Pinacidil suppresses contractility and preserves energy but glibenclamide has no effect during muscle fatigue

W. MATAR,1 T. M. NOSEK,2 D. WONG,1 AND J.-M. RENAUD1
1Department of Cellular and Molecular Medicine, University of Ottawa, Ottawa, Ontario, Canada K1H 8M5; and 2Department of Physiology and Endocrinology, Medical College of Georgia, Augusta, Georgia 30912-3000

Matar, W., T. M. Nosek, D. Wong, and J.-M. Renaud. Pinacidil suppresses contractility and preserves energy but glibenclamide has no effect during muscle fatigue. Am. J. Physiol. Cell Physiol. 278: C404–C416, 2000.—The effects of 10 µM glibenclamide, an ATP-sensitive K+ (KATP) channel blocker, and 100 µM pinacidil, a channel opener, were studied to determine how the KATP channel affects mouse extensor digitorum longus (EDL) and soleus muscle during fatigue. Fatigue was elicited with 200-ms-long tetanic contractions every second. Glibenclamide did not affect rate and extent of fatigue, force recovery, or 86Rb+ fractional loss. The only effects of glibenclamide during fatigue were: an increase in resting tension (EDL and soleus), a depolarization of the cell membrane, a prolongation of the repolarization phase of action potential, and a greater ATP depletion in soleus. Pinacidil, on the other hand, increased the rate but not the extent of fatigue, abolished the normal increase in resting tension during fatigue, enhanced force recovery, and increased 86Rb+ fractional loss in both the EDL and soleus. During fatigue, the decreases in ATP and phosphocreatine of soleus muscle were less in the presence of pinacidil. The glibenclamide effects suggest that fatigue, elicited with intermittent contractions, activates few KATP channels that affect resting tension and membrane potentials but not tetanic force, whereas opening the channel with pinacidil causes a faster decrease in tetanic force, improves force recovery, and helps in preserving energy.

skeletal muscle; tetanic force; resting potential; action potential; rubidium-86; phosphocreatine; extensor digitorum longus; adenosine 5’-triphosphate

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

There are two mechanisms by which opening KATP channels could reduce force. First, opening the channels increases K+ conductance, which shortens action potential duration (19, 21). Second, opening the channels increases K+ efflux, which increases extracellular K+ concentration (11, 19), depolarizes the membrane (24), and inactivates Na+ channels (1). Together, shortening of the action potential and inactivation of Na+ channels reduces membrane excitability, decreases Ca2+ release from intracellular stores, and decreases force production (13). As the amount of Ca2+ released and force decrease, Ca2+-ATPase and myosin ATPase utilize less energy. Indeed, blocking KATP channels with glibenclamide has been shown to cause larger decreases in ATP and phosphocreatine (PCr) levels during ischemia in cardiac muscle (31) and during metabolic inhibition in skeletal muscle (21).

The role of the KATP channel during fatigue in skeletal muscle is still not well understood. We have previously reported that blocking KATP channels with glibenclamide impaired force recovery of frog sartorius muscle following fatigue even though it did not affect the decrease in force during fatigue (29). Van Lunteren et al. (41) also found that glibenclamide did not affect force production of diaphragm muscle during continuous stimulation at 5 or 100 Hz. In these two studies, however, the glibenclamide concentration was 100 µM, a concentration that increases the Ca2+ sensitivity of contractile components (13), i.e., glibenclamide may have had some nonspecific effects that masked the expected slowing of the rate of fatigue.

In another study, glibenclamide did not affect force of rat extensor digitorum longus (EDL) muscle continuously stimulated at 0.2 Hz (43). However, in this case, the glibenclamide concentration was only 1 µM, which is not large enough to block all KATP channels (2, 3). Finally, Duty and Allen (13) reported that glibenclamide (50–100 µM) affects the Ca2+ transient and force during fatigue, but only in three out of six single fibers from mouse flexor digitorum brevis (FDB) muscle. It is thus not clear whether KATP channels contribute to the decrease in force during fatigue.

The overall objective of this study was to determine how modulating KATP channel activity with both a channel agonist (pinacidil) and antagonist (glibenclamide) during fatigue affects muscle function (tetanic force).
force, resting and action potentials, 86Rb+ efflux, ATP and PCr contents). Two muscles were used for this study: the mouse soleus muscle, which is predominantly composed of slow fiber types (40% type I and 60% type IIA fibers) (44), and the mouse EDL muscle, which is predominantly composed of fast fiber types (68% type IIB, 20% type IIX, and 12% type IIA fibers) (36). The strategy of this study was to use concentrations of glibenclamide and pinacidil that were effective in, respectively, blocking or activating K<sub>ATP</sub> channels (see MATERIALS AND METHODS) and that had no effect on the contractile apparatus (see RESULTS). The effects of pinacidil observed in this study suggest that activating K<sub>ATP</sub> channels can preserve energy and protect muscle function as originally proposed by Noma (34). However, the effects of glibenclamide suggest that a significant number of K<sub>ATP</sub> channels is not activated when mouse EDL and soleus are fatigued with one tetanic contraction every second.

MATERIALS AND METHODS

Animals and Muscle Preparation

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

Pinacidil and Glibenclamide as Modulators of K<sub>ATP</sub> Channel Activity

Pinacidil (100 µM) was used to activate K<sub>ATP</sub> channels because it is the most effective channel opener in skeletal muscle. Pinacidil has a dissociation constant (K<sub>d</sub>) of 31.5 µM and a maximum effect near 100 µM (6). Other channel openers such as diazoxide have no effect in skeletal muscle, whereas levromakalim has a K<sub>d</sub> of 186 µM, with no maximum effect at 400 µM. Pinacidil also does not affect other K<sub>ATP</sub> channels, except for the K<sub>ATP</sub> channel (6). Glibenclamide was used to block the K<sub>ATP</sub> channel, since its effectiveness and specificity are well characterized in skeletal muscle. In most experiments, glibenclamide was used at 10 µM, because at that concentration it blocks most (~95%) K<sub>ATP</sub> channels under patch-clamp conditions (2) and during metabolic inhibition (3). A concentration of 100 µM was used for action potential measurements (see Measurement of Resting and Action Potentials). At 100 µM, glibenclamide has no effect on voltage-sensitive K<sup>+</sup> channels, Ca<sup>2+</sup>-activated K<sup>+</sup> channels, and whole cell currents in voltage-clamp (~60 mV to +32 mV) experiments on rat skeletal muscle (6, 30). Duty and Allen (13) have reported that 50–100 µM glibenclamide shifts the pCa-force curve of intact muscle fiber of mouse FDB muscle toward lower Ca<sup>2+</sup> concentrations. Glibenclamide is highly soluble in lipid membranes and has a poor solubility in water (14, 16). It is thus not possible to ascertain what was the intracellular concentration of glibenclamide that affected the contractile components in those experiments.

Measurement of Force

In Skinned Muscle Fibers

Membrane disruption was accomplished by exposing small bundles of EDL and soleus muscle fibers to a skinning solution for 30 min at 22°C. The skinnning solution contained 0.1% wt/wt Triton X-100 (a nonionic detergent that permeabilizes the sarcotendinous membrane and all subcellular organelles), 1.0 mM Mg<sup>2+</sup>, 5.0 mM MgATP, 15 mM PCr, 140.0 mM potassium methanesulfonate, 50.0 mM imidazole (200 ionic strength), 10.0 mM EGTA (pCa > 8.5, pH 7.0 at 22°C). The skinnning solution also contained a cocktail of protease inhibitors to protect the fibers from the damaging effects of proteolysis (0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM leupeptin, 1.0 mM benzamidine, 10 µM aprotinin, and 1.0 mM dithiothreitol). Skinned fibers prepared this way were used on the same day the animal was killed.

Single EDL and soleus muscle fibers (~4,000 × 25–50 µm) were then isolated by holding one end of the muscle with a pair of forceps and pulling on the other end to free single fibers. Single fibers were mounted between an optoelectric force transducer (Scientific Instruments, Heidelberg, Germany) and a movable arm by wrapping the fibers several times around small stainless steel wires followed by a brief exposure to pCa 4.0 to secure the fibers to the wire. The length of the fiber between the wires after mounting was ~2,000 µm. The fibers were bathed in solutions contained in 2.5-ml troughs milled in a spring-loaded Plexiglas plate. The composition of all solutions was calculated using a computer program (Borland International, Scotts Valley, CA) employing the stability constants commonly used (20). To avoid any possible source of transitional metals contaminating the bathing media, all solutions were prepared with nanopure water run through a Chelex column.

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

Force Measurements in Intact EDL and Soleus Muscle

Muscle chamber and solutions. Force measurements from intact EDL and soleus muscles were carried out using a chamber that was 0.9 cm wide, 1.7 cm long, and 1.0 cm deep. One muscle tendon was tied to a lightweight stainless steel wire that was hooked to a force transducer. The other tendon was held in place between two Teflon clamps. The flow of fresh physiological saline solution was 15 ml/min through tubing located in both Teflon clamps. This allowed the solution to flank the top and bottom of the muscle alongside its length (as observed with a blue dye). The composition of the physiologi-
cal saline solution for control conditions contained (in mM) 118.5 NaCl, 4.7 KCl, 2.4 CaCl₂, 3.1 MgCl₂, 25 Na₂HCO₃, 2 NaH₂PO₄, and 5.5 d-glucose, pH 7.4 (95% O₂-5% CO₂). Glibenclamide- and pinacidil-containing solutions were prepared by first dissolving the drugs in DMSO and then adding the proper volume to the control solution. The final DMSO concentration was 0.1% (vol/vol) in all solutions, including control solution. The experimental temperature was 37°C at all times.

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

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

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

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

Measurement of Resting and Action Potentials

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

Measurement of K<sub>ATP</sub> channel current using voltage-clamp or patch-clamp techniques is impossible during muscle contraction because of possible damage to the cell membrane by microelectrodes. We therefore measured 86Rb<sup>+</sup> efflux before, during, and after fatigue to estimate the activity of K<sub>ATP</sub> channels. 86Rb<sup>+</sup> is qualitatively a satisfactory marker for K<sup>+</sup> movements through several K<sub>ATP</sub> channels, including the K<sub>ATP</sub> channel (9).

Solutions, muscle stimulation, and the fatigue protocol were all as described for force measurements. The muscle chamber was a methyl acrylate cuvette in which muscles were attached vertically at one end to a hook and the other to a Grass FT03 force transducer. Tetanic forces were recorded on a Grass Polygraph (Model 7D). Muscles were immersed throughout the experiment in 1.7 ml of solution constantly bubbled with 95% O₂-5% CO₂ and maintained at 37°C. Muscles were loaded with 86Rb<sup>+</sup> (4–8 µCi/ml) for 60 min (with a change of fresh solution after 30 min). 86Rb<sup>+</sup> loading was followed by an initial 40-min washout in a series of three cuvettes (15, 15, and 10 min) containing 1.7 ml of nonradioactive saline solution (preliminary washout experiments showed that a constant 86Rb<sup>+</sup> efflux was reached after 40 min, results not shown). Glibenclamide or pinacidil were added during the initial washout so muscles would be exposed to either drug 30 min before fatigue as described above for the experiments on force measurements.

Basal 86Rb<sup>+</sup> efflux was measured before fatigue by changing the cuvette three times every 5 min. During the 3-min fatigue period and the first 3 min of recovery, cuvettes were changed every minute. The last two cuvettes were used to measure 86Rb<sup>+</sup> efflux between the 3rd and 5th and between the 5th and 10th minutes of recovery. From each cuvette, 1.0 ml of incubation fluid was added to 10.0 ml of biodegradable counting scintillant (CBS; Amersham) for radioactive counting. The 86Rb<sup>+</sup> content remaining in muscles was determined by homogenizing muscles in 2 ml of 6% perchloric acid. The resulting solution was centrifuged at 10,000 g for 10 min, and 1.0 ml of the supernatant was added to the counting scintillant. 86Rb<sup>+</sup> counting was done using a WinSpectral liquid scintillation counter (model 1414; Wallac Instruments). Quenching was corrected by counting 1 µCi of 86Rb<sup>+</sup> in 1.0 ml of physiological solution and another 1 µCi in 1.0 ml of 6% perchloric acid.

Measurement of 86Rb<sup>+</sup> Efflux Measurements

Solutions, fatigue protocol, muscle stimulation, and experimental chamber were all as described for 86Rb<sup>+</sup> efflux measurements. All muscles were first allowed to equilibrate 30 min in the absence (control) or presence of glibenclamide or pinacidil. Muscles were then freeze-clamped in liquid nitrogen immediately after the 30-min equilibrium or after the 3-min fatigue period. All muscles were stored at −80°C until analyzed. The extraction of ATP and PCR was as described by Passoneau and Lowry (35), with some modifications. Briefly, muscles were freeze-dried overnight with a freeze drier (model Freezemobile 6; Virtis). Tendons were then removed and muscle tissues broken in small pieces under a microscope. A weighed amount of dried muscle tissue (0.8–2.0 mg)
was added to 400 µl of ice-cold 6% perchloric acid. The solution was then sonicated with a Microson ultrasonic cell disruptor (Heat Systems-Ultrasonics) at maximum power for 15 s. After centrifugation at 10,000 g and 4°C for 30 min, the supernatant was neutralized with ice-cold 3 M K₂CO₃. K⁺ salt was precipitated at 10,000 g and 4°C for 15 min. ATP and PCr content was measured from the supernatant according to the enzymatic test described by Passoneau and Lowry (35) using a spectrophotometer (model DU 640; Beckman). All assays contained 10 µM P₁₁P₅⁻di(adenosine-5`)pentaphosphate to inhibit any myokinase that resisted the perchloric acid precipitation (35).

Statistical Analysis

ANOVA was used to determine significant differences. Split plot designs were used when muscles were tested at all levels of a treatment (e.g., time effect during fatigue and recovery). In all other cases, a two-way ANOVA design was used. ANOVA calculations were made using the General Linear Model procedures of the SAS statistical software (SAS Institute, Cary, NC). When a main effect or an interaction was significant, the least square difference was used to locate any significant difference (40). The word “significant” refers only to a statistical difference (P < 0.05).

RESULTS

Effects of Pinacidil and Glibenclamide on Contractile Components

Neither 100 µM pinacidil nor 10 µM glibenclamide had an effect on the pCa-force curve of skinned soleus muscle fibers (Fig. 1, A and B). As a positive control, we measured the effect of 100 µM glibenclamide. At that concentration, glibenclamide shifted the pCa-force curve toward lower Ca²⁺ concentration. Ca₅₀ values in the absence and presence of 100 µM glibenclamide were, respectively, 1.5 and 1.0 µM. The effects of 100 µM pinacidil and 10 and 100 µM glibenclamide were the same in EDL skinned muscle fibers (results not shown). To determine the concentration at which glibenclamide affects the contractile components, a dose-response curve was measured while soleus skinned muscle fibers were exposed at pCa 6.2. Glibenclamide had no effect at 10 µM, whereas it significantly increased force when its concentration equaled or exceeded 20 µM (Fig. 1C).

The above experiments were carried out at 22°C because of the instability of skinned fibers at 37°C (the temperature used for the fatigue experiments). To further test that 10 µM glibenclamide has no effect on the contractile apparatus of intact EDL and soleus muscle at 37°C, we measured the glibenclamide effect on twitch force and force-frequency curve over a 2-h period. KATP channels are inactive in unfatigued muscle (3). Thus any increase in twitch force or shift in force-frequency curve toward lower frequencies would be evidence that glibenclamide increases the Ca²⁺ sensitivity of the contractile components. Glibenclamide at 10 µM had no effect on twitch force, whereas at 100 µM it caused the expected significant increase in both EDL (Fig. 2) and soleus muscle (results not shown). Glibenclamide at 10 µM also had no effect on the force-frequency curves of unfatigued EDL (Fig. 3).
and soleus muscle (results not shown). Thus the results from Figs. 1–3 demonstrate that using 10 µM glibenclamide to block and 100 µM pinacidil to activate K\textsubscript{ATP} channels during fatigue (next experiments) does not affect the contractile apparatus of EDL and soleus muscle.

**Effects of Glibenclamide and Pinacidil on Contractility During Fatigue**

**Effect before fatigue.** Measured at the beginning of each experiment, mean tetanic forces of control EDL and soleus muscles were, respectively, 36.5 ± 0.5 and 30.1 ± 0.2 N/cm² (n = 10 muscles). Mean tetanic forces of the pinacidil and glibenclamide groups were not significantly different from those of control (data not shown). The tetanic force of control EDL muscles decreased by 2.18 ± 0.36% (n = 10) during the 30-min equilibrium period before fatigue. In the presence of 10 µM glibenclamide or 100 µM pinacidil, the decreases were, respectively, 4.82 ± 2.62% and 4.21 ± 2.65% (n = 5). For soleus muscles, the decreases were 0.10 ± 0.55% (control), 2.31 ± 1.05% (glibenclamide), and 0.50 ± 0.75% (pinacidil). None of those decreases were significant (ANOVA P > 0.05).

**Rate of fatigue.** The rate of fatigue was measured from the decrease in tetanic force when muscles were stimulated with one tetanic contraction every second. The rate of fatigue was faster in control EDL muscle than in control soleus muscle. For example, after 1 min of stimulation, the tetanic force of soleus muscle was 74.1% of the prefatigue force compared with 9.3% for EDL muscle (Fig. 4). The extent of fatigue was also greater in EDL: at the end of the 3-min fatigue period, tetanic force of EDL muscle was 6.7% compared with 29.7% for soleus muscle. Glibenclamide at 10 µM had no effect on the decrease in tetanic force during fatigue for both EDL and soleus. On the other hand, 100 µM pinacidil caused a faster decrease in tetanic force when compared with control muscles. In EDL muscle, the pinacidil effect was significant only during the first 30 s of stimulation (Fig 4A). Mean tetanic forces of pinacidil-exposed EDL after 15 and 30 s were, respectively, 15.7 and 8.55% less than the mean forces of control EDL.

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

**Resting tension.** A resting tension developed during fatigue when muscles failed to completely relax between contractions. The increase in resting tension was quite variable and not observed in all control muscles. Despite the variability, two noticeable effects were observed in the presence of glibenclamide and pinacidil. To best analyze these effects, we pooled together data from all muscles used in this study (see fatigue kinetics of Fig. 4, \textsuperscript{86}Rb\textsuperscript{+} fractional loss in Fig. 8, and ATP and PCR content in Fig. 9).

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

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

Recovery of tetanic force. Tetanic force increased toward its prefatigue value when the fatigue stimulation was stopped. The initial rate and extent of force recovery was greater in control soleus than in control...
EDL muscle (Fig. 6). After 200 ms of recovery, the tetanic force of EDL muscle was 20.3% of the prefatigue force compared with 62.5% in soleus muscle. Tetanic force reached a maximum of 89.9% after 15 min of recovery compared with 39.8% after 30 min of recovery in EDL muscle. For both EDL and soleus muscle, the recovery of tetanic force in the presence of 10 µM glibenclamide was slightly less than in control. This difference was not significant.

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

Effects of Glibenclamide and Pinacidil on Membrane Potential

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

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

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

To determine whether an activation of KATP channels by pinacidil was large enough to alter membrane potentials, we studied how pinacidil affects resting and action potentials in soleus muscle at rest. Pinacidil significantly reduced the mean overshoot by 8.8 mV (Table 1) but did not cause a shortening of the action potential. In fact, the mean half-repolarization time was significantly longer by 0.019 ms in the presence of pinacidil. These effects were observed while the resting

![Fig. 6. Effects of glibenclamide and pinacidil on tetanic force recovery of EDL (A) and soleus (B) muscles. Force recovery was measured after fatigue had been elicited with one 200-ms-long tetanic contraction (200 Hz for EDL and 140 Hz for soleus) every second for 3 min. Glibenclamide or pinacidil was added 30 min before fatigue and remained present during recovery. Experimental temperature was 37°C. Tetanic force is expressed as a percentage of the tetanic force measured before fatigue. ○, Control; □, 10 µM glibenclamide; △, 100 µM glibenclamide; ○, 100 µM pinacidil. Vertical error bars represent SE of 5 muscles (absent when smaller than symbols). *Mean tetanic force in presence of glibenclamide or pinacidil was significantly different from mean tetanic force in control muscle during same time period (ANOVA, LSD, $P < 0.05$).]
potential was unaffected by pinacidil. In the presence of 10 µM glibenclamide, pinacidil did not affect the overshoot, whereas the half-repolarization time was still prolonged.

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

Effect of Glibenclamide and Pinacidil on $^{86}$Rb$^+$ Efflux

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

The $^{86}$Rb$^+$ fractional losses at rest (results not shown) and during fatigue (Fig. 8) were not affected by 10 µM glibenclamide. Even at 100 µM, glibenclamide failed to reduce $^{86}$Rb$^+$ fractional loss during fatigue (results not shown). Pinacidil also had no effect on $^{86}$Rb$^+$ fractional losses in resting EDL (0.0079 ± 0.0005, n = 7) and soleus (0.0078 ± 0.0007, n = 8) muscle. However, it significantly increased the losses during fatigue. In EDL muscle, $^{86}$Rb$^+$ fractional loss during the first minute was 1.3 times greater in the presence of pinacidil than in control muscles (Fig. 8A). The differences increased to 2.0 times for the last 2 min of the fatigue period. $^{86}$Rb$^+$ fractional losses in the presence of pinacidil also continued to remain above those of control until the third minute of recovery. In soleus muscle, pinacidil significantly increased $^{86}$Rb$^+$ fractional losses by 1.4–1.5 times above those of control only during the first 2 min of fatigue. Thereafter, the $^{86}$Rb$^+$ fractional losses were similar in control and pinacidil-exposed muscle.

### Table 1. Effect of pinacidil on resting and action potentials of soleus muscle

<table>
<thead>
<tr>
<th>Experimental Condition</th>
<th>Resting Potential, mV</th>
<th>Overshoot, mV</th>
<th>Half-repolarization Time, ms</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>A:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>−74.8±1.9</td>
<td>23.4±1.6</td>
<td>0.222±0.008</td>
<td>4/24</td>
</tr>
<tr>
<td>100 µM Pinacidil</td>
<td>−76.6±4.1</td>
<td>14.6±1.4*</td>
<td>0.241±0.004*</td>
<td>4/25</td>
</tr>
<tr>
<td>Washout</td>
<td>−77.4±1.1</td>
<td>19.2±2.0</td>
<td>0.225±0.001</td>
<td>4/18</td>
</tr>
<tr>
<td>B:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>−76.4±1.9</td>
<td>19.2±1.1</td>
<td>0.219±0.008</td>
<td>3/12</td>
</tr>
<tr>
<td>10 µM Glibenclamide</td>
<td>−77.3±1.8</td>
<td>18.7±1.4</td>
<td>0.236±0.003*</td>
<td>3/15</td>
</tr>
<tr>
<td>100 µM Pinacidil</td>
<td>−77.4±2.0</td>
<td>20.4±1.5</td>
<td>0.231±0.013</td>
<td>3/16</td>
</tr>
</tbody>
</table>

Values are means ± SE. Action potentials were measured first in the absence of pinacidil and glibenclamide (Control). A: action potentials were measured 20 min after the addition of 100 µM pinacidil. B: action potentials were measured after exposing muscle 20 min to 10 µM glibenclamide, and then 20 min to 10 µM glibenclamide and 100 µM pinacidil. Action potentials were also measured 2 h after drug washout. Experimental temperature was 37°C. n = Number of muscles/number of fibers. *Mean value was significantly different from the “Control value,” ANOVA, LSD, P < 0.05.

### Effects of Glibenclamide and Pinacidil on ATP and PCr Content

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

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

Contrary to the situation observed in EDL muscle, both glibenclamide and pinacidil significantly altered ATP and PCr content of soleus muscle. Glibenclamide had no effect before fatigue but caused a significantly greater depletion of ATP during fatigue. After fatigue, the ATP content of control soleus muscle was 3.5 µmol/g dry wt lower than the value at rest, whereas for glibenclamide-exposed muscle the decrease was 11.6 µmol/g dry wt. Before fatigue, the ATP and PCr contents of pinacidil-exposed muscles were, respectively, 4.2 and 23.7 µmol/g dry wt less than in control soleus.
During fatigue, PCr content decreased by 38.4 µmol/g dry wt in the presence of pinacidil compared with 50.1 µmol/g dry wt in control muscles. For ATP, the content increased by 1.6 µmol/g dry wt in the presence of pinacidil, whereas it decreased by 3.8 µmol/g dry wt in control soleus.

DISCUSSION

The major findings of this study are that, during fatigue, glibenclamide-exposed muscles rarely showed any physiological difference when compared with control EDL and soleus muscles, whereas pinacidil had several effects. During fatigue, glibenclamide 1) caused greater increase in resting tension, 2) caused greater depletion of ATP content (only in soleus), and 3) abolished the cell membrane hyperpolarization and shortening of action potential duration. Glibenclamide did not affect ⁸⁶Rb⁺ fractional loss, rate of fatigue, and force recovery. Pinacidil 1) increased the rate of fatigue, 2) abolished the increase in resting tension, 3) increased the rate of force recovery, 4) increased ⁸⁶Rb⁺ fractional loss, 5) reduced the action potential overshoot, and 6) reduced ATP and PCr depletion. The effects of pinacidil then suggest that K<sub>ATP</sub> channels can influence tetanic force during fatigue. However, the effects of glibenclamide suggest that they do not influence tetanic force when mouse EDL and soleus muscles are fatigued with one tetanic contraction every second.

Specificity of Glibenclamide and Pinacidil

So far, there is no evidence that glibenclamide (10 and 100 µM) and pinacidil (100 µM) affect channels other than the K<sub>ATP</sub> channel in mammalian skeletal muscle (6, 30). Although glibenclamide increases the Ca²⁺ sensitivity of contractile components of intact muscle fibers (13), the current study using skinned muscle fibers shows that this effect occurs only when the glibenclamide concentration is >10 µM (Fig. 1C). At 10 µM, glibenclamide did not affect twitch force (Fig. 2) or the force-frequency curve (Fig. 3) of intact EDL or soleus muscles. Pinacidil also had no effect on the pCa-force curve of EDL or soleus skinned muscle fibers (Fig. 1A). Therefore, the effects of glibenclamide and pinacidil observed in this study are believed to be related to a modulation of K<sub>ATP</sub> channel activity, i.e., a block by glibenclamide and an activation by pinacidil.

Activation of K<sub>ATP</sub> Channels During Fatigue

Activity of K<sub>ATP</sub> channels during fatigue was estimated from the effect of glibenclamide and pinacidil on membrane potentials and ⁸⁶Rb⁺ fractional loss [as a marker for K⁺ movement across the channel (9)]. Here, we discuss the activation of the K<sub>ATP</sub> channel during fatigue and by pinacidil and, in Physiological Role of K<sub>ATP</sub> Channel During Fatigue, the importance of this activation on force.

Activation of K<sub>ATP</sub> channels during fatigue in control muscle. A hyperpolarization and shorter repolarization phase were observed in fatigued soleus muscle fibers compared with unfatigued fibers (Fig. 7). This is contrary to most in vitro studies, which have reported the reverse [see for example Balog et al. (4) and Juel (27)]. Although there is no study reporting shorter repolarization phase in fatigued muscle, there is at least one study reporting a hyperpolarization in rat soleus muscle (23). In the latter study, evidence was provided for a contribution of the Na⁺-K⁺ pump to the hyperpolarization. The current study now shows that both the hyperpolarization and shortening of action potential repolarization phase caused by fatigue were abolished by glibenclamide. These glibenclamide effects can be explained by a block of K<sub>ATP</sub> channels that decreases K⁺ conductance. A reduction in K⁺ conductance prevents the hyperpolarization by reducing the outward K⁺ current that maintains a negative resting potential [which means that both Na⁺-K⁺ pump (23) and K<sub>ATP</sub> channel are important in the hyperpolarization process]. A smaller K⁺ conductance also reduces the K⁺ current that contributes to the repolarization phase.

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

Activation of K<sub>ATP</sub> channels by pinacidil. Contrary to the effects of fatigue stimulation, pinacidil at 100 µM is effective in activating K<sub>ATP</sub> channels. Three observations support this conclusion. First, Barrett-Jolley and McPherson (6) reported maximal activation of K<sub>ATP</sub> current by pinacidil at 100 µM in intact resting FDB muscle fibers. Second, we observed that pinacidil reduced the action potential overshoot in resting soleus muscle fiber, an effect that was blocked by glibenclamide (Table 1). The pinacidil effect on the overshoot can be explained by an activation of the K<sub>ATP</sub> channel that increases K⁺ conductance, which in turn reduces the depolarization effect of Na⁺ channels and overshoot. Third, we observed that, during fatigue, pinacidil significantly increased ⁸⁶Rb⁺ fractional loss to a greater extent than that observed in control muscles (Fig. 8).

Physiological Role of K<sub>ATP</sub> Channel During Fatigue

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

Although K\(_{ATP}\) channels did not contribute to the decrease in force in our fatigue model, it does not imply that these channels are not important in skeletal muscle during other metabolic stresses. We found that an activation of K\(_{ATP}\) channels by pinacidil increased the rate of fatigue, abolished the increase in resting tension, and increased the rate of force recovery. The pinacidil effects support Noma's (34) hypothesis that the channel can contribute to the decrease in force (rate of fatigue) and protect muscle function (lower resting tension and faster force recovery). However, more studies are necessary to determine what physiological conditions activate a sufficient number of K\(_{ATP}\) channels to affect muscle contractility and protect muscle function.

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

If in skeletal muscle an effect on action potential is not important, then an effect on K\(^+\) efflux must be a major mechanism of action when K\(_{ATP}\) channels were activated by pinacidil. This is indeed supported by the fact that pinacidil significantly increased \(^{86}\)Rb\(^+\) fractional loss during fatigue (Fig. 8). However, the pinacidil effect on \(^{86}\)Rb\(^+\) fractional loss (or K\(^+\) efflux) was greater in EDL. This cannot explain how the rate of fatigue in the presence of pinacidil was increased to a greater extent in soleus muscle (Fig. 4), unless other events are considered.

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

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

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

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

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

This study was supported by grants from the Ontario Thoracic Society and the National Science and Engineering Research Council (to J.-M. Renaud), by National Institute of Arthritis and Musculoskeletal and Skin Diseases Grant AR-40598 (to T. M. Nosek), and by a summer studentship from the Heart and Stroke Foundation (to D. Wong).

Present address of T. M. Nosek: Case Western Reserve Univ., 10900 Euclid Ave., Cleveland, OH 44106-4963.

Address for reprint requests and other correspondence: J.-M. Renaud, Dept. of Cellular and Molecular Medicine, Univ. of Ottawa, 451 Smyth Rd., Ottawa, Ontario, Canada K1H 8M5 (E-mail: jmrenaud@uottawa.ca).

Received 11 june 1999; accepted in final form 9 September 1999.

REFERENCES