HIGH-FREQUENCY INITIAL PULSES DO NOT AFFECT EFFICIENCY IN RAT FAST SKELETAL MUSCLE

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Summary

This study investigated the effects of high-frequency initial pulses on the efficiency (=total work output/high-energy phosphate consumption) of rat fast skeletal muscle. *In situ* rat medial gastrocnemius muscles performed 15 repeated shortening contractions ($2\,s^{-1}$; velocity $50\,mm\,s^{-1}$) with occluded blood flow while activated with triplets of $400\,Hz$ followed by $60\,Hz$ trains (T400;60) or with constant-frequency trains of either 60 or $91\,Hz$. All stimulation patterns consisted of six pulses. After the last contraction, the muscles were quickly freeze-clamped and analysed for metabolite levels. The calculated efficiencies were $20.4\pm3.0\,mJ\,\mu mol^{-1}\,P$ ($N=7$), $19.4\pm1.8\,mJ\,\mu mol^{-1}\,P$ ($N=8$) and $19.6\pm2.5\,mJ\,\mu mol^{-1}\,P$ ($N=7$; means ± S.D.) for T400;60, 60 and $91\,Hz$ stimulation respectively ($P>0.05$). It is concluded that, although high-frequency initial pulses can enhance muscle performance, the efficiency of rat fast skeletal muscle did not differ from that for submaximal constant-frequency stimulation patterns.

Key words: skeletal muscle, performance, energy turnover, excitation/contraction coupling, efficiency, rat, muscle.

Introduction

The neural activation of a muscle consists of the recruitment and rate modulation of the population of single motor units that compose skeletal muscle. According to the size principle of Henneman (Henneman et al., 1974), the force generated by the muscle can be progressively increased by the recruitment of larger motor units. Another way to modulate the force generation of skeletal muscle is to change the frequency of stimulation (rate coding). It is known that force increases in a sigmoidal manner as the stimulation frequency is raised. *In vivo*, however, the discharge patterns of single motor units often start with a few high-frequency initial pulses (HFIPs), e.g. doublets and triplets. These HFIPs have been reported in freely moving rats (Hennig and Lømo, 1985; Gorassini et al., 1999) and in humans (e.g. Bawa and Calancie, 1983; Van Cutsem et al., 1998). HFIPs have been shown to increase the rate of force development as well as the peak force (e.g. Burke et al., 1970; Sandercock and Heckman, 1997) and peak power output during concentric contractions (Abbate et al., 2000). Moreover, the latter study indicated that the increases in peak power output with HFIPs resembled those achieved with high(er) constant-frequency stimulation. However, high stimulation frequencies have been shown to lead to a higher rate of fatigue (e.g. Jones et al., 1979; Binder-Macleod and Barker, 1991), an increased consumption of high-energy phosphate (HEPC) and a lower efficiency than low-stimulation

frequencies (Abbate et al., 1999). Surprisingly, stimulation patterns starting with HFIPs have been shown to reduce fatigue during repeated contractions (Binder-Macleod and Barker, 1991). This decrease in fatigue may indicate that muscle HEPC with respect to work output is reduced and consequently that efficiency (=total work output/HEPC) is greater when HFIPs are used. Thus, the aim of the present investigation was to study whether HFIPs increase the efficiency of fast skeletal muscle. The efficiency of fast skeletal muscle was therefore compared between a stimulation pattern starting with HFIPs, i.e. a triplet at $400\,Hz$ followed by a $60\,Hz$ train, and two constant-frequency patterns of stimulation at either $60\,Hz$ or $91\,Hz$.

Materials and methods

Muscle preparation

Experiments were performed using male Wistar rats ($N=16$; body mass $287\pm12\,g$; mean ± S.D.) anaesthetised with urethane ($1.5\,g\,kg^{-1}$ body mass; given intraperitoneally). Supplementary intraperitoneal injections of $0.63\,g\,kg^{-1}$ body mass were given if necessary. The experimental procedures have been described previously (e.g. de Haan, et al., 1989; de Haan et al., 1993; Abbate et al., 2000) and will be summarised below. The medial gastrocnemius muscle/tendon complex was separated from the

surrounding muscles without compromising the blood supply. The animal was placed prone on a heated pad (35 °C) with the femur of the operated leg clamped in a vertical position and the muscle held horizontally. The tendon was connected to a force transducer, which was part of an isovelocity measuring system. Stimulation was performed through the severed sciatic nerve, with only the branch to the medial gastrocnemius left intact. The current of each stimulation pulse was 1 mA, which was approximately 30 % higher than needed for maximal force development. Pulse duration was 0.05 ms. Motor movements and stimulation were computer-controlled. Force and length data were digitised (1000 Hz) and stored on disc for later analyses. At the end of the experiments, the rats were killed by cervical dislocation.

Muscle optimum length and temperature

Tetanus optimum length (L_0) was first estimated by determining twitch optimum length. L_0 was usually approximately 1 mm below twitch optimum length, and only two or three tetani (120 Hz, 150 ms duration) were therefore needed to determine L_0. After L_0 had been assessed, the muscle was allowed a rest period of 15 min before the experiments. Thus, the assessment of L_0 did not compromise energy metabolism prior to the experimental exercise. Muscle temperature was controlled by a flow of water-saturated air around the muscle at 34±0.5 °C. Using this technique, a previous study has shown that muscle temperature was within 1 °C of the temperature of the airflow (de Haan, 1998).

Stimulation patterns

Measurements of single motor units revealed that discharge patterns in freely moving rats are characterised by short bursts of activity (Hennig and Lømo, 1985). Therefore, all stimulation patterns used in this study consisted of bursts of six pulses only. The following three stimulation patterns were used. The first pattern, the HFIP pattern, consisted of a triplet of stimuli at 400 Hz followed by a 60 Hz train (T400;60). The second pattern consisted of a 60 Hz constant-frequency train (pulse interval 16.7 ms), which in our preparation leads to nearly fused contractions. The third pattern was a constant-frequency stimulation at 91 Hz. This frequency was chosen because it had the same average pulse interval as the T400;60 pattern; i.e. 11.0 ms.

Shortening contractions

In studies of efficiency, energy turnover is expressed with respect to the work done. Therefore, the contractions were optimised such that each stimulation pattern resulted in the highest work output. A pilot study was performed to determine which contraction type resulted in maximal work output for each stimulation pattern. In this pilot study, the start of shortening was varied in steps of 5 ms during stimulation of the muscle. The contraction protocol that resulted in the highest work output was used in the main experiments. The contractions started at $L_0+2.5$ mm, to which the muscle was passively stretched prior to the start of stimulation. The motor

performed a movement from $L_0+2.5$ mm to $L_0-5.5$ mm for all three stimulation frequencies. Examples of force signals for the three stimulation patterns are shown in Fig. 1. To study efficiency, a series of 15 repeated ($2\,s^{-1}$) shortening contractions was used during which the muscle was stimulated with T400;60, 60 or 91 Hz. The contractions were performed at a shortening velocity of $50\,mm\,s^{-1}$. Care was taken to ensure that the relaxation of the muscle was completed before the end of the motor movement.

Before the start of experiments, the blood flow to the medial gastrocnemius was occluded to minimise aerobic metabolism and to prevent the removal of metabolites from the muscle. Immediately following occlusion of the blood flow, the series of 15 contractions was started. After the last contraction, the muscle was quickly freeze-clamped with a pair of tongs precooled in liquid nitrogen. Subsequently, the muscle was
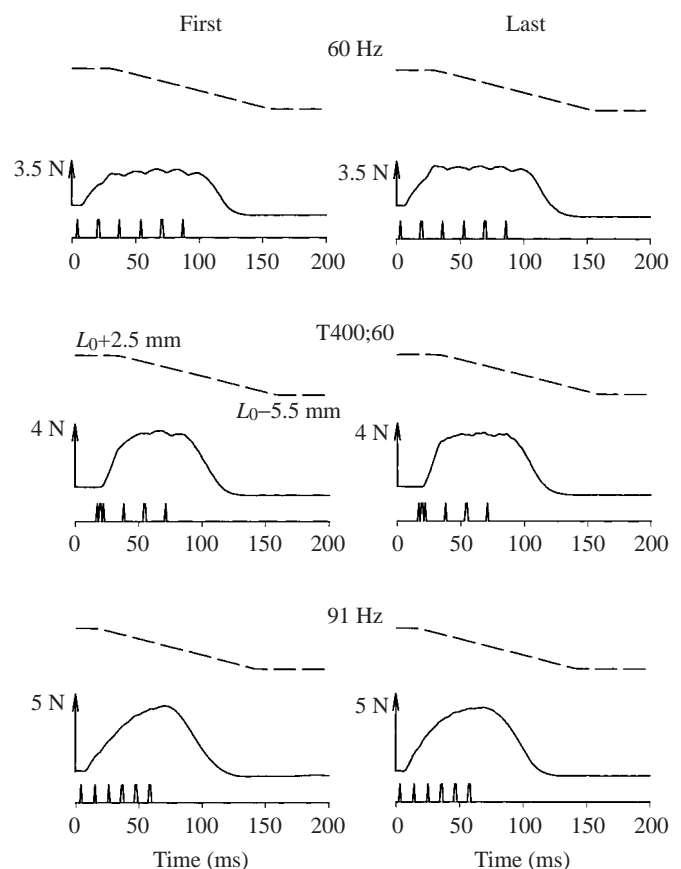

Fig. 1. Examples of force recordings of the first and last contractions (of a series of 15) obtained with the three stimulation patterns used in this study. The start of stimulation and the start of shortening were matched to give maximal work output for each stimulation pattern. The stimulation patterns used were as follows: 60 Hz stimulation (top panel); a triplet of 400 Hz followed by a 60 Hz train (T400;60) (middle panel); 91 Hz stimulation (lower panel). The dashed line shows the length recording, which varies from $L_0+2.5$ mm before to $L_0-5.5$ mm after each contraction. L_0 is the optimum length for tetanus. The stimulation pulses are shown superimposed on the time scale.

excised from the animal and stored in liquid nitrogen until further analysis.

Work output

Work was calculated for each contraction by integrating force over the shortening distance (and corrected for passive forces), while total work output was calculated as the sum of the work produced in the 15 individual contractions.

Electromyogram (EMG) recordings

In a pilot study, EMG signals were monitored to verify whether neuromuscular failure occurred following stimulation with the triplet at 400 Hz. EMG recordings were obtained with two stainless-steel wire electrodes (diameter 100 μm) bared of insulation for the last 2 mm and bent at the tip. These electrodes were inserted into the muscle using thin needles. The EMG signals were amplified, bandpass-filtered (from 10 Hz to 8 kHz), digitized (sample frequency 10 kHz) and stored on computer disc. The pilot study differed in two minor ways from the main experiments of this study. First, the time interval between the triplet and the first pulse of the 60 Hz train was somewhat shorter than used the main experiments. As a result of the shorter pulse interval after the triplet, the risk of neuromuscular fatigue would be higher in the pilot experiment than in the main study. However, Fig. 2 shows that no neuromuscular fatigue occurred in either the first or the last contraction of the pilot experiment. Second, in the pilot study, 10 contractions were performed instead of the 15 contractions used in the main experiments. No changes in the rate of force build-up or in relaxation times were found during the series of contractions in the main experiments. It is therefore very unlikely that failure of neuromuscular transmission would have occurred in the main experiments.

Analysis of metabolites

The frozen muscles were ground in a mortar under constant addition of liquid nitrogen. The muscle powder was then freeze-dried and stored at −80 °C until further analysis. Metabolites were separated and quantified using a high-performance liquid chromatography (HPLC) system that consisted of a Binary LC pump (model 250; Perkin-Elmer, USA), an autosampler with cooling tray and automatic injector (Basic Marathon, Spark Holland, The Netherlands) and a variable-wavelength ultraviolet spectrophotometric detector (model 759A; Applied Biosystems, The Netherlands). The size of the injection loop was 20 μl. For ATP/IMP and phosphocreatine/creatine (PCr/Cr) separations, ultraviolet absorption was measured at 254 and 210 nm, respectively. Peaks were identified and quantified using a chromatography data system (model 717; Axxiom Chromatography, CA, USA) by comparing the peak heights of samples with those of external standards. Lactate concentration was measured enzymatically using a Beckman DU 640 spectrophotometer. The time between experiments and metabolite analyses was between 1 and 3 months.
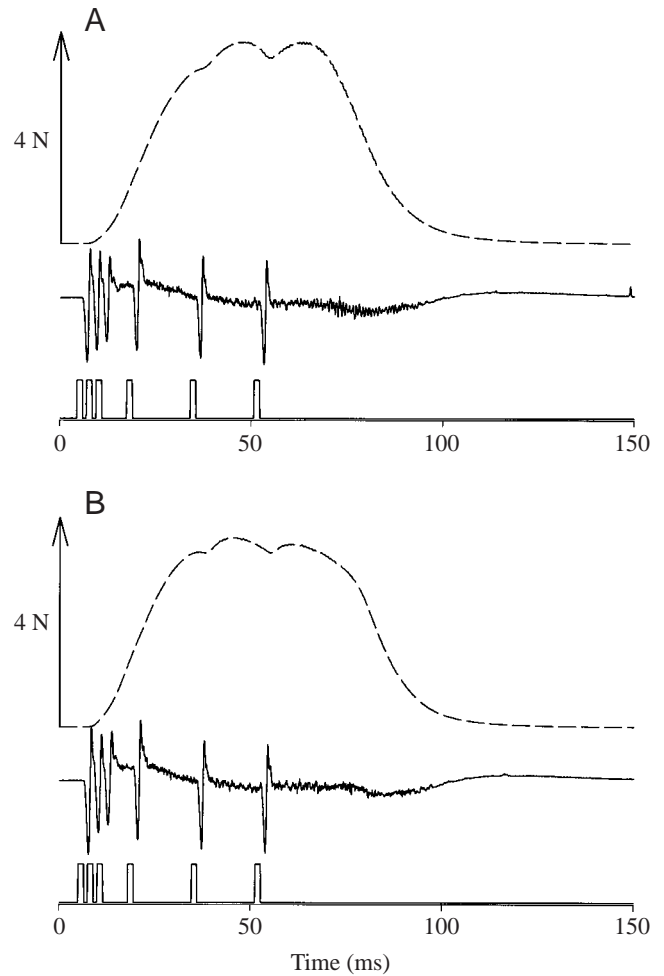

Fig. 2. Force and electromyogram (EMG) traces recorded in a pilot study during stimulation with a pattern using the triplet at 400 Hz. (A) First contraction and (B) last contraction of a series of 10 contractions. The upper recording (broken line) is the force signal. EMG recordings are shown in the middle trace, and stimulation pulses are superimposed on the time scale (bottom line). Note that the stimulation protocol differed in two minor ways from the protocol used in the main experiments (see Materials and methods).

Nucleotides and creatine compounds

Prior to HPLC analysis, metabolites were extracted by adding 1 μl of methanol (60 %, v/v) per microgram of muscle powder (total approximately 200 μg). Extraction was performed overnight at −80 °C. Preliminary work (Karatzaferi et al., 1999) did not identify any significant loss of metabolites from muscle after prolonged storage at −80 °C.

Analysis was carried out as described previously for single fibres (Karatzaferi et al., 1999). Separation was performed at controlled room temperature (20 °C) under isocratic conditions using a reversed-phase 125 mm×4 mm analytical column protected by a 4 mm×4 mm guard cartridge (both 5 μm particle size; RP-18 LiChrosphere 100, Hewlett-Packard, The Netherlands). The mobile phase was pumped at a flow rate of 1 ml min⁻¹. For ATP and IMP analysis, the mobile

phase consisted of $215\,\mathrm{mmol\,l^{-1}}$ KH_2PO_4, $2.3\,\mathrm{mmol\,l^{-1}}$ tetrabutylammonium hydrogensulphate (TBAHS) and 2% acetonitrile aqueous solution adjusted to pH 6.5 with $5\,\mathrm{mol\,l^{-1}}$ KOH. For phosphocreatine (PCr) and creatine (Cr) analysis, the mobile phase consisted of $14.7\,\mathrm{mmol\,l^{-1}}$ KH_2PO_4, $1.15\,\mathrm{mmol\,l^{-1}}$ TBAHS aqueous solution adjusted to pH 5.3 with $5\,\mathrm{mol\,l^{-1}}$ KOH. Solutions were degassed before use and were kept under helium during analysis. Before each assay, the column was washed and equilibrated with the mobile phase.

Lactate

Duplicate extractions were made by homogenising approximately 5 mg of dry muscle tissue in 0.5 ml of cold perchloric acid (5%, v/v). The muscle tissue was sonicated for 1 min, and the homogenate was centrifuged at $0.6\,\boldsymbol{g}$ for 15 s at 0 °C (Biofuge 22R from Heraeus Sepatech), after which 400 µl of the supernatant was neutralized with 50 µl of K_2CO_3/Tris solution (2.8 mol l^{-1} K_2CO_3, 0.1 mol l^{-1} Tris). The neutralized homogenate was centrifuged for 20 min ($0.7\,\boldsymbol{g}$ at 4 °C). The supernatant was stored in an Eppendorf tube at −80 °C until further analysis. Lactate was measured enzymatically (as described by Bergmeyer, 1970).

HEPC and efficiency

The concentrations of ATP, IMP, PCr and Cr were all normalised to the mean total amount of creatine (PCr+Cr) in each muscle. High-energy phosphate consumption (HEPC) was calculated from the differences in metabolite concentrations between the experimental and the mean of the control muscles (N=9) using the following formula:

$$\mathrm{HEPC} = 1.5\Delta[\mathrm{lactate}] - \Delta[\mathrm{PCr}] - \Delta[\mathrm{ATP}] + \Delta[\mathrm{IMP}].$$

HEPC was then multiplied by 0.23 (dry/wet mass ratio; de Haan et al., 1986) and by muscle mass to obtain HEPC per muscle. Efficiency was calculated from the total work output and HEPC per muscle and is expressed as mJ µmol^{-1} phosphate used.

Statistical analyses

A one-way analysis of variance (ANOVA) was used to test for differences between the three groups. Bonferroni *post-hoc* tests were used to test for significant differences between the group means (P<0.05). Results are presented as means ± S.D.

Results

Work output

Fig. 3A shows the work output during each of the 15 repeated contractions. Work output per contraction did not change significantly over time for all three stimulation patterns, indicating that no fatigue was occurring. However, during the latter part of the series of contractions, the work output using T400;60 was significantly lower (P<0.05) than the work output using 60 Hz stimulation. Furthermore, the total work output at T400;60 (210.4±13.4 mJ, N=7) was significantly lower (P<0.05) than that at 60 Hz (254.6±26.9 mJ, N=8), while total
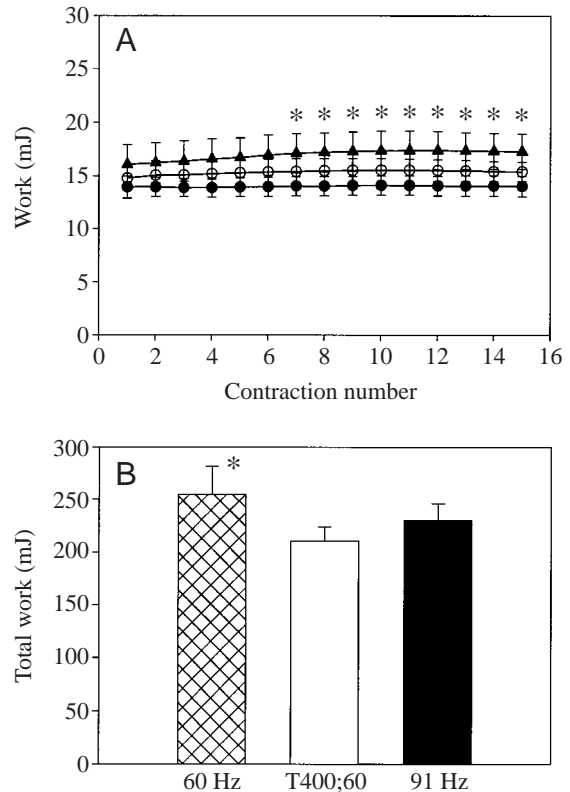

Fig. 3. (A) Mean work output per contraction during 15 repeated concentric contractions with stimulation at 60 Hz (filled triangles, N=8), T400;60 (filled circles, N=7) or 91 Hz (open circles, N=7). (B) Mean total work output after 60 Hz, T400;60 and 91 Hz stimulation. An asterisk indicates a significant difference from the value for T400;60 (P<0.05). Values are means ± S.D. T400;60 indicates stimulation with a triplet at 400 Hz followed by a train at 60 Hz.

work at 91 Hz (230.2±15.6 mJ, N=7) was not significantly different from that of the two other stimulation patterns (Fig. 3B).

Note that the total stimulation time of the muscle differed depending on the stimulation pattern used, i.e. all stimulation patterns consisted of the same number of stimulation pulses but had different (average) pulse intervals of either 11.0 ms (T400;60 and 91 Hz) or 16.7 ms (60 Hz). As a consequence, the stimulation duration was longer at 60 Hz and thus resulted in an increased shortening distance over which the muscle was activated (see Fig. 1), which accounts for the higher total work output compared with stimulation at T400;60. These differences in stimulation duration are unlikely to have affected efficiency because previous experiments in which activation duration was varied revealed that economy (de Haan et al., 1986) and efficiency (de Haan, 1996) did not change with time during repeated short-lasting contractions.

HEPC and efficiency

The calculated HEPC values for the three stimulation patterns were 10.5±1.7 µmol phosphate muscle^{-1} (N=7) for T400;60, 13.2±1.7 µmol phosphate muscle^{-1} (N=8) for 60 Hz
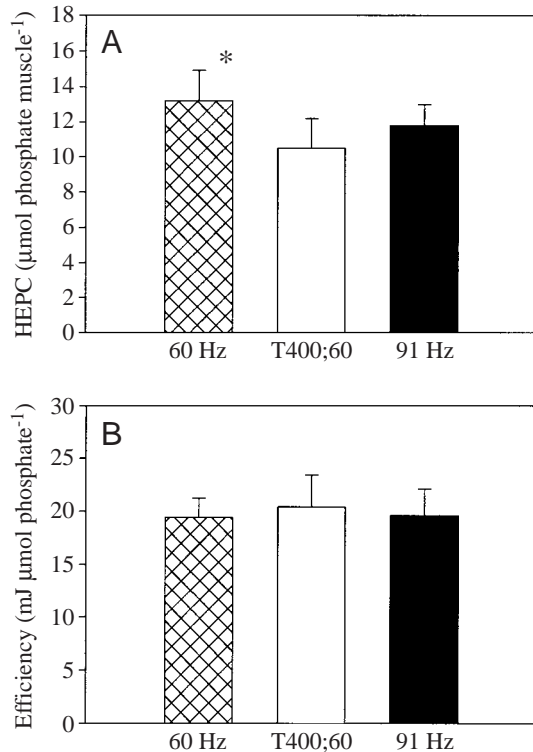
Fig. 4. (A) Mean high-energy phosphate consumption (HEPC) after 15 repeated concentric contractions at 60 Hz (*N*=8), T400;60 (*N*=7) or 91 Hz (*N*=7) stimulation of the muscle. (B) Mean efficiency at which the series of contractions was performed. Efficiency was calculated as total work output/HEPC. An asterisk denotes a value significantly different from the value for T400;60 (*P*<0.05). Values are means + S.D. T400;60 indicates stimulation with a triplet at 400 Hz followed by a train at 60 Hz.

and 11.8±1.2 μmol phosphate muscle^{-1} (*N*=7) for 91 Hz. HEPC was significantly higher for 60 Hz than for T400;60, but no differences were found between T400;60 and 91 Hz or between 60 and 91 Hz (Fig. 4A).

No significant differences were found among the efficiencies of the three stimulation patterns: 20.4±3.0 mJ μmol^{-1} phosphate (*N*=7) for T400;60, 19.4±1.8 mJ μmol^{-1} phosphate (*N*=8) for 60 Hz and 19.6±2.5 mJ μmol^{-1} phosphate (*N*=7) for 91 Hz (Fig. 4B).

Discussion

It is known that discharge patterns of single motor units *in vivo* often start with HFIPs during (ballistic) movements. Previous studies have revealed that HFIPs enhanced muscle performance in skeletal muscle (e.g. Burke et al., 1970; Sandercock and Heckman, 1997; Abbate et al., 2000). The present study investigated whether the efficiency (=total work output/HEPC) of rat fast skeletal muscle is also changed when stimulation is started with HFIPs compared with two submaximal stimulation patterns consisting of constant-frequency trains. Therefore, the efficiency of rat medial

gastrocnemius muscles stimulated with triplets of 400 Hz followed by 60 Hz stimuli was compared with the efficiency obtained with constant-frequency trains of either 60 or 91 Hz.

Efficiency has been investigated in many species under various conditions. During such studies in isolated muscles and single fibres, stimulation has been performed using constant-frequency trains. To our knowledge, this is the first study that investigates efficiency using stimulation patterns with HFIPs, which may be a better representation of the *in vivo* situation. Recordings of single motor units show that the *in vivo* discharge patterns consist of short-lasting bursts, and we therefore used only six pulses in each stimulation pattern. Surprisingly, in the present study, a higher work output was generated at 60 Hz than when HFIPs were used. However, as explained above, this was due to the difference in stimulation durations that results from the equal number of stimulation pulses used.

Previous studies have shown that increasing muscle performance through high stimulation frequencies reduced muscle efficiency (Abbate et al., 1999) and resulted in a higher rate of fatigue compared with submaximal stimulation levels (e.g. Jones et al., 1979). Binder-Macleod and Barker (Binder-Macleod and Barker, 1991) showed that HFIPs reduced the effects of fatigue during a prolonged series of repeated contractions. Numerous studies have indicated the importance of metabolic changes in the development of fatigue (for a review, see Miller et al., 1995), so a reduction in the rate of fatigue with HFIPs may suggest that energy turnover is reduced as well. Therefore, if HFIPs result in an increased performance at a relatively low energetic cost, a higher efficiency may be expected. One way in which HFIPs could lead to a relatively lower energetic cost has been hypothesized (Zajac and Young, 1980; Stevens, 1996). These authors reasoned that HFIPs may lead to an initial high Ca^{2+} level so that the subsequent pulses have only to maintain that Ca^{2+} level to continue the same level of performance. Continuous high-frequency stimulation would lead to a higher release of Ca^{2+}. Because Ca^{2+} is reaccumulated at the expense of ATP, a relatively higher energetic cost may then be expected. Our results showed that, under the present experimental conditions, HFIPs led to a significant decrease in energy turnover compared with constant 60 Hz stimulation but not compared with stimulation at 91 Hz. However, the differences in energy turnover were proportional to the differences in work output among the three stimulation patterns and, consequently, no effects on efficiency were found. It is therefore concluded that, under the present experimental conditions, the effects of HFIPs on energy turnover are too small to affect the efficiency of whole skeletal muscle.

Does HFIP potentiation require an effect on muscle energetics to decrease the effects of fatigue? Experiments by Westerblad and Allen (Westerblad and Allen, 1992a; Westerblad and Allen, 1992b) revealed that Ca^{2+} release from the sarcoplasmic reticulum is decreased late in fatigue, resulting in a reduction in crossbridge formation and, hence, force generation. Therefore, if HFIPs prevent such a decrease

in crossbridge formation, possibly by causing an initial large release of Ca^{2+}, the effects of fatigue may be reduced without necessarily affecting efficiency. However, it remains to be investigated whether this is the case.

Another possible advantage of starting stimulation with HFIPs may be the prevention of activation failure during a series of contractions (see Binder-Macleod and Barker, 1991). Activation failure has been shown to occur after prolonged high-frequency electrical activation and leads to considerable force loss and a slowing and ultimately the failure of the muscle action potential during electrical stimulation (Bigland-Ritchie et al., 1979). Thus, the use of HFIPs and the subsequent submaximal stimulation frequency may help to optimize mechanical output while reducing the possibility of the failure of neuromuscular transmission. Indeed, Fig. 2 shows that no neuromuscular failure occurred under the present experimental conditions. Moreover, because work output did not further potentiate during the series of contractions and no changes in relaxation time occurred, it may be concluded that the triplet induced the maximal potentiated state. Although the contribution of activation failure to the decrease in performance during voluntary contractions is very small (Baker et al., 1993), the effects may be larger with the use of electrical stimulation in, for example, functional electrical stimulation, in which stimulation of muscles lasts longer and higher stimulation frequencies are required than the short bursts measured during in vivo movement.

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