the effect of glibenclamide during metabolic inhibition is consistent with the idea that $K^+_{(ATP)}$ channels are activated and contribute to the repolarization phase in these conditions. However, this is not in agreement with the measurement of the membrane conductivity which gave no evidence for activation of $K^+_{(ATP)}$ channels after 10 minutes of metabolic inhibition. One possible explanation for the discrepancy is the fact that $K^+_{(ATP)}$ channels of frog sartorius muscle have a slight voltage dependency, a property not observed in other tissues such as cardiac muscle. This was observed in another study in which lowering the $pH_i$ increased the outward current at positive membrane potentials while it had little effect at negative membrane potentials. It is therefore possible that after 10 minutes of metabolic inhibition the degree of $K^+_{(ATP)}$ channel activation is such that it can be observed only at positive membrane potentials and not at the negative potentials used to estimate $K^+_{(ATP)}$ channel activity in this study. An effect of blocking $K^+_{(ATP)}$ channels on the membrane conductance near the resting potential became evident after 20 minutes probably because at that time there was a very large number of active channels.

Other mechanisms for the effect on the action potential shortening in the presence of glibenclamide added 60 minutes prior to metabolic inhibition can not, however, be

ignored. The dV/dT and the overshoot of the action potential are significantly reduced in the presence of glibenclamide and this reduction in the depolarization phase of the action potential most likely slows down activation of the $K^+$ delayed rectifiers contributing to a slower rate of repolarization. It is therefore suggested that $K^+_{(ATP)}$ channels contribute to the shortening of the repolarization phase during metabolic inhibition, but other mechanisms are possible.

### 3. *Effect of $K^+_{(ATP)}$ channel modulation during metabolic inhibition on the tetanic force*

According to the hypothesis that $K^+_{(ATP)}$ channels contribute to a faster decrease in force, blocking the channel should lead to a slower decline in force. This was observed for a short period of time when glibenclamide was added after 8 minutes of metabolic inhibition, but not when glibenclamide was added 60 minutes prior to or 18 minutes after the onset of metabolic inhibition. In order to understand how the effect of adding glibenclamide at different times affects the tetanic force, the effect of adding glibenclamide 60 minutes prior to metabolic inhibition will be explained first and then the effect of adding glibenclamide 8 and 18 minutes into metabolic inhibition will be explained.

Three mechanisms can be proposed for the faster decrease in tetanic force in the presence of glibenclamide added 60 minutes prior to metabolic inhibition. First, glibenclamide affects a step of the excitation-contraction coupling mechanism and/or the generation of force by the myofibrils. However, this is unlikely since glibenclamide has no effect prior to metabolic inhibition (this study, and Comtois *et al.*, 1993). Second,

the increase in resting tension is greater in the presence of than in the absence of glibenclamide. Since the increase in resting tension is observed while the PCr content has decreased by 20% and while ATP levels are still at normal levels, it is then unlikely that the increase in resting tension is due to some cross-bridges in rigor state; a state normally observed at low ATP content. An increase in intracellular $Ca^{2+}$ concentration is most likely the reason for the increase in resting tension. This higher $Ca^{2+}$ level in glibenclamide-exposed muscle may be a consequence of the large membrane depolarization since it is known that a $K^+$ induced depolarization causes an increased intracellular $Ca^{2+}$ concentration (Westerblad *et al.*, 1993). Both the increase in resting tension and $Ca_{2+}$ concentration then increase the rate of energy utilization of myosin ATPase and calcium ATPase pumps.

However, is the difference in bioenergetic state between control and glibenclamide exposed muscles large enough (Figures 20 to 23 and Table IV) to explain the difference in force at 20 and 30 minutes of metabolic inhibition between the two groups of muscles? Previously, Godt & Nosek (1989) have demonstrated that decreases in ATP and PCr, as well as increases in levels of ADP all lead to slight increase in force production, not a decrease; i.e., the metabolic changes during the metabolic inhibition can not explain the greater decrease in tetanic force in the presence of glibenclamide. On the other hand, an increase of inorganic phosphate by 15 mM leads to an 18% reduction in tetanic force development (Godt & Nosek, 1989). Although inorganic phosphate levels were not measured in this study, the greater depletion in ATP and PCr and the greater increase in ADP during a metabolic inhibition in the presence of glibenclamide also suggest a greater increase in $P_i$. From the small changes (during the first 20 minutes of metabolic inhibition)

of ATP, ADP, and PCr it can be calculated that the increase in $P_i$ is less than 15 mM. This was calculated assuming a 1:1 ratio for $P_i$ production from PCr degradation. In this study, in the presence of glibenclamide, the tetanic force had decreased by 85% after thirty minutes of metabolic inhibition compared to 45% in controls, a difference of 40%. Therefore, the greater increase in inorganic phosphate in glibenclamide-exposed muscles can only partially account for a larger decrease in tetanic force compared to controls.

Third, the large depolarization associated with glibenclamide treatment leads to inactivation of sodium channels within the muscle (Bigland-Ritchie *et al.*, 1979). Large and sudden decreases in the tetanic force in frog skeletal muscle are observed when the membrane is depolarized from -70 to -65 mV as the force decreases from 100% to 35%, while the contractility becomes completely abolished at -60 mV (Renaud & Light, 1992). In the presence of glibenclamide added 60 minutes prior to metabolic poisoning, the membrane potential depolarized to -68 mV within 30 minutes. However, there was a large variability amongst the fibers where some fibers depolarized to a greater extent (to -40 mV) and most likely became rapidly inexcitable, while other fibers depolarized at a slower rate. Considering that the large decrease in tetanic force corresponded in time with the membrane depolarization, it is more than likely that the membrane depolarization largely contributes to the decrease in tetanic force.

Whatever the mechanism these results support the notion that $K^+_{(ATP)}$ channels have a myoprotective effect. In metabolic inhibition, this is observed with large depolarizations of the cell membrane, larger increases in resting tension, faster decrease in tetanic force and

greater energy depletion when the channels are blocked with glibenclamide. Thus, this is consistent with Noma's notion (1983) that $K^+_{(ATP)}$ channels protect a muscle against an impairment of its bioenergetic state and in its functioning.

When glibenclamide was added after 8 minutes of metabolic inhibition, there was no immediate depolarization or increase in resting tension; the depolarization starts after 12 minutes and the increase in resting tension starts after 24 minutes. This would suggest that the deleterious effect of blocking $K^+_{(ATP)}$ channels with glibenclamide did not start immediately. This delay then allowed time for other effects of blocking $K^+_{(ATP)}$ channels to be observed; namely, effects on force. The lack of a decrease and the slight increase in tetanic force for 12 minutes after the addition of glibenclamide 8 minutes into metabolic inhibition is evidence that $K^+_{(ATP)}$ channels contribute to the decrease in force in frog sartorius muscle. Furthermore, this effect occurred while the bioenergetic state of the cell was within physiological limits. After 10 minutes of metabolic inhibition, the ATP concentration does not change, the ADP concentration increases by 80% and PCr concentrations decrease by 29%. During fatigue in frog sartorius muscle ATP decreases by 11%, ADP increases by 40% and PCr decreases by 70% (Mainwood *et al.*, 1972), i.e., the changes in ATP and PCr are greater during fatigue than during 10 minutes of metabolic inhibition while the changes in ADP are fairly similar. According to these results $K^+_{(ATP)}$ channels should be activated and contribute to a decrease in force during fatigue. However, no such evidence is observed as further activating $K^+_{(ATP)}$ channels has no effect on the rate of fatigue (Chapter 4). Thus, perhaps metabolic inhibition and fatigue are different on how $K^+_{(ATP)}$ channels are activated and affect contractility. There is evidence that cardiac $K^+_{(ATP)}$

channels are more sensitive to the ATP produced from glycolysis than oxidative phosphorylation (Weiss & Lamp, 1987). Therefore, it is possible that under metabolic inhibition there is a greater activation of $K^+_{(ATP)}$ channels than under fatigue because under the latter condition glycolysis is not blocked. Consequently, a modulation of $K^+_{(ATP)}$ channel activity will then have a greater effect on muscle contractility in metabolic inhibition but not in fatigue.

The deleterious effect of blocking $K^+_{(ATP)}$ channels with glibenclamide was still evident when glibenclamide was added 8 minutes into metabolic inhibition, since the increase in force development was short lived. A rapid decrease in tetanic force was observed when the cell membrane started to depolarize and later when the resting tension started to increase.

Adding glibenclamide after 18 minutes did not slow down the decrease in tetanic force as it did when added after 8 minutes of metabolic inhibition. However, it was observed that the depolarization and increased resting tension were almost immediate after the addition of glibenclamide, causing an immediate decrease in tetanic force. This was probably because at 18 minutes the number of open $K^+_{(ATP)}$ channels is such that blocking them with glibenclamide caused an immediate depolarization resulting in an increase in resting tension.

In summary, evidence was provided showing that $pH_i$ is not the only important factor causing activation of $K^+_{(ATP)}$ channels, because activity of these channels was observed with

small decreases in ATP and no evidence for a decrease in $pH_i$. This study also gave evidence that $K^+_{(ATP)}$ channels contribute to a decrease in force under physiological conditions; i.e., under a bioenergetic state that is within physiological limits. However, it must be pointed out that metabolic inhibition is distinct to that of normal muscle fatigue. Finally, this study gave further evidence for the myoprotective role of $K^+_{(ATP)}$ channels as blocking the channels causes greater energy depletion, greater decrease in tetanic force, greater increase in resting tension and greater depolarization.

# CHAPTER 6: DISCUSSION

$K^+_{(ATP)}$ channel activity is regulated by changes in intracellular metabolites. It was proposed, based on their characteristics under excised membrane patch clamp conditions, $K^+_{(ATP)}$ channels protect muscle against deleterious energy depletion and irreversible impairment of muscle function (Noma, 1983); i.e., $K^+_{(ATP)}$ channels provide a mechanism which is sensitive to the bioenergetic state of the cell and which reduce muscle contractility to reduce energy utilization. In cardiac tissue $K^+_{(ATP)}$ channels reduce energy depletion by reducing muscle contractility when exposed to ischemia (Auchampach *et al.*, 1992; Yao *et al.*, 1993). It was further shown that $K^+_{(ATP)}$ channels have a cardioprotective effect as cardiac muscles recover better from ischemia when $K^+_{(ATP)}$ channels are activated (Auchampach *et al.*, 1992; Yao *et al.*, 1993).

Considering that $K^+_{(ATP)}$ channels are also present in skeletal muscle, it was proposed that $K^+_{(ATP)}$ channels are activated and contribute to the decrease in force during fatigue (Standen, 1992). Despite evidence that $K^+_{(ATP)}$ channels are indeed activated during fatigue (Comtois *et al.*, 1995; Light *et al.*, 1994), studies failed to show an effect on the rate of fatigue under normoxic conditions, as measured by the decrease in force, when the activity of $K^+_{(ATP)}$ is modulated. The purpose of this thesis was to examine the effect of modulating $K^+_{(ATP)}$ channel activity in order to determine under what metabolic stress $K^+_{(ATP)}$ channels contribute to the decrease in force during fatigue.

The first approach used was to test the effect of two $K^+_{(ATP)}$ channel openers, pinacidil and levcromakalim, on the rate of fatigue in frog sartorius muscle. This muscle was used because there is evidence for an activation of $K^+_{(ATP)}$ channels when frog sartorius muscles are stimulated with one tetanic contraction every second for three minutes (Light *et al.*, 1994; Comtois *et al.*, 1995). Channel openers were used instead of blockers because blocking $K^+_{(ATP)}$ channels is deleterious, and the deleterious effects may counteract the expected slower decrease in force expected when $K^+_{(ATP)}$ channels are blocked.

In this study, however, it was shown that pinacidil up to 500 $\mu mole \cdot L^{-1}$ fails to activate $K^+_{(ATP)}$ channels in unfatigued muscle fibers. Although a decrease in ATP levels is known to increase the efficiency of pinacidil in activating the channel, pinacidil still fails to activate $K^+_{(ATP)}$ channels under fatigue and under a metabolic inhibition, the latter condition causing large decreases in ATP. Thus contrary to mammalian muscle (Fan *et al.*, 1990), pinacidil is ineffective in activating $K^+_{(ATP)}$ channels in frog muscle.

On the other hand, levcromakalim at 100 $\mu mole \cdot L^{-1}$ can activate $K^+_{(ATP)}$ channels in frog skeletal muscle causing increases in membrane conductance and $K^+$ efflux (Benton & Haylett, 1992). However, contrary to the effect of channel openers on the action potential of cardiac muscle, levcromakalim fails to shorten the action potential in both fatigued and unfatigued muscles. Also, levcromakalim had no effect on the rate of fatigue as measured from the decrease in tetanic force.

Since neither $K^+_{(ATP)}$ channel blockers nor openers affected the rate of fatigue under conditions known to activate $K^+_{(ATP)}$ channels, it became important to better understand the relationship among the bioenergetic state, $K^+_{(ATP)}$ channel activity and force in an attempt to determine whether or not there is a bioenergetic state within physiological limits which can activate enough $K^+_{(ATP)}$ channels to affect force development. In a second approach, frog sartorius muscles were exposed to a metabolic inhibition induced with cyanide and iodoacetate.

Under these conditions, it was found that within 30 minutes the membrane conductance increases by 64% and that almost two thirds of this increase was due to an activation of $K^+_{(ATP)}$ channels. More importantly, this activation of $K^+_{(ATP)}$ channels occurs while the $pH_i$, a factor considered as an important activator during fatigue, is not expected to change since glycolysis was blocked. Further evidence for the lack of a $pH_i$ decrease was the lack of an effect on the relaxation phase which is very sensitive to a decrease in $pH_i$; although a decrease in the rate of relaxation was observed, the decrease was due to the decrease in tetanic force and not to a prolongation of the relaxation phase. This suggests that the increase in $K^+_{(ATP)}$ channel activity during the first 30 minutes is somehow related to the changes in ATP, PCr and ADP levels. It remains to be determined how this activation occurs, but considering the small changes in these metabolites during the first 30 minutes of metabolic inhibition, it suggests that in intact muscle, $K^+_{(ATP)}$ channels may be quite sensitive to even a small change in the bioenergetic state.

Regarding the physiological role of the $K^+_{(ATP)}$ channel, strong evidence was obtained for the myoprotective effect and the contribution of these channels to a decrease in force during metabolic stress. The myoprotective of $K^+_{(ATP)}$ channels was observed from numerous physiological aspects. First, blocking $K^+_{(ATP)}$ channels resulted in faster decrease in ATP and PCr, as well as greater increases in ADP and AMP. Thus, like Noma (1983) suggested, $K^+_{(ATP)}$ channels play an important role in preventing large decreases in energy. During a metabolic inhibition, this protection of the bioenergetic state by $K^+_{(ATP)}$ channels involves a maintenance of the resting potential at normal values which then helps in reducing any increase in calcium levels and thereby resting tension, two effects which contribute to a large energy utilization. Second, blocking $K^+_{(ATP)}$ channels caused greater impairment of muscle function as observed by the faster decrease in tetanic force. This faster decrease in force may be related i) to a greater increase in $P_i$ and more importantly ii) to the large membrane depolarization which makes several fibers inexcitable. Third, blocking $K^+_{(ATP)}$ channels gives rise to an earlier and faster increase in resting tension which is most likely due to increased intracellular calcium levels, and elevated calcium is a very well known cause for cellular damage (Fitts, 1994).

The most important finding of this study was that $K^+_{(ATP)}$ channels can contribute to a decrease in force under physiological conditions. When glibenclamide is added 8 minutes into metabolic inhibition there was no immediate sign of the deleterious effect of blocking $K^+_{(ATP)}$ channels (i.e., large depolarization and increase in resting tension), and this allowed for a slower rate of decline in force as expected. This effect on force was observed while in control muscles (no glibenclamide) the changes in ATP, ADP, and PCr were similar to,

if not less than, those observed during fatigue development. Therefore, one would expect that $K^+_{(ATP)}$ channels when activated contribute to a decrease in force during fatigue. However, modulating $K^+_{(ATP)}$ channel activity fails to affect the rate of fatigue in frog sartorius muscles stimulated with 100 msec long tetanic contractions every sec for 180 sec, even though there is evidence for an activation of $K^+_{(ATP)}$ channels under these fatigue conditions from three points of view: i) the decrease in $pH_i$ is large enough to activate the channels (Standen *et al.*, 1992); ii) the changes in ATP, ADP, and PCr are also large enough (this study); iii) the repolarization phase is prolonged in fatigued muscle fibers when $K^+_{(ATP)}$ channels are blocked with either glibenclamide or tolbutamide (Comtois *et al.*, 1994; Light *et al.*, 1995).

The lack of an effect on the rate of fatigue when $K^+_{(ATP)}$ channels are blocked with either glibenclamide or tolbutamide is most likely related to the removal of the myoprotection of the channel. Thus, when $K^+_{(ATP)}$ channels are blocked at the onset of fatigue or metabolic inhibition a slower rate in the force decrease can not be observed because the deleterious effects of blocking $K^+_{(ATP)}$ channels counteract the expected slower decrease in force.

In the presence of levcromakalim, an effective activator of $K^+_{(ATP)}$ channels, no deleterious effects are expected and a further activation of $K^+_{(ATP)}$ channels was expected to increase the rate of fatigue. Such a result was not observed, possibly due to the number of $K^+_{(ATP)}$ channels activated by levcromakalim may be too small to significantly increase the $K^+$ efflux and $K^+$ accumulation in the interstitium above what is observed during fatigue.

Considering that the shortening of the action potential and the increase in $K^+$ efflux are two mechanisms by which $K^+_{(ATP)}$ channels are expected to cause a decrease in force, it may be the reason why levcromakalim fails to increase the rate of fatigue.

In summary, this study has provided evidence that i) $K^+_{(ATP)}$ channels are capable of contributing a decrease in force in skeletal muscle under physiological conditions, and ii) $K^+_{(ATP)}$ channels have an important myoprotective effect on both the bioenergetic state and functioning of skeletal muscle.

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# APPENDIX:

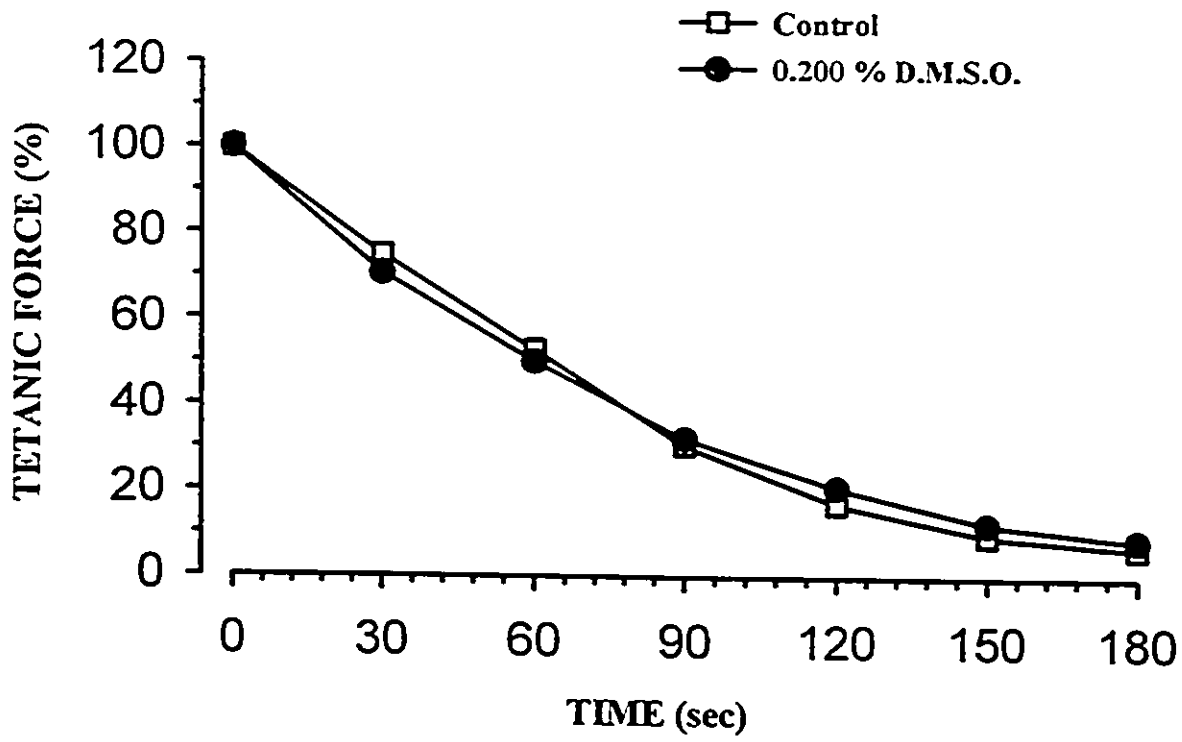
| Time (min) | Tetanic Force (%) | Resting Potential (mV) | Half-Repolarization Time (msec) | Overshoot (mV) | dV/dT (%) |
|---|---|---|---|---|---|
| 0 | 100 | -82.3 ± 1.4 | .76 ± .05 | 16.7 ± 2.5 | 100 |
| 30 | 94 ± 1.4 | -87.8 ± 1.2 | .72 ± .02 | 18.3 ± 4.4 | 113 ± 7 |
| 60 | 93 ± 1.6 | -84.7 ± 2.4 | .71 ± .03 | 15.6 ± 2.5 | 111 ± 7 |

**Appendix I: The effect of 0.200 % DMSO on unfatigued frog sartorius muscle.**

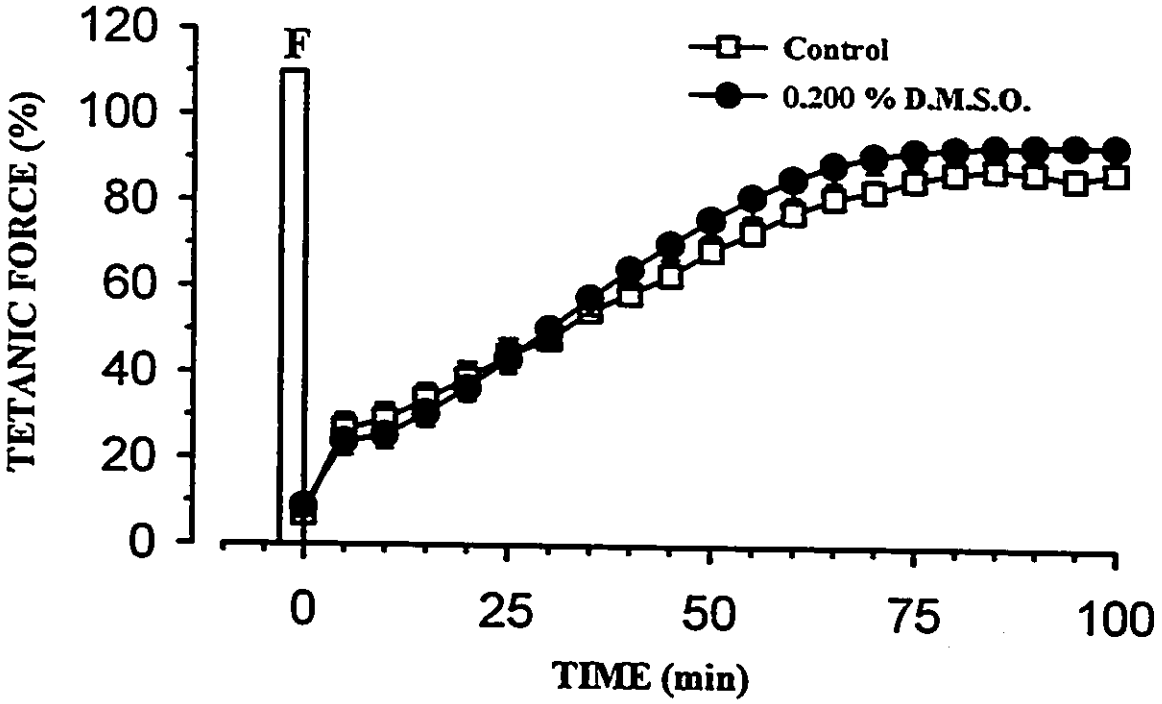
Muscles were equilibrated for 30 minutes in control solutions as outlined in the Methods and Materials (Chapter 3). 0.200% V/V DMSO was added at time 0. Tetanic force was measured by stimulating the entire muscle. The slight decrease in the presence of DMSO was not significantly different from muscles that were not exposed to DMSO during the 60 minute incubation (results not shown). For each time period, action potentials were measured in three different fibers. For each muscle, values from the three fibers were averaged and the means were then calculated from these averages. Values represent the mean ± S.E.M for 6 muscles. ANOVA, $p > 0.05$. DMSO had no significant effects on the resting membrane potential, action potential overshoot, the action potential half-repolarization time, and the action potential maximal rate of depolarization (dV/dT).

Appendix II: The effect of 0.2 % V/V dimethyl sulfoxide on frog sartorius muscle during A) fatigue and B) recovery. DMSO was added 60 minutes prior to fatigue. Muscles were fatigued with 100 msec long tetanic contractions every second for 180 seconds. Every 30 seconds the train duration was increased to 200 msec to measure the tetanic force. During the recovery period muscles were stimulated every 5 minutes. In B, the fatigue period is represented by an open vertical bar labelled F. DMSO had no significant effect on the rate of fatigue and recovery of the tetanic force (ANOVA, $p > 0.05$).

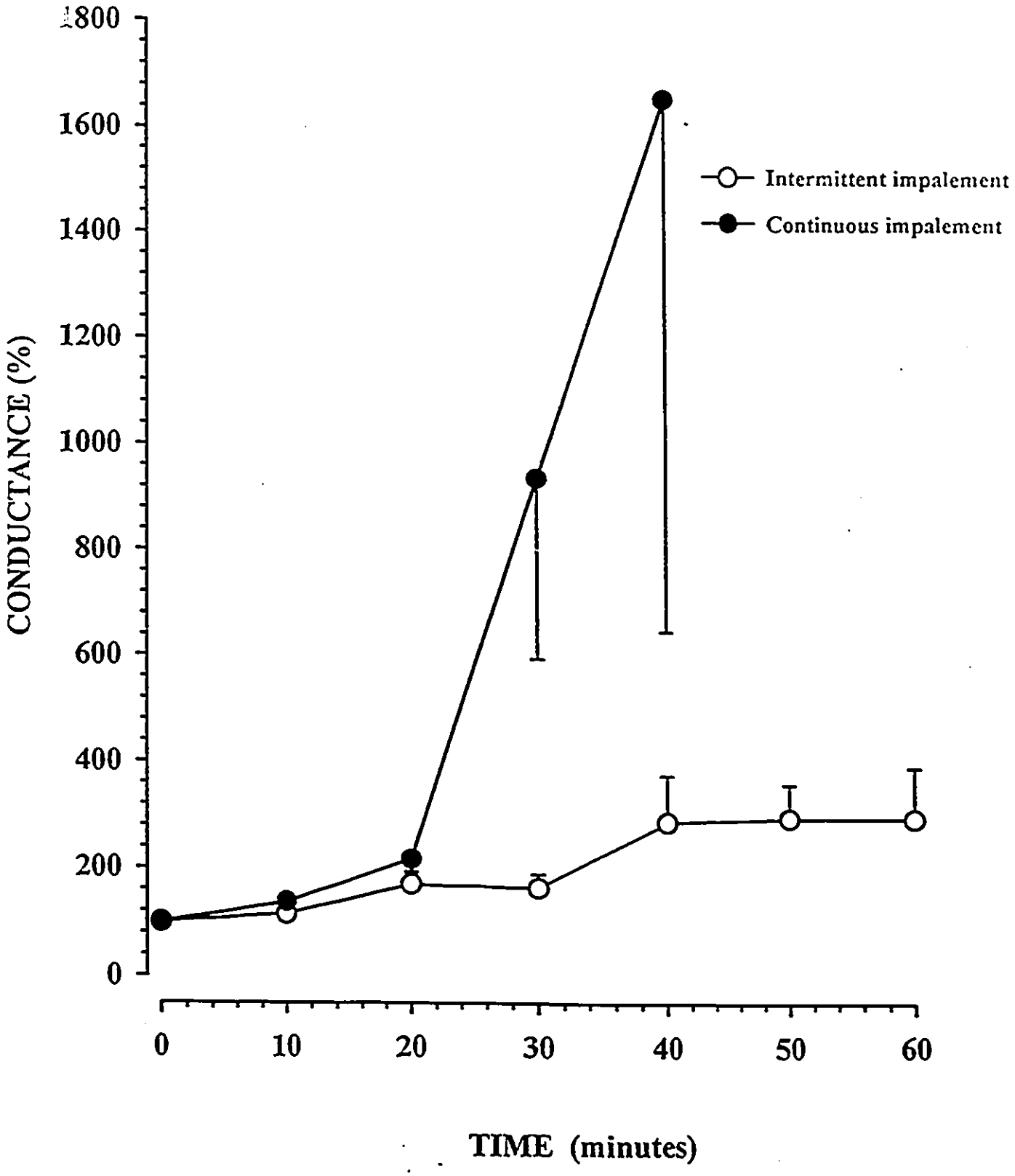
A)


Control
0.200 % D.M.S.O.
TETANIC FORCE (%)
120
100
80
60
40
20
0
0
30
60
90
120
150
180
TIME (sec)

B)


F
Control
0.200 % D.M.S.O.
TETANIC FORCE (%)
120
100
80
60
40
20
0
0
25
50
75
100
TIME (min)

Appendix III: The effect of intermittent and continuous recording of the membrane conductance during metabolic inhibition. The intermittent measurement of the membrane conductance was measured from the low frequency cable properties as described in Chapter 3. As described in Chapter 5, membrane conductance was measured in a few fibers prior to metabolic inhibition, and then each fiber was used only once during the metabolic inhibition in order to calculate a % change. For the continuous recording of membrane conductance, the method of Fink & Luttgau, 1976, was used. Briefly, two microelectrodes (50 - 100 μm apart) were used, one to inject a current and the other to measure the change in membrane potential. As the microelectrodes are close to one another, it allows a good estimation of $V_o$, the change in membrane potential at the injecting site. From the change in $V_o$, the % change in membrane conductance was calculated, assuming a constant cytosolic resistance over time. As it can be observed the continued presence of the microelectrode, lead to a much higher increase in membrane conductance compared to the intermittent measurements. Finally, continuous recordings gave rise to larger depolarization (results not shown) which is not in agreement with those of Fink & Luttgau, 1976, or Chapter 5. These depolarizations suggest that with continuous recordings during metabolic inhibition leads to cell membrane damage.


1800
1600
1400
1200
1000
800
600
400
200
0
CONDUCTANCE (%)
Intermittent impalement
Continuous impalement
0
10
20
30
40
50
60
TIME (minutes)