The Journal of Experimental Biology 214, 3915-3923

doi:10.1242/jeb.061150

RESEARCH ARTICLE

The effect of work cycle frequency on the potentiation of dynamic force in mouse fast twitch skeletal muscle

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Accepted 30 August 2011

SUMMARY

The purpose of this study was to test the hypothesis that the potentiation of concentric twitch force during work cycles is dependent upon both the speed and direction of length change. Concentric and eccentric forces were elicited by stimulating muscles during the shortening and lengthening phases, respectively, of work cycles. Work cycle frequency was varied in order to vary the speed of muscle shortening and/or lengthening; all forces were measured as the muscle passed though optimal length (L_o). Both concentric and eccentric force were assessed before (unpotentiated control) and after (potentiated) the application of a tetanic conditioning protocol known to potentiate twitch force output. The influence of the conditioning protocol on relative concentric force was speed dependent, with forces increased to 1.19±0.01, 1.25±0.01 and 1.30±0.01 of controls at 1.5, 3.3 and 6.9 Hz, respectively (all data N=9–10 with P<0.05). In contrast, the conditioning protocol had only a limited effect on eccentric force at these frequencies (range: 1.06±0.01 to 0.96±0.03). The effect of the conditioning protocol on concentric work (force × distance) was also speed dependent, being decreased at 1.5 Hz (0.84±0.01) and increased at 3.3 and 6.9 Hz (1.05±0.01 and 1.39±0.01, respectively). In contrast, eccentric work was not increased at any frequency (range: 0.88±0.02 to 0.99±0.01). Thus, our results reveal a hysteresis-like influence of activity-dependent potentiation such that concentric force and/or work were increased but eccentric force and/or work were not. These outcomes may have implications for skeletal muscle locomotor function *in vivo*.

Key words: eccentric, concentric, myosin phosphorylation.

INTRODUCTION

Skeletal muscle tissue performs a variety of roles crucial for organism survival. Chief among these is the ability to transduce chemical energy into mechanical energy, a conversion that powers locomotion. Important in this regard is the ability of skeletal muscle to generate the necessary concentric and eccentric forces to produce and absorb work, respectively. The rapid and rhythmical changes in muscle length and speed that characterize locomotion directly constrain the work-generating ability of skeletal muscle *via* the length–tension and force–velocity relationships; in addition, skeletal muscle performance is highly dependent upon adequate activation of available muscle fibres/motor units regardless of mechanical load (Marsh, 1999; Josephson, 1993). Importantly, prolonged activity leading to substrate depletion, metabolite and ion accumulation and/or compromised structural integrity (i.e. damage) may challenge the force-generating ability of skeletal muscle during locomotion. Hence, vertebrate skeletal muscle must operate within a wide envelope of mechanical and metabolic conditions that either directly or indirectly modulate performance.

Based on the myriad factors interacting with skeletal muscle performance it is perhaps not surprising that intracellular mechanisms have evolved to aid skeletal muscle function. An example is phosphorylation of the myosin regulatory light chains (RLC), widely reported to coincide with activity-dependent potentiation, i.e. the increased isometric twitch force observed during or following contractile activity of vertebrate fast twitch skeletal muscle (MacIntosh, 2010). In rodent hindlimb muscles, for example, an increased isometric twitch force is often observed during low frequency contractile activity or following brief, high frequency contractile activity (i.e. staircase and post-tetanic potentiation, respectively) (Close and Hoh, 1968a; Krarup, 1981a). Biochemical studies illustrate that the same cytosolic Ca^{2+} signal that activates muscle contraction also generates a signalling cascade that phosphorylates the RLC in a stimulation frequency- and duration-dependent manner (Klug et al., 1982; Manning and Stull, 1982; Moore and Stull, 1984). In this process, a Ca^{2+}–calmodulin signalling cascade activates a skeletal isoform of the myosin light chain kinase (skMLCK) enzyme to catalyse the transfer of a phosphate moiety from ATP to a serine residue contained by the RLC subunit of fast skeletal myosin isoforms (reviewed by Stull et al., 2011). In mechanistic terms, studies using permeabilized skeletal muscle fibres have demonstrated that skMLCK catalysed phosphorylation of the RLC increases the sensitivity of the contractile apparatus to suboptimal Ca^{2+} activation (Persechini et al., 1985; Sweeney and Kushmerick, 1985). Although redundant mechanisms may exist, recent work using fast muscles from the hindlimb of mice devoid of skMLCK verify that RLC phosphorylation is a primary mechanism for activity-dependent potentiation. For example, extensor digitorum longus (EDL) muscles from skMLCK knockout mice, which cannot phosphorylate the RLC above basal levels, exhibit no post-tetanic potentiation and only reduced staircase responses compared with wild-type muscles (Gittings et al., 2011;

Zhi et al., 2005). Moreover, muscles from mice in which skMLCK is overexpressed exhibit increased activity-dependent potentiation relative to wild-type responses (Ryder et al., 2007). Based on these results, it seems justified to suggest that contraction-induced elevations in RLC phosphorylation operate as a molecular memory to modulate the contractile function of fast twitch skeletal muscle (Stull et al., 2011).

The relationship between RLC phosphorylation and activity-dependent potentiation has largely been established using isometric contractions. More recent studies, however, demonstrate dynamic muscle function involving muscle shortening, work and power are also subject to modulation by activity-dependent potentiation. Relevant in this regard are studies showing that RLC phosphorylation is associated with increased dynamic force, work and power in both rat and mouse muscle (Abbate et al., 2000; Grange et al., 1995; Grange et al., 1998; MacIntosh and Bryan, 2002; MacIntosh et al., 2008; Xeni et al., 2011). In general, these studies show that the rate and extent of isotonic shortening and/or work/power are enhanced during or following contractile activity that phosphorylates the myosin RLC. Important questions remain, however, regarding how RLC phosphorylation-mediated force potentiation interacts with the speed and direction of muscle length change. For example, relatively few studies have examined the influence of activity-induced potentiation on concentric *versus* eccentric forces in response to different but comparable length change speeds. It thus seems incumbent to investigate the influence of activity-dependent potentiation on both concentric and eccentric forces of intact skeletal muscle in order to better understand the functional utility of this phenomenon in skeletal muscle locomotor function.

The purpose of the present study was to test the effect that the speed and direction of muscle length change has on dynamic twitch force potentiation during muscle work that mimics locomotion. To this end we used the work cycle technique pioneered by Josephson (Josephson, 1985) to assess concentric and eccentric force potentiation at different frequencies in mouse EDL muscle stimulated *in vitro* (25°C). We hypothesized that concentric force would be potentiated more than isometric force and that this potentiation would vary with the speed of muscle length change. Moreover, we hypothesized that eccentric force potentiation would be negligible and independent of the speed of muscle length change.

MATERIALS AND METHODS

All procedures received full ethical approval from the Brock University Animal Care and Use Committee. Adult female C57Bl/6 mice (17–22 g) were housed in the Brock animal care facility and provided with standard chow and water *ad libitum*. Mice were anaesthetized with an intra-peritoneal injection of sodium pentobarbitol (6 mg 100 g^{-1} body mass) and the EDL muscle was surgically excised. Muscles were suspended in a jacketed vertical bath, containing continuously oxygenated Ringer solution maintained at 25°C. The proximal tendon was attached to the arm of a dual mode servomotor (Model 305B, Aurora Scientific Inc., Aurora, ON, Canada) by non-absorbable silk suture, while the distal tendon was fixed in a clamp. Following a 45 min equilibration period optimal length for isometric twitch force (L_o) was determined. Muscle stimulation was applied using flanking platinum electrodes, provided by a Model 701B biphasic stimulator (Aurora Scientific Inc.) with voltage set to 1.25 times the minimum threshold value required to elicit a twitch. Muscle length was controlled by LINUX-based software (Aurora Scientific Inc.) and force was monitored by the servomotor. All data were collected on-line at 2000 Hz and saved to a hard drive for future analysis.

Work cycle construction and design

Work cycles were performed by cycling muscle length about L_o using a software-generated sine-wave function (Grange et al., 1998; Xeni et al., 2011). Sine excursion (i.e. amplitude) was 5% of muscle length centred on L_o (±2.5%). Concentric and eccentric forces were produced by applying a single stimulus pulse while the muscle was being shortened or lengthened, respectively. In each case, the stimulus pulse was timed to produce peak twitch force at or near L_o. A key feature of these experiments was the use of different work cycle frequencies in order to alter the speed of muscle length change (concentric and eccentric). Accordingly, the work cycle frequencies used in this study were 1.5, 3.3 and 6.9 Hz. The amplitude and frequency of the work cycles fall within the range previously reported for mouse muscle (Askew et al., 1997; Grange et al., 1998; Stevens and Syme, 1993b; Syme and Stevens, 1989). Although a sine-wave function does not produce constant speed shortening or lengthening over total excursion amplitude, length change speed immediately around L_o is relatively linear (Josephson, 1985). Based on previous published values (Brooks and Faulkner, 1988) for maximal shortening velocity (V_{max}) of mouse EDL (25°C) of 9.8 fibre lengths s^{-1}, work cycle frequencies of 1.5, 3.3 and 6.9 Hz produced muscle length change speeds corresponding to ~5, 12 and 25% V_{max} as the muscle passed through L_o. A plot of active dynamic force output against length over the duration of the sine-wave creates a work loop the area of which represents total work (in J) (Grange et al., 1998; Josephson, 1985). Work performed was then normalized to wet muscle mass (J kg^{-1}). Multiplying net work per cycle by sine frequency yielded mechanical power output (W kg^{-1}).

Experimental protocol

The experimental time line is shown in Fig. 1. Each muscle was cycled at a specific sine frequency for ~30 s without stimulation

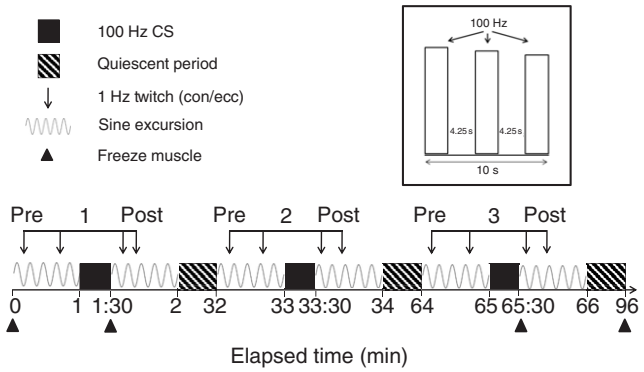

Fig. 1. Time line showing various events during each experiment. Each experiment had three phases, with each phase representing one work cycle frequency (i.e. 1.5, 3.3 or 6.9 Hz). During each phase, dynamic parameters at a specific work cycle frequency were assessed ~20 s before and ~20 s after a conditioning protocol designed to elevate myosin regulatory light chain (RLC) phosphorylation and potentiate muscle twitch forces (see inset). Work cycle frequency was altered from phase to phase with the sequence randomized from muscle to muscle to minimize order effects. Each phase was separated by ~30 min, a time during which muscle state slowly decayed from potentiated to unpotentiated, as assessed by contractile and chemical data. Inset: with length fixed at optimal length for twitch force (L_o) the muscle received three volleys of 100 Hz stimulation with each volley lasting 500 ms. The time between volleys was ~4.25 s. Peak forces attained during the first and last tetanus of the protocol were compared to assess the presence of fatigue. In addition, isometric twitches (pre and post) were elicited in the time available between sinusoidal length changes and the conditioning protocol. Dynamic and isometric potentiation were assessed by comparing post- with pre-protocol data (1.00=no change).

(passive). Muscles then received single pulses during shortening and lengthening during alternate sine waves (for ~30 s). Upon completion of this 60 s time window the sine function was halted and the muscle held at L_o for ~30 s. During this time an isometric twitch was elicited shortly before (~5 s) and after (~15 s) receiving a conditioning protocol consisting of three brief (500 ms), high frequency (100 Hz) volleys spaced evenly apart (4.2 s) within a 10 s window. This protocol has been shown to potentiate isometric and concentric twitch force with minimum fatigue (Vandenboom et al., 1997). Immediately following this the muscle was again cycled around L_o at the same frequency as before the conditioning protocol. Because of the rapid dissipation of potentiation, only responses obtained within 5 s of the conditioning protocol were analysed, however. In all cases, passive responses were subtracted from stimulated responses to obtain active responses for both concentric and eccentric force as well as total work.

Myosin phosphorylation

RLC phosphate content was assessed at four different time points throughout the experimental sequence. RLC phosphorylation levels in resting muscles were obtained by freezing muscles immediately after equilibration before any stimulation (no contractile data obtained) as well as 30 min after the final conditioning protocol (contractile data obtained) of some experiments. RLC phosphorylation levels in response to the conditioning protocol were also assessed in two different groups. In some experiments, muscles were frozen after a single conditioning protocol (no contractile data obtained) while others were frozen after the third and final conditioning protocol of an experiment (contractile data obtained). All frozen muscles were stored at –80°C until being packed on dry ice and shipped to the laboratory of Dr James Stull (University of Texas, Southwestern Medical Center, Dallas, TX, USA) for quantification of RLC phosphorylation. Details regarding these methods and procedures have been presented elsewhere (Xeni et al., 2011).

Statistics

A Univariate ANOVA was used to test the main effect for work cycle frequency and conditioning stimulus (CS) on dynamic force. Similarly, a univariate ANOVA was used to test the main effect for work cycle frequency and CS on dynamic work/power. For comparison, all relative means were assessed using a one-way ANOVA. Tukey's HSD *post hoc* test was used to assess differences between treatment means. In all cases, differences were considered significant at $P<0.05$. All data are reported as means ± s.e.m.

RESULTS

Dynamic forces

Representative records showing the effect of the conditioning protocol on concentric and eccentric responses are shown in Fig. 2 (A and B, respectively). These records show how passive and total (passive + active) forces were altered during work cycles at 1.5, 3.3 and 6.9 Hz. Because passive forces were not influenced, the increase in concentric force shown for each condition was an increased active force. Concentric and eccentric forces determined during different frequency work cycles before and after the conditioning protocol are shown in Table 1. These data show that, consistent with known force–velocity properties, concentric forces decreased and eccentric forces increased as work cycle frequency increased from 1.5 to 6.9 Hz. The overall effect of the conditioning protocol on relative concentric and eccentric forces during different work cycle frequencies is summarized in Fig. 3. This plot shows that although concentric force was increased at all work cycle frequencies by the conditioning protocol, there was a speed dependence for this increase. For example, the relative response at 1.5 Hz was similar to the isometric response; however, higher frequencies produced progressive increases in the magnitude of concentric force potentiation. In contrast, eccentric force potentiation was either severely attenuated (1.5 Hz) or absent (3.3–6.9 Hz) following the conditioning protocol.

Work and power

Representative work loops from these experiments are shown in Fig. 4 with concentric and eccentric data from all experiments compiled in Tables 2 and 3, respectively, with relative changes to these parameters plotted in Fig. 5. The traces show the relative change in total work (and thus power) by the conditioning protocol at each frequency tested for concentric (Fig. 4A–C) and eccentric (Fig. 4D–F) responses. Interestingly, the concentric work loops in Fig. 4A–C reveal how, despite robust increases in concentric force, total work loop area was not increased at all work cycle frequencies. For example, it was only at 6.9 Hz that concentric work was increased in accordance with the concentric force response. Interestingly, the concentric work loops shown in Fig. 4A–C reveal a frequency-dependent shift in the 'cross-over' point, i.e. the point during shortening where post-stimulus force is reduced compared with pre-stimulus force during the twitch relaxation phase. The importance of this point is that its position relative to the length axis influences the change in total work (i.e. force × length) caused by the conditioning protocol. It is noteworthy that the cross-over point shifts to the left, away from L_o and peak concentric force, with increasing work cycle frequency. As an example, at 6.9 Hz the position of the cross-over point at the very shortest length means that concentric force was increased throughout the work cycle, unlike the response at lower frequencies. This analysis accounts for why concentric work was not increased in accordance with the potentiation of peak concentric force at 1.5 and 3.3 Hz. In contrast to concentric responses, the eccentric work loops shown in Fig. 4D–F reveal that, in addition to the greatly increased force, work and power relative to concentric forces, there was little difference in these parameters when pre- and post-stimulus responses were considered.

Isometric responses

The influence of the conditioning protocol on isometric twitch forces is shown in Table 1. It is noteworthy that absolute values for isometric twitch force obtained either before (unpotentiated) or after (potentiated) the conditioning protocol were similar over the course of our experiments. Moreover, the magnitude of potentiation was constant across all conditions, varying by less than 5% (pooled mean, 1.20±0.1 of unpotentiated controls). In terms of the conditioning protocol itself, the last tetanic volley produced 0.97±0.002 of the force produced in the first tetanic volley (i.e. 255±6.8 to 246±6.6 mN, respectively; N=27 trials all muscles), with little variation between trials. These data thus show that the baseline twitch performance of mouse EDL muscle was constant over the duration of our experiment and that interpretation of our work cycle results was not complicated by the presence of fatigue.

RLC phosphorylation

The effect of the conditioning protocol on RLC phosphorylation is shown in Fig. 6. These data show that RLC phosphorylation levels were low in quiescent muscles frozen either at the beginning or at the end of the experiments and that the conditioning protocol increased this approximately 3-fold. These data thus show that

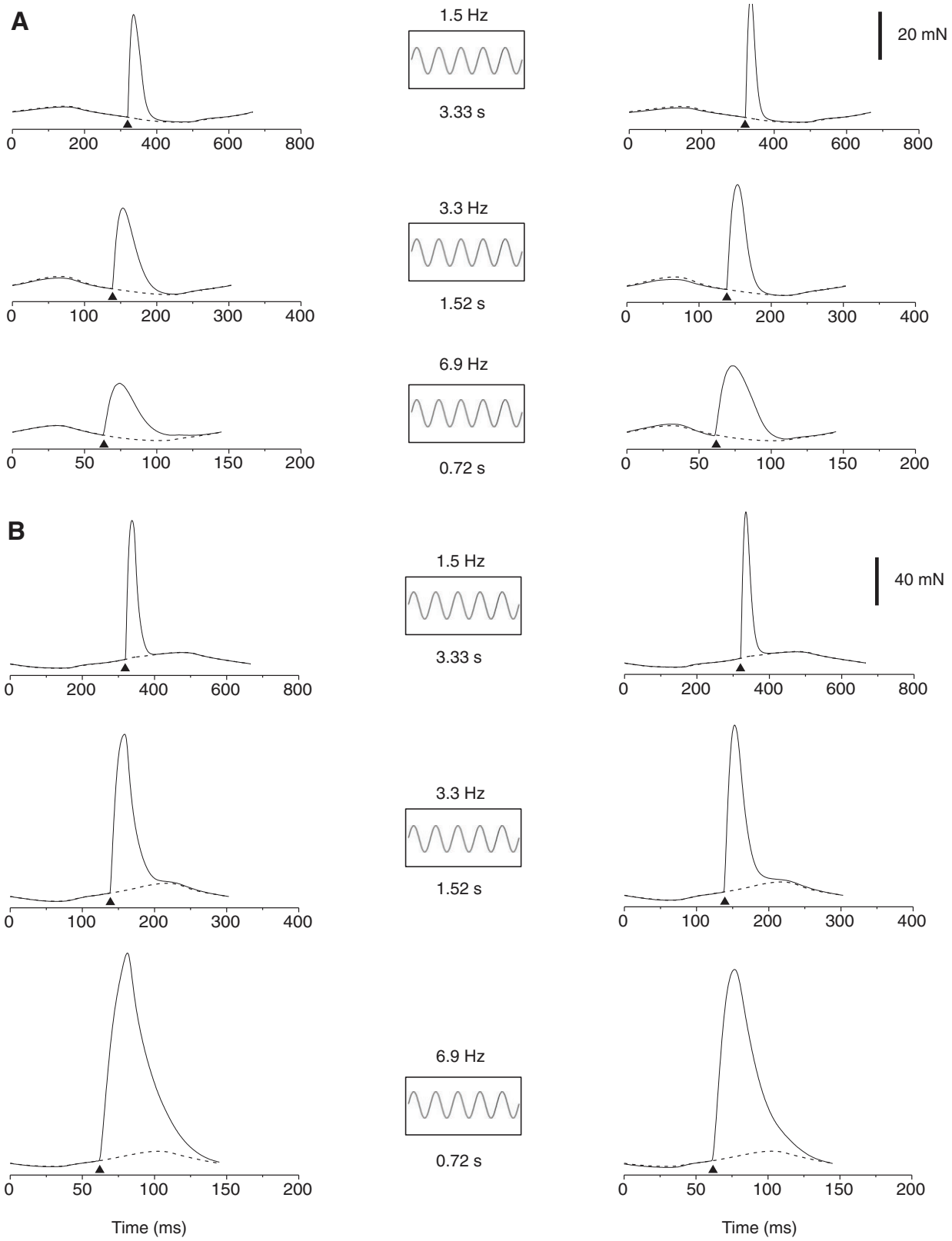

Fig. 2. Example of passive and stimulated responses during sinusoidal length changes when stimulated during (A) shortening phase and (B) lengthening phase to produce concentric responses. Traces show responses to passive (dotted) and stimulated (solid) work cycles (traces are overlayed) at sinusoidal length change frequencies of 1.5 (top), 3.3 (middle) and 6.9 Hz (bottom) before and after a conditioning protocol. Active force was obtained by subtracting passive from stimulated traces; work loops were constructed by plotting active force *versus* length (see Figs 4 and 5 for examples relating to A and B, respectively). Note the change in time scale from top to bottom, reflecting the change in sine-wave period. The period for five complete sinusoidal functions is shown for each work cycle frequency (box). Force scale bar applies to all records in A or B. Arrowhead in each panel indicates when a single pulse was delivered during the stimulated work cycle. All results are from the same muscle for A or B.

Table 1. Twitch forces before and after a conditioning protocol

	Concentric		Eccentric		Isometric	
Frequency (Hz)	Pre	Post	Pre	Post	Pre	Post
1.5	57.1±2.8	67.6±3.4*	78.5±3.7	82.9±4.1	67.0±3.2	79.9±4.0*
3.3	45.5±2.2†	56.6±2.9*,†	95.4±5.3†	96.8±5.0†	67.3±3.5	80.8±4.3*
6.9	28.2±1.7‡	36.4±2.2*,‡	126.6±7.4‡	120.6±7.1‡	66.2±3.8	79.6±4.5*

Means ± s.e.m.; N=9–10 for all observations. Forces are expressed in mN and represent active force (total force minus passive force). For dynamic forces, muscles were stimulated during the shortening or lengthening phase of sinusoidal shortening to produce concentric or eccentric force, respectively. In each case, force was measured as the muscle passed through the optimal length for twitch force (L_o). Isometric data were obtained during separate, fixed length contractions at L_o. All data were obtained before and after a tetanic conditioning protocol (see Fig. 1). Interactions between concentric, eccentric and isometric forces were not analysed. *Post-stimulus value significantly different from respective pre-stimulus value (P<0.05). †Significantly different from 1.5 Hz values (P<0.05). ‡Significantly different from 1.5 and 3.3 Hz values (P<0.05).

repeated application of our standard conditioning protocol throughout a ~90 min experiment produces repeatable levels for RLC phosphorylation. When data from these respective conditions were pooled they yielded RLC phosphorylation levels of 0.17±0.02 and 0.52±0.02 mol phosphate mol^{-1} RLC, respectively (N=10 and 13, respectively).

DISCUSSION

The purpose of this study was to investigate the speed dependence of the potentiation of dynamic force following a standard conditioning protocol known to phosphorylate the myosin RLC and potentiate concentric twitch force in mouse EDL muscle *in vitro* (25°C). Our results confirm previous results showing an association between RLC phosphorylation and the potentiation of dynamic force and work (Abbate et al., 2000; Grange et al., 1995; Grange et al., 1998; MacIntosh and Bryan, 2002; MacIntosh et al., 2008; Xeni et al., 2011). Moreover, the present results extend these observations by showing that the potentiation of concentric force and work is shortening speed dependent and that eccentric force and work are relatively insensitive to potentiation. These results highlight the possibility that while activity-dependent potentiation enhances concentric forces, eccentric forces are relatively unaffected, a hysteresis-like effect that may have important implications for working skeletal muscle *in vivo*.

Work cycle technique

The method of rapidly cycling muscle length about L_o whilst stimulating muscle to produce active forces, pioneered by Josephson (Josephson, 1985; Josephson, 1999), provides a more realistic mechanical model for assessing locomotor muscle function than do isometric contractions (James et al., 1996; Stevens and Syme, 1993a). Stimulating a muscle to produce concentric force during the shortening phase of a work cycle causes the muscle to perform work against the servomotor arm; in contrast, stimulating a muscle to produce eccentric force during the lengthening phase of a work cycle causes the muscle to absorb work from the servomotor arm (Josephson, 1993). These responses adequately replicate *in vitro* the force–velocity constraints imposed upon *in vivo* skeletal muscle during locomotion (Askew et al., 1997; James et al., 1995; Marsh, 1999). Further to this, the work cycle frequencies used in our experiments were selected for comparison with locomotion rates reported for mouse muscle *in vivo* (Clarke and Still, 1999; James et al., 1996; Leblond et al., 2003). Although single stimulus pulses producing twitch responses (isometric, concentric or eccentric) are not a realistic representation of neural activation *in vivo*, there is evidence that the influence of activity-dependent potentiation on concentric force is present at both low and high frequencies of stimulation (e.g. Abbate et al., 2000; MacIntosh et al., 2008). That is, it is possible that despite obvious discrepancies in terms of total force magnitude, the relative changes observed at the level of the twitch may be replicated during tetanic contractions. The rapid cycling and small amplitude excursions used in our experimental model necessitated the use of twitch *versus* tetanic contractions, however. Despite this limitation, we maintain that our work cycle model remains a viable experimental technique for studying the activity-dependent potentiation influence on dynamic aspects of skeletal muscle function.

Concentric responses

Because work cycle parameters of excursion amplitude and frequency were fixed, the only possible cause of increased work and power is an increased force (Askew et al., 1997; Brooks and Faulkner, 1991). Indeed, the increased concentric force we observed coincident with a ~3-fold elevation in RLC phosphorylation accords

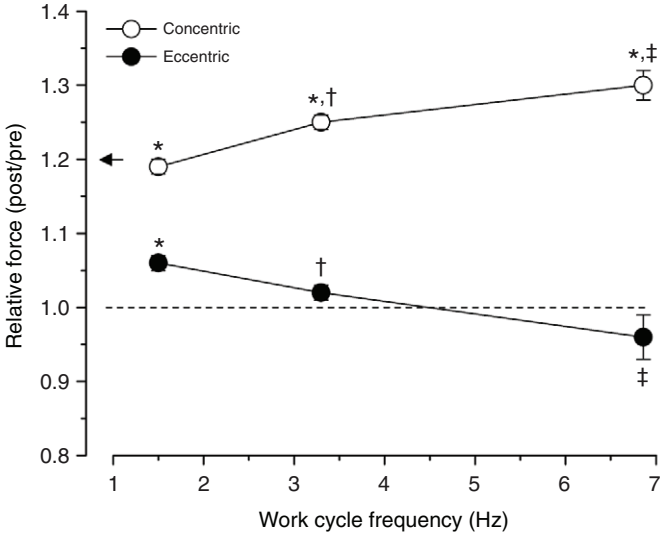

Fig. 3. Relative (post/pre) change in concentric and eccentric force during work cycles as a function of work cycle frequency. Note that although concentric forces tended to increase and eccentric forces tended to decrease with increasing length change speed, the range of responses was similar (i.e. concentric: 1.19 to 1.29; eccentric: 1.06 to 0.96). The effect of the conditioning protocol on relative isometric twitch force was similar for all conditions and thus these data were pooled (mean: 1.20±0.01; arrow). *Isometric, concentric or eccentric different from 1.00 (P<0.05); †3.3 Hz different from 1.5 Hz; ‡6.9 Hz different from 1.5 and 3.3 Hz (P<0.05). All concentric values were greater than corresponding eccentric values (P<0.05). Values calculated from Table 1.

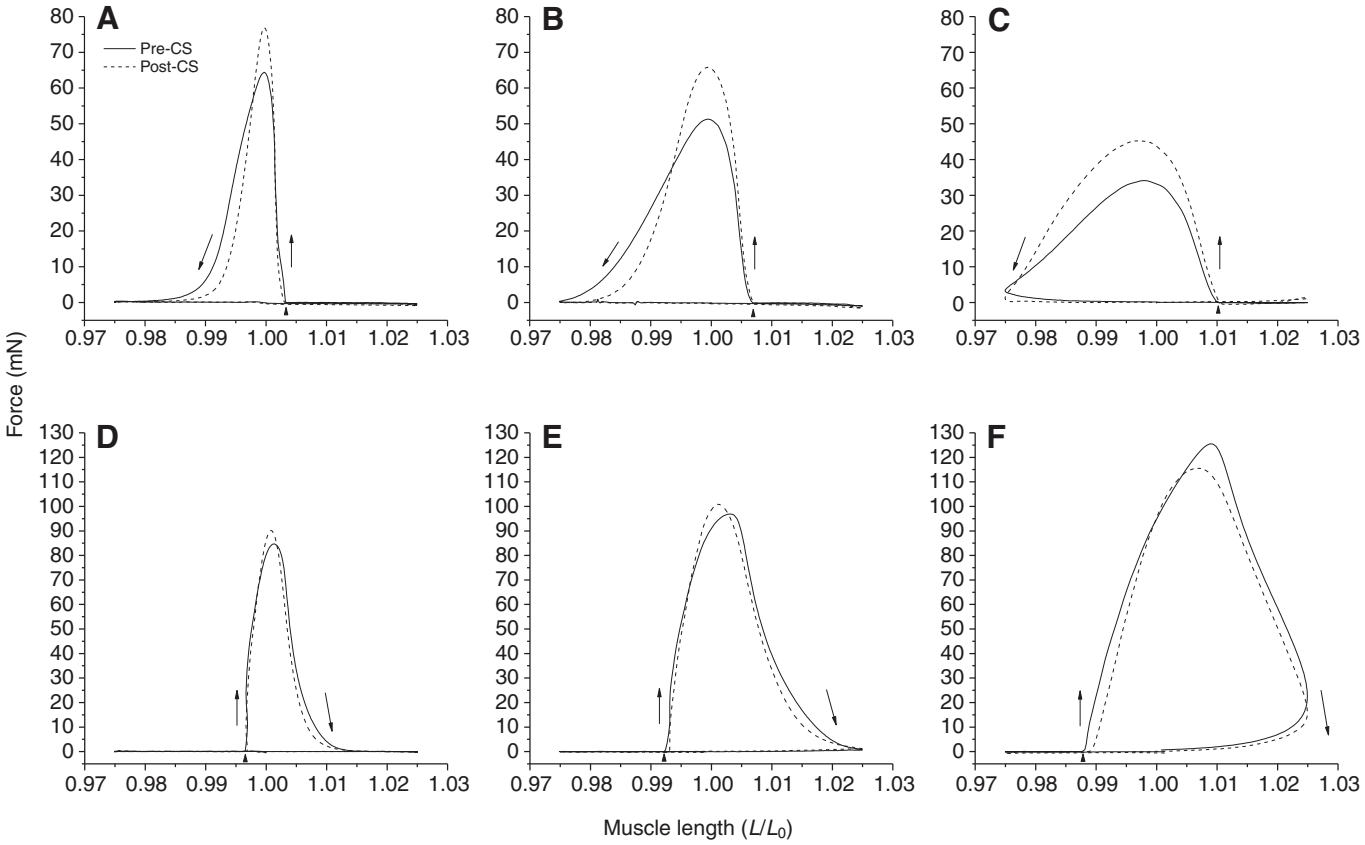

Fig. 4. Work loops of mouse extensor digitorum longus (EDL) muscle *in vitro* (25°C) before and after a conditioning protocol (CS, conditioning stimulus). (A–C) Representative traces at 1.5, 3.3 and 6.9 Hz (left to right) when a single stimulus pulse was applied (arrowhead) during shortening to produce concentric force at or near L_o. In each panel, active concentric force plotted *versus* muscle length yields an anti-clockwise loop (arrows), the area of which represents work. Note the frequency-dependent shift in cross-over point during twitch relaxation (i.e. when potentiated response becomes less than unpotentiated response). (D–F) Representative traces at 1.5, 3.3 and 6.9 Hz when a single stimulus pulse was applied (arrowhead) during lengthening to produce eccentric force at or near L_o. In each panel, active eccentric force plotted *versus* muscle length yields a clockwise loop (arrows), the area of which represents work. Note the speed-dependent shift in the peak of eccentric twitch force development (both pre and post) to longer lengths. See Materials and methods for details on sine-wave function and determination of dynamic work and power. All traces are from the same muscle.

with previous studies using mouse fast twitch muscle (Xeni et al., 2011). Interestingly, however, work at 1.5 and 3.3 Hz was either not increased or increased only marginally relative to concentric force. Detailed analysis of the concentric work loops shown in Fig. 4A–C reveal that although concentric force was increased throughout the entire work cycle period at 6.9 Hz, this was not evident at lower frequencies. Accelerated relaxation (25–35%) of potentiated twitches has been reported previously for EDL muscles from both wild-type and skMLCK knockout mice (Gittings et al., 2011). With regard to the present results, although not perhaps a direct result of RLC phosphorylation *per se*, a potentiated concentric twitch with accelerated relaxation produces a work loop with distinct segments of increased and decreased relative work for each work cycle frequency. Total work is reduced in the potentiated state if relaxation rate is increased more than peak concentric force (1.5 and 3.3 Hz) but total work is increased if peak concentric force is increased more than relaxation rate (6.9 Hz).

Eccentric responses

One of the main findings of our study was that eccentric force was not increased under the same conditions that increased concentric force by up to 30%. Interestingly, the disparate effect of potentiation on concentric and eccentric force may be considered an ideal response for high-speed cyclic contractions *in vivo*. For example, disregarding relaxation kinetics *per se*, potentiation of eccentric forces could increase the work required of antagonistic muscles to re-lengthen agonist muscles during the stretch–shortening cycle of muscles across a joint. In addition, eccentric force-induced damage has been implicated in both short- and long-term reductions in muscle function (Allen, 2001). Thus, activity-dependent potentiation of eccentric forces could exacerbate muscle damage and dysfunction arising from high force, lengthening contractions. Interesting in this regard are results from permeabilized rabbit psoas skeletal fibres showing that RLC phosphorylation-mediated potentiation of maximal calcium-activated eccentric force was associated with a ~30% increase in the subsequent contraction-induced force deficit (Childers and McDonald, 2004). Although our results do not support these conclusions it is important to realize that our data do not exclude the possibility of more general potentiation of eccentric forces during higher frequency stimulation producing unfused or fused tetani. In this event, concurrent potentiation of tetanic concentric and eccentric responses would accord more closely with the results of Childers and McDonald (Childers and McDonald, 2004). Interestingly, inspection of the eccentric work loops shown in Fig. 4D–F shows that the peak of eccentric force development at 6.9 Hz was shifted to longer lengths following the conditioning

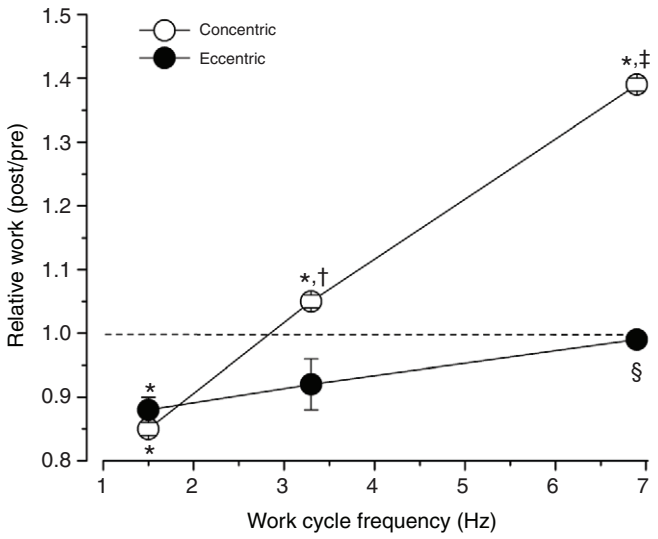

Fig. 5. Relative change in concentric and eccentric work/power as a function of work cycle frequency. For each parameter, relative change was calculated by dividing post-stimulation values by pre-stimulation values during each work cycle frequency. Figure is based on absolute data compiled in Tables 2 and 3. *Concentric or eccentric different from 1.00 (P<0.05); †3.3 Hz different from 1.5 Hz; ‡6.9 Hz different from 1.5 and 3.3 Hz (P<0.05); §6.9 Hz different from 1.5 Hz (P<0.05). Concentric response was greater than eccentric response at 3.3 and 6.9 Hz only (P<0.05).

protocol. Although this shift resembles that reported for damaged muscle (Whitehead et al., 2003), this effect dissipated with time (data not shown). Based on the relatively low eccentric forces in our experiments, as well as the general absence of eccentric twitch force potentiation, it seems more likely that this shift was due to an undetected change in either optimal length for eccentric force development or muscle stiffness caused by passive cycling at 6.9 Hz.

Temperature considerations

Our experiments were performed at a relatively cool temperature in order to comply with the requirements for metabolic stability, i.e. matching O_2 supply to O_2 demand, provided by Barclay (Barclay, 2005). In this regard it should be pointed out that activity-dependent potentiation of rodent hindlimb muscle is highly temperature dependent. For example, previous studies (Close and Hoh, 1968b; Krarup, 1981b) have shown that isometric twitch force potentiation of rat EDL muscle increases linearly as temperature increases from 20 to 37.5°C. Similar results were shown for mouse EDL muscle at 25–35°C (Moore et al., 1990). In addition, because RLC phosphorylation levels were shown to decline, higher temperatures may increase the sensitivity of the contractile apparatus to the potentiating influence of this molecular event (Moore et al., 1990). Thus, based on the assumption that concentric force displays the same temperature dependence as isometric force, our results may underestimate the influence of activity-dependent potentiation on dynamic forces for working muscle *in vivo*.

Molecular mechanism for speed and direction dependence

The putative influence of RLC phosphorylation on muscle contractility may best be understood in terms of how this molecular event affects the structure and function of the actomyosin complex. For example, studies by Levine and colleagues using permeabilized rabbit psoas muscle fibres showed that phosphorylation of the RLC reversibly disrupts the normally well-ordered, helical arrangement of myosin heads close to the surface of the thick filament (Levine et al., 1996; Levine et al., 1998). Sweeney and colleagues showed that this disruption was due to charge repulsion between the myosin head and the thick filament, as the addition of a negative charge to the RLC mimicked this effect of phosphorylation (Sweeney et al., 1994). In terms of myosin function, the lateral displacement and/or increased mobility of the myosin head relative to the thick filament may facilitate formation of the actomyosin complex and the strongly bound, force-generating cross-bridge state in a variety of species (Levine et al., 1996; Levine et al., 1998; Miller et al., 2011; Sweeney et al., 1994). In support of this idea, Yang and colleagues (Yang et al., 1998) showed that RLC phosphorylation effects on isometric force were mimicked by myofilament lattice spacing compression. Overall, these alterations to myosin structure and function by RLC phosphorylation account mechanistically for the increase in Ca^{2+} sensitivity of steady-state force development observed in permeabilized skeletal fibres (Sweeney et al., 1993).

The divergent effects of activity-dependent potentiation on concentric and eccentric forces may be accounted for on the basis of how a population of phosphorylated myosin heads interacts with the overall cross-bridge distribution as determined by load and velocity parameters (for a review, see Edman, 2010). For example, a phosphorylation-mediated increase in the ability of myosin heads to attain force-generating cross-bridge states against the thin filament would be expected to exert a relatively large effect on concentric force at high speeds of shortening when cross-bridge distribution generally favours non-force-generating over force-generating states. On the other hand, at slow speeds of shortening when cross-bridge distribution favours force-generating over non-force-generating states, phosphorylation-mediated alterations to myosin structure and function would be expected to exert little effect on force. In this context, the relative absence of eccentric force potentiation is explained by work showing that active lengthening of intact frog skeletal fibres

Table 2. Concentric work and power during different frequency work cycles before and after a conditioning protocol

	Work		Power	
Frequency (Hz)	Pre	Post	Pre	Post
1.5	0.62±0.06	0.52±0.04*	0.94±0.08	0.79±0.07*
3.3	0.94±0.07†	0.99±0.07†	3.11±0.23†	3.25±0.26†
6.9	0.85±0.06†	1.18±0.08*,†	5.85±0.40‡	8.14±0.58*,‡

Means ± s.e.m.; N=9–10 for all observations. Work is expressed in J kg^{-1} wet muscle mass and represents the dimension of force × excursion per work cycle. Power is expressed as W kg^{-1} wet muscle mass and represents the dimension of work per unit time (s). Muscles were stimulated during the shortening phase of sinusoidal shortening to produce concentric force and work. Data were obtained before and after a tetanic conditioning protocol that did not cause fatigue. *Post-stimulus value different from respective pre-stimulus value (P<0.05). †Significantly greater than 1.5 Hz values (P<0.05). ‡Significantly different from 1.5 and 3.3 Hz values (P<0.05). For more details regarding calculation of work and power see Materials and methods.

Table 3. Eccentric work and power during different frequency work cycles before and after a conditioning protocol

	Work		Power	
Frequency (Hz)	Pre	Post	Pre	Post
1.5	0.83±0.07	0.73±0.06	1.24±0.11	1.09±0.09
3.3	1.96±0.17†	1.78±0.15†	6.54±0.54†	5.86±0.48†
6.9	3.43±0.32‡	3.37±0.29‡	23.67±2.1‡	23.24±2.00‡

Means ± s.e.m.; N=9 for all observations. Work is expressed in J kg^{-1} wet muscle mass. Power is expressed as W kg^{-1} wet muscle mass. Muscles were stimulated during the lengthening phase of the work cycle to produce active eccentric force and work. Data were obtained before and after a conditioning protocol that did not cause fatigue. Neither eccentric work nor power was increased at any frequency by the conditioning protocol. †Significantly different than 1.5 Hz values (P<0.05). ‡Significantly different from 1.5 and 3.3 Hz values (P<0.05).

increases the proportion of force-generating cross-bridges, perhaps *via* binding of both myosin heads, compared with the isometric distribution (Brunello et al., 2007).

An important point regarding our experiments is that we used twitch contractions to assay the influence of activity-dependent potentiation on dynamic performance. As a result, all concentric and eccentric forces measured were submaximal in nature. Consistent with results from permeabilized skeletal fibres showing that the potentiating effect of RLC phosphorylation is inversely proportional to Ca^{2+} activation, results from intact skeletal muscle show that activity-dependent potentiation is evident at low but not at high stimulation frequencies (Vandenboom et al., 1993; Vandenboom et al., 1997). In addition, the magnitude of isometric twitch force potentiation of rodent hindlimb muscle has been shown to be increased or decreased by pharmaceutical interventions that decrease or increase, respectively, the Ca^{2+} activation of the contractile apparatus (MacIntosh, 1991; Palmer and Moore, 1989; Vandenboom and Houston, 1996). These results indicate that thin filament activation level is an important factor governing the magnitude of activity-dependent potentiation. Interesting in this regard is evidence that cycling cross-bridges cooperatively activate the thin filament [see Gordon et al. (Gordon et al., 2000) and references therein]. In principle, reductions in thin filament activation secondary to cross-bridge detachment may contribute to activity-dependent potentiation. Although experimentally difficult to parse out the differential effects of variations in cross-bridge distribution from shortening deactivation influences *per se*, a length change direction and speed dependence for activity-dependent potentiation suggests that the effects of myosin phosphorylation on dynamic force are mediated, at least in part, by cross-bridge influences on thin filament activation level (Xeni et al., 2011). Interesting in this regard is the possibility that enhanced cross-bridge binding helps abolish eccentric force potentiation *via* cooperative effects on thin filament activation (e.g. Kad et al., 2005).

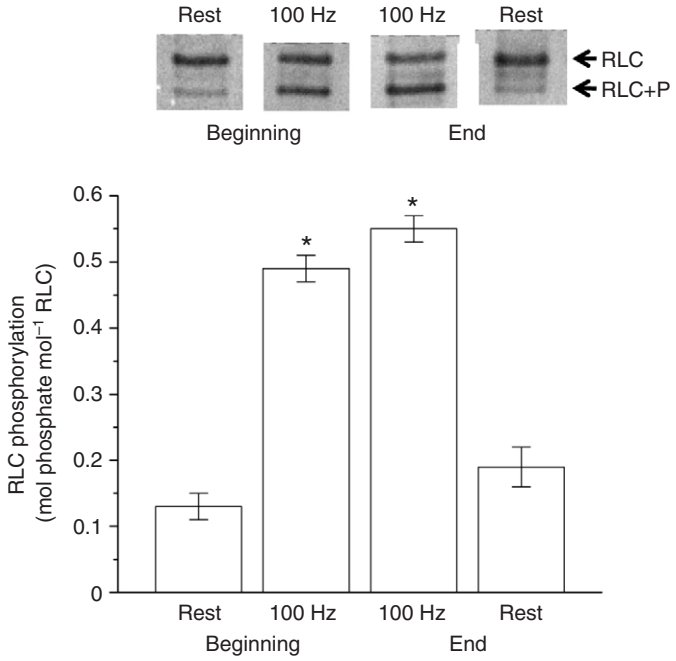

Fig. 6. RLC phosphate content before and after the conditioning protocol. (Top) Representative blots showing phosphorylated (RLC+P) and unphosphorylated (RLC) bands in muscle samples. Resting samples depicting low levels of RLC phosphorylation were obtained from quiescent muscles frozen at the beginning (Rest, left) and the end (Rest, left) of the experiment. Stimulated samples depicting high levels of RLC phosphorylation were obtained from muscles frozen immediately after the potentiating stimulus at the beginning (100 Hz, left) and the end (100 Hz, right) of the experiment. Note that RLC phosphorylation was similar in quiescent muscles frozen at the beginning and at the end of an experiment (range: 0.13–0.19 mol phosphate mol^{-1} RLC) as well as in stimulated muscles frozen at the beginning and at the end of an experiment (range: 0.49–0.55 mol phosphate mol^{-1} RLC). This figure was constructed using lanes cut from different blots for the purposes of comparison. (Bottom) Summary of all blots showing RLC phosphorylation (expressed as mol phosphate mol^{-1} RLC as quantified by optical density) in quiescent and stimulated muscles. *Stimulated value greater than resting value (P<0.001). Values are means ± s.e.m. (N=5–8 per group).

CONCLUSIONS

The potentiation of dynamic twitch forces during work cycles is highly dependent upon both the direction and speed of muscle length change. In our model, an increasing speed of muscle shortening increased the relative potentiation of concentric force without, however, greatly altering eccentric responses from control. This hysteresis-like response may increase the work output of agonist muscles without increasing the work required of antagonist muscles to re-extend them during relaxation. This effect may be particularly important for enhancing high duty cycle muscle function during locomotion.

ACKNOWLEDGEMENTS

We would like to thank Dr Jim Stull for analysis of myosin RLC phosphate content in our EDL muscle samples.

FUNDING

This study was supported by funds provided by the Natural Sciences and Engineering Research Council of Canada [Discovery grant no. 312012 to R.V.].

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