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Myosin light chain phosphorylation during staircase in fatigued skeletal muscle

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Abstract. It has been reported that the peak of the staircase or the enhanced tension response during low frequency stimulation is delayed in fatigued fast muscle. Our purpose was to determine if the rate and extent of regulatory myosin light chain (P-LC) phosphorylation, a molecular mechanism associated with the positive staircase, are also altered by fatigue. The staircase contractile response, muscle metabolites and phosphate incorporation by the P-LC were assessed at 0, 5, 10 or 20 s of 10-Hz stimulation, in either non-fatigued (control) or fatigued (10 Hz for 5 min, followed by 20 min of recovery) rat gastrocnemius muscle in situ. The concentration of adenosine triphosphate (ATP) in fatigued muscles, 21 $\pm$ 0.9 mmol $\cdot$ kg^{-1} (dry weight) was significantly lower ($P < 0.05$) than in the control muscles, 26.1 $\pm$ 1.5 mmol $\cdot$ kg^{-1}. In both groups, ATP content was significantly lower after 20 s of 10 Hz stimulation. The P-LC phosphate content (in mol phosphate $\cdot$ mol^{-1} P-LC) was 0.10, 0.38, 0.60 and 0.72 after 0, 5, 10 or 20 s of 10 Hz stimulation in control muscles, but only 0.03, 0.08, 0.11 and 0.19 at these times in fatigued muscles. Although the absolute magnitude of tension potentiation was attenuated in proportion to the depressed twitch amplitude, these surprisingly low levels of phosphorylation were associated with 0, 48, 79 and 86% potentiation of the developed tension at these times in contrast with 0, 71, 87 and 49% potentiation in control muscles. These data demonstrate that while the rate and extent of phosphate incorporation is depressed in fatigued muscle, tension potentiation is still evident. The persistence of potentiation in the fatigued state indicates that either this condition results in greater potentiation for a given level of P-LC phosphorylation, or that factors in addition to P-LC phosphorylation are responsible for the staircase response.

Key words: Potentiation – Myosin light chain kinase – Muscle stimulation – Fatigue

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Introduction

Repetitive low frequency stimulation of mammalian fast-twitch skeletal muscle in situ elicits a tension staircase response, in which twitch developed tension is progressively potentiated over several seconds compared to developed tension of a control twitch [6, 9, 15]. In the rat gastrocnemius muscle, which is composed primarily of fast-twitch fibres [3], stimulation at 10 Hz results in a tension staircase which peaks after about 10 s [21, 22], and this is followed by a decrease in developed tension, fatigue [19, 21].

Although several factors (amplitude of Ca^{2+} transients, alkalinization, etc.) may contribute to potentiation, it has been proposed that the primary mechanism of staircase potentiation in mammalian fast or mixed fast skeletal muscle is phosphorylation of the regulatory (or phosphorylatable) myosin light chains (P-LC) [22]. The P-LC are phosphorylated by the specific enzyme myosin light chain kinase (MLCK) which is activated during the Ca^{2+} transient through binding of the Ca^{2+} $\cdot$ calmodulin complex by the enzyme [29]. The time course of phosphorylation and dephosphorylation corresponds closely with that of potentiation during the staircase and during the subsequent dissipation of enhanced isometric twitch tension in the period following the staircase [22]. In contrast, the magnitude of phosphorylation and potentiation are both low in red skeletal muscle [22]. Furthermore, it has been demonstrated that phosphorylation of the P-LC is associated with a leftward shift in the pCa^{2+}/tension relationship in skinned rabbit skeletal muscle fibres [26, 30]. This relationship suggests that force potentiation may result from increased sensitivity of the contractile proteins to subsaturating levels of Ca^{2+} as a result of P-LC phosphorylation.

The time needed to reach the peak of the staircase response (i. e. the number of individual twitches) is increased in muscle previously subjected to prolonged repetitive stimulation [19]. If potentiation during the staircase is tightly coupled with P-LC phosphorylation, the time course of phosphorylation should also be affected

in fatigued compared with non-fatigued muscle subjected to the same potentiating stimulus (i. e. 10 Hz for 10−20 s). However, if there is an apparent uncoupling in this relationship in fatigued muscle, this might suggest that either there is a change in the sensitivity of the relationship between P-LC phosphorylation and potentiation, as seen in muscles treated with dantrolene sodium [25], or that additional mechanisms may be responsible for, or contribute to, the tension staircase. In this regard, a transient alkalosis, associated with net creatine phosphate hydrolysis [2], might increase the sensitivity of the contractile proteins to Ca^{2+} [10]. Conversely, increased availability of Ca^{2+}, due either to transsarcolemmal movement [33] or to saturation of available intracellular Ca^{2+}-binding sites, could account for some degree of force potentiation.

Our purpose was to determine if the rate and extent of P-LC phosphorylation are altered in fatigued skeletal muscle. To test this possibility, fatigued and non-fatigued rat gastrocnemius muscles were stimulated at 10 Hz for 20 s. At intervals during this stimulation protocol, both tension response and P-LC phosphate content were determined.

Materials and methods

Muscle preparation. All procedures followed in this study were approved by a University committee for the ethical use of animals in research. Sprague-Dawley rats (175−220 g) were given rat chow and water ad libitum and maintained on a 12 h light : 12 h dark cycle. Animals were anaesthetized with an intraperitoneal injection of sodium pentobarbital (Somnotol, 50 mg/kg); with supplemental injections as needed. The left gastrocnemius muscle was surgically isolated while vascular connections and the muscle origin were left intact. The sciatic nerve was cleared of connective tissue for a length of 1−1.5 cm and cut proximally. The calcaneus was severed leaving a piece of bone attached to the Achilles tendon and the soleus and plantaris muscles were dissected free. A ligature was tied tightly around the leg midway between the ankle and the knee, and the distal half was cut off. A dissecting probe was inserted into the medulla of the tibia, and a drill bit placed perpendicularly through the femur and these were rigidly fixed to anchor the gastrocnemius muscle origin.

Once the leg was fixed in the myograph apparatus, the gastrocnemius muscle was secured via the Achilles tendon to an isometric tension transducer (Grass, FT.10 with black/blue springs, Grass Instruments, Quincy, Mass., USA, with a CWE PM 1000 pressure amplifier module, CWE, Ardmore, Pa., USA). The transducer was mounted on a rack and pinion device and oriented to measure strain in line with the pull of the muscle (approximately 45° from the horizontal). The connective tissue between the skin and the leg was disrupted, and the loosened skin was pulled up around the sides to form a bath which was filled with warmed mineral oil. Rectal and bath temperatures were monitored (YSI, model 43 telethermometer, Yellow Springs, Ohio, USA) and both were kept near 37°C with radiant heat.

The distal stump of the cut sciatic nerve was placed across a pair of silver wire hook electrodes, and stimulated with square pulses of 0.14 ms duration at supramaximal (1−5 V) voltage with a Grass Model S88 stimulator. Output of the tension transducer was visually displayed (Tektronix oscilloscope, Model TEK 2235), and monitored with a microcomputer (Apple II E, Apple Computers, Cupertino, Calif., USA) following analog-to-digital conversion at 7500 Hz.

Procedures. When rectal and muscle bath temperatures had stabilized at 37°C, muscle length was adjusted to yield maximal developed tension for double pulse contractions (delay between pulses = 5 ms). Double pulse contractions were used to determine optimal length, since single pulse contractions may interfere with this procedure, due to length-dependent activation [17]. All further stimulations were with single pulses at the specified frequency. The gastrocnemius muscles of rats randomly assigned to control (non-fatigued) and fatigued groups were subjected to 25 min of stimulation as follows. Control muscles were initially stimulated at 10 Hz for 20 s and thereafter for the remainder of the 25 min period at 0.1 Hz. For the fatigued group, the gastrocnemius muscle was stimulated at 10 Hz for 5 min followed by 20 min of test contractions at 0.1 Hz. This stimulation pattern has been used previously to study contractile response to activity, and has been shown to result in a depression of the contractile response which seems to be analogous to low frequency fatigue [19−21]. Low frequency fatigue is characterized by a prolonged depression of contractions in response to low, but not high, frequency stimulation. Following the recovery period, twitch amplitude remains attenuated but tetanic contractions are not different from prefatigue amplitude [20]. For both groups following their respective 25 min protocols, a 10 Hz stimulation pattern for up to 20 s was employed to elicit the staircase response. Muscles were freeze clamped prior to (0 s) or after 5, 10 or 20 s of this 10 Hz stimulation. To facilitate rapid freezing, the oil was removed from around the muscle just prior to the start of the (10 Hz) staircase stimulation. The frozen muscle was stored at −80°C for later analysis.

Contractions were sampled by the microcomputer at intervals during the stimulation and analysed for developed tension, time from the onset of tension development to the peak of the contraction, and peak rate of relaxation which was calculated as the maximal decline in tension (steepest slope) over 14% of the falling phase of the twitch. Twitch contractile characteristics were determined using a program written by one of the authors on an Apple IIE computer. It was anticipated that these measurements of the twitch contraction characteristics would be useful in evaluating the possible role of factors other than P-LC phosphorylation in the staircase response. For example, saturation of the Ca^{2+}-binding sites in the myoplasm should result in prolonged contraction time and slowed peak rate of relaxation [11, 31].

Biochemical analysis. Frozen muscle samples were analysed for P-LC phosphorylation and metabolite content. Analysis of P-LC phosphorylation was performed according to the procedures of Silver and Stull [27], with modifications as described by Moore and Stull [22] and Stuart et al. [28]. Briefly, 5−10 mg of frozen tissue were homogenized and diluted and then subjected to pyrophosphate polyacrylamide gel electrophoresis to isolate the myosin. The band representing myosin was identified with Coomassie blue stain, cut from the gel, denatured and subjected to isoelectric focusing over the pH range 4.5−6.0. Gels from isoelectric focusing were fixed and silver stained and then scanned using an LKB 2202 Ultroscan laser densitometer with attached LKB 2220 recording integrator to establish the relative proportions of the phosphorylated and non-phosphorylated P-LC.

The frozen muscle samples were lyophilized for metabolite assays, and the freeze-dried samples were stored under a vacuum at −80°C. Before extraction of metabolites, the samples were brought to room temperature under a vacuum, visible connective tissue was teased away and the muscle samples were weighed. Muscle metabolites were extracted with 0.5 M $HClO_4$ and neutralized with 2.2 M $KHCO_3$. After centrifugation, the neutralized supernatant was assayed for metabolite concentrations and the pellet frozen for later determination of glycogen content. The contents of ATP, phosphocreatine (PCr), lactate and glycogen for each muscle were determined in triplicate using established fluorometric techniques [18]. These metabolite measurements were necessary to establish the energetic state of the fatigued muscle relative to the control muscles, and specifically the measurement of PCr was anticipated to provide insight into the possible role of PCr hydrolysis contributing to staircase potentiation.

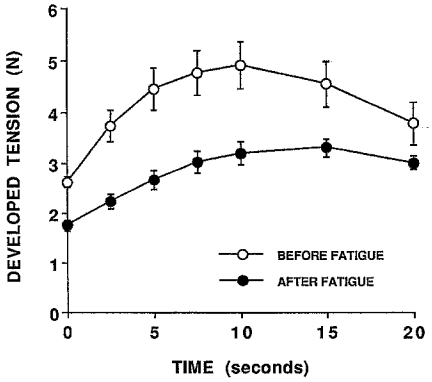

Fig. 1. Developed tension versus time for 20 s of repetitive stimulation at 10 Hz before and after the 5 min fatigue (plus 20 min of recovery). Values are means ± SEM ($n = 4$)

Statistical methods. A two factor analysis of variance (group and time) was employed to determine significant differences in the phosphorylation and metabolic data. Simple main effects were assessed by a one-way analysis of variance for each group. A repeated measures analysis of variance was used to assess the staircase responses of the rested and fatigued muscles which were stimulated at 10 Hz for 5 min and also stimulated at 10 Hz for the complete 20 s after the recovery period. Significant differences between means were determined with the Newman-Keuls test, modified for repeated measures where appropriate [14]. Differences were considered significant for $P < 0.05$. Data are presented as means ± SEM.

Results

Contractile responses

For the control group (non-fatigued), twitch developed tension was initially 3.38 ± 0.24 N and was unchanged (3.40 ± 0.23 N) after the 25 min stimulation protocol. During the initial 20 s of 10 Hz stimulation for both the control and fatigued groups, muscle staircase responses were similar, demonstrating a peak force potentiation of 85% by 10 s. When 10 Hz stimulation was continued for 5 min, the potentiated response was followed by a rapid decrease to 39% of the initial developed tension at 60 s. There was little further change in developed tension to the end of the 5 min of repetitive stimulation. Within seconds of changing the stimulation frequency to 0.1 Hz, a partial recovery in force was observed; however, an additional 20 min at 0.1 Hz led to only minor additional tension changes. Over the 25 min protocol for the fatigue group, developed tension which was initially 3.53 ± 0.27 N, decreased ($P < 0.05$) to 1.99 ± 0.16 N.

To illustrate the staircase responses of rested and fatigued muscles, Fig. 1 presents the developed tension of twitch contractions from the initial 20 s of the 5 min 10 Hz fatigue stimulation (before fatigue) with tension responses from the 20 s of 10 Hz stimulation following the fatigue plus recovery protocol (after fatigue). These observations were obtained from the only four muscles for which contractile responses could be obtained for the complete 20 s of staircase both before and following fatigue. Therefore, the same muscles are represented before and after fatigue. Peak developed tension occurred earlier before fatigue than it did after fatigue. The actual degree of potentiation observed at the time of freezing for muscles of both groups (control and fatigue) is presented in Table 1.

Table 1. Twitch developed tension prior to freezing[a]

Time (s)	Tension			
	n	Control	n	Fatigue
5	6	71 ± 4.4	5	48 ± 8.5
10	6	87 ± 3.4	4	79 ± 5.5
20	4	49 ± 11.7	4	86 ± 14.2

[a] Developed tension is expressed as $(F_D^* \times F_{DO}^{-1} - 1) \times 100$, where F_D^* is developed tension just prior to freezing, and F_{DO} is developed tension of the first contraction in the 10 Hz (staircase) series

The contraction time and peak rate of relaxation of twitch contractions during staircase before and after fatigue were also measured. Before fatigue, contraction time increased ($P < 0.05$) during the initial 7.5 s of the tension staircase (Fig. 2). In contrast, during staircase following recovery there was a significant decrease in contraction time during the initial 2.5 s, with a subsequent increase back to the control value by 10–20 s of repetitive stimulation. Figure 2 B illustrates the transient changes in the peak rate of relaxation during 20 s of stimulation at 10 Hz before and after fatigue. In both cases, the peak rate of relaxation increased during staircase from 0 to 7.5 or 10 s. Following this, peak rate of relaxation decreased (20 s < 15 s < 10 s, $P < 0.05$) before fatigue but did not change in the fatigued muscles (10 s = 15 s = 20 s, $P > 0.05$). Therefore, slowing of relaxation is evident late in the staircase response before fatigue but not apparently when the muscles have been fatigued.

P-LC phosphate content

The observed values for phosphorylation of the P-LC are shown in Fig. 3. In control muscles, the phosphate content of the P-LC was significantly increased at the three sampling points compared to the 0 s value. In fatigued muscle, only 20 s of stimulation resulted in a significant increase in phosphorylation over the 0 s value. P-LC phosphate content in the control muscles was significantly different from that of the fatigued muscles at all corresponding time points except for the 0 s time.

Metabolites

The concentrations of ATP, PCr, lactate and glycogen in the muscles frozen during the final tension staircase are presented in Table 2. In the control muscles, the concen-

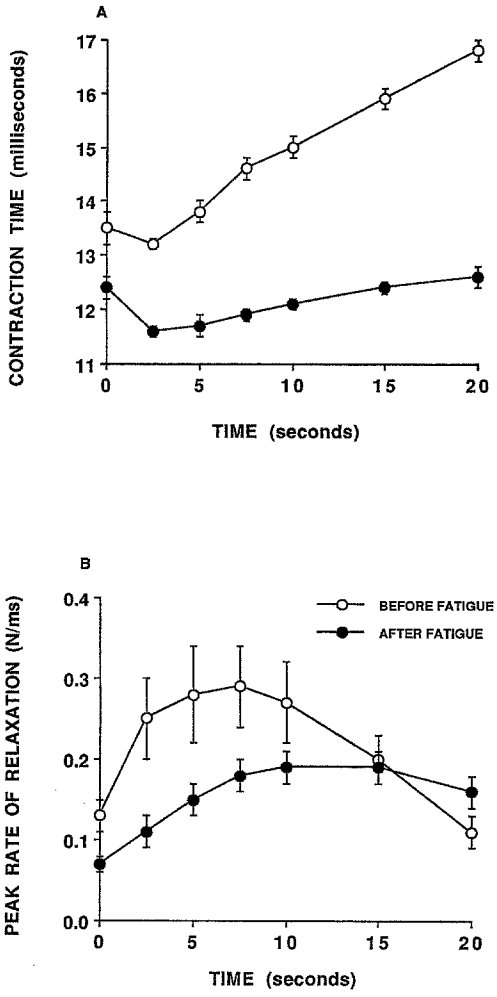

Fig. 2. **A** Contraction time versus time for 20 s of repetitive stimulation at 10 Hz before and after 5 min of fatiguing stimulation plus 20 min of recovery. Values are means ± SEM ($n = 4$). **B** Peak rate of relaxation versus time for 20 s of repetitive stimulation at 10 Hz before fatigue and after the 5 min of 10 Hz stimulation plus 20 min of recovery. Values are means ± SEM ($n = 4$)

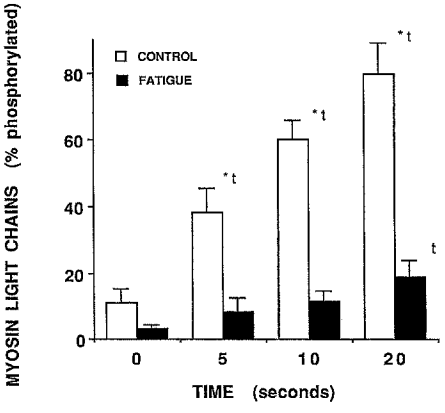

Fig. 3. Phosphorylation of the regulatory light chains of myosin during 20 s of staircase with stimulation at 10 Hz in non-fatigued and fatigued muscle is shown. See Table 1 for number of experiments for each condition. Symbols represent significant differences ($P < 0.05$) between groups (control versus fatigued). * Significant difference from the corresponding zero time measurement within the same group (t)

tration of ATP was significantly decreased relative to the 0 s value only at 20 s of stimulation but was still 70% of the control value at this time. PCr was significantly decreased by 5 s and was less than 10% of the initial level after 20 s of 10 Hz stimulation in the control muscles. Lactate was significantly increased threefold and nearly fourfold at 10 s and 20 s respectively; and glycogen was significantly decreased by 10 s and depleted 50% after 20 s. Compared to the 0 s concentrations of the control group, the fatigued muscles after 20 min of recovery demonstrated similar PCr and lactate values, but significantly lower concentrations of ATP and glycogen. In the fatigued group the changes in the metabolite concentrations during the 20 s of 10 Hz stimulation followed a pattern similar to that of the control group. The exception was lactate which increased moderately but not significantly over the 20 s stimulation.

Discussion

Phosphate incorporation into the P-LC of myosin accompanied by force potentiation has been consistently observed in response to brief tetani or more prolonged lower frequency stimulation in fast and mixed fast skeletal muscle [12, 13, 22–25]. In the present study our purpose was to investigate the phosphorylation/force potentiation relationship in fatigued muscle in order to determine if the rate and extent of myosin P-LC phosphorylation are altered during the 20 s of 10 Hz stimulation in fatigued compared to non-fatigued muscle. While our values for phosphorylation and potentiation are similar to those reported by Moore and Stull [22] in non-fatigued muscle, our data suggest an apparent uncoupling in this relationship since we observed a lower level of phosphorylation for a similar degree of potentiation in fatigued compared to non-fatigued muscle.

In agreement with the close temporal relationship between P-LC phosphorylation and staircase potentiation reported by Moore and Stull [22], our control muscles demonstrated a linear relationship between these two measured (Fig. 4 A), at least for the first 10 s of 10 Hz stimulation. The relationship between developed tension and P-LC phosphorylation in the fatigued muscles over this initial stimulation period differs from that of the control muscles in that the combined changes in potentiation and phosphorylation merely bring the fatigued muscle to values which correspond with the resting (zero time) condition of the control muscles. In particular, the slopes of the two relationships are different. This difference between fatigued and control muscles is more evident in Fig. 4 B, where developed tension is plotted as % of its initial value. It is clear that the relative potentiation which is associated with a given level of phosphorylation is much greater in muscles of the fatigued group. In fact, the fatigued muscles attained 86% potentiation with only 19% P-LC phosphorylation. If P-LC phosphorylation is the principle mechanism responsible for staircase potentiation, then the strong relationship between level of phosphorylation and potentiation should

Table 2. Concentrations of metabolites during staircase (mmol · kg^{-1} dry weight)

Group	Time (s)	n	Metabolite			
			ATP	PCr	Lactate	Glycogen
C	0	6	26.1 ± 1.5	65.6 ± 7.0	19.6 ± 5.7	120.4 ± 8.2
C	5	6	25.0 ± 1.0	45.2 ± 2.4*	32.4 ± 2.9	105.9 ± 4.9
C	10	6	23.9 ± 1.2	33.5 ± 9.6*	60.9 ± 12.2*	90.5 ± 7.2*
C	20	4	18.1 ± 1.2*	5.9 ± 2.9*	73.1 ± 9.8*	59.0 ± 13.1*
F	0	8	21.0 ± 0.9	74.2 ± 6.7	21.2 ± 6.2	61.7 ± 4.2
F	5	5	22.8 ± 1.6	48.7 ± 7.5*	40.4 ± 7.0	50.7 ± 10.1
F	10	5	18.9 ± 0.9	49.7 ± 12.8*	36.5 ± 9.9	51.4 ± 6.8
F	20	4	15.9 ± 1.3*	13.2 ± 1.1*	39.9 ± 2.5	34.2 ± 2.7*

* Significantly different from the time zero measurement ($P < 0.05$); C, control; F, fatigued; PCr, phosphocreatine

be preserved in fatigued muscle. Our results suggest, however, that either there was an increased responsiveness of the contractile apparatus to the potentiating effect of phosphorylated P-LC, and/or that additional potentiating mechanisms were operating.

A greater degree of potentiation at a given level of P-LC phosphorylation would be expected if smaller Ca^{2+} transients (lower Ca^{2+} concentration during activation) occurred in these fatigued muscles. The discrepancy apparent between the extent of relative potentiation and phosphate incorporated into the P-LC in the fatigued muscle is similar to that observed for in vitro mouse fast muscles treated with dantrolene sodium [25], a drug which attenuates sarcoplasmic Ca^{2+} release [9, 16]. Depressed absolute twitch force values for dantrolene-treated muscles still demonstrate a greater relative post-tetanic potentiation despite only modest increases in P-LC phosphate content compared with control muscles [25]. Furthermore, this interpretation is analogous with the effect of increased temperature on Ca^{2+} availability. For example, there is less P-LC phosphorylation, yet a greater relative twitch potentiation at 35°C compared to 25°C in fast muscles of mouse stimulated at 5 Hz for 20 s [24]. As explained by Moore et al. [24], the diminished and faster tension profiles that occur at high compared with low temperatures [7] are consistent with temperature-dependent effects on the amplitude and time course of the Ca^{2+} transient [4]. These temperature-dependent changes result in less activaton of the contractile element as well as less phosphate incorporation by the P-LC, consistent with the Ca^{2+}-dependent nature of this process [5]. Similar to these observations, it is quite possible that the greater potentiation at a given level of P-LC phosphorylation in the fatigued muscles of this study, is associated with an attenuated Ca^{2+} transient, an event which accompanies some forms of fatigue [1, 32].

There is some evidence that 10 Hz stimulation of the rat gastrocnemius muscle in situ for 5 min results in attenuated Ca^{2+} transients. This type of fatigue is characterized by a prolonged attenuation of the twitch contraction, while tetanic contraction is not different from that in the control (rested) condition [20]. The depressed twitch could be the result of either a smaller Ca^{2+} transient with each activation, or a depressed sensitivity of the

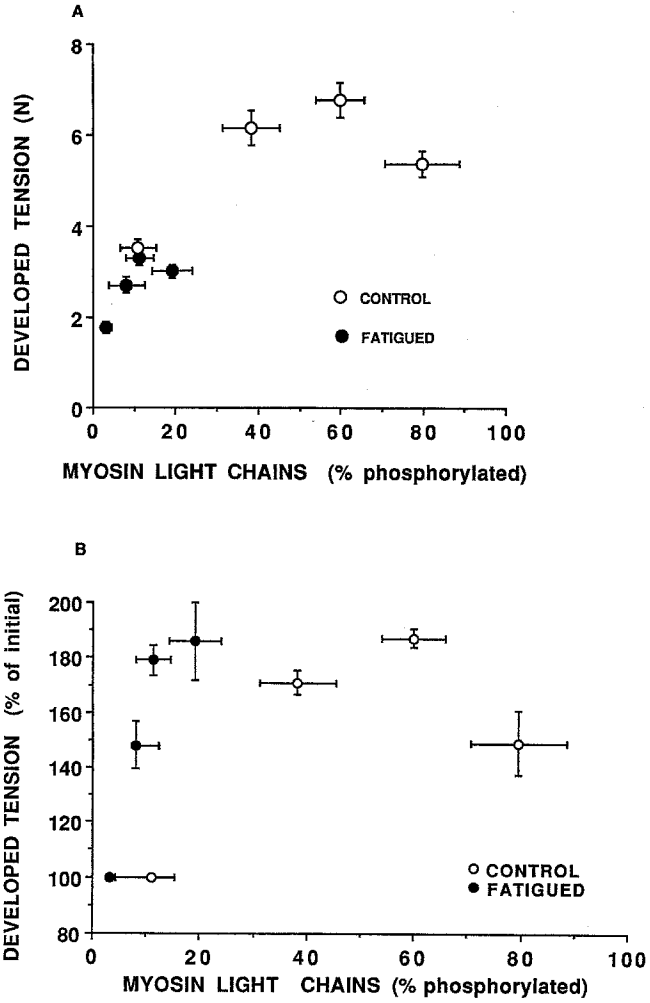

Fig. 4. Developed tension in absolute (**a**) and relative (**b**) units versus percentage myosin light chain phosphorylation is shown. *Points* correspond to measurements at 0, 5, 10 and 20 s representing progressively greater phosphorylation. Note that the values of developed tension (**A**) and % phosphorylation for the initial contraction in the non-fatigued muscle (0 s), is almost superimposed with the value for the fatigued muscle at 10 s. Regression analysis of the mean values for tension potentiation versus phosphorylation relationship over the first 10 s of stimulation gave the following: F_D (rested) = 3.02 ± 0.07 (% phosphorylated); r = 0.95 and F_D (fatigued) = 1.19 ± 0.19 (% phosphorylated); r = 1.0

contractile proteins to Ca^{2+}. The major factors which have been identified to alter contractile sensitivity to Ca^{2+} in fatigue are increased concentration of inorganic phosphate [8] and/or decreased pH [10]. The metabolic measurements presented in Table 2 show that PCr is fully restored after the 20 min recovery period, therefore the concentration of inorganic phosphate was not likely to be elevated at this time. Lactate content is also back to the control (rested) level, so the fatigued muscles were unlikely to be acidotic. Sensitivity of the contractile proteins to Ca^{2+} is probably not affected in the fatigued muscle after 20 min of recovery. This leaves attenuation of Ca^{2+} transients as the most likely cause of this fatigue. In further support of this contention, muscles treated with dantrolene sodium mimic many of the contractile features of fatigue resulting from 5 min of 10 Hz stimulation, including prolongation of the time taken to reach the peak of the staircase response and the transient changes in half-relaxation time during staircase [19].

Smaller Ca^{2+} transients for each activation can possibly explain the greater relative potentiation of these fatigued muscles for a given level of P-LC phosphorylation, and could certainly explain the apparent slower rate of P-LC phosphorylation, which was observed in these muscles since this is a Ca^{2+}-dependent process [5]. Alternatively, some other potentiating factor may have been involved, and this other factor was more effective in potentiating fatigued than non-fatigued muscles. Transient alkalosis due to PCr hydrolysis or saturation of Ca^{2+}-binding sites in the myoplasm may contribute to staircase potentiation. However, since PCr hydrolysis was greater in the control muscles than in the fatigued muscles, and since prolongation of contraction time was more apparent in control than in fatigued muscles, it seems unlikely that these processes contributed to the enhanced staircase in the fatigued muscles. Nevertheless, the involvement of other mechanisms in the staircase potentiation process of fatigued muscles cannot be ruled out without further experimentation.

In this paper, we have reported that there is a slower rate of P-LC phosphorylation in fatigued muscle compared with non-fatigued muscle and that relative potentiation for a given level of P-LC phosphorylation is greater in the fatigued muscle. This enhanced potentiation and attenuation of phosphorylation of P-LC is consistent with the theory that fatigue results in smaller Ca^{2+} transients. However, it is unclear whether or not phosphorylation of the P-LC can account for all of the potentiation which was observed in the fatigued muscles.

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