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## Sarcomere length-dependence of activity-dependent twitch potentiation in mouse skeletal muscle

Dilson E Rassier and Brian R MacIntosh\*

Address: Human Performance Laboratory, Faculty of Kinesiology, University of Calgary, 2500 University Drive, T2N 1N4, Calgary (AB), Canada

Email: Dilson E Rassier - [rassier@kin.ucalgary.ca](mailto:rassier@kin.ucalgary.ca); Brian R MacIntosh\* - [brian@kin.ucalgary.ca](mailto:brian@kin.ucalgary.ca)

\* Corresponding author

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### Abstract

**Background:** It has been reported that potentiation of a skeletal muscle twitch response is proportional to muscle length with a negative slope during staircase, and a positive slope during posttetanic potentiation. This study was done to directly compare staircase and posttetanic responses with measurement of sarcomere length to compare their length-dependence.

**Methods:** Mouse extensor digitorum longus (EDL) muscles were dissected to small bundles of fibers, which permit measurement of sarcomere length (SL), by laser diffraction. *In vitro* fixed-end contractions of EDL fiber bundles were elicited at 22°C and 35°C at sarcomere lengths ranging from 2.35 μm to 3.85 μm. Twitch contractions were assessed before and after 1.5 s of 75 Hz stimulation at 22°C or during 10 s of 10 Hz stimulation at 22°C or 35°C.

**Results:** Staircase potentiation was greater at 35°C than 22°C, and the relative magnitude of the twitch contraction ( $Pt^*/Pt$ ) was proportional to sarcomere length with a negative slope, over the range 2.3 μm – 3.7 μm. Linear regression yielded the following:  $Pt^*/Pt = -0.59 \cdot SL + 3.27$  ( $r^2 = 0.74$ );  $Pt^*/Pt = -0.39 \cdot SL + 2.34$  ( $r^2 = 0.48$ ); and  $Pt^*/Pt = -0.50 \cdot SL + 2.45$  ( $r^2 = 0.80$ ) for staircase at 35°C, and 22°C and posttetanic response respectively. Posttetanic depression rather than potentiation was present at long SL. This indicates that there may be two processes operating in these muscles to modulate the force: one that enhances and a second that depresses the force. Either or both of these processes may have a length-dependence of its mechanism.

**Conclusion:** There is no evidence that posttetanic potentiation is fundamentally different from staircase in these muscles.

### Background

Activity-dependent potentiation is the enhancement of contractile response, which occurs as a consequence of prior activation. Staircase, the progressive increase in developed tension (DT) during low frequency stimulation, and posttetanic potentiation, the enhancement of twitch DT following tetanic stimulation are two common forms of activity-dependent potentiation [1–3]. It is generally

considered that the enhancement of DT during staircase and following a tetanic contraction occurs as a result of phosphorylation of the regulatory light chains of myosin [4,5].

It has been reported that there is a length-dependence of potentiation in skeletal muscle. Experiments conducted at 37°C have demonstrated that the magnitude of staircase

potentiation is proportional to muscle length with a negative slope [6–9]. This is the case, whether the staircase is conducted at the different lengths, or the poststaircase twitch contractions are evaluated at different lengths after staircase at a given length [6,10]. Length-dependence of posttetanic potentiation has been evaluated *in vitro* at room temperature, and it has been reported that there is a length-dependence of potentiation, with the fractional enhancement of force being proportional to muscle length with a positive slope [11].

Considering these studies of length-dependence of potentiation, it would appear that the impact of muscle length on the force potentiation of a twitch is different between staircase and posttetanic potentiation. This would suggest that there is a fundamental difference in the mechanism of posttetanic potentiation relative to staircase. Alternatively, this difference in length-dependence could be a function of the different temperatures at which the experiments were conducted.

In this study, the length-dependence of staircase and post-tetanic twitch contraction amplitude was evaluated in skeletal muscle *in vitro*, at room temperature with measurement of sarcomere length. In addition, staircase was assessed at a warmer temperature, to permit comparison with the responses previously done with staircase. The results indicate that there is a length-dependence of force modulation in the twitch contraction for both staircase and posttetanic response, and that in both cases the twitch potentiation is proportional to sarcomere length with a negative slope. Therefore there is no justification for ascribing a mechanism for posttetanic response that is any different from the mechanism for staircase.

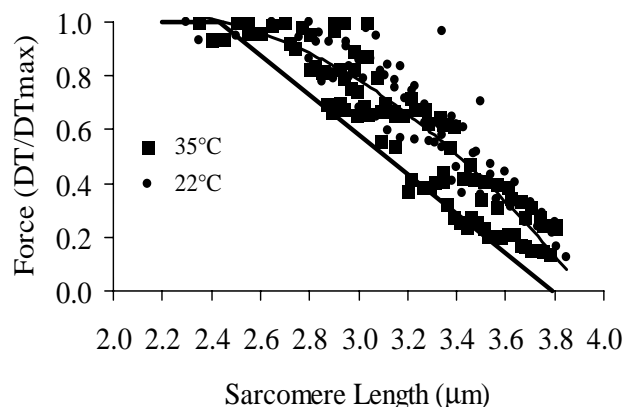
## Results

### Sarcomere length measurements

In many preparations, after obtaining a clear laser diffraction pattern at rest, the tracing was lost during the contractions. This was probably caused by translations of the muscle, which resulted in connective tissue, and/or adherent damaged fibers moving into the laser beam preventing a consistent diffraction pattern. Due to this variability of sarcomere length during contractions, all results presented herein are related to sarcomere length readings made prior to the contractions (passive sarcomere length), at one position along the length of the fiber. Results of a given contraction were included only when the sarcomere length after the contraction was the same as it was in the beginning (i.e., the tracing was still clear and without apparent change).

### Force-sarcomere length relation of tetanic contractions

The force-sarcomere length relation for tetanic contractions is presented in Figure 1. The results obtained at the



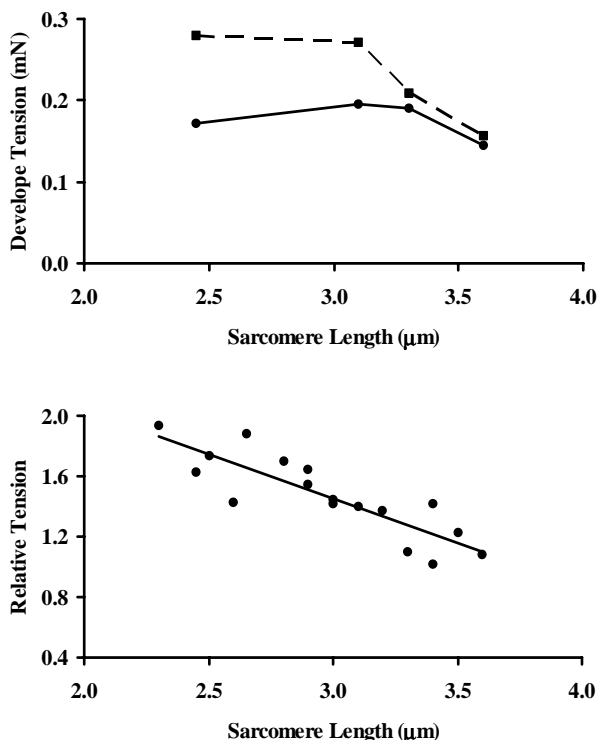
**Figure 1**

Force-length relation for tetanic contractions ( $n = 10$ ). The theoretical (thick line) relationship is obtained from ter Keurs et al. (1984) with a plateau extended from 2.26  $\mu\text{m}$  to 2.43  $\mu\text{m}$  and a zero overlap intercept on the descending limb occurring at 3.79  $\mu\text{m}$ . The DT is given relative to the maximum force obtained in each preparation, and the passive force has been subtracted, as explained in the methods section. Note that the shape of force-length relation does not change substantially with temperature. Force appears to drop to zero at a sarcomere length of approximately 3.9  $\mu\text{m}$  (thin line, representing 2nd order polynomial least squares fit).

two temperatures used in this study depict virtually the same force-length relation [ $DT = -1.20 + 1.86 \cdot SL - 0.39 \cdot SL^2$  ( $r^2 = 0.87$ ) and  $DT = 0.85 + 0.55 \cdot SL - 0.20 \cdot SL^2$  ( $r^2 = 0.86$ ), for 22°C and 35°C respectively]. The force is plotted relative to the highest force obtained at any length in a given experiment. By extrapolating the results obtained on the descending limb of the force-sarcomere length relation, the force appears to decrease to zero at approximately 3.8  $\mu\text{m}$  – 3.9  $\mu\text{m}$ , consistent with previous findings with intact [12] or skinned fibers [13] of EDL muscle of the rat (3.79  $\mu\text{m}$  and 3.93  $\mu\text{m}$ , respectively). Similar to previous studies, the descending limb of the force-length relation shows a shift to the right when compared to the theoretical predictions based on the length of myofilaments, obtained from ter Keurs et al. [12].

### Activity-dependent potentiation

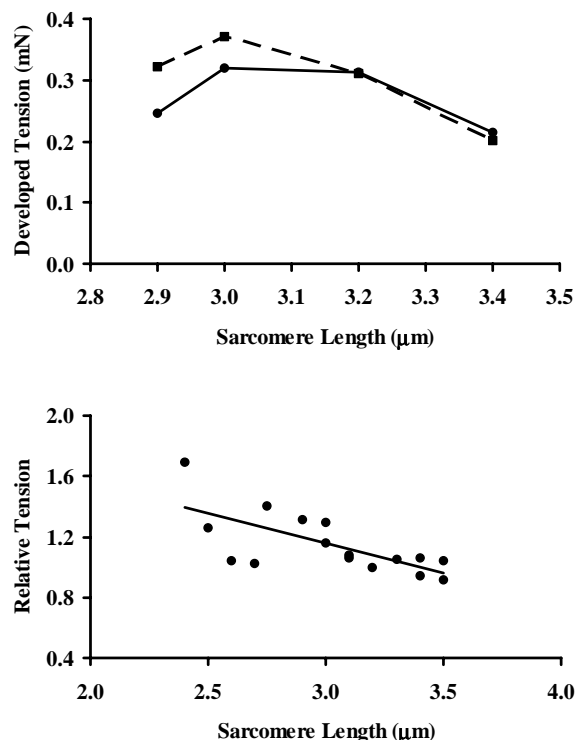
Twitch amplitude was evaluated after each of the following: 10 Hz stimulation for 10 s at 35°C, 10 Hz stimulation for 10 s at room temperature, and 75 Hz for 1.5 s at room temperature. In each case, the amplitude of the potentiated twitch DT expressed relative to a control twitch ( $Pt^*/Pt$ ) was proportional to sarcomere length, with a negative slope.



**Figure 2**

An example of the force-length relation (A) observed in the experiments conducted at 35°C. Each pair of points (pre-staircase, solid line vs. poststaircase, dashed line) was obtained at the start (Pt) and end (Pt\*) of 10 s of 10 Hz stimulation at a given sarcomere length. In the lower panel (B), the relative force (Pt\*/Pt) is presented as a function of sarcomere length. Data from 4 muscles are shown. Regression analysis gave  $r^2 = 0.74$  and  $Pt^*/Pt = -0.59 \cdot SL + 3.27$  (where SL is sarcomere length).

When stimulation was 10 Hz for 10 s, at 35°C, there was a staircase potentiation ( $Pt^*/Pt > 1$ ) which was evident at all sarcomere lengths studied (range = 2.4 µm – 3.6 µm). Figure 2A illustrates the length-tension relation for twitch contractions obtained at the start and end of 10 s of 10 Hz stimulation at each of 4 different sarcomere lengths in a given muscle preparation. Two features of the length-tension relation should be noted. The obvious point is that enhancement of DT, whether expressed in absolute or relative ( $Pt^*/Pt$ ) terms, is much greater at short sarcomere lengths than at long sarcomere lengths. The second point is that the shape of the length-tension relation relative to sarcomere length has shifted, such that optimal length (peak of the length-tension relation) for potentiated contractions is less than optimal length for nonpotentiated contractions. This pattern is apparent as an increase in DT going from 2.4 µm to 3.1 µm pre-staircase, as opposed to a decrease over this length range for poststaircase twitch

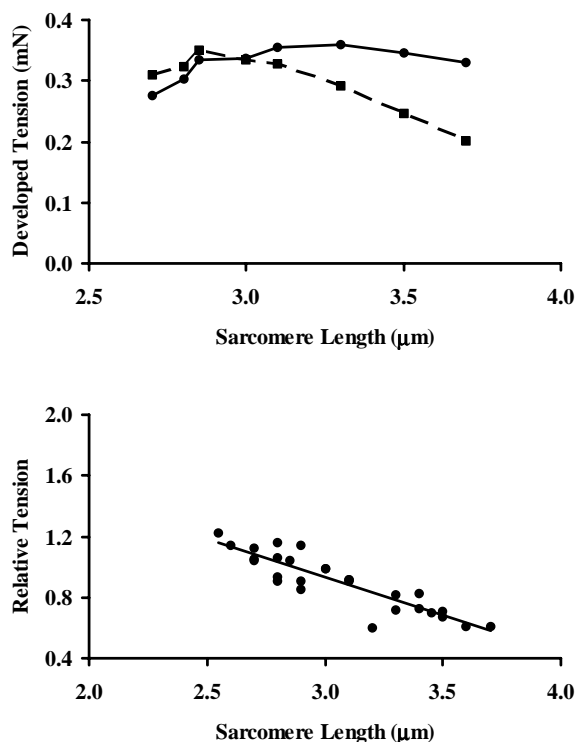


**Figure 3**

An example of the force-length relation (A) observed in the experiments for staircase at 22°C. Individual points represent twitch contractions either prior to (Pt, solid line) or after (Pt\*, dashed line) 10 s of 10 Hz stimulation at selected sarcomere lengths. Similar to the situation at 35°C, potentiation was greater at short sarcomere lengths than at long sarcomere lengths. In B, the results from 5 muscles are shown. In this case (as in Figure 2B), the force is expressed as  $Pt^*/Pt$ . Regression analysis gave  $r^2 = 0.48$  and  $Pt^*/Pt = -0.39 \cdot SL + 2.34$  (where SL is sarcomere length).

contractions. The relative enhancement of force ( $Pt^*/Pt$ ) at a variety of sarcomere lengths is presented in Figure 2B, showing the strong length-dependence of the staircase response, with very little potentiation at long sarcomere lengths.

Stimulation at 10 Hz for 10 s at 22°C always resulted in potentiation at short sarcomere lengths, but potentiation was not always observed at long sarcomere lengths. A representative experiment is shown in Figure 3A. There is a shift to the left in the length at which the peak DT occurs after staircase. Figure 3B presents  $Pt^*/Pt$  for staircase at a variety of sarcomere lengths in muscles at 22°C. The magnitude of potentiation at 22°C is less than the magnitude of potentiation at 35°C, but the length-dependence is still evident.



**Figure 4**

The twitch DT at various sarcomere lengths is shown in A for contractions obtained prior to (Pt) and after (Pt\*) a 1.5 s tetanic contraction at 75 Hz when bath solution temperature was 22°C. In this case, like for staircase, there was enhancement of DT at short sarcomere lengths, but unlike the case for staircase, there was a clear force depression at long sarcomere lengths. As seen in B, the Pt\*/Pt decreases with increases in sarcomere length. The results of 4 muscles are shown. Regression analysis gave  $r^2 = 0.80$  and  $Pt^*/Pt = -0.50 \cdot SL + 2.45$  (where SL is sarcomere length).

Twitch contractions following tetanic contractions were potentiated only at short sarcomere lengths (22°C). At longer sarcomere lengths, posttetanic depression was apparent, as the posttetanic twitch was smaller than the pre-tetanic twitch ( $Pt^*/Pt < 1$ ) (Figure 4A). In studying posttetanic potentiation, the length tension relation for twitch contractions was obtained with sequential contractions at different lengths, both prior to and after a tetanic contraction at optimal length. For this reason, the length-tension relation is more clearly defined in these experiments than in the staircase experiments, for which twitch contractions were obtained at a given length prior to and after repetitive stimulation at that sarcomere length. In

Figure 4A, the length associated with maximal twitch amplitude is shifted to the left in the posttetanic contractions. In spite of the posttetanic depression which was evident in these experiments, there was a marked length-dependence of the posttetanic twitch amplitude ( $Pt^*/Pt$ ). The ratio of the posttetanic twitch to the pre-tetanic one decreased with increased SL (see Figure 4B).

## Discussion

The primary purpose of this study was to determine if there was a fundamental difference in the length-dependence of staircase and posttetanic twitch contractions. The results of the experiments reported in this paper confirm that both staircase and the posttetanic response have a length-dependence, and that the activity-dependent change in twitch amplitude is proportional to sarcomere length with a negative slope in both cases. These observations are consistent with previous reports at 35°C [6,7,9,10], and one report of frog muscle at room temperature [1], but are in contradiction to the report of Moore and Persechini [11] who studied mouse skeletal muscle posttetanic potentiation at room temperature.

### The force-length relation

In order to discuss the results regarding the length-dependence of potentiation, we had to first define the force-length relation for the muscle preparation used in this study. The force-length relation for tetanic contractions had a descending limb, which was deviated to the right when compared to the theoretical force-length relation. It also showed an extended plateau where the force did not decrease until well beyond estimated optimal filament overlap. This extended plateau and deviation from the theoretical force-length relation (Gordon et al., [14] has been observed in other studies that used fixed-end contractions [15–17]. This augmented force observed at a given sarcomere length is due either to inhomogeneity of sarcomere lengths or to a compliance within the muscle or measurement system.

Forces that are higher than predicted could be explained if there is an inhomogeneity of sarcomere lengths during the contraction, as proposed by Gordon et al. [14]. Inhomogeneity has been shown to occur during tetanic contractions elicited on the descending limb of the force-length relation [18,19], where some sarcomeres are being stretched while others are shortening. The sarcomeres that are shortening are likely situated near the end of the fibers. In our study, sarcomeres near the tendons may have been operating at a shorter sarcomere length during the contraction, when compared to the passive sarcomere length that we actually measured.

Any compliance in the muscle or measuring system would also cause the sarcomere length during the contractions to

be shorter than that before (or after) the contraction. Assuming all of the discrepancy between the theoretical and actual length tension relation is due to compliance, a correction factor can be generated. The data in Figure 1 would center around the line representing the theoretical length-tension relationship if sarcomere length for each contraction was corrected by subtracting  $0.19 \mu\text{m}$ . This correction could relate to a short range compliance that could be due to movement of the aluminum clips during each contraction.

The force-length relationship observed in this study is consistent with previous investigations on the force-length relation for fixed-end tetanic contractions of skeletal muscle. Therefore, our measurements of sarcomere length in the experiments on the length-dependence of potentiation are a reasonable expression of the actual (initial) sarcomere length for these conditions.

#### **Length-dependence of potentiation**

The observation that the magnitude of the posttetanic twitch response ( $P_t^*/P_t$ ) was proportional to length but with a negative slope is in direct contrast with a previous report [11]. However, this observation is consistent with data of Roszek et al. [20], who reported enhanced low-frequency responses when these were preceded by tetanic stimulation in the medial rat gastrocnemius muscle (at  $37^\circ\text{C}$ ). They observed that this enhancement was greater at short muscle lengths. Our results provide evidence that the discrepancy between [11] and those of [20] is not a function of temperature. Furthermore, it would appear that it is unnecessary to propose different mechanisms for posttetanic potentiation and staircase.

There are other differences between the study by [11] and this one. They used a whole mouse EDL, while we used a fiber bundle. While it is tempting to suggest that they may have had a central core of fatigued or damaged cells, it is not clear how this could account for the reversal of the relationship between length and potentiation. Another difference is the apparent length range which was studied. In the paper by Moore & Persechini [11], length is expressed relative to a reference length which appears to be the length at which tetanic developed tension is greatest. Contractile response was measured at this length, and up to 25% longer, at which length tetanic developed tension was apparently less than 40% of the peak value at the reference length. At this same length, twitch tension was just over 30% of its respective peak value at the reference length. In contrast, in our experiments, the length range studied was from a sarcomere length of  $2.3 \mu\text{m}$  to  $3.5 \mu\text{m}$ . The relative length increase here is over 50%. We would appear to be studying a greater range of lengths, but the range of twitch amplitudes is much less. In fact twitch amplitude at the longest length studied in this paper, has a

developed tension that is not substantially different from that at  $2.5 \mu\text{m}$ . The greatest twitch amplitude occurred at an intermediate length. Of interest, our tetanic developed tensions were about 40% of the maximum at a sarcomere length of  $3.5 \mu\text{m}$ . This difference in the length-dependence of tetanic and twitch contractions has been reported previously and is attributed to length dependence of activation [21].

It is interesting that the twitch contraction after a brief tetanic contraction at  $22^\circ\text{C}$  in our experiments demonstrated potentiation when the response was measured at short sarcomere lengths, and depression when the response was measured at long sarcomere lengths. This is consistent with the observation of Krarup [22] that in the poststimulation period at room temperature, there are both factors which enhance subsequent contractile response and factors that diminish the contractile response. Krarup [22] reported that the dissipation of the depression of force was faster than the dissipation of enhancement, such that after a period of time, the enhancement became evident. In the case of the posttetanic contractions reported here, the enhancement apparently predominated at a short length, while depression was evident at long sarcomere length. This indicates that the factor(s) resulting in depression may have a mechanism that is length-dependent. It is also possible that the depression may be similar at all lengths, and the potentiating effects may be the only factor that is length-dependent. It is important to point out that our posttetanic twitch contractions were obtained at a variety of lengths after a tetanic contraction at optimal length. The metabolic disturbance, or level of regulatory light chain phosphorylation would not have been different between these lengths.

The depression of twitch amplitude after a tetanic contraction may be a consequence of hydrogen ion or inorganic phosphate ( $\text{P}_i$ ) accumulation. These factors are likely to be more evident after the tetanic contraction (75 pulses per s) than after staircase (10 pulses per s). It is known that decreased pH or increased  $\text{P}_i$  results in a rightward shift in the force- $\text{pCa}^{2+}$  relation [23–25]. It has been shown for both pH and  $\text{P}_i$  that the shift is more evident at room temperature than at physiological temperature [26–28]. This factor may also explain why potentiation is less during staircase at  $22^\circ\text{C}$  than at  $35^\circ\text{C}$  – in fact Martyn and Gordon [29] have shown an interdependence of changes in  $\text{Ca}^{2+}$  sensitivity associated with pH, and length.

The mechanism for the length-dependence of potentiation may be related to length-dependence of activation, as suggested previously [6]. It was argued that the rightward shift in the control force-length relation during twitch (in comparison with tetanic) contractions is apparently due to enhanced sensitivity to  $\text{Ca}^{2+}$  as muscle length is in-

creased [13,30]. Since regulatory light chain phosphorylation also results in increased sensitivity to  $\text{Ca}^{2+}$  [31], then it may be that there is a ceiling effect, and the two mechanisms are not simply additive. Light chain phosphorylation may be less effective in enhancing the contractile response when it is already enhanced by length-dependent activation.

This interpretation relies on the assumption that the magnitude of light chain phosphorylation was similar after staircase elicited at different lengths. Myosin light chain kinase, the enzyme responsible for phosphorylating the regulatory light chain, is activated by  $\text{Ca}^{2+}$  bound to calmodulin. A similar level of light chain phosphorylation would be expected if  $\text{Ca}^{2+}$  transients during this stimulation were not affected by length. Balnave and Allen [32] have shown that  $\text{Ca}^{2+}$  transients in mouse muscle are independent of length. Furthermore, a length-dependence of potentiation similar to that reported here has been reported for rat muscle after staircase at one length [10], an approach similar to that used in this study for the posttetanic potentiation. It seems unlikely that the length-dependence of potentiation described here is due to length-dependent differences in regulatory light chain phosphorylation.

The data available in this study cannot discern the possible mechanisms that are operating to result in a length-dependence of  $\text{Pt}^*/\text{Pt}$ . However, the observations in this paper clearly demonstrate that this length-dependence is operative at the sarcomere level, and  $\text{Pt}^*/\text{Pt}$  decreases as sarcomere length increases whether it is studied after high frequency (tetanic contraction) or after low frequency (staircase) stimulation. These observations are consistent with the proposed mechanism by which light chain phosphorylation enhances developed tension. It has been proposed that light chain phosphorylation causes individual cross-bridges to swing out from the myosin back-bone [5], thereby bringing the actin binding site of the cross-bridge in close proximity to the actin filament. This would increase the probability of cross-bridge binding to actin, resulting in a greater number of cross-bridges exerting force during the twitch contraction. This mechanism is apparently ineffective when the myofilaments are in close proximity as would be the case at a long sarcomere length. There is no need to invoke a different explanation for posttetanic potentiation than for staircase.

## Methods

Male Swiss-Webster mice weighing approximately 30 grams were housed in a room with a 12:12 h light:dark cycle, and standard mouse chow and water were provided *ad libitum*. Care and treatment of these animals were according to the Canadian Council on Animal Care and all pro-

cedures were approved by a University committee for the ethical use of animals for research.

## Muscle preparation

The animals were deeply anaesthetized with an intraperitoneal injection of pentobarbital ( $60 \text{ mg} \cdot \text{kg}^{-1}$ ). The right hindlimb was shaved and an incision was made along the surface of the hindlimb. The tibialis anterior muscle was removed to expose the extensor digitorum longus (EDL) muscle. The EDL muscle was then transferred to a dissecting chamber, through which Krebs Henseleit solution ( $\text{NaCl}$ , 125 mM;  $\text{KCl}$ , 4.7 mM;  $\text{NaH}_2\text{PO}_4$ , 1.78 mM;  $\text{MgCl}_2$ , 1 mM;  $\text{CaCl}_2$ , 1.9 mM;  $\text{NaHCO}_3$ , 24 mM; Dextrose, 10 mM) flowed. This solution was bubbled continuously with 95%  $\text{O}_2$  and 5%  $\text{CO}_2$  ( $\text{pH} = 7.4$ , temperature =  $22^\circ\text{C}$ ).

Experiments were performed with small fiber bundles (approximately 6 to 30 fibers, 10–12 mm long) of the EDL muscle. Care was taken to clear the fibers from connective tissue as much as possible. After dissection, the tendons of the dissected fibers were gripped with small pieces of T-shaped aluminum foil as close to the ends of the fibers as possible. The bundle of fibers was then transferred to an experimental chamber, with continuous superfusion of the same solution as in the dissection chamber. One tendon clip was attached to a force transducer hook and the other to a Cambridge motor arm (model 300B), allowing the fiber bundle to be suspended horizontally and adjusted to a specific sarcomere length, as desired during the experiment.

## Sarcomere length measurements

The sarcomere length was measured by laser diffraction technique. A 10 mW He-Ne laser (Model 1125, Uniphase, Manteca, California) with a beam diameter of 0.8 mm was directed through the experimental chamber perpendicular to the long axis of the muscle fibers. The sarcomere length was established in a clear region of the bundle, close to the force transducer. This region was chosen because in most experiments it was not possible to obtain a clear diffraction pattern in all regions of the fiber due to adherent damaged fibers and/or connective tissue.

The position and intensity distribution of the first-order diffraction pattern was monitored by a photodiode array which was scanned electronically every 0.5 ms. The diffraction pattern was collected in a 10X objective lens (N.A. 0.5, Nikon) and reflected through an access port in the microscope. The transmitted laser light was projected via a telescopic lens and three cylindrical lenses onto a photodiode array (RL-128A, Reticon Corp., Sunnyvale, California). An amplifier was used to produce an analog signal, the voltage of which was proportional to the sarcomere length, based on the median intensity profile of

the first-order diffraction pattern [12]. The system was calibrated before every experiment with a test grating (12.5  $\mu\text{m}$ ).

Muscle bundles were adjusted to the desired length with the servo Cambridge motor during experiments. Muscle tension and median sarcomere length were displayed on the computer screen during the experiments. Muscle force was measured with a semiconductor strain gauge transducer (ENTRAN, Intertechnology Inc., Calgary) connected to an amplifier in a half-bridge configuration. Stimulation (Grass S88, Grass Instruments), was with supramaximal (10–50 V) square pulses, 0.5 ms duration, through two platinum wires in the experimental chamber, parallel to the muscle fibers.

Muscle bundles were divided into two groups, one for the establishment of the force-length relation during submaximal and maximal stimulation, and the other for measurement of staircase and posttetanic potentiation. Muscles were first studied at 22°C and then at 35°C. A 20–30 min equilibration period was allowed at each temperature.

In the first group of fiber bundles, tetanic contractions (200 Hz stimulation rate; 200–400 ms duration) were elicited at sarcomere lengths that were changed in steps of 0.1  $\mu\text{m}$  to 0.4  $\mu\text{m}$ , with 2 min rest between contractions. Sometimes during the course of the experiments, the muscle bundles were set at a reference sarcomere length where maximal force was obtained to verify absence of fatigue and/or damage in the preparation. In all cases, the force was not decreased when compared to previous contractions recorded at this same reference length, indicating that fatigue or damage did not occur in these experiments.

In the second group of fibers, each fiber bundle was used to assess either staircase (10 Hz for 10 s) or posttetanic response (75 Hz for 1.5 s). When staircase was induced, sarcomere length was set, and then the stimulation was applied. Three to four sarcomere lengths were tested in each preparation (10 min intervals between each period of repetitive stimulation). The first (Pt) and last (Pt\*) contraction in each series were collected (4000 Hz analogue to digital conversion) for assessment of the effects of repetitive stimulation (Pt\*/Pt). The sarcomere length was always the same after the 10 s of stimulation as it had been prior to the stimulation. During the 10 minute intervals between stimulation, control twitch contractions were elicited to verify absence of force decline due to fatigue and/or fiber damage.

To assess the length-dependence of posttetanic response, twitch contractions at 10 s intervals were obtained at a variety of sarcomere lengths both prior to (Pt) and following

(Pt\*) a tetanic contraction which was elicited at optimal length (the length which gave the strongest tetanic contraction). The first twitch contraction was obtained 30 s after the tetanic contraction, and the subsequent twitches were collected with 10 s intervals. These procedures follow closely what was done by Moore & Persechini [11], who have shown that at room temperature regulatory light chain phosphorylation and Pt\*/Pt following a tetanic contraction return to baseline very slowly at room temperature.

### Author contributions

Author DER contributed in the design of the experiments, and was primarily responsible for conducting the experiments and analyzing the data. He completed the first draft of the manuscript. Author BRM was the supervisor of author DER (doctoral candidate) and contributed to the design of the experiments and edits to the manuscript. Both authors have read and approved the final version of the manuscript.

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