Short communication

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Prolonged activity evokes potentiation and the “sag” phenomenon in slow motor units of rat soleus

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Slow motor units (MUs) have no sag in their unfused tetani. This study in anesthetized rats shows that the sag can be observed in slow soleus MUs after prolonged activity. Twitches and unfused tetanic contractions were recorded from male (n=35) and female (n=39) MUs before and after the four minutes of the fatigue test (trains of 13 pulses at 40 Hz repeated every second). After this activity twitch contractions potentiated and a shift in the steep part of the force-frequency curve towards lower frequencies was observed in both sexes. Initially no sag was visible in unfused tetani, but after the fatigue test the phenomenon was observed in 77% of male and in 13% of female MUs, with the sex difference possibly related to a higher content of IIa myosin and faster MU contraction in male soleus. Decomposition of tetani with sag into trains of separate twitches elicited by successive stimuli revealed higher forces for the initial than subsequent twitches. The newly revealed enhancement of the sag in force development following long-lasting activation is more pronounced in males than in females.

Key words: sag, force potentiation, slow motor unit, soleus, sex differences, mathematical decomposition, rat

The soleus is a typical homogenous muscle, composed predominantly of slow motor unis (MUs) (Close 1967, Drzymała-Celichowska and Krutki 2015). In most of physiological studies performed on MUs of cat and rat hindlimb muscles the division of MUs into fast (F) and slow (S) has been based on the sag profile, a rapid increase of tetanic tension followed by a decrease of tension present in unfused tetani of fast units (Burke et al. 1973) whereas this phenomenon has never been observed in slow MUs. In previous papers we have revealed that basic contractile properties of MUs in rat soleus muscle are sex-related, and male MUs are faster (Drzymała-Celichowska and Krutki 2015), consistent with higher contents of IIa myosin in the latter (Eason et al. 2000, Drzymała-Celichowska et al. 2012). It is known that a substantial portion of diversity of physiological properties can be attributed to the presence of distinct sets of myofibrillar proteins that define general classes of muscle fibers (Pette and Staron 1990, Stephenson et al. 1998, Carp et al. 1999). Slow MUs have very high fatigue resistance and are participating in long-lasting tonic activity necessary to maintain the body posture against gravity. However, the effects of their long-lasting activity are not well known. Therefore, the purpose of this paper was to analyze the effects of long-lasting activity on twitch properties, profiles of unfused tetani, as well as changes in the force-frequency relationships of slow MUs in rat soleus. The twitches and unfused tetanic contractions before and immediately after four minutes of a standardized fatigue test were recorded and compared. It was observed that the single twitch force potentiated and the force of unfused tetani increased and, in some MUs, a sag typical for fast MUs appeared at low frequency of stimulation.

The present study was performed on 6-months old Wistar rats: four females and five males (body mass 270.0±20.0 g and 484.8±21.7 g, respectively), all coming from one breeder. Rats were anesthetized with sodium pentobarbital (initial dose of 60 mg kg^{-1} i.p., supplemented after 2 hours with additional doses of 10 mg kg^{-1} h^{-1} i.p.), and adequacy of the anesthesia was verified by the lack of withdrawal and pinna reflexes. At the end of the experiment the animals were killed by an overdose of sodium pentobarbital (180 mg kg^{-1} i.p.). All the procedures were approved by the Local Ethics Committee and followed the European Union guidelines on animal care as well as the Polish Law on the Protection of Animals.

The surgical procedures were described in detail in previous paper (Drzymała-Celichowska and Krutki 2015).

To record the evoked activity of single MUs, L5 or L4 ventral roots were split into very fine bundles of axons

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that were placed on a silver wire electrode and electrically stimulated (0.1 ms rectangular pulses of amplitude up to 0.5 V). The "all or none" character of evoked twitch contractions and action potentials were used as criteria for a single MU isolation.

All investigated MUs were stimulated according to the following protocol:

1) 5 single twitches (5 stimuli at 1 Hz, the averaged twitch was estimated);

2) series of stimuli at 5, 10, 20, 30, 40, 50, 60, 75, 100 and 150 Hz frequencies and 500 ms duration to determine the force-frequency relationship;

3) 5 single twitches (5 stimuli at 1 Hz, the averaged twitch was estimated);

4) series of stimuli at 5, 10, 15, 20, 30, 40 Hz frequencies and 4000 ms duration;

5) the fatigue test (tetanic contractions evoked by trains of 14 stimuli at 40 Hz, repeated every second for 4 minutes) (Burke et al. 1973);

6) 5 single twitches (5 stimuli at 1 Hz, the averaged twitch was estimated);

7) series of stimuli at 5, 10, 15, 20, 30, 40 Hz frequencies and 4000 ms duration;

8) series of stimuli at 5, 10, 20, 30, 40, 50, 60, 75, 100 and 150 Hz frequencies and 500 ms duration to determine the force-frequency relationship.

10-second time intervals were applied between consecutive steps of the protocol.

The twitch force potentiation (TP) was calculated as a ratio of the twitch force recorded in point 6 of the protocol to the initial force (point 1 of the protocol, TP) and additionally the force increase was presented as a percentage of the initial twitch force.

The MUs were accepted as slow (S) if no sag was observed in tetani at several different stimulation frequencies (10, 20, 30 and 40 Hz, point 2 of the protocol). All isolated slow MUs were included in the sample. For all twitch recordings (point 1, 3 and 6 of the protocol) the twitch force (the force at a peak of recording) and twitch contraction time (measured from the beginning of a twitch to the force peak) were calculated. Finally, the fatigue index was calculated as a ratio of the forces of the 120th tetanus to the force of the first tetanus within the fatigue test (Burke et al. 1973). The mean value of the index for studied MUs amounted to 0.99±0.01 (0.96–1.0) and 0.98±0.02 (0.96–1.0) for males and females, respectively.

Additionally, for five male motor units the decomposition of the tetanic contractions evoked within the point 4 and 7 of the protocol was performed (Fig. 2). The mathematical method for decomposition of the unfused tetanic curve into twitch-shape contractions, which are mechanical responses to individual pulses, was described in details previously (Raikova et al. 2007).

All data are expressed as means ± standard deviations (SDs), and the minimum and maximum values are given. For statistical evaluation of significances of differences between the properties of male and female slow motor units the Mann-Whitney *U*-test was used, and the differences in sag appearance between males and females were evaluated by the proportion test. Moreover, values for non-potentiated and potentiated twitches within male or female MUs were compared with Wilcoxon signed-rank test.

The prolonged activity evoked considerable changes in the twitch properties of studied MUs ($P<0.05$ for both, the twitch force and contraction time), which were not visible when the twitches recorded within point 3 and 6 of the protocol were compared. For all motor units the twitches potentiated after the fatigue test and the potentiation increased both the force and the contraction time (Table 1). The potentiation-related changes in the contraction time were reflected in the force-frequency relationships of MUs in both, males and females (Fig. 1). The leftward shift of the

Table 1. The mean values, standard deviations and variability ranges for twitch properties of MUs in male and female soleus

	TwF [mN]	CT [ms]	TwFp [mN]	CTp [ms]	TP [%]
Male	11.98±5.56	30.90±8.60	16.56±7.14	35.80±9.90	42.70±22.00
n=35	7.00–31.00	20.00–52.00	8.79–37.36	20.00–61.00	12.30–95.50
			###	#	
Female	10.72±3.73	33.40±5.80	13.88±4.75	39.60±7.80	31.10±17.80
n=39	5.00–21.70	23.00–50.00	6.84–30.28	29.00–65.00	4.00–72.80
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Data for the initial twitch (point 1 of the stimulation protocol): the twitch force (TwF), the contraction time (CT). Data for the potentiated twitch (point 6 of the protocol): the twitch force (TwFp), the contraction time (CTp). In the last column the twitch force potentiation (TP) is presented as a percentage of the initial twitch force. Significance of male/female differences: * – difference significant at $P<0.05$, *** – difference significant at $P<0.001$ (Mann-Whitney *U*-test). Significance of non-potentiated/potentiated twitch differences: # – difference significant at $P<0.05$, ### – difference significant at $P<0.001$ (Wilcoxon signed-rank test).

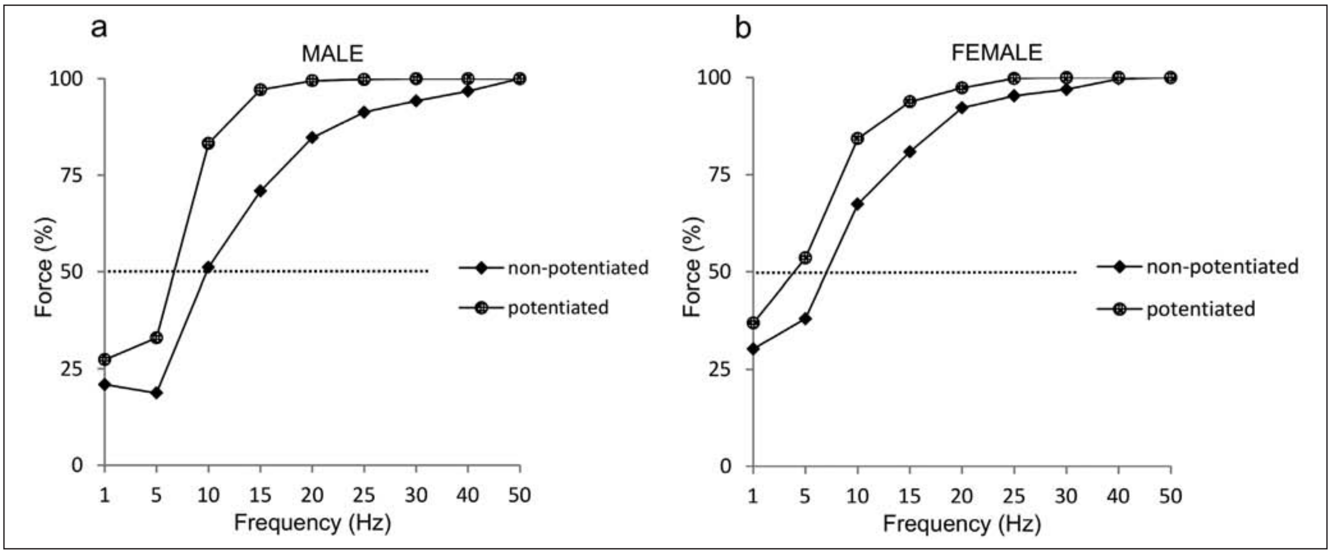

Fig. 1. The averaged force-frequency of stimulation relationships for studied MUs in soleus muscle before (diamonds) and after (circles) the fatigue test. Data presented separately for male (A) and female (B) soleus. The dotted horizontal lines indicate 50% of the maximum tetanus force.

force-frequency curves was observed in all MUs. Following potentiation, 50% of the maximal force was achieved at significantly lower frequencies of stimulation: 10.6±1.8 *vs.* 6.8±0.7 Hz in males and 9.1±4.6 *vs.* 5.0±3.4 Hz in females for non-potentiated and potentiated MUs, respectively ($P<0.05$). The long-lasting activity also evoked surprising changes in profiles of sub-fused tetani – the appearance of a sag (Fig. 2B). It should be stressed that this phenomenon was visible in contractions evoked at 5 or 10 Hz frequency (with the mean fusion indices 0.80±0.20 for males and 0.70±0.19 for females) that in non-potentiated MUs were less fused (fusion indices 0.50±0.30 and 0.13±0.10 for males and females, respectively) or even evoked non-overlapping twitches. The sag was more frequent in male MUs and it was noted for 27 out of 35 male MUs (77.1%) and for 5 out of 39 female MUs (12.8%) after the fatigue test ($P<0.001$). The relative force drop during sag amounted to 16.0±7.0% (6.0–29.3%). Examples of 15 Hz stimulation of a MU without sag effects recorded before the fatigue test – point 4 of the protocol, and with sag recorded after the fatigue test at 10 Hz (point 7 of the protocol) are shown in Fig. 2A, 2B.

Among 74 MUs recorded during the experiments, the unfused tetanic contractions recorded before (no sag) and after fatigue test (sag present) for 5 male units were chosen for the mathematical decomposition. The selection criteria were a high signal-to-noise ratio and a lack of artifacts in force recordings. The values of relative force drop in sag for these 5 units covered a similar range as for the whole group of MUs: 14.7±8.1% (6.1–28%). The effect of the decomposition was a train of twitch-shape responses to all successive stimuli. The first decomposed twitch was always the weakest and had the shortest duration in non-potentiated MUs as well as in the potentiated ones with visible sag. Changes in force and time parameters of the following decomposed twitches were different for non-sagging tetani recorded and those with the sag (Fig. 2C-2D, 2E–2F). The duration of decomposed twitch responses for non-potentiated tetani was evidently prolonged in relation to the first twitch, whereas in potentiated tetani time differences between first and remaining decomposed twitches were smaller. The ratio of the longest contraction time noted for the decomposed twitches to the shortest one (the first) was different for units without sag and for units with visible sag and amounted to 2–4.3 and 1.3–2.0, respectively (Fig. 2E-2F). Moreover, for potentiated tetani with a sag the 3rd–8th response was the strongest and for the following responses a decrease of force (by 6–20%) was observed, while in tetani without sag this force decrease was not observed. The ratio of the strongest decomposed twitch response to the first response was lower in potentiated tetani (2.1–2.5) than in non-potentiated ones (2.9–4.5).

The present study confirmed the classical observation that there is no sag in tetanic contractions of S type MUs (Burke et al. 1973, Burke 1981), which was commonly used as a specific criteria for MU type identification (e.g. Gordon et al. 1990, Kanda and Hashizume 1992). Unexpectedly, we have found the sag appears in low-frequency tetani of potentiated MUs in rat soleus. Additionally this result differs from an earlier observation that in fast MUs of rat gastrocnemius the sag is most evident just at the beginning of their activity (Celichowski et al. 2005). The analysis of the time course of unfused tetani with sag in fast fatigable and fast resistant MUs in rat medial gastrocnemius muscle revealed better summation of responses to, in some cases, first stimuli leading to early peak in force followed by decrease of further responses i.e. sag. These processes occurred most evidently within the first unfused tetanus,

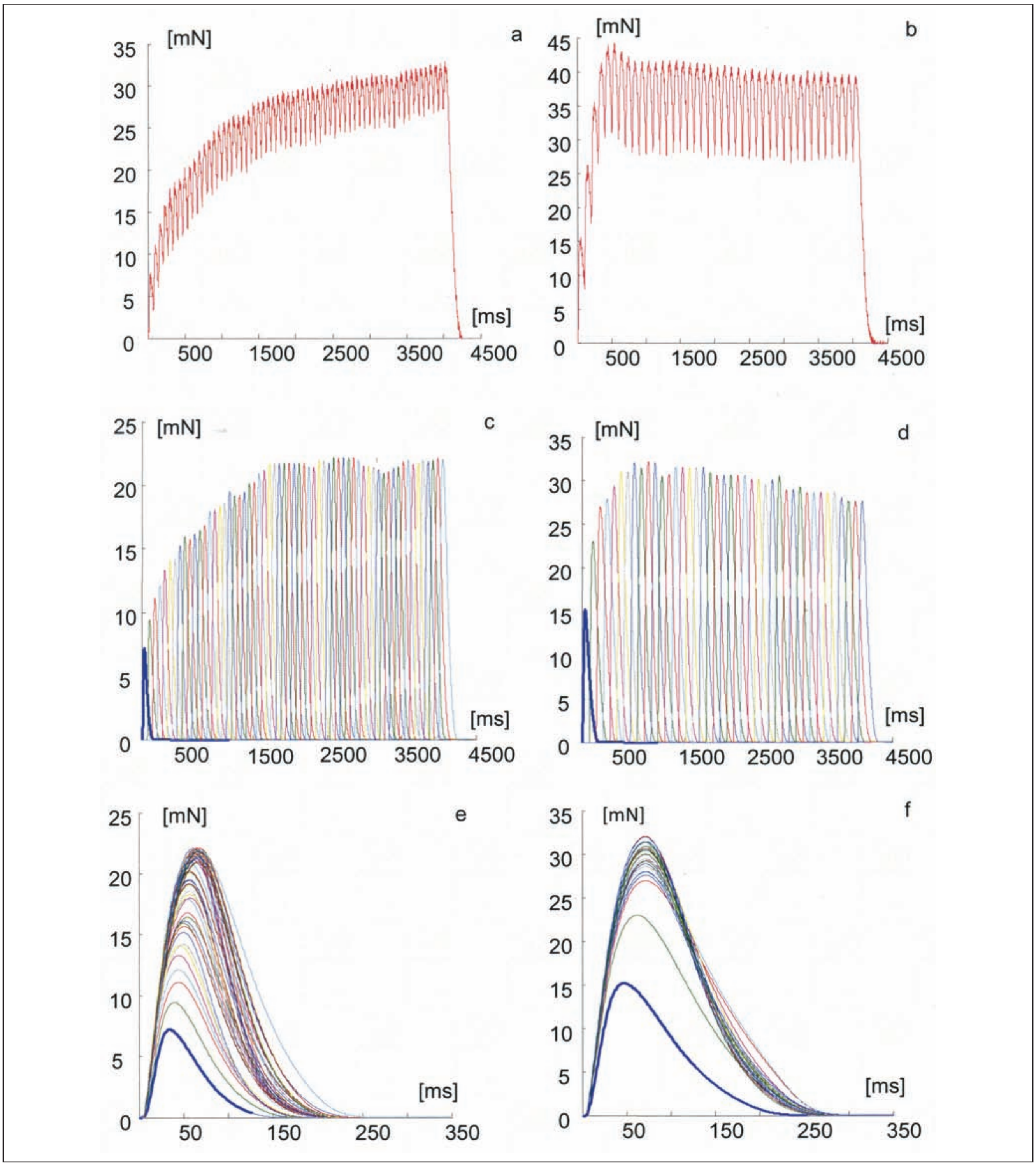

Fig. 2. The decomposition of unfused tetanic curves recorded before (left column) and after the fatigue test (right column) for the same slow MUs. The applied stimulation frequencies: 15 Hz (left column) and 10 Hz (right column). The observed twitch force potentiation resulted in considerably different fusion indices, so to compare contractions with similar fusion indices (0.86 and 0.76, respectively) frequencies could not be identical.
The first row (A, B) shows the recorded forces. The force drop in b amounted to 6.1%. The second row (C, D) shows the decomposed contractions presented according to their time position. The third row (E, F) shows the same decomposed twitch-shape responses to consecutive stimuli presented as superimposed assuming the time appearance for each stimulus as 0 ms at abscissa. In C–F, the first (the weakest) decomposed twitches are distinguished by thick dark blue lines. For the non-potentiated conditions (left column) the first decomposed twitch parameters are: the contraction time 29 ms, the twitch force 7.26 mN whereas for the potentiated conditions (right column) the first decomposed twitch parameters are: the contraction time 37 ms, the twitch force 14.2 mN.

whereas in subsequently repeated tetani the phenomenon disappeared but the force progressively potentiated.

The observation concerning the appearance of sag was different for male and female MUs. Considerably higher body and muscle mass of male rats indicate that baseline conditions for contractions were unequal for both sexes (e.g. higher demands with respect to MU force levels for a male soleus muscle), which might consequently affect conditions for sag appearance. Additionally, the ability to develop a sag following the long-lasting activity seems to be a property partly dependent on the force potentiation, observed in all examined MUs, but stronger for males. On the other hand, the muscle-body mass ratios, and changes of twitch parameters after the prolonged activation of MUs were comparable between sexes (0.041% for males *vs.* 0.046% for females, P>0.05), in line with previously demonstrated data (Mierzejewska-Krzyżowska et al. 2012). Force decrease was not observed after the fatigue test in any of MUs, so one should exclude fatigue itself as a reason of sag appearance. The long-lasting activity evoked the force potentiation and prolongation of the contraction time for all studied slow MUs whereas the shorter duration of very high-frequency stimulation (up to 150 Hz, point 2 of the stimulation protocol) did not potentiate the twitch. Therefore, it seems that the time of activity is a crucial factor in evoking the force potentiation in slow MUs. The high frequency stimulation caused also a transitory post-tetanic potentiation of the isometric twitch contractions of mammalian fast skeletal muscles, whereas there was a depression in a response of slow muscles (Standaert 1964, Bagust et al. 1974). However, it is worth noting that Burke and others (1973) observed no differences in the potentiation of slow and fast MUs of cat gastrocnemius evoked by repeated tetanic stimulations although they have not specified the expected differences in the potentiation procedure duration for different types of MUs. The prolongation of potentiated twitches noted for the studied MUs was also observed for fast as well as for slow MUs of cat gastrocnemius (Burke et al. 1973). It is known that the contraction time correlates to a course of force-frequency of stimulation curve (Kernell et al. 1983). Therefore, most probably as an effect of this prolongation the force-frequency curves of male and female MUs were shifted to the left, a result similar to that found for potentiated fast MUs (Celichowski and Grottel 1997). Interestingly, in potentiated tetani time differences between the first and remaining decomposed twitches were smaller than in non-potentiated ones (Fig. 2 E–2F). The decomposition of recorded non-sagging initial tetanic contractions and the potentiated, sagging ones revealed a difference in profiles of changes in parameters of responses to successive stimuli. This analysis revealed that sag is related to the extra force of several first twitch responses within the sub-fused tetanus (Fig. 2C–2D). The latter may, in turn, be related to increased release of calcium ions within these responses caused by the preceding long activity and transient changes in the phosphorylation state of the myosin light chain subunits responsible for twitch potentiation (see Vandenboom et al. 2013 for a review).

In conclusion, this study shows that mechanisms of the force development in slow MUs may be altered due to their long-lasting activation, revealing force potentiation and a sag, previously not observed in unfused tetanic contractions of slow MUs. Moreover, the occurrence of sag is sex-dependent, as under our experimental conditions it was present significantly more often in male than in female rats.

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