Denervation enhances the physiological effects of the $K_{\text{ATP}}$ channel during fatigue in EDL and soleus muscle

W. MATAR, J. A. LUNDE, B. J. JASMIN, AND J.-M. RENAUD

Department of Cellular and Molecular Medicine, University of Ottawa, Ottawa, Ontario, Canada K1H 8M5

Received 9 June 2000; accepted in final form 12 February 2001

Matar, W., J. A. Lunde, B. J. Jasmin, and J.-M. Renaud. Denervation enhances the physiological effects of the $K_{\text{ATP}}$ channel during fatigue in EDL and soleus muscle. Am J Physiol Regulatory Integrative Comp Physiol 281: R56–R65, 2001.—The objective was to determine whether denervation reduces or enhances the physiological effects of the $K_{\text{ATP}}$ channel during fatigue in mouse extensor digitorum longus (EDL) and soleus muscle. For this, we measured the effects of $100 \mu$M of pinacidil, a channel opener, and of $10 \mu$M of glibenclamide, a channel blocker, in denervated muscles and compared the data to those observed in innervated muscles from the study of Matar et al. (Matar W, Nosek TM, Wong D, and Renaud JM. Pinacidil suppresses contractility and preserves energy but glibenclamide has no effect during fatigue in skeletal muscle. Am J Physiol Cell Physiol 278: C404–C416, 2000). Pinacidil increased the 86Rb$^+$ fractional loss during fatigue, and this effect was 2.6- to 3.4-fold greater in denervated than innervated muscle. Pinacidil also increased the rate of fatigue; for EDL the effect was 2.5-fold greater in denervated than innervated muscle, whereas for soleus the difference was 8.6-fold. A major effect of glibenclamide was an increase in resting tension during fatigue, which was for the EDL and soleus muscle 2.7- and 1.9-fold greater, respectively, in denervated than innervated muscle. A second major effect of glibenclamide was a reduced capacity to recover force after fatigue, an effect observed only in denervated muscle. We therefore suggest that the physiological effects of the $K_{\text{ATP}}$ channel are enhanced after denervation.

tetanic force; resting tension; recovery; pinacidil; glibenclamide; 86Rb$^+$; adenosine 5'-triphosphate; phosphocreatine; Kir6.2

WHEN A SKELETAL MUSCLE is brought from rest to full activity, its metabolic rate increases considerably (44). Although muscles have mechanisms to increase ATP production during muscular activity, they do not always produce enough ATP to meet the demand, especially during strenuous activity. Thus muscles must have mechanisms that protect them against a large decrease in ATP to prevent impairment of muscle function. One of these mechanisms involves the $K_{\text{ATP}}$ channel.

$K_{\text{ATP}}$ channels are inactive at rest and become activated during fatigue when the concentration of ATP decreases and when the concentration of ADP, H$^+$, and adenosine increases (3, 4, 12, 38). Once activated, the $K_{\text{ATP}}$ channels have two physiological effects: 1) they reduce the force generated by the contractile apparatus during contraction; and 2) they prevent a large increase in resting tension, which occurs when muscles fail to completely relax between contractions (15, 17, 28, 43). The mechanisms by which $K_{\text{ATP}}$ channels affect the resting tension is unknown, but there are indications as to how they affect force development in skeletal muscle. $K_{\text{ATP}}$ channels allow for greater K$^+$ efflux, especially during action potentials, leading to increases in extracellular K$^+$ concentration (8, 11, 28). As the K$^+$ concentration increases, the cell membrane depolarizes (34), which inactivates Na$^+$ channels reducing the action potential overshoot (18). As an outcome of the effect on action potential, the amount of Ca$^{2+}$ released by the sarcoplasmic reticulum (28) and the force developed by the contractile apparatus decrease (7, 28, 43). Smaller Ca$^{2+}$ release and less activation of cross-bridges then result in less activity of the Ca$^{2+}$ ATPase and myosin ATPase allowing for the preservation of ATP (17, 28).

It is now well established that denervation affects the expression of several K$^+$ channels. For example, decreases in K$^+$ conductance resulting in falls in resting membrane potential and increases in membrane resistance are observed when muscle activity is completely abolished by denervation (1, 19, 33). A more recent study has now shown a decreased expression of the inwardly rectifying K$^+$ channel (IRK1 or Kir2.1; Ref. 36), which is important in maintaining resting membrane potential. Denervation also affects the expression of other K$^+$ channels, including the large conductance Ca$^{2+}$-activated K$^+$ channel (BK channel; Ref. 13), the small conductance Ca$^{2+}$-activated K$^+$ channel (SK channel; Ref. 32), and the Kv3.4 channel (41). However, little is known about the effect of denervation on $K_{\text{ATP}}$ channels.

Based on the important function of the channel, we hypothesized that the degree of muscle activity affects the expression of the $K_{\text{ATP}}$ channel where the most active muscles have the greatest level of $K_{\text{ATP}}$ channel.
activity and/or expression. Based on this hypothesis, it is then expected that denervation, which abolishes muscle activity, decreases the expression, activity, and physiological effects of the K$_{ATP}$ channel. One study has shown that lemakalim, a K$_{ATP}$ channel opener, hyperpolarizes the cell membrane and reduces twitch force of denervated diaphragm muscle whereas it has no effect in innervated muscle (19). Thus contrary to our hypothesis, denervation appears to enhance the efficacy of lemakalim to activate K$_{ATP}$ channel and/or the physiological effects of the channel. However, the study was carried out using resting unfatigued muscle, and under those conditions K$_{ATP}$ channels are normally inactive (3, 17, 25, 38). Therefore, it is still unknown as to how denervation alters the physiological effects of the K$_{ATP}$ channel during a metabolic stress, such as muscle fatigue.

The objective of this study was therefore to determine whether denervation reduces or enhances the physiological effects of K$_{ATP}$ channels. For this, we first determined during fatigue the effects of pinacidil, a K$_{ATP}$ channel opener, and glibenclamide, a channel blocker, in denervated muscle. We then compared our results to those from the study of Matar et al. (28) who recently studied the effects of these drugs in normal innervated muscles. Like Matar et al., we measured tetanic force, resting tension, ATP, and phosphocreatine (PCr) during fatigue as well as during recovery after fatigue using the extensor digitorum longus (EDL) and soleus muscles from a 2- to 3-mo-old CD-1 mice. These muscles have different fiber-type compositions (35) and different fatigue characteristics (28), and they sometimes have different responses to denervation as previously reported for the Kv3.4 mRNA content (41). We also measured the effect of denervation on the Kir6.2 mRNA content in EDL and soleus muscle, the Kir6.2 subunit being the protein that makes the pore of the K$_{ATP}$ channel (21). Our results and comparisons between denervated and innervated muscles showed that despite a decrease in Kir6.2 mRNA content in EDL and no change in soleus, the effects of pinacidil and glibenclamide are enhanced after denervation in both EDL and soleus muscles, suggesting that the K$_{ATP}$ channel becomes physiologically more important.

METHODS AND MATERIALS

Animals and Muscle Denervation

The Animal Care Committee of the University of Ottawa approved all experimental procedures. Two- to three-month-old female CD-1 mice weighing 25–30 g were obtained from Charles River and housed according to the guidelines of Canadian Council for Animal Care. The animals were fed ad libidum. Before surgery, animals were injected with 0.01 ml of the angesics buprenorphine (0.03 mg/ml). Muscle denervation was performed under anesthesia with halothane. The left hindlimb was denervated by excision of a 5-mm segment of the sciatic nerve at the thigh region. Subsequently, two more analgesic injections were administered to the animal within 24 h after the operation. Before any experiment, mice were tested for any reinnervation occurrences. The test consisted of suspending the animal by its tail and checking for the absence of a reflex extension of the foot and spreading of the toes. Preliminary experiments (as described Force Measurements and Fatigue and Recovery Protocol) showed that the effects of 1- and 2-wk denervation on the rate of fatigue (measured from the decrease in tetanic force) and force recovery after fatigue were not significantly different (ANOVA, P > 0.05, data not shown). Therefore, all subsequent experiments were carried out using the 1-wk denervation period.

Experimental Groups

One-week denervated muscles were divided into three experimental groups: 1) denervated control (no drug), 2) 10 µM of pinacidil, and 3) 10 µM of glibenclamide. Pinacidil activates K$_{ATP}$ channels with a near maximal effect at 100 µM in intact flexor digitorum muscle fibers (5). Glibenclamide, at 10 µM, blocks most (>95%) K$_{ATP}$ channel under patch-clamp condition (2) and during metabolic inhibition (5). At these respective concentrations, neither pinacidil nor glibenclamide affects other K+ channels (5, 26), the pCa-force curve of skinned muscle fibers (28), and the kinetics of fatigue in K$_{ATP}$ channel deficient mice (Kir2.2−/− mice; B. Gong and J.M. Renaud, unpublished results).

All denervated muscles were simultaneously tested with their contralateral innervated muscles, except that the latter were not exposed to glibenclamide or pinacidil. Data from the innervated muscles were first compared with those obtained from the control denervated group to determine that the denervation effects observed in this study were similar to those previously reported (see RESULTS and DISCUSSION). Also, for consistency between the three experimental conditions of denervated muscles, we compared the data among the innervated muscles. In this case, we found no significant difference among the three groups of innervated muscles (ANOVA, P > 0.05, data not shown). For statistical analyses (i.e., to keep the sample size to 5 muscles for all groups), we used the data from one of the three groups of innervated muscles; i.e., the contralateral innervated muscles of the denervated control.

Muscle Preparation and Solutions

Mice were anesthetized with an intraperitoneal injection of pentobarbital sodium (Somnotol), delivered at a dose of 0.8 mg/10 g body wt. EDL and soleus muscles were excised, and both tendons were tied with surgical silk (6.0) to allow attachment of muscle to the experimental apparatus. Muscles were always immersed in physiological saline solution containing (in mM): 118.5 NaCl, 4.7 KCl, 2.4 CaCl$_2$, 3.1 MgCl$_2$, 25 Na$_2$HCO$_3$, 2 NaH$_2$PO$_4$, and 5.5 n-glucose. The solution was continuously bubbled with 95% O$_2$–5% CO$_2$ and had a pH 7.4. The temperature of all experiments was 37°C. Glibenclamide and pinacidil containing solutions were prepared by first dissolving the drugs in DMSO before adding the proper volume to the saline solution. The final concentration of DMSO (including control solutions) was 0.1% (vol/vol).

Force Measurement

Measurements of tetanic force were carried out as described by Matar et al. (28). Briefly, tetanic force was measured with a Cambridge ergometer (model 300) and digitized with a Keithley Metabyte A-D board (model DAS550). Sampling rate was 5 kHz. Muscles were stimulated by passing a current between parallel platinum wires located on opposite sides of the muscle. Tetanic contractions were elicited with 200-ms long train of 0.3-ms long rectangular pulses of 8 V (supramaximal voltage). Stimulation frequencies were 140 Hz for soleus muscle and 200 Hz for EDL muscle.
Fatigue and Recovery Protocol

Muscle length was adjusted to give maximal tetanic force and allowed a 30-min equilibration period. During this period, tetanic contractions were elicited every 2 min. For all pinacidil- and glibenclamide-exposed muscles, drugs were added at the beginning of the equilibration period (i.e., 30 min before fatigue) and were present throughout the remainder of the experiment. After equilibration, fatigue was elicited with one tetanic contraction every second for 3 min. The decreases in tetanic force under this fatigue protocol are >70% (28) and are largely due to a failure of the sarcoplasmic reticulum to release adequate amount of Ca^{2+} upon stimulation (10, 14). If $K_{ATP}$ channels contribute to this failure as mentioned in the introduction, then pinacidil and glibenclamide will affect the kinetics of fatigue. After fatigue, muscles were stimulated at 10, 20, 100, and 200 s and 5 min and every 5 min thereafter until 30 min to measure the recovery of tetanic force.

ATP and PCR Measurement

These measurements were done as described by Matar et al. (28). Briefly, muscles were freeze-clamped in liquid nitrogen immediately after the equilibration period to establish rest-state values or immediately after fatigue to obtain fatigued values. Muscles were stored at −80°C until analyzed. The extraction of ATP and PCR was as described by Passoneau and Lowry (31) with some modifications as described in Matar et al. (28).

$^{86}$Rb$^+$ Fractional Loss Measurement

The methodology was as described by Matar et al. (28). Briefly, we used $^{86}$Rb$^+$ fractional loss to estimate the activity of $K_{ATP}$ channel, as the latter permeates $^{86}$Rb$^+$ at a similar permeability as K$^+$ (30). Muscles were loaded with $^{86}$Rb$^+$ (4–8 μCi/ml) for 60 min (with a change of fresh solution at 30 min). The $^{86}$Rb$^+$ loading was followed by four washout periods (15, 15, 5, and 5 min). The basal $^{86}$Rb$^+$ fractional loss was then obtained from three 5-min washout periods. During fatigue and the first 3 min of recovery, washout periods were 1-min long. $^{86}$Rb$^+$ fractional loss was also measured between the third and fifth and between the fifth and tenth minute of recovery. At the end of the experiment, muscles were homogenized in 2 ml of 6% perchloric acid. The homogenate was centrifuged at 10,000 g for 30 min. $^{86}$Rb$^+$ counting was done using a WinSpectral liquid scintillation counter (model 1414, Wallac Instruments), and quenching was corrected by counting 1 μCi of $^{86}$Rb$^+$ in 1 ml of physiological solution and another 1 μCi of $^{86}$Rb$^+$ in 1 ml of 6% perchloric acid.

RNA Extraction and Reverse-Transcription and Polymerase-Chain Reaction

Total RNA was isolated from EDL and soleus muscles by using TriPure Isolation Reagent (Life Sciences/Gibco) according to the manufacturer’s instructions. Briefly, after homogenization of the samples with a Polytron set at maximum speed, chloroform was added and the solution was vortexed vigorously before centrifugation at 12,000 g for 15 min at 4°C. The RNA contained in the resulting aqueous layer was precipitated with isopropanol, and the pellet was washed one time with 70% ethanol. The RNA was then resuspended in RNase-free water and stored at −80°C.

Quantification of the amount of total RNA in each sample was performed using the Pharmacia Gene Quant II RNA/DNA spectrophotometer. To assess the accuracy of these measurements, we have performed control experiments in which serial dilutions of total RNA ranging in concentration over one order of magnitude, i.e., 25, 50, 100, 200, and 400 ng as determined spectrophotometrically, were subjected to reverse-transcription (RT) and polymerase-chain reaction (PCR) using primers that selectively amplified S12 rRNA (see below). Because the greater proportion of total RNA is ribosomal, using primers for S12 should under these conditions reflect the total RNA content. Under our experimental RT-PCR conditions, we routinely obtained a $r^2 > 0.90$ between the amount of input total RNA and the relative intensity of the corresponding S12 rRNA PCR products for data plotted logarithmically (data not shown).

Each muscle sample was therefore adjusted to a final concentration of 50 ng of total RNA per microliter. Semi-quantitative RT-PCR assays were carried out as previously described in detail elsewhere (6, 9, 16, 22, 29, 40) using the GeneAmp RNA PCR kit (Perkin-Elmer). RT, using MuLV reverse transcriptase and random hexamers as primers, was performed for 45 min at 42°C with 100 ng of total RNA. The reaction was then terminated with a 5-min incubation at 99°C. PCR was then used to amplify cDNAs corresponding to Kir6.2 and S12 RNA. Primers that amplify Kir6.2 transcripts were synthesized according to their previously published sequence (17). These were designed as primer A (5’-3’: CTGGGGTCCGGATCGTGTAACCC) at position 697 bp and primer B (5’-3’: TCACAGCTCTAGACGCTCCTG) at position 1108 bp. The PCR cycling parameters included 1-min denaturation at 94°C followed by 3 min of primer annealing at 70°C. Subcloning and sequencing of the Kir6.2 RT-PCR product confirmed its identity. The rRNA primers used previously by others in RT-PCR assays were synthesized based on this previous report (13a). The 5’ (5’-3’: GGAAGGATAGCTGCTG) and 3’ (5’-3’: CCTCGATGACATCCTTG) primers amplified a 368 bp target. The cycling parameters for rRNA were 1 min denaturation at 94°C, 1 min primer annealing at 54°C, and 2-min extension at 72°C. For each reaction, a final 10-min elongation step was carried out at 72°C after the last cycle of amplification.

Quantification of the relative abundance of the PCR products was performed after agarose gel electrophoresis and visualization of the amplified cDNAs using the fluorescent dye VistraGreen (Amersham, Arlington Heights, IL). The analysis was performed using a Storm PhosphorImager and the accompanying ImageQuant software (Molecular Dynamics, Sunnyvale, CA). The intensity of the RT-PCR signals for Kir6.2$^{-/}$ was linearly related to the amount of specific RNAs amplified as it has been observed for other RNA (see Refs. 6, 16). The values obtained for Kir6.2 were standardized relative to the corresponding level of rRNA present in the same sample. In these experiments, negative controls consisted of reactions in which total RNA was replaced by RNase-free water.

All RT-PCR measurements aimed at determining the relative abundance of Kir6.2 mRNA, and rRNA was performed during the linear range of amplification (see examples of amplification curves in Refs. 9, 22, 29). Typically, the cycle numbers were 24 for Kir6.2 and 24 for rRNA. RT-PCR conditions (primer concentrations, input RNA, choice of RT primer, cycling conditions) were always run in parallel. In all experiments, PCR products were never detected for the negative controls.
Statistical Analysis

Data are reported as means ± SE. ANOVA was used to determine significant differences. Split plot designs were used when muscles were tested at all levels of a treatment (e.g., time effect during fatigue and recovery). In all other cases, a two-way ANOVA design was used. ANOVA calculations were made using the General Linear Model procedures of the Statistical Analysis Software (SAS Institute, Cary, NC). When a main effect or an interaction was significant, the least square difference was used to locate the significant differences (39). The word “significant” refers only to a statistical difference (P < 0.05).

RESULTS

Muscle Weight

One-week denervation caused significant decrease in muscle weight in both EDL and soleus muscle (ANOVA, P < 0.05). Denervated EDL muscles weighed an average 8.31 ± 0.24 mg (n = 15) compared with 9.38 ± 0.23 mg (n = 15) for the contralateral innervated EDL. For the soleus muscles, the respective values were 5.10 ± 0.24 mg (n = 15) and 7.13 ± 0.23 mg (n = 15).

Tetanic Force

Denervation had no significant effect on the tetanic forces measured before fatigue (ANOVA, P > 0.05). The tetanic forces of denervated and innervated EDL muscles were 39.4 ± 4.3 and 40.6 ± 4.8 N/cm² (n = 15), respectively. For the soleus muscles, the values were 32.6 ± 4.0 and 33.1 ± 3.2 N/cm² (n = 15), respectively. The addition of pinacidil, a K$_{ATP}$ channel opener, or glibenclamide, a channel blocker, 30 min before fatigue did not affect the tetanic force (data not shown), which is similar to the lack of effect in innervated muscles (28).

When fatigue was elicited with one tetanic contraction every second, the decrease in tetanic force was significantly less in denervated than in innervated EDL muscles. For example, during the first 30 s of stimulation, the tetanic force decreased to 46.0% of prefatigue force in denervated EDL compared with 34.8% in innervated EDL (Fig. 1A). The difference between denervated and innervated EDL remained significant until the end of the fatigue period. A reverse situation was observed with soleus, as the initial decrease in tetanic force was greater in denervated than innervated muscles. After 60 s of stimulation, the tetanic force of denervated soleus was 43.6% of prefatigue value compared with 65.3% for innervated soleus; a difference of 21.7% (Fig. 1B).

Ten micromolar of glibenclamide, a K$_{ATP}$ channel blocker, did not affect the decrease in tetanic force during fatigue for both denervated EDL and soleus muscles (Fig. 1). The K$_{ATP}$ channel opener pinacidil (100 μM), on the other hand, caused significantly greater reduction in tetanic force. In denervated EDL, the pinacidil effect lasted 30 s. At that time, the tetanic force of pinacidil-exposed muscle was 28.0% of the prefatigue value compared with 46.0% in denervated control muscle, a difference of 18.0%. In denervated soleus, the pinacidil effect lasted 60 s, as the differences in tetanic force between control and pinacidil-exposed muscles were 16.7 and 8.4% after 30 and 60 s of stimulation, respectively. Thus in denervated EDL and soleus, glibenclamide had no effect on tetanic force, while pinacidil affected the initial decrease but not the final extent of fatigue.

Resting Tension

A resting tension developed when muscles failed to fully relax between contractions during fatigue. The increases in resting tension in innervated EDL and soleus muscle were small and not significant, being 0.1 and 2.5%, respectively, of the prefatigue tetanic force.
at the end of the stimulation period (Fig. 2). Resting tension increased significantly more in denervated muscles, reaching final values of 4.3% in EDL and 4.8% in soleus. In the presence of glibenclamide, the increases in resting tension were greater and sooner as they became significant after 60 s in EDL and 30 s in soleus. At the end of the fatigue period, resting tension of glibenclamide-exposed EDL muscle was 10.1%, which was 2.3-fold greater than in denervated control. In glibenclamide-exposed denervated soleus, the resting tension reached 34.8%, representing a 7.3-fold increase compared with denervated control soleus. Pinacidil completely abolished the development of the resting tension observed in control denervated EDL and soleus muscle. Thus in denervated EDL and soleus muscle, glibenclamide, a K<sub>ATP</sub> channel blocker, caused greater increase in resting tension while pinacidil, a channel opener, had the reverse effect.

**Tetanic Force Recovery**

After fatigue, the tetanic force increased back toward its initial value as muscles recovered. Force recovery was significantly greater in denervated than innervated EDL and soleus muscle (Fig. 3). The final extent of force recovery measured after 30 min was 68.4% of the prefatigue tetanic force in denervated EDL compared with 44.0% in innervated EDL. For soleus muscle, the respective values were 91.5 and 76.8%. Glibenclamide significantly reduced the capacity of denervated muscles to recover their tetanic force. The effect was especially large in denervated EDL, which recovered only 43.9% of its prefatigue force. In soleus muscle, the glibenclamide effect was smaller and was observed only between the fifth and tenth minute of recovery when the tetanic forces of glibenclamide-exposed muscle were 9.8 and 12.9%, respectively, less than the force of denervated control muscle. Pinacidil did not affect force recovery in denervated EDL, whereas it increased the initial recovery during the first 5 min in denervated soleus by 6.9–13.3%.

**86Rb<sup>+</sup> Fractional Loss**

Basal 86Rb<sup>+</sup> fractional losses were measured to estimate the activity of K<sub>ATP</sub> channels as Rb<sup>+</sup> diffuses through the channel as K<sup>+</sup> does. The fractional losses of innervated and denervated EDL muscles measured before fatigue were similar, as the values were 0.0092 ± 0.0007/min and 0.0091 ± 0.0006/min, respectively. Basal 86Rb<sup>+</sup> fractional losses for soleus muscles were similar to those of EDL (data not shown). For EDL, there was no difference in the fractional losses between innervated and denervated muscles (Fig. 4A). For soleus, on the other hand, the 86Rb<sup>+</sup> fractional losses were 1.7- to 1.8-fold greater in denervated than innervated muscles throughout the fatigue period (Fig. 4B).
Fig. 3. Effects of glibenclamide and pinacidil on tetanic force recovery in 1-wk denervated EDL (A) and soleus muscles (B). Tetanic force is a percentage of tetanic force measured before fatigue. Fatigue was induced with one 200-ms-long tetanic contraction (200 Hz for EDL and 140 Hz for soleus) every second for 3 min. Experimental temperature was 37°C. Glibenclamide or pinacidil was added 30 min before fatigue and remained present during recovery.

Fig. 4. Effects of glibenclamide and pinacidil on 86Rb\(^{1}\) fractional loss in 1-wk denervated EDL (A) and soleus muscles (B). Muscles were loaded 1 h with 4–8 \(\mu\)Ci 86Rb\(^{1}\). After 4 initial washouts (15, 15, 10, and 5 min), the 86Rb\(^{1}\) fractional loss was measured at rest from 3 successive periods of 5-min washout. Muscles were then fatigued (at time \(\sim\) 3 min) with 200-ms-long tetani (200 Hz for EDL and 140 Hz for soleus) every 1 s for 3 min. Washout periods were 1-min long during fatigue and during 1st 3 min of recovery; last 2 washouts were 2- and 5-min long. Experimental temperature was 37°C. Data are change in fractional losses measured during fatigue and recovery (basal 86Rb\(^{1}\) fractional losses measured before fatigue are given in text). Symbols are plotted in middle of time period when washouts were taken. \(\circ\), denervated control muscle; \(\odot\), denervated muscle exposed to 10 \(\mu\)M glibenclamide; \(\odot\), denervated muscle exposed to 100 \(\mu\)M pinacidil; \(\bullet\), contralateral innervated muscle; horizontal bar, fatigue period. Verticals bars are SE of 7–8 muscles (absent when smaller than symbols). *Mean 86Rb\(^{1}\) fractional loss of denervated muscle was significantly greater than innervated muscle at same time period; ANOVA and LSD, \(P < 0.05\). Dotted line indicates level of fractional loss that is significantly greater than 0, ANOVA and LSD, \(P < 0.05\).
Glibenclamide did not affect the \(^{86}\text{Rb}^+\) fractional loss in both denervated EDL and soleus muscles (data not shown). Pinacidil significantly increased \(^{86}\text{Rb}^+\) fractional loss. The increases were 1.6- to 1.7-fold in denervated EDL and 1.3- to 1.5-fold in denervated soleus.

**ATP and PCr contents**

In EDL muscle, both ATP and PCr contents before fatigue were 25 and 32\%, respectively, less in denervated than innervated EDL muscles (Fig. 5). The decreases in ATP and PCr contents during fatigue were smaller in denervated than innervated EDL muscles, so that after fatigue there was no longer a difference between the two groups of muscles. Neither glibenclamide nor pinacidil affected the ATP and PCr content before fatigue. Pinacidil prevented the significant decrease in ATP contents observed in denervated control EDL, whereas glibenclamide had no effect. The effects of denervation, glibenclamide, and pinacidil on the ATP and PCr contents of soleus muscle were similar to those observed in EDL, except that pinacidil did not reduce the ATP depletion during fatigue as it did in EDL (data not shown).

**Kir6.2 mRNA Content**

As a first indication as to whether denervation affects the expression of the \(K_{\text{ATP}}\) channel, we measured the Kir6.2 mRNA contents in 1-wk denervated EDL and soleus muscles vs. the contralateral innervated muscles. As illustrated in Fig. 6A, denervation did not appear to affect expression of Kir6.2 transcripts in soleus muscles. In contrast, denervated EDL muscles showed an apparent reduction in Kir6.2 mRNA levels compared with innervated muscles. As measured by semiquantitative RT-PCR, denervated EDL had 50\% less Kir6.2 mRNA than innervated EDL, while in soleus, the expression of Kir6.2 was relatively unaffected by denervation (Fig. 6B). These results, therefore, are consistent with our hypothesis that expression of Kir6.2 is affected by muscle denervation but only in

---

Fig. 5. Effects of glibenclamide and pinacidil on ATP (A) and phosphocreatine (PCr; B) content in 1-wk denervated EDL muscles. Muscles were incubated for 30 min in the absence or presence of glibenclamide or pinacidil. Muscles were freeze-clamped immediately after the 30-min incubation period (muscles at rest) or after fatigue. Fatigue was induced with one 200-ms-long tetanic contraction (200 Hz for EDL and 140 Hz for soleus) every second for 3 min. Experimental temperature was 37\°C. □, ATP and PCr content in muscle at rest; ■, ATP and PCr content after fatigue. CON, control denervated muscle; GLI, denervated muscles exposed to 10-\(\mu\)M glibenclamide; PIN, denervated muscle exposed to 100-\(\mu\)M pinacidil; INN, contralateral innervated muscle. Vertical bars are SE of 8–10 muscles. §Mean ATP or PCr content was significantly less after than before fatigue; ANOVA and LSD, \(P < 0.05\). *Mean ATP or PCr contents of innervated muscles were significantly greater than in denervated muscles; ANOVA and LSD, \(P < 0.05\).

Fig. 6. Effect of denervation on Kir6.2 mRNA levels in mouse EDL and soleus muscles. A: shows an ethidium bromide-stained agarose gels containing RT-PCR products corresponding to Kir6.2 mRNA in innervated (I) and denervated (D) EDL and soleus (SOL) mouse muscles. B: Kir6.2 mRNA content in each muscle sample was quantified using fluorescent dye VistraGreen and a Storm PhosphorImager. Signal corresponding to Kir6.2 was determined and normalized to content of S12 rRNA, which acted as an internal control, present in same sample. Note that 1 wk of denervation induces a –50\% decrease in levels of Kir6.2 mRNA in fast EDL muscles while leaving expression of this \(K_{\text{ATP}}\) channel intact between innervated and denervated soleus muscles. Data are means ± SE of 6 muscles.
muscles containing primarily type IIX and IIB fibers, i.e., EDL.

**DISCUSSION**

This study shows that a 1-wk denervation period affects the muscle weight, the $^{86}$Rb$^+$ fractional loss, the decrease in tetanic force, the development of resting tension, and the ATP contents during fatigue as well as force recovery after fatigue. Pinacidil, a K$_{ATP}$ channel opener, affects all these parameters in denervated EDL and soleus muscle, whereas glibenclamide, a channel blocker, affects only the resting tension and the recovery of tetanic force.

Three of the denervation effects observed here are in agreement with those in previous studies. They include 1) the muscle weight loss (27); 2) the increased fatigue resistance in EDL and the decreased fatigue resistance in soleus (42); and 3) the lower ATP and PCr contents in resting, unfatigued denervated muscle compared with innervated muscles (23).

Denervation had no effect on the $^{86}$Rb$^+$ fractional loss during fatigue in EDL while it caused greater losses in soleus. $^{86}$Rb$^+$ was used as a marker for K$^+$ movement across the cell membrane (28). Thus the denervation effects on $^{86}$Rb$^+$ fractional loss suggest that denervation does not result in smaller K$^+$ permeability during fatigue. This is in contrast with the fact that the K$^+$ permeability in resting muscle decreases after denervation (24, 33). So, in general the denervation effects observed in this study are in agreement with those previously reported, except that the $^{86}$Rb$^+$ permeability does not decrease during fatigue as it does in unfatigued muscle.

**The Physiological Effects of the K$_{ATP}$ Channel in Denervated and Innervated Muscle**

On a qualitative basis, the glibenclamide and pinacidil effects were in most cases the same between denervated EDL and soleus muscle. That is, pinacidil increased the $^{86}$Rb$^+$ fractional losses (Fig. 4), which is in agreement with the fact that pinacidil activates K$_{ATP}$ channels (5, 7, 28). It also caused faster decrease in force (Fig. 1) and prevented an increase in resting tension (Fig. 2) during fatigue, while it improved force recovery after fatigue (Fig. 3). Glibenclamide, on the other hand, had no effect on the rate of fatigue and $^{86}$Rb$^+$ fractional loss, caused greater increased in resting tension, and reduced the capacity to recover force. These similarities between denervated EDL and soleus suggest that K$_{ATP}$ channels accomplish similar functions in both muscles.

On a quantitative basis, there were differences in the pinacidil and glibenclamide effects between denervated EDL and soleus muscle. The most striking difference was the increase in resting tension in the presence of glibenclamide, which was greater in soleus than EDL (Fig. 2). Another difference was the smaller decrease in ATP contents in the presence of pinacidil during fatigue in EDL (Fig. 5) but not in soleus muscle (data not shown). These differences, however, cannot be explained based on the results presented here. Further studies will be required to explain these differences.

The main objective of this study was to determine whether denervation modifies the physiological effects of the K$_{ATP}$ channel in skeletal muscle. For this we will now compare the pinacidil and glibenclamide effects observed in this study to those that have been reported for innervated muscles by Matar et al. (28).

**Pinacidil effects.** On the one hand, the pinacidil effects on the $^{86}$Rb$^+$ fractional loss, the initial rate and final extent of fatigue, and the resting tension and force recovery after fatigue in denervated (this study) muscle are qualitatively similar to those in innervated muscle (28). On the other hand, there are quantitative differences. First, the differences in $^{86}$Rb$^+$ fractional losses between pinacidil-exposed and control muscle ranged between 0.0101 and 0.0156/min for denervated soleus compared with 0.0032–0.0061/min for innervated soleus; i.e., the pinacidil effects are 2.6- to 3.2-fold greater in denervated than innervated soleus. For EDL, the pinacidil-dependent $^{86}$Rb$^+$ fractional losses were 3.4-fold greater during the first minute and 1.5-fold greater during the last 2 min of fatigue in denervated than innervated muscles.

Second, in denervated EDL and soleus muscles the decreases in tetanic force during the first 30 s of stimulation was 18% greater in the presence than in the absence of pinacidil. In innervated EDL and soleus the differences were 7.1 and 2.1%, respectively. Thus the pinacidil effects on force decay during the first 30 s are 2.5-fold greater in denervated EDL and 8.6-fold greater in denervated soleus compared with their innervated counterparts.

Pinacidil prevented the development of resting tension during fatigue that is observed in control muscle. However, in this case, it is not possible to compare the pinacidil effect between denervated and innervated muscles because we cannot estimate the true effect of pinacidil as resting tension is always zero in the presence of pinacidil (28). Pinacidil also improves force recovery after fatigue in denervated (Fig. 3) and innervated muscles. Again, a comparison between denervated and innervated muscle is not possible as force recovery is close to 100% for denervated EDL, Fig. 3A and 100% for denervated soleus, Fig. 3B). Finally, the decrease in ATP was less in the presence than in the absence of pinacidil. However, for innervated muscles this effect was observed only in soleus, whereas for denervated muscles the effect was observed only in EDL.

Thus except for the ATP contents, pinacidil has qualitatively similar effects in denervated and innervated EDL and soleus muscles. On a quantitative basis, the pinacidil effects on $^{86}$Rb$^+$ fractional loss and the decrease in force during fatigue are greater in denervated than innervated muscle.

**Glibenclamide effects.** In innervated EDL and soleus muscle, glibenclamide has no effect on the $^{86}$Rb$^+$ fractional loss and decay in tetanic force while it causes greater development of resting tension during fatigue
(28). Shivkumar et al. (37) have shown that blocking K_{ATP} channels during hypoxia does not reduce K^+ and Rb^+ effluxes. They argued that the effluxes depend primarily on an accumulation of intracellular Na^+, which forces K^+ and Rb^+ throughout other K^+ channels. Thus the lack of a glibenclamide effect on ^{86}Rb^+ fractional loss is not indicative of a lack of K_{ATP} channel activation during fatigue (in the absence of pinacidil). Gong et al. (15) showed that a K_{ATP} channel deficiency (from Kir6.2^{−/−} mouse) like glibenclamide fails to affect the rate of fatigue in EDL and soleus muscle. They also showed that the increases in resting tension were quantitatively similar between glibenclamide-exposed wild-type muscles and Kir6.2^{−/−} muscles. Finally, glibenclamide has no effect on resting tension during fatigue in K_{ATP} channel deficient muscle (B. Gong and J.-M. Renaud, unpublished results).

We therefore suggest that denervation enhances the physiological effects of the K_{ATP} channel during fatigue and that the only effect of the K_{ATP} channel is to reduce the development of resting tension.

In denervated EDL and soleus, glibenclamide also fails to affect ^{86}Rb^+ fractional loss (Fig. 4) and the decrease in tetanic force (Fig. 1), while it caused greater development of resting tension (Fig. 2). We therefore suggest that K_{ATP} channels are also activated during fatigue in denervated EDL and soleus muscle and that their effect is limited to an effect on resting tension as observed in innervated muscle. Resting tension in the presence of glibenclamide reached 37% of the prefatigue tetanic force in denervated soleus (Fig. 2B) compared with only 20% in innervated soleus (28), which represents a 1.9-fold difference. Likewise, the glibenclamide-sensitive increase in resting tension was 2.7-fold greater in denervated than innervated EDL.

Glibenclamide also reduced the capacity to recover tetanic force after fatigue in denervated muscle (Fig. 3). This effect was especially large for denervated EDL muscle where the difference between control and glibenclamide-exposed muscles varied between 20 and 25% throughout the recovery period. Interestingly, glibenclamide has no effect on force recovery in innervated EDL and soleus muscle (28). Thus like for the pinacidil effects, glibenclamide had greater effects in denervated than innervated EDL and soleus muscle. We therefore suggest that denervation enhances the physiological effects of the K_{ATP} channel during fatigue in mouse EDL and soleus muscle. Our observations are also in agreement with those of Hong and Chang (19) who showed in unfatigued muscle that the effects of activating K_{ATP} channels on resting potential and twitch force are greater in denervated than innervated diaphragm (19).

One possible explanation for this denervation effect would be an increased expression of the K_{ATP} channels in the cell membrane. To address this question, we used semiquantitative RT-PCR to examine the level of Kir6.2 mRNA, which encodes for the protein that makes the pore of the channel. However, we find that levels of Kir6.2 message do not appear to be affected by denervation in soleus muscle, while in EDL, 1-wk denervation seems to elicit a reduction in Kir6.2 mRNA (Fig. 6).

In summary, the pinacidil effects on ^{86}Rb^+ fractional loss and tetanic force during fatigue and the glibenclamide effects on resting tension and force recovery were greater in denervated than in innervated EDL and soleus muscle. Therefore, we suggested that denervation enhances the physiological effects of the K_{ATP} channel during fatigue and recovery.

**Perspectives**

It was interesting to note that the physiological effects of the K_{ATP} channel were enhanced after 1-wk denervation despite a decrease in the mRNA content of Kir6.2 (at least in EDL muscle). It is therefore possible that the decrease in mRNA content is overcompensated by greater translation of the Kir6.2 mRNA resulting in more of the K_{ATP} channel proteins in the cell membrane. However, it is also possible that the number of K_{ATP} channels in the cell membrane is less in denervated than innervated muscle. In this case, the enhancement of the physiological effects of pinacidil and glibenclamide would be because 1) a greater proportion of K_{ATP} channels are activated during fatigue in denervated than innervated muscle; or 2) the decrease in the number of K_{ATP} channel is relatively less than for other K^+ channels, like the Kir2.1, BK, and Kv3.4 channel (13, 36, 41). In both cases, the relative contribution of K_{ATP} channels to the total K^+ conductance becomes greater in denervated than innervated muscle resulting in an enhanced physiological effect. It will therefore be necessary to determine in future studies how denervation affects 1) the K_{ATP} channel protein content in the cell membrane and 2) to compare the change in protein content to that of other K^+ channels to better understand how denervation (or a change in muscle activity) affects the expression of the K_{ATP} channel.

This study was supported by a grant from the National Science and Engineering Research Council to J.-M. Renaud.

**REFERENCES**


