Time-dependent and indirect effect of inorganic phosphate on force production in rat gastrocnemius exercising muscle determined by ^{31}P-MRS

Benoît Giannesini, Marguerite Izquierdo, Sylviane Confort-Gouny, Patrick J. Cozzone, David Bendahan*

Centre de Résonance Magnétique Biologique et Médicale (CRMBM), UMR CNRS 6612, Faculté de Médecine de Marseille, 27 Boulevard Jean Moulin, 13005 Marseille, France

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Abstract The relationship of inorganic phosphate (P_i) and its diprotonated form ($H_2PO_4^-$) to isometric force (F) was analyzed non-invasively using ^{31}P-magnetic resonance spectroscopy. Rat gastrocnemius muscles were electrically stimulated at six different frequencies in order to produce different levels of fatigue. A curvilinear relationship was demonstrated between force production and [P_i] and [$H_2PO_4^-$] accumulation. [P_i] and [$H_2PO_4^-$] were correlated with F at the end of the stimulation period but not when F was maximal at the early stage of the stimulation period. Interestingly, the respective [P_i] and [$H_2PO_4^-$] did not differ significantly between these two stages demonstrating that [P_i] and [$H_2PO_4^-$] cannot be considered as direct effectors of fatigue. This time-dependent and indirect effect of [P_i] and [$H_2PO_4^-$] on force production might be mediated by calcium ions. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Rat skeletal muscle; Fatigue; Electrical stimulation; ^{31}P-Magnetic resonance spectroscopy; Inorganic phosphate; Calcium

1. Introduction

In skeletal muscle, intermittent fatiguing stimulation results in a progressive depletion of phosphocreatine (PCr) associated with an accumulation of inorganic phosphate (P_i) and a decrease in pH as a result of lactate accumulation. Myoplasmic accumulation of P_i has been frequently proposed to play a key role in fatigue development, defined as a decline in force production during prolonged skeletal muscle activity [1–7]. As an illustration, mouse skeletal muscle deficient in creatine kinase (CK) have been shown to be more fatigue-resistant than wild type muscle and it has been suggested to be due to a lesser P_i accumulation [8]. It has been initially proposed that fatigue is not related to P_i per se but rather to its diprotonated form, $H_2PO_4^-$ [1–3,9,10]. $H_2PO_4^-$ might directly inhibit contractile protein function by decreasing the proportion of force-producing cross-bridges [2,5,11]. However, the exact nature of this relationship is still a matter of debate. Several studies have reported a decline in force with no changes in [$H_2PO_4^-$] suggesting then, no role of P_i in fatigue [3,10,12]. On the other hand, relationships between P_i or its diprotonated form and force production have been reported for a limited number of exercise protocols making difficult to decide whether the relationships are causative or incidental. Our purpose was to investigate non-invasively, using ^{31}P-magnetic resonance spectroscopy (^{31}P-MRS), the possible relationships of [P_i] and [$H_2PO_4^-$] to isometric force (F) in rat gastrocnemius muscle for a variety of experimental protocols. Muscles were stimulated at six stimulation frequencies, ranging from 0.8 to 7.6 Hz, in order to obtain a wide range of metabolic and mechanical changes.

2. Materials and methods

2.1. Surgical preparation

Forty-four male Wistar rats (CERJ, Le Genest Saint Isle, France) weighing 350–375 g were used for these experiments, following the guidelines of the National Research Council Guide for the care and use of laboratory animals and the French Law on Animal Handling and Protection. Anesthesia was induced by an intraperitoneal injection of pentobarbital sodium (50 mg kg^{-1} body weight) and was maintained throughout the experiment by repeated administration of anesthetics (10 mg kg^{-1} body weight, every 30 min) through an intraperitoneal catheter. The left hindlimb was surgically prepared for in situ sciatic nerve stimulation. The Achilles tendon was exposed and removed from the foot at the site of the calcaneus bone attachment, leaving intact the neurovascular supply of the muscle. The distal part of the gastrocnemius tendon was attached to a home-built force transducer via an inextensible silk thread.

The left sciatic nerve was exposed in its gluteal course and was carefully cleared of connective tissue. A home-built bipolar electrode connected to an electrical stimulator (SI-10, Narco, USA) was placed around the sciatic nerve, which was cut proximally to the electrode attachment. The part of the sciatic nerve connected to the bipolar electrode was put back in place and the incision was closed. The rat was placed backwards in a custom-made Perspex cradle integrating a warm-water heating pad. Body temperature (typically 35–36°C) was monitored throughout the experiment using a rectal probe. The left leg was firmly immobilized by securing the foot with straps and by inserting a non-magnetic brass pin into the tibia head. In that position, the belly of the gastrocnemius muscle was located above the MR surface coil. Muscle was passively loaded at rest (typically 1.3–1.5 N) to give maximum isometric twitch tension in response to supramaximal square-wave pulses (1–10 V, 1 ms duration) delivered to the sciatic nerve.

2.2. Experimental protocol

Animals were randomly assigned to one of the six stimulation protocols: 0.8 Hz ($n=7$), 2 Hz ($n=7$), 3.2 Hz ($n=9$), 4 Hz ($n=9$), 5.2 Hz

*Corresponding author. Fax: (33)-4-91 25 65 39.
E-mail address: david.bendahan@medecine.univ-mrs.fr (D. Bendahan).

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($n=6$) or 7.6 Hz ($n=6$). The stimulation protocol consisted in 6 min of repeated isometric contractions electrically induced via the sciatic nerve with supramaximal square-wave pulses (1–10 V, 0.2 ms duration).

2.3. Force measurements

Force measurements were conducted with a home-built force transducer. The electrical signal coming out from the force transducer was amplified, converted to a digital signal and processed on a personal computer using ATS software (Sysma, France). Isometric twitch force (in Ns twitch^{-1}) was calculated every 15 s of stimulation by integrating isometric tension (in N) relative to time (in s).

2.4. MRS and data processing

^{31}P-MRS investigations were performed in a horizontal superconducting magnet (Brüker 47/30 Biospec system, Karlsruhe, Germany) operating at 4.7 T. MR data were collected with a home-built ^{31}P-MRS surface coil (10×14 mm). Magnetic field homogeneity was optimized by monitoring the water signal until its width at half height was below 0.25 ppm. ^{31}P-MR signals were acquired at 81 MHz following a 20-μs radiofrequency pulse applied with a repetition time (TR) of 2.4 s. MR data acquisition was synchronized to stimulation. Free-induction decays (FIDs) were continuously recorded in 30-s blocks (12 scans, 4000-Hz sweep width) during rest (6 min), stimulation (6 min) and recovery (30 min) periods. FIDs were transferred to an IBM RISC 6000 workstation and processed using the NMR1 spectroscopy processing software (New Methods Research, Syracuse, NY, USA) as previously described [13]. Signal areas corresponding to [PCr], P_i and ATP were measured after curve fitting to a Lorentzian function [13], and were corrected for magnetic saturation effects using fully relaxed spectra collected at rest with a TR of 20 s. Intracellular pH (pH_i) was calculated from the chemical shift of P_i relative to PCr (−2.45 ppm) [14]. Absolute concentrations were expressed relative to a resting β-ATP concentration of 5.8 mM reported from fluorimetrical measurements in the same muscle from the same strain of rat [15]. [$H_2PO_4^-$] was calculated from pH_i and [P_i], assuming a dissociation constant (pK) of 6.75 for $H_2PO_4^-$ [16]: $[H_2PO_4^-]=[P_i]/(1+10^{(pH_i-6.75)})$. Time-points for the time course of pH_i and phosphorylated metabolite concentrations were assigned to the midpoint of the acquisition interval.

2.5. Statistics

All results are presented as mean ± S.E.M. Statistical differences were tested within each stimulation protocol using two-tailed Student's t-test for paired observations. The level of significance was set at $P<0.01$.

3. Results

3.1. Isometric twitch force

Throughout the protocols with stimulation frequencies ranging from 0.8 to 5.2 Hz, F increased transiently to reach a maximal value (F_{max}) sooner when stimulation frequency was higher (Fig. 1A). Such a transient increase did not occur for protocol at 7.6 Hz and F_{max} was reached right at the onset of stimulation (Fig. 1A). F_{max} did not differ significantly among the six protocols and averaged 0.183 ± 0.006 Ns twitch^{-1}. Within each protocol, F measured at the end of the stimulation period (F_{end}) was significantly reduced when compared to F_{max} as a sign of fatigue (Fig. 1A). The extent of this reduction, expressed as the ratio (in percent) between F_{end} and F_{max}, increased with the stimulation frequency: 18.5 ± 5.3% of reduction at 0.8 Hz, 49.9 ± 3.9% at 2 Hz, 64.7 ± 2.9% at 3.2 Hz, 68.9 ± 2.6% at 4 Hz, 75.6 ± 2.7% at 5.2 Hz and 81.9 ± 4.0% at 7.6 Hz.

3.2. pH_i

Resting pH_i did not differ significantly among the six groups and averaged 7.04 ± 0.02 (Fig. 1B). Within each protocol, pH_i decreased throughout the stimulation period (Fig. 1B). At 0.8 Hz, pH_i decreased continuously throughout the 6 min of contractions. At 2 Hz and beyond, pH_i decreased rapidly to reach a steady-state, which was maintained until the end of the stimulation period. The extent of acidosis at the end of the stimulation period increased with stimulation frequency (Fig. 1B).

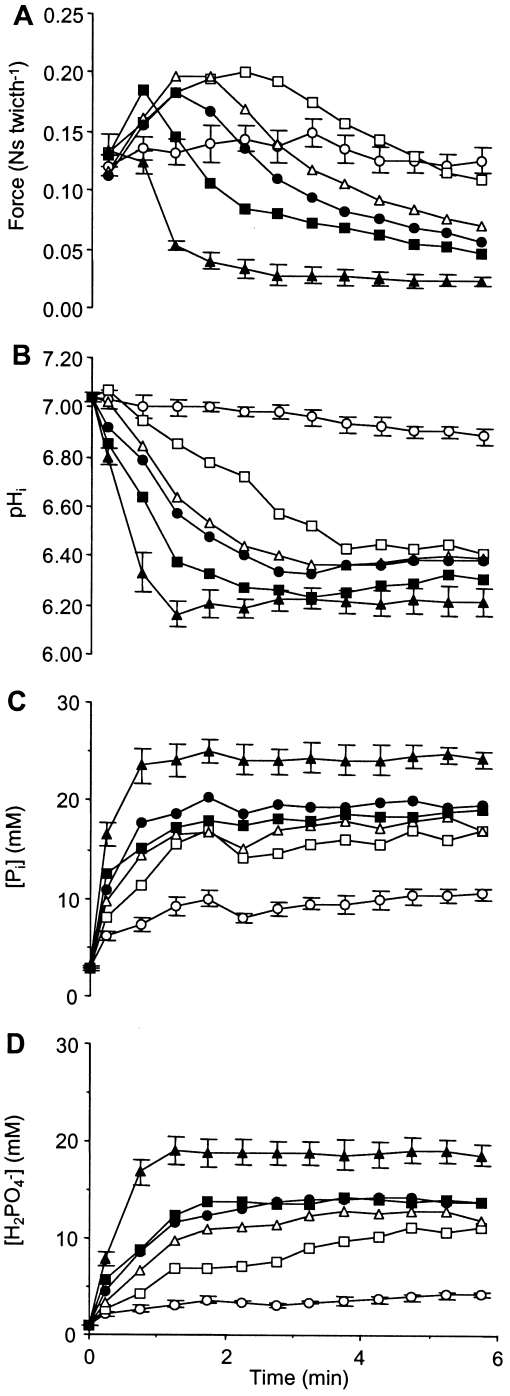

Fig. 1. Isometric twitch force (A), pH_i (B), [P_i] (C) and [$H_2PO_4^-$] (D) as a function of time throughout the six stimulation protocols. Values are means ± S.E.M. For the sake of clarity, error bars are only shown for stimulation protocols at 0.8 and 7.6 Hz. White circles: 0.8 Hz, white squares: 2 Hz, white triangles: 3.2 Hz, black circles: 4 Hz, black squares: 5.2 Hz, black triangles: 7.6 Hz.

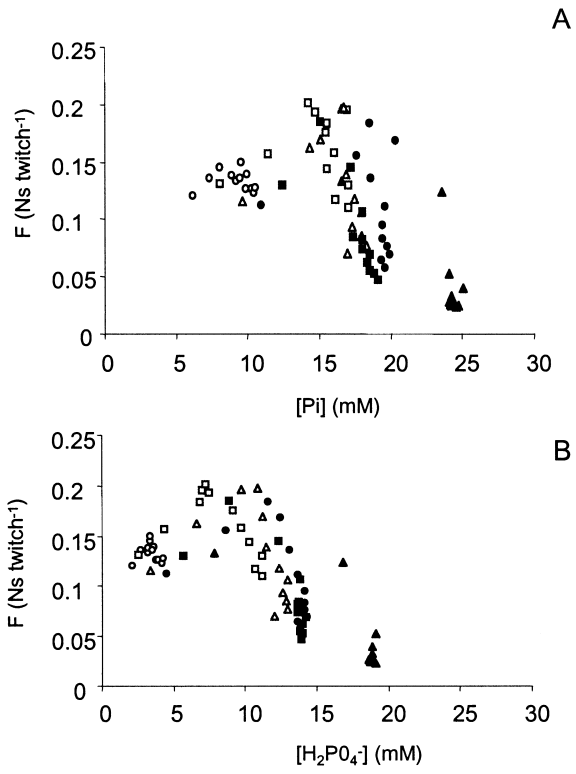

Fig. 2. Relationships between force production and [P_i] accumulation (A) and [$H_2PO_4^-$] accumulation (B). For the sake of clarity, error bars have been omitted. White circles: 0.8 Hz, white squares: 2 Hz, white triangles: 3.2 Hz, black circles: 4 Hz, black squares: 5.2 Hz, black triangles: 7.6 Hz.

3.3. P_i and $H_2PO_4^-$

At rest, [P_i] and [$H_2PO_4^-$] did not differ significantly among the six groups and averaged respectively 2.89 ± 0.05 and 0.94 ± 0.02 mM (Fig. 1C,D). The time-related changes in [P_i] were qualitatively similar whatever the protocol with an initial rapid phase leading to a large [P_i] increase followed by a second phase during which [P_i] reached a steady-state (Fig. 1C). Due to pH changes related to muscle stimulation, the accumulation of [$H_2PO_4^-$] lagged behind the total phosphate and its time-related changes were slower and smaller in amplitude (Fig. 1D).

3.4. Relationships of $[P_i]$ and $[H_2PO_4^-]$ to force production

A curvilinear relationship was observed when [P_i] and [$H_2PO_4^-$] changes were expressed with respect to force production (Fig. 2A,B). An increase in force production was measured while [P_i] increased from the resting value up to 15 mM and for [$H_2PO_4^-$] close to 10 mM. Then, a dramatic reduction of force was measured while [P_i] and [$H_2PO_4^-$] reached their maxima i.e. 25 and 20 mM respectively. The relationships between force, [P_i] and [$H_2PO_4^-$] were also analyzed at two particular time-points i.e. when F was maximum (respectively [P_i]F_{max} and [$H_2PO_4^-$]F_{max}) and at the end of the stimulation period (respectively [P_i]end and [$H_2PO_4^-$]end). [P_i]F_{max} ranged between 9.5 ± 0.9 mM at 0.8 Hz and 19.2 ± 2.3 mM at 7.6 Hz (Fig. 1C); [P_i]end ranged between 10.6 ± 0.6 mM at 0.8 Hz and 24.2 ± 0.8 mM at 7.6 Hz (Fig. 1C); [$H_2PO_4^-$]F_{max} ranged between 3.5 ± 0.3 mM at 0.8 Hz and 11.5 ± 2.7 mM at 7.6 Hz (Fig. 1D); [$H_2PO_4^-$]end ranged between 4.3 ± 0.3 mM at 0.8 Hz and 18.7 ± 1.1 mM at 7.6 Hz (Fig. 1D). Within each pro-

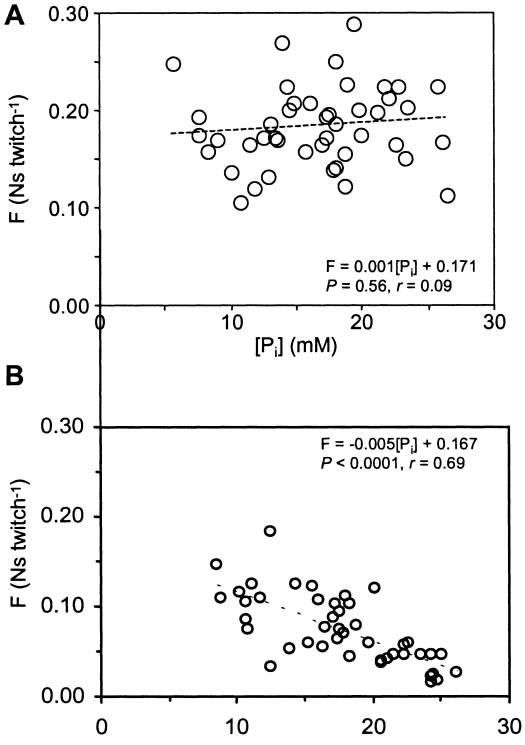

Fig. 3. Relationship between [P_i] and isometric force at the time when isometric force was maximal (A) and at the end of the 6-min period of repeated isometric contractions (B). Data are pooled from the six experimental protocols.

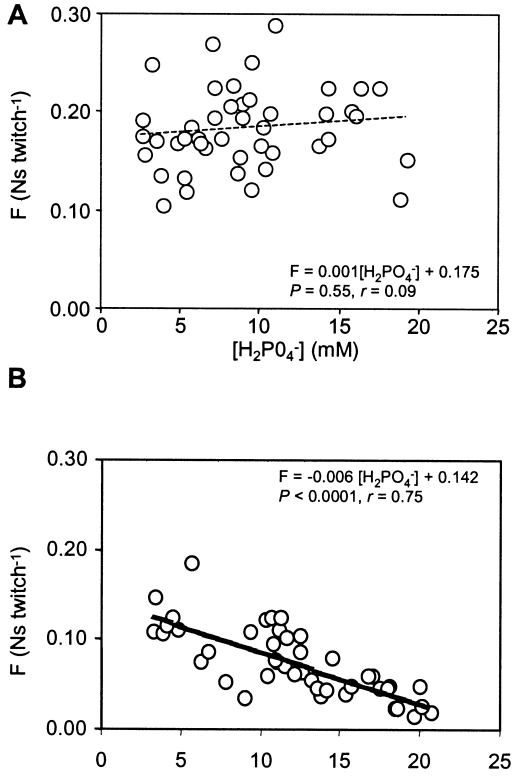

Fig. 4. Relationship between [$H_2PO_4^-$] and isometric force at the time when isometric force was maximal (A) and at the end of the 6-min period of repeated isometric contractions (B). Data are pooled from the six experimental protocols.

tocol, $[P_i]F_{max}$ and $[H_2PO_4^-]F_{max}$ did not differ significantly from $[P_i]end$ and $[H_2PO_4^-]end$ respectively. Relationships were analyzed on data pooled from the six protocols. No significant correlation was found either between F_{max} and $[P_i]F_{max}$ ($P = 0.56$) (Fig. 3A) nor between F_{max} and $[H_2PO_4^-]F_{max}$ ($P = 0.55$) (Fig. 4A). On the contrary, F_{end} was linearly correlated with $[P_i]end$ ($P < 0.0001$; $r = 0.69$) (Fig. 3B) and to $[H_2PO_4^-]end$ ($P < 0.0001$; $r = 0.75$) (Fig. 4B).

4. Discussion

We have investigated in vivo the relationship of $[P_i]$ and $[H_2PO_4^-]$ to force production. Possible correlations were analyzed on data pooled from the six stimulation protocols at two major stages of the stimulation period: (i) at the end of the stimulation period, when F was reduced as a sign of fatigue, and (ii) at the time when F reached a maximal value (F_{max}). F_{max} was reached transiently as soon as the onset of the stimulation period for the protocol at 7.6 Hz and throughout the stimulation period for protocols ranging from 0.8 to 5.2 Hz. This transient increase in F has been largely documented and has been referred as activity-dependent activation (staircase) resulting from the enhancement of twitch amplitude during repeated low-frequency stimulations [17,18]. It is generally accepted that activity-dependent activation is primarily due to increased calcium sensitivity of the contractile mechanism resulting from the phosphorylation of the regulatory myosin light chain [19–21].

We have showed that $[H_2PO_4^-]$ was correlated with F ($P < 0.0001$; $r = 0.75$) at the end of the stimulation, suggesting, in agreement with previous correlative studies, that $H_2PO_4^-$ would inhibit force production [1–3,5,9,11]. Such a mechanism has been suggested in NaCN poisoned frog muscle undergoing repeated isometric contractions [22], skinned rabbit muscle fibers [9] and in humans throughout maximum voluntary contraction [2]. However, we found no significant correlation between $[H_2PO_4^-]$ and F ($P = 0.55$) at the time when F was maximal although $[H_2PO_4^-]$ was in the same range. This indicates that the inhibitory effect of P_i on force production is affected by time, being effective at the end but not at the onset of stimulation. This effect is further illustrated by the curvilinear relationships between $[H_2PO_4^-]$, $[P_i]$ and force which has been already reported in humans [23]. In contracting muscle, $[H_2PO_4^-]$ accumulates close to the myofilaments because P_i production resulting from PCr hydrolysis occurs mainly at the myosin ATPase level. Therefore, $H_2PO_4^-$ should theoretically affect F equally well at the time when F was maximal and at the end of the stimulation period. In keeping with our observations, it has been previously reported that the relationship between $[H_2PO_4^-]$ and force decline is affected by the exercise protocol [2], is different when analyzed in fatiguing or recovering muscle [23] and in patients with glycogen phosphorylase deficiency [23]. These observations are in line with our results and opposite to the classical hypothesis of a simple inhibitory effect of $H_2PO_4^-$ on force production as a result of impaired contractile protein function [2,5,11]. From a quantitative point of view, similar results have been reported previously with a reduction in force ranging from 25 to 70% for a 20-mM P_i concentration [9,24,25].

The time-dependent effect of $[P_i]$ and/or $[H_2PO_4^-]$ demonstrated in the present study is in agreement with recent reports indicating the involvement of calcium ions in the inhibitory effect of $[P_i]$ and/or $[H_2PO_4^-]$ on force production. P_i has been proposed to cause fatigue indirectly by altering Ca^{2+} fluxes throughout the sarcoplasmic reticulum (SR) membrane. Recent in vitro studies have demonstrated that P_i could enter the SR and binds Ca^{2+} to form transient highly-insoluble CaP_i species that precipitate within the SR [6,26–29]. This phenomenon has been proposed to account for the development of fatigue by reducing the amount of releasable Ca^{2+} from SR upon electrical stimulation, hence leading to the decrease in force-producing cross-bridges [6,26–29]. Our data are consistent with the hypothesis that fatigue could be indirectly caused by P_i precipitation into the SR. Direct micro-injection of P_i into single fibers from mouse muscle have evidenced that the entry of P_i into the SR was time-related and lasted several minutes for myoplasmic $[P_i]$ ranging from 10 to 50 mM [26,27] i.e. for concentrations similar to those measured in the present study. The fact that P_i movements between the myoplasm and the SR are time-related [26,27] could therefore explain why we found that $[P_i]$ was inversely correlated with F at the end of the stimulation period but not at the time when F was maximal, although $[P_i]$ was in the same range at these two stages of stimulation. An additional argument supporting this hypothesis is that a significant (12–29%) reduction in SR Ca^{2+} release has been reported in vitro for myoplasmic $[P_i]$ similar to those reported here [28].

In conclusion, given the curvilinear relationship between force production and $[P_i]$ and $[H_2PO_4^-]$ we have demonstrated that P_i and its diprotonated form cannot be considered as direct effectors of fatigue. P_i and $H_2PO_4^-$ affects force production only at the end of the stimulation period suggesting an indirect effect related to time, which might be mediated by an alteration of Ca^{2+} fluxes throughout the SR membrane.

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