Time course of changes in *in vitro* sarcoplasmic reticulum Ca^{2+}-handling and Na^{+}-K^{+}-ATPase activity during repetitive contractions

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Abstract

Time-dependent changes in sarcoplasmic reticulum (SR) Ca^{2+}-handling and Na^+-K^+-ATPase activity, as assessed *in vitro*, were investigated in the superficial (GS) and deep regions (GD) of rat gastrocnemius muscles undergoing short-term (up to 30 min) electrical stimulation. There was a rapid and progressive loss of force output during the first 5 min of stimulation. For GS, significant depressions ($P < 0.05$) in SR Ca^{2+}-uptake rate and Ca^{2+}-ATPase activity were observed during only the first 1 min. No further reductions occurred with stimulation time. SR Ca^{2+}-release rate was significantly ($P < 0.05$) decreased at 3 min. For GD, significant reductions ($P < 0.05$) in Ca^{2+}-uptake rate, Ca^{2+}-release rate and Ca^{2+}-ATPase activity were manifested after 3, 5 and 5 min, respectively. A decay in Na^+-K^+-ATPase activity was found only in 1-min stimulated GD and 30-min stimulated GS. After 30 min, the depressed functions reverted to resting levels in GD but not in GS. The alterations in any variables examined were not parallel with changes in force output. These results suggest that, at least under the conditions used in this study, *in vivo* disruptions in cation regulation mediated by vigorous contractile activity would be attributable primarily to events other than structural alterations to the respective proteins.

Introduction

Vigorous muscle contraction ultimately results in an inability to produce a desired force, known as muscle fatigue. Although the mechanistic basis of fatigue is complex and involves a variety of metabolic and nonmetabolic factors that influence different excitation and contraction processes in muscle [1, 7, 31], recent years accumulating evidence has implicated an impairment of cation [e.g., potassium (K^+), sodium (Na^+), and calcium (Ca^{2+})] regulation, as a major contributor to muscular fatigue [17, 18]. In skeletal muscle, the vectoral transport of Na^+ and K^+ across the plasma membrane by Na^+-K^+-ATPase, or Na^+-K^+-pump, restores ionic gradients after action potential, and the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_f$) regulation responsible for activation and inactivation of the myofibrillar complex is primarily dependent on the kinetics of Ca^{2+} release and uptake by the sarcoplasmic reticulum (SR).

Numerous studies reported to date, using different experimental protocols including intense and prolonged exercise [11, 14, 16, 17] and short- and long-term electrical stimulation [10, 15], have indicated that fatiguing contraction disturbs Na^+-K^+- and/or Ca^{2+}-cycling behavior in working muscles. In most cases, SR function and Na^+-K^+-ATPase activity were assessed *in vitro* under optimal conditions that resemble the environment in rested muscles. It seems most likely, therefore, that the observed alterations would represent structural modifications to the respective proteins regulating these functions. Despite the extensive research, the precise mechanisms for the structural

disturbances resulting from muscle contraction are not as yet known, but some possibilities can be considered. With regard to SR Ca^{2+}-ATPase, the enzyme responsible for Ca^{2+}-sequesteration, reductions in its activity that occur with exercise have been shown to be mediated by oxidation and nitrosylation of proteins secondary to accumulation of reactive oxygen species (ROS) [12, 16]. In contrast, single fiber study has described that oxidation of the calcium-release channel (CRC) results in little or no change in the amount of Ca^{2+} released by the SR [21]. For Na^+-K^+-ATPase, evidence has been provided to suggest that the diminishing activity may be ascribed to the elevated $[Ca^{2+}]_f$ as well as ROS accumulation [18, 27]. In addition, the increased muscle temperature that can reach 43ºC during exercise accounts for protein unfolding leading to a decrease in its function [25].

It is conceivable that changes in these possible factors, which could exert the deleterious effects on the structure of proteins regulating cation, may not be necessarily simultaneous and that the susceptibilities of different proteins to these factors may not be necessarily the same. If this is the case, perturbations in the Ca^{2+}-cycling capacities by the SR and the catalytic activity of Na^+-K^+-ATPase as assessed *in vitro* must follow different time courses. It would also be anticipated that the effect of repetitive contractile activity on proteins involved in cation regulation would depend, to some extent, on the oxidative potential of muscles, because the antioxidant abilities are higher in aerobically adapted muscle fibers [22]. Although the time course of any such depression in cation regulation

has important implications for understanding the significance of these alterations in muscle fatigue, studies of these issues are sparse.

Thus the present study was undertaken in order to elucidate the time course of changes in SR Ca^{2+}-handling and sarcolemma Na^+-K^+-cycling properties, as assessed *in vitro*, in muscles of distinct oxidative potential during repetitive contractile activity. We tested the hypotheses that *1)* depressions in SR Ca^{2+}-uptake and release rates and Na^+-K^+-ATPase activity would follow different time courses; and *2)* these changes would occur more rapidly in less oxidative muscles than in oxidative muscles. To gain insight into the role of the cellular redox state in alterations in the structure state of proteins, we also measured the reduced glutathione (GSH) content that is an index of oxidative stress [24].

Methods

Animal care

Experiments were performed on adult male Wistar rats (n = 32) weighing 278 ± 3.0 g (mean ± SE). The animals were fed on water and laboratory chow ad libitum and housed in a thermally controlled room maintained at 20-24°C with a 12-h light/dark cycle until the time of study. All experiments were conducted at approximately the same time, between 10:00 AM and 3:00 PM, to limit diurnal variation in muscle glycogen. All procedures were approved by the Animal Care Committee of Hiroshima University.

Stimulation

The experiments were done on electrically stimulated intact gastrocnemius (GAS) muscles working *in situ*. To prevent the extensor digitorum longus, tibialis anterior, plantaris and soleus muscles from contributing to force production, tenotomy of these muscles was performed alternately on the right and left legs. After the animals were deeply anaesthetized using an intraperitoneal injection of pentobarbital sodium (6 mg/100 g body wt), the tendons of four muscles were cut by making two incisions parallel to the long axis of the tibia on the anterior and posterior aspects of the lower leg, followed by suturing. The sciatic nerve on the side where tenotomy was performed was then exposed so that electrode could be attached. Care was taken to avoid damaging any vessels and nerves during the surgical procedure. The muscles from contralateral legs were used as controls. The animal

was placed in a supine position and the experimental limb was attached to a home-made foot holder connected with an isometric transducer. The knee and ankle were stabilized at 90° on the foot holder using a strap. The experimental muscles were stimulated via the sciatic nerve with 100-msec trains of pulses (0.1-msec pulse, 75 Hz) delivered once every 2 sec for 1, 3, 5 or 30 min (n = 8 per each group). Force was continuously recoded on a personal computer and analyzed using dedicated software. Control and stimulated GAS muscles were removed immediately after the termination of repeated tetani. The superficial (GS) and deep (GD) regions of the GAS were separated visually by color, and samples were always collected from similar areas. Oxidative potential estimated by the maximal activity of citrate synthase, a rate-limiting enzyme of the Krebs cycle, has been reported to be 2.72 ± 0.26 and 7.50 ± 0.34 µmol min^{-1} g^{-1} protein (mean $\pm$ SE) for GS and GD, respectively [10].

SR Ca^{2+}-ATPase activity

Muscle pieces of approximately 100 mg were diluted in 9 volumes (mass vol^{-1}) of ice-cold homogenizing buffer composed of (in mM) 300 sucrose, 20 Mops/KOH, 0.0014 pepstatin, 0.83 benzamidine, 0.0022 leupeptin and 0.2 phenylmethanesulfonyl-fluoride (pH 7.4) and were homogenized on ice for 3×30 s at 5,000 rpm using a hand-held glass homogenizer. For analysis of Na^{+}-K^{+}-ATPase activity, 20 µL of homogenate were aliquoted and the remaining homogenates were then centrifuged at 5,000 g for 10 min. The

resulting supernatant was used for SR Ca^{2+}-handling analyses, i.e., Ca^{2+}-uptake and release

rates and ATPase activity. SR Ca^{2+}-ATPase activity was spectrophotometrically determined

in triplicate at 37°C according to Simonides and van Hardeveld [26]. The assay mixture

was composed of 1 mM EGTA, 20 mM *N*-2-hydroxyethylpiperazine- *N''*-2-ethanesulfonic

acid (HEPES), 200 mM KCl, 15 mM $MgCl_2$, 10 mM NaN_3, 0.4 mM NADH, 10 mM

phosphoenolpyruvate, 0.8 mM $CaCl_2$, 18 U mL^{-1} pyruvate kinase, 18 U mL^{-1} lactate

dehydrogenase, and 4 μg mL^{-1} Ca^{2+} ionophore A23187 (pH 7.1). After the addition of a 20

μL aliquot of homogenate to 2 mL of assay mixture, the assay mixture was pre-incubated at

37°C for 4 min. The reaction was started by adding ATP to give a final concentration of 4

mM. Finally a $CaCl_2$ concentration was increased to 20 mM in order to selectively inhibit

SR Ca^{2+}-ATPase activity. The remaining activity was defined as the background ATPase

activity. The activity of SR Ca^{2+}-ATPase was calculated as the difference between total

ATPase and the background ATPase activities.

SR Ca^{2+}-uptake and release rates

SR Ca^{2+}-uptake and release rates were measured in triplicate using the Ca^{2+}

fluorescent dye indo-1 according to Ward et al. [32]. The assay mixture consisted of (in

mM) 100 KCl, 20 HEPES, 6.8 potassium oxalate, 0.5 $MgCl_2$, 10 NaN_3, and 0.001 indo-1

(pH 7.1). Without the addition of extra $CaCl_2$, the $[Ca^{2+}]_f$ of the assay mixture containing

muscle homogenates was 1.2–1.4 μM. It was adjusted to approximately 2 μM by the

addition of small amounts of $CaCl_2$ solution (0.25 mM stock), as in preliminary experiments, this level of initial $[Ca^{2+}]_f$ was observed to yield reproducible values for Ca^{2+}-uptake rate between repeated trials. After the addition of a 20 μL aliquot of homogenate to 1 mL of the assay mixture, the assay mixture was pre-incubated at 37°C for 4 min. Uptake was initiated by adding 1 mM MgATP and allowed to continue until little or no change in extravesicular $[Ca^{2+}]_f$ was observed. Ca^{2+} release was then initiated by the addition of 10 mM 4-chloro-*m*-cresol. For calibration purposes, at the completion of each assay, the maximum and minimum fluorescence ratios were measured by the addition of 10 μL of 0.1 M EGTA, followed by 10 μL of 0.25 M $CaCl_2$. During these procedures, the buffer solution was continually stirred and temperature was maintained at 37°C. Extravesicular $[Ca^{2+}]_f$ was monitored flurometrically using a fluorometer (CAF-110, Nihon-Bunkko, Kyoto, Japan). Excitation light came from a high-pressure lamp equipped with a monochromator and filtered at 349 nm. Emission fluorescence was determined by a pair of photomultipliers using 410 and 500 nm filters. Photon counts were simultaneously recorded for both emission wavelengths. The $[Ca^{2+}]_f$ was computed using the ratiometric method of Grynkiewicz et al. [9].

Na$^+$-K$^+$-ATPase activity.

The catalytic activity of Na$^+$-K$^+$-ATPase was assessed using the K$^+$-stimulated 3-O-methylfluorescein phosphatase (3-O-MFPase) assay according to Fraser and McKenna

[6]. Homogenates for Na^+-K^+-ATPase activity were freeze-thawed four times and diluted in 9 volumes (mass vol^{-1}) of ice-cold homogenate buffer composed of (in mM) 250 sucrose, 2 EDTA, 5 NaN_3, and 10 Tris (pH 7.4). After the addition of a 30 μL aliquot of homogenate to 1 mL of assay mixture composed of (in mM) 5 $MgCl_2$, 1.25 EDTA, 5 NaN_3, and 100 Tris (pH 7.4), the assay mixture was pre-incubated at 37°C for 3 min. The reaction was started by adding 3-O-MFP to give a final concentration of 200 μM. Finally, 10 mM KCl was added in order to selectively increase Na^+-K^+-ATPase activity. Changes in fluorescence were quantified by fluorescence spectrophotometry (excitation wavelength = 475nm; emission wavelength = 515 nm). The 3-O-MFPase activity was based on the difference in slope before and after the addition of KCl.

Glutathione content

Muscle pieces of approximately 50 mg were minced, placed on ice in 9 volumes of 5% (vol vol^{-1}) 5-sulfosalicylic acid for 30 min, and then centrifuged at 16,000 g for 10 min to remove precipitated materials. For total glutathione (GSH + GSSG), triehanolamine was added to the supernatant to give a final concentration of 6% (vol vol^{-1}). For GSSG, 2% (vol vol^{-1}; final concentration) 2-vinylpyridine was additionally added. The amount of GSH and GSSG was spectrophotometrically determined in triplicate at 37°C according to Baker et al. [3]. The assay buffer consisted of 1.52 mM NaH_2PO_4, 7.6 mM Na_2HPO_4, 0.485 mM EDTA, 1 U mL^{-1} glutathione reductase and 0.1 mM NADPH (pH 7.5). After the addition of a 20

µL aliquot of the sample to 2 mL of the assay mixture, the assay mixture was pre-incubated at 37°C for 3 min. The reaction was started by adding 5,5'-dithiobis-(2-nitrobenzoic acid) to give a final concentration of 0.4 mM. The GSH content was calculated as the difference between total and GSSG contents.

Lactate and glycogen contents

The lactate and glycogen concentrations were measured using fluorometric techniques according to procedures previously described [11]. Muscle samples ranging from 25 to 30 mg were freeze-dried at -70ºC for 24 h. The metabolites were extracted in 2 M perchloric acid (PCA) and neutralized by 2 M $KHCO_3$. The supernatant obtained by centrifugation at -4ºC for 15 min at 4,000 g was used for analysis of lactate. The glycogen concentration was measured in PCA-denatured protein pellets.

Statistical analyses

All data are presented as means ± SE. For metabolites, SR Ca^{2+}-handling capacity, Na^{+}-K^{+}-ATPase activity, Student's *t* test for paired samples was used to determine the significance of differences between control and experimental muscles. A one-way ANOVA was used to investigate the effect of stimulation time. For analysis of force, a one-way ANOVA for repeated measures was used to test for statistical differences. If an overall significant F-value was obtained, Fisher's least significant difference analysis was used to

isolate the significant different means. The acceptable level of significance was set $P <$ 0.05.

Results

Force output

After the onset of 30-min stimulation, there was a rapid and progressive loss of force during the first 5 min. With 1, 3 and 5 min of stimulation, force declined to 82.7, 64.1 and 51.2% of the initial value, respectively (Fig. 1). However, beyond 5 min, pronounced alterations were not observed. At 30 min of stimulation, force amounted to 45.8% of the initial.

Glycogen and lactate contents

For GS, glycogen was rapidly declined by 59.7% during the first 1 min of stimulation (Fig. 2). With continued stimulation, further depressions were observed. It reached 17.4% of control levels at 30 min of stimulation. For GD, glycogen was decreased by 22.8% at 1 min of stimulation and maintained with a minimal decrease until the end of stimulation. Lactate in GS was increased to 296.2% of the control value during the first 1 min and remained elevated until 5 min (Fig. 3). Thereafter, it decayed, reaching values similar to control after 30 min. For GD, significant increases were observed only at 1 min of stimulation.

SR Ca^{2+}-uptake rate and Ca^{2+}-ATPase activity

For GS, a significant decrease in SR Ca^{2+}-uptake rate to 87.4% of control was observed during the first 1 min of stimulation (Fig. 4). No further changes were

observed, but until the cessation of stimulation, the uptake rate remained depressed. SR Ca^{2+}-ATPase activity displayed changes similar to those of Ca^{2+} uptake, but was compromised to a great extent (Fig. 5). Stimulation for 1, 3, 5 and 30 min elicited a 15.5, 25.9, 19.9 and 16.1% reduction, respectively. For GD, SR Ca^{2+}-uptake rate was significantly reduced at 3 and 5 min of stimulation (Fig. 4). The reduction at 5 min amounted to 30.8% compared to control levels. Later on, it was recovered, reaching values similar to control at 30 min of stimulation. In contrast to GS, the percentage decline of SR Ca^{2+}-ATPase activity in GD was smaller than that of uptake (Fig. 5). Significant reductions, which amounted to 12.2%, were observed only at 5 min of stimulation.

SR Ca^{2+}-release rate

For GS, 3-min stimulation evoked a 30.4% decline (Fig. 6). With continued stimulation, it was partially recovered, but remained decreased after 30 min. SR. For GD, Ca^{2+}-release rate was unaltered by 3 min. However, a sudden decline to 69.5% of the control value was found at 5 min of stimulation. With continued stimulation, the release rate was restored.

Na^+-K^+-ATPase activities

For GS, a trend towards a decline in Na^+-K^+-ATPase activity was seen for 3- and 5-min stimulated muscles, but the changes did not reach significant level ($P = 0.08$ and

0.09, respectively; Fig. 7) A significant decrease, which amounted to 11.0%, was found at 30 min of stimulation. For GD, the activity was depressed by 16.0% at 1 min of stimulation, but recovered to control levels at 3 min. Later on, it was kept constant.

GSH content

It is well known that GSH, a low-molecular-weight thiol, regulates the cellular redox state and alteration in the GSH content can be a hallmark for oxidative stress [24]. For GS, 1-min stimulation elicited a 9.7% decline (Fig. 8). No further changes were found with continued stimulation. In contrast, for GD, significant reductions amounting to 13.1% were found only at 30 min of stimulation.

Discussion

This study reveals novel results on time-dependent changes in stimulated rat fast-twitch muscle, not previously recognized. According to the present results, our first and second hypotheses seem to be justified but only in oxidative muscle and only with regard to SR Ca^{2+}-handling, respectively (see below). The unexpected results are that *1)* in GS, repetitive tetani caused decreases in SR Ca^{2+}-ATPase activity and Ca^{2+}-uptake rate during only the first 1 min of stimulation; *2)* despite a rapid loss of force until 5 min, no further reductions in the activity and uptake rate occurred beyond 1 min; and *3)* the depressed SR Ca^{2+}-handling and Na^+-K^+-ATPase activity observed in GD during 1-5 min reverted to resting levels at 30 min of stimulation. It is important to note that any alterations observed in the current study do not directly reflect the *in vivo* function of the respective proteins, but do represent structural modifications.

In situ study by de Ruiter et al. [4], in which rat GS and GD were separately stimulated, has revealed that 4-min stimulation induces the much larger decline in tetanic force in GS than in GD (73% vs 37%). The results of de Ruiter et al. [4] and the present study seem likely to be comparable, since we employed the stimulation protocol similar to that (*in situ* model using electrical stimulation via the sciatic nerve) used in their study. Taking their findings into account, it appears likely that a rapid and progressive decline of force in whole muscle during the initial 5 min would be attributable primarily to a decrease in force produced by GS. The steep and large increase in lactate, concomitant with glycogen breakdown, emphasizes the importance

of anaerobic energy supply in GS. In addition, these changes imply that the intracellular environment in GS during this phase may resemble that of muscles undergoing intense contractions, e.g., several-min running to exhaustion [19] or repeated maximal tetani that halves the force within a few min [2]. It is well accepted that intense contraction-induced muscle fatigue is intimately related to both an inability of the SR to regulate Ca^{2+} and a rundown in the chemical Na^+/K^+ gradients across the sarcolemma [2, 19]. If such is the case in GS stimulated in this study, these perturbations should develop progressively during the first 5 min.

The present results that any alterations in three variables examined in GS, i.e., Na^+-K^+-ATPase activity and SR Ca^{2+}-uptake and release rates, were not necessarily parallel with the changes in force output suggest that at least under the conditions used in this study, structural alterations to the respective proteins may not contribute largely to disruptions in cation regulation that would presumably occur *in vivo*. This could be especially true of Na^+-K^+-ATPase because unlike the SR, an appreciable depression in its function was not observed *in vitro*. It seems most likely that alterations in the metabolic environment may be involved in *in vivo* disruptions, given previous studies showing that glycogen depletion, inorganic phosphate and Mg^{2+} accumulations, and inorganic phosphate and ADP accumulations are closely associated with the impaired functions of Na^+-K^+-ATPase [20], CRC [5, 13] and SR Ca^{2+}-ATPase [30], respectively. More pronounced changes in some of these metabolites during the first several min of stimulation are characteristic of less oxidative muscles as compared to oxidative

muscles [8, 33].

Generally, structural alterations to Na^+-K^+-ATPase, CRC and SR Ca^{2+}-ATPase in GS appear to follow similar time courses although depressions in Na^+-K^+-ATPase activity in 3- and 5-min stimulated muscles did not reach significant level ($P = 0.08$ and 0.09, respectively; Fig. 7). It is possible that the result of Na^+-K^+-ATPase activity might reflect the assay variability, small sample size, and consequently likelihood of a Type II error. If so, our results of GS would suggest a common mechanism by which repetitive contraction evokes structural modifications to the SR and Na^+-K^+-ATPase. Recently, McKenna et al. [18] investigated the impact of N-acetylcysteine (NAC), an antioxidant compound, on Na^+-K^+-ATPase activity in human skeletal muscle and found that NAC intravenous infusion was capable of attenuating depressions in the catalytic activity induced by prolonged exercise (at 71% of maximal O_2 consumption for 45 min). Their findings are strongly suggestive that oxidative stress, probably due to ROS accumulation, would be responsible for the decreased activities during muscle contraction. In addition to Na^+-K^+-ATPase, the ATP-binding site of SR Ca^{2+}-ATPase has been demonstrated to represent a target for modification of ROS [34], leading to a decay of its activity. The action of ROS appears to be more complex on the CRC, compared to SR Ca^{2+}-ATPase and Na^+-K^+-ATPase. The CRC comprises approximately 50 free thiol (SH) groups that regulate the gating of the CRC [28]. Oxidation of up to one-half of SH groups activates the CRC, whereas more extensive oxidation inactivates it [28]. The ~10% decrease in the GSH content partially supports the plausible involvement of ROS

in impaired function of the SR and Na^+-K^+-ATPase. However, the magnitude of decreases observed in the present study is smaller than shown in previous study on exercised rodent muscles [24].

The time-dependent changes found in GD differed from those of GS in several aspects; *1)* declines in Na^+-K^+-ATPase activity occurred earlier than those of SR Ca^{2+}-handling; *2)* decreases in SR Ca^{2+}-handling abilities were manifested later, compared to GS; *3)* muscles stimulated for up to 5 min did not display a hallmark of oxidative stress; and *4)* in spite of a significant decrease in the GSH content at 30 min of stimulation, the depressed SR Ca^{2+}-handling and Na^+-K^+-ATPase activity were restored. These results of GD and the subtle decline in the GSH content in GS point out that events other than ROS may be related mainly to the changes observed in three proteins. At present, the acute mechanisms by which the *in vitro* function of SR Ca^{2+}-handling and Na^+-K^+-ATPase activity fluctuates during repeated muscle contractions remain incompletely understood. However, as the possible candidates, previous observations have implicated; *1)* sarcolipin that associates with fast isoform of SR Ca^{2+}-ATPase and decreases its activity [30]; *2)* phospholamban that lowers the affinity of slow isoform of SR Ca^{2+}-ATPase for Ca^{2+} [30]; *3)* phosphorylation of calmodulin kinase II that increases the open status of the CRC [29]; and *4)* tyrosine phosphorylation of Na^+-K^+-ATPase that increases its catalytic activity [23]. The restoration of Na^+-K^+-ATPase activity in GD consisting of slow- and fast-twitch fibers appears likely to be ascribed, at least partly, to *4)*, given that 15-min stimulation (500-msec trains at 30 Hz every 1.5 sec) of

slow-twitch soleus muscle brings about increased phosphorylation of α-subunit, the catalytic subunit of the enzyme [23]. It may be speculated that varying degrees of alterations in some of these factors are linked to the differences in the time-course changes between GS and GD. Whatever the reason for alterations in the *in vitro* function, the fact that the diminished functions reverted to normal levels after 30 min of stimulation implies that stimulation may produce only a minor conformational changes that can be reversed.

In the present study, we examined changes in the *in vitro* function of major cation transport regulatory proteins, i.e., Ca^{2+}-ATPase and CRC of the SR and sarcolemma Na^+-K^+-ATPase, in rat gastrocnemius muscle undergoing repetitive tetanic contraction for up to 30 min. Although it is well accepted that intense contraction-induced muscle fatigue is intimately related to disturbances in the cation regulation, the alterations in any variables examined are not parallel with mechanical changes as assessed by force output. These results suggest that, at least under the conditions employed in this study, *in vivo* disruptions in the cation regulation mediated by vigorous contractile activity would be attributable mainly to events other than structural alterations to the respective proteins.

Grants

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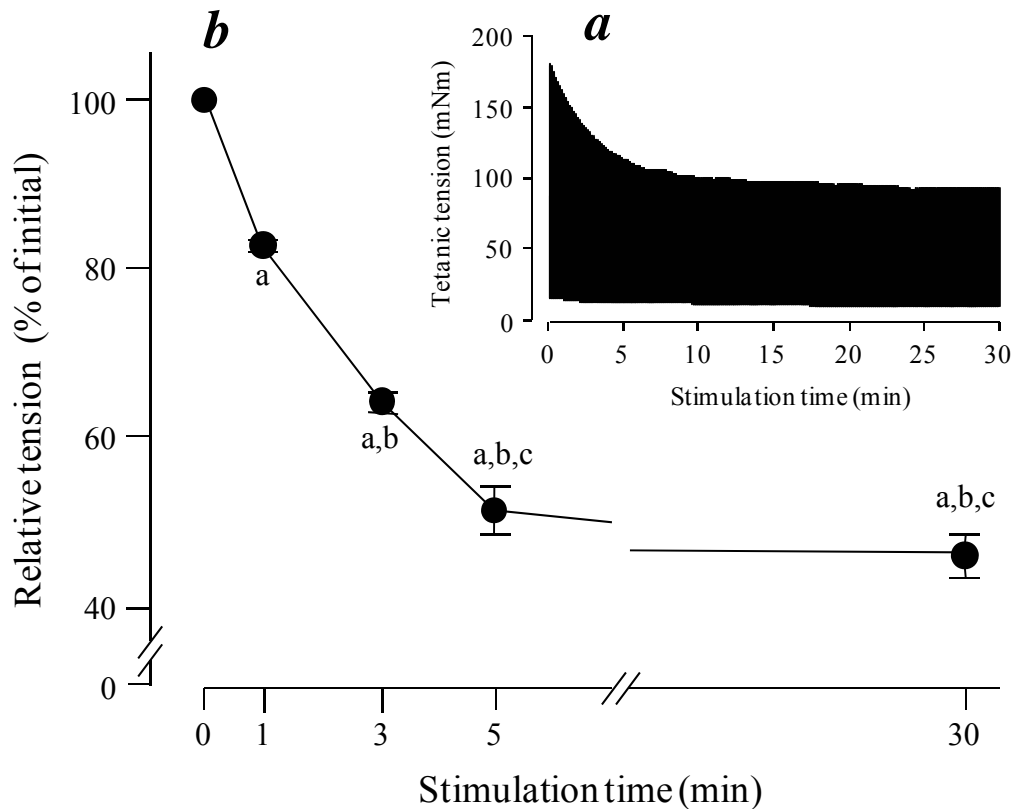
Fig. 1. **A typical example of force output (*a*) and time-course changes in tetanic tension (*b*) in 30-min stimulated gastrocnemius muscles.** The intact gastrocnemius muscle from the one leg of each rat was fatigued by intermittent tetani evoked by electrical stimulation (100-msec trains at 75 Hz every 2 sec,) via the sciatic nerve. Values are means ± SE (n = 8), as expressed as percentages of the initial values. [a]$P < 0.05$, vs 0 min (initial). [b]$P < 0.05$, vs 1 min. [c]$P < 0.05$, vs 3 min.
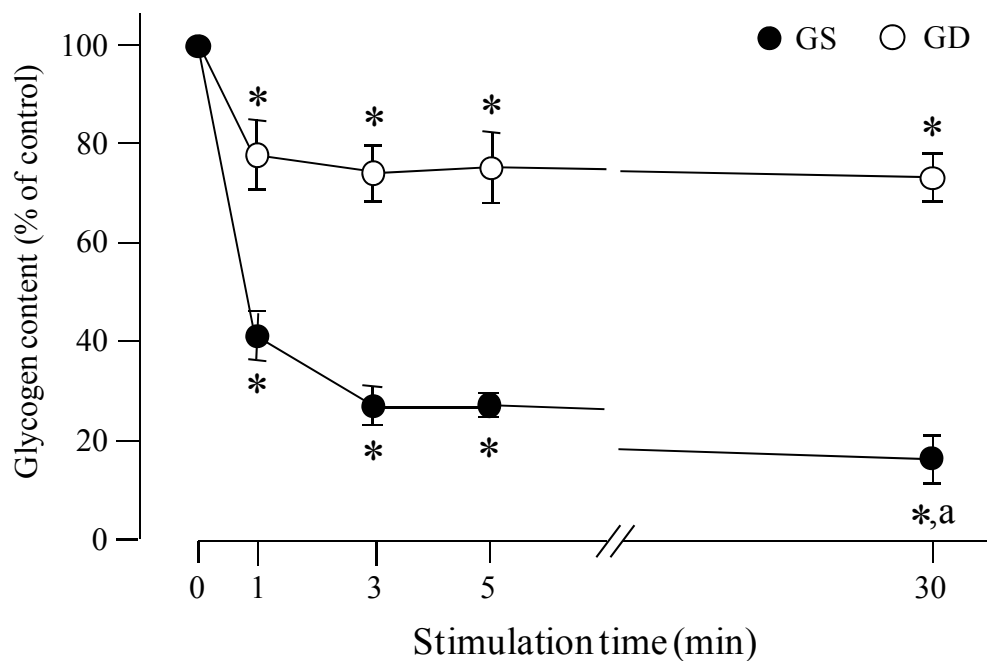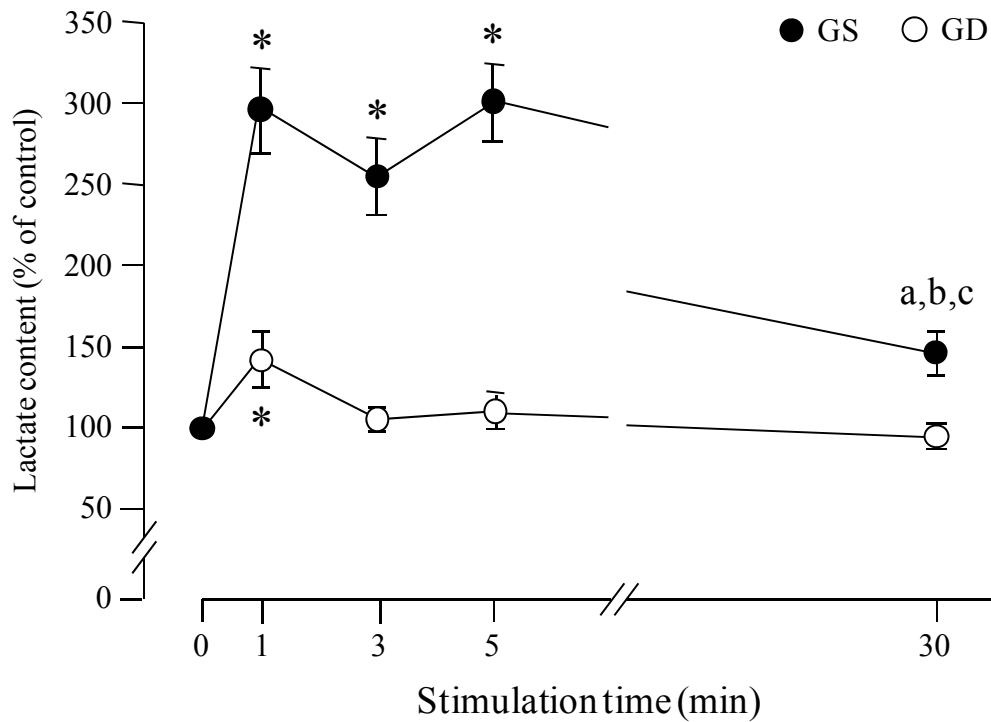
Fig. 2. Changes in glycogen content in superficial (GS) and deep (GD) regions of stimulated gastrocnemius muscles. For the stimulation protocol, see legend to Fig. 1. Values are means ± SE (n = 8 per each time point), as expressed as percentages of the values in unstimulated contralateral (control) muscles. For control GS from 1-, 3-, 5- and 30-min stimulated rats, the absolute values (means ± SE) were 119.3 ± 3.0, 118.5 ± 4.3, 118.2 ± 5.3 and 119.1 ± 3.2 μmol glycosyl units g^{-1} dry wt, respectively, and for control GD, they were 113.0 ± 6.8, 117.3 ± 4.2, 112.8 ± 5.9 and 113.1 ± 3.3 μmol glycosyl units g^{-1} dry wt, respectively. *$P < 0.05$, vs control. [a]$P < 0.05$, vs 1 min.

Fig. 3. Changes in lactate content in superficial (GS) and deep (GD) regions of stimulated gastrocnemius muscles. For the stimulation protocol, see legend to Fig. 1. Values are means ± SE (n = 8 per each time point), as expressed as percentages of the values in unstimulated contralateral (control) muscles. For control GS from 1-, 3-, 5- and 30-min stimulated rats, the absolute values (means ± SE) were 4.6 ± 0.2, 4.9 ± 0.5, 3.9 ± 0.2 and 3.9 ± 0.2 μmol g^{-1} dry wt, respectively, and for control GD, they were 4.2 ± 0.3, 4.3 ± 0.3, 3.9 ± 0.2 and 3.9 ± 0.2 μmol g^{-1} dry wt, respectively. *$P < 0.05$, vs control. [a]$P < 0.05$, vs 1 min. [b]$P < 0.05$, vs 3 min. [c]$P < 0.05$, vs 5 min.
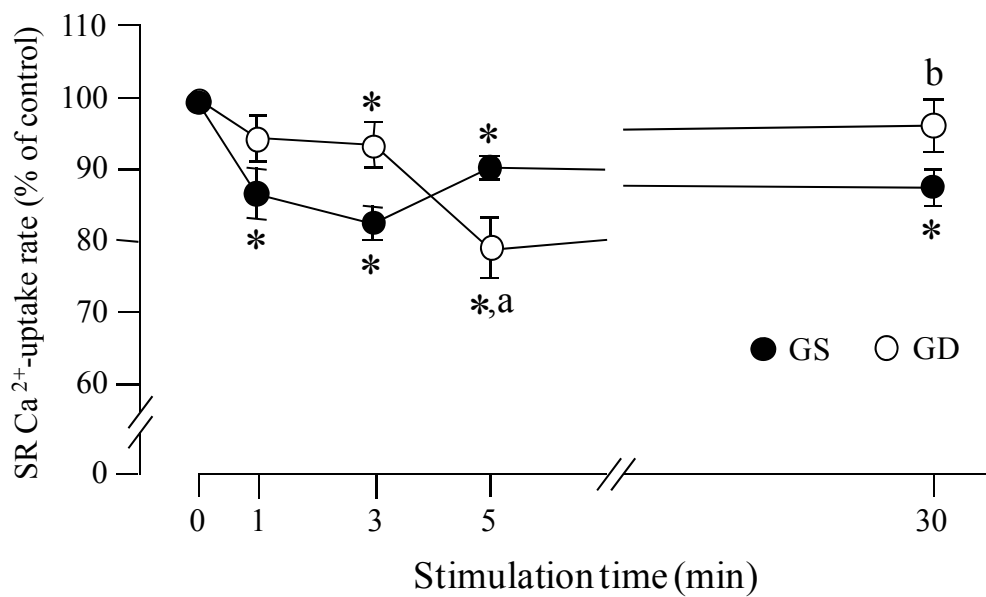
Fig. 4. Changes in sarcoplasmic reticulum (SR) Ca^{2+}-uptake rate in superficial (GS) and deep (GD) regions of stimulated gastrocnemius muscles. For the stimulation protocol, see legend to Fig. 1. Values are means ± SE (n = 8 per each time point), as expressed as percentages of the values in unstimulated contralateral (control) muscles. For control GS from 1-, 3-, 5- and 30-min stimulated rats, the absolute values (means ± SE) were 2.12 ± 0.07, 2.18 ± 0.05, 2.16 ± 0.06 and 2.19 ± 0.05 μmol min^{-1} g^{-1} wet wt, respectively, and for control GD, they were 1.76 ± 0.08, 1.76 ± 0.07, 1.82 ± 0.07 and 1.77 ± 0.06 μmol min^{-1} g^{-1} wet wt, respectively. *$P < 0.05$, vs control. [a]$P < 0.05$, vs 1 min. [b]$P < 0.05$, vs 5 min.
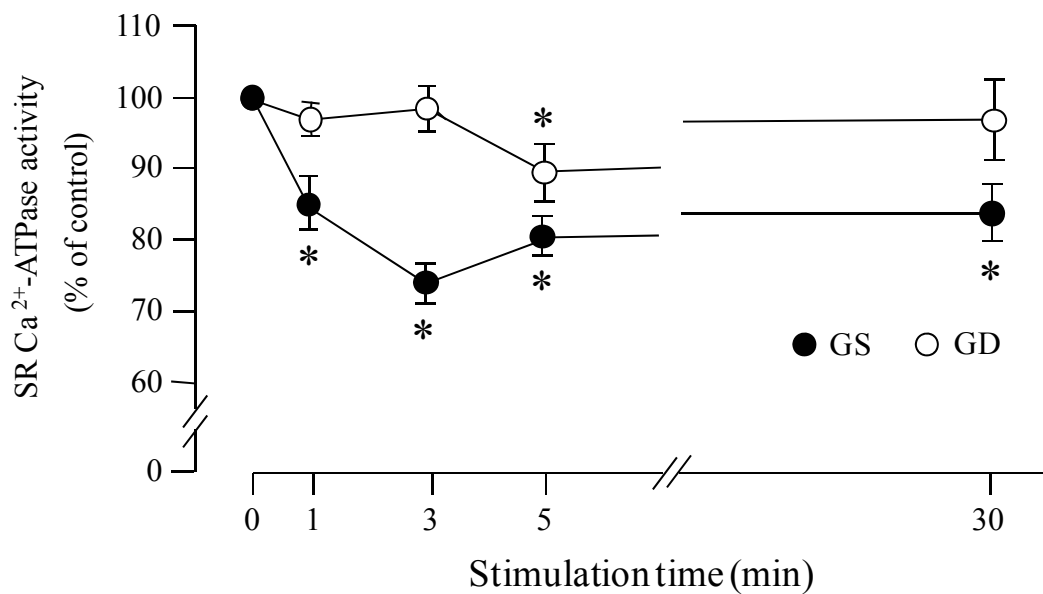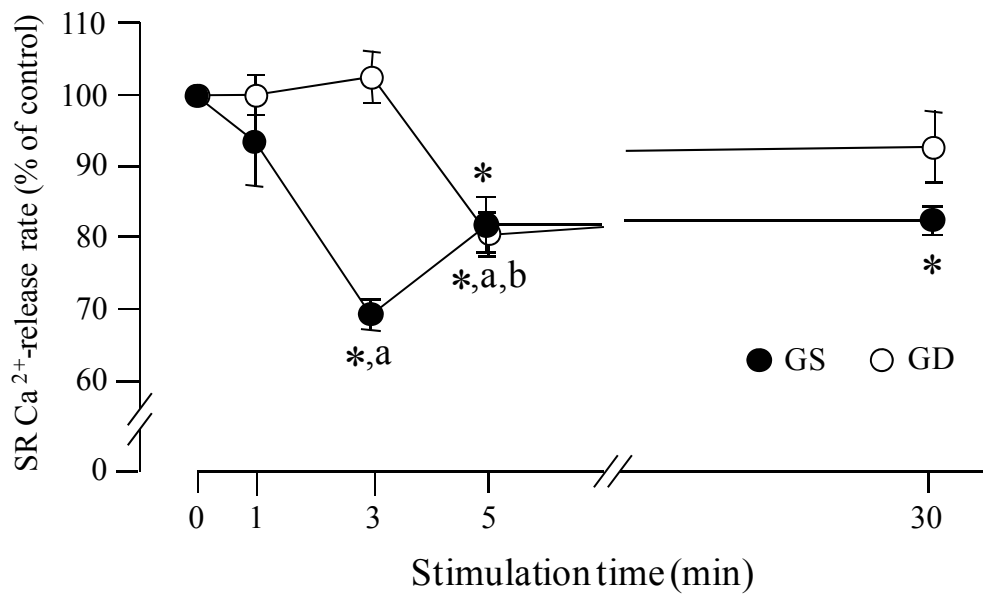
Fig. 5. Changes in sarcoplasmic reticulum (SR) Ca^{2+}-ATPase activity in superficial (GS) and deep (GD) regions of stimulated gastrocnemius muscles. For the stimulation protocol, see legend to Fig. 1. Values are means ± SE (n = 8 per each time point), as expressed as percentages of the values in unstimulated contralateral (control) muscles. For control GS from 1-, 3-, 5- and 30-min stimulated rats, the absolute values (means ± SE) were 80.4 ± 5.8, 82.3 ± 5.5, 80.8 ± 6.6 and 80.6 ± 4.9 μmol min^{-1} g^{-1} wet wt, respectively, and for control GD, they were 65.0 ± 4.0, 64.3 ± 2.9, 67.8 ± 3.3 and 66.4 ± 3.2 μmol min^{-1} g^{-1} wet wt, respectively. *P < 0.05, vs control.

Fig. 6. **Changes in sarcoplasmic reticulum (SR) Ca^{2+}-release rate in superficial (GS) and deep (GD) regions of stimulated gastrocnemius muscles.** For the stimulation protocol, see legend to Fig. 1. Values are means ± SE (n = 8 per each time point), as expressed as percentages of the values in unstimulated contralateral (control) muscles. For control GS from 1-, 3-, 5- and 30-min stimulated rats, the absolute values (means ± SE) were 2.20 ± 0.10, 2.18 ± 0.05, 2.16 ± 0.06 and 2.23 ± 0.07 μmol min^{-1} g^{-1} wet wt, respectively, and for control GD, they were 1.85 ± 0.08, 1.87 ± 0.11, 1.93 ± 0.09 and 1.94 ± 0.10 μmol min^{-1} g^{-1} wet wt, respectively. *$P < 0.05$, vs control. $^{a}P < 0.05$, vs 1 min. $^{b}P < 0.05$, vs 3 min.
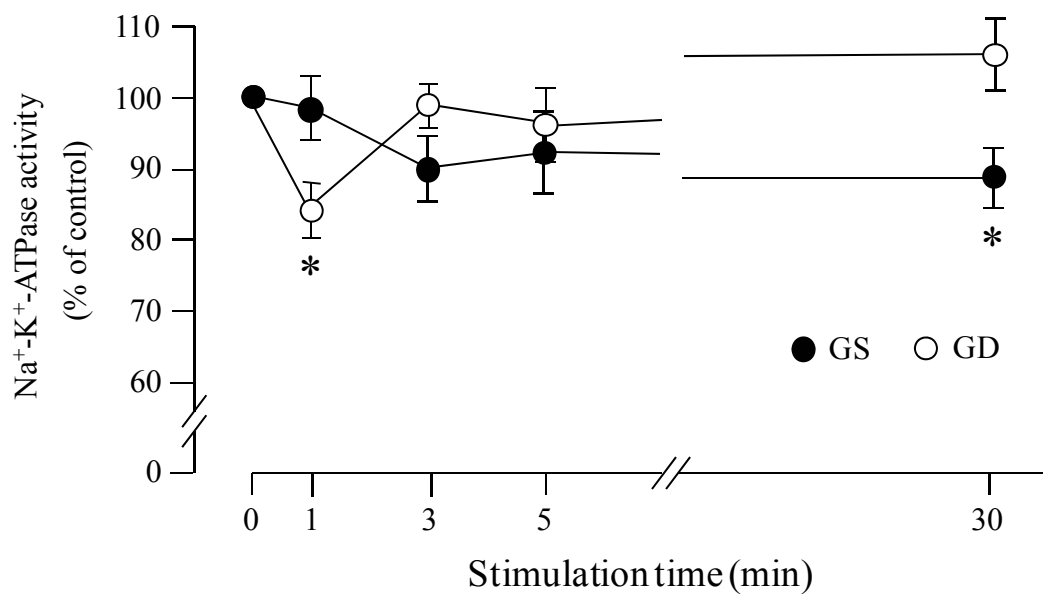
Fig. 7. Changes in Na$^+$-K$^+$-ATPase activity in superficial (GS) and deep (GD) regions of stimulated gastrocnemius muscles. For the stimulation protocol, see legend to Fig. 1. Values are means ± SE (n = 8 per each time point), as expressed as percentages of the values in unstimulated contralateral (control) muscles. For control GS from 1-, 3-, 5- and 30-min stimulated rats, the absolute values (means ± SE) were 405.7 ± 23.9, 388.8 ± 21.4, 399.5 ± 35.8 and 395.5 ± 23.5 nmol min^{-1} g^{-1} wet wt, respectively, and for control GD, they were 461.8 ± 22.5, 458.0 ± 25.9, 480.8 ± 26.5 and 455.3 ± 21.4 nmol min^{-1} g^{-1} wet wt, respectively. *$P < 0.05$, vs control.
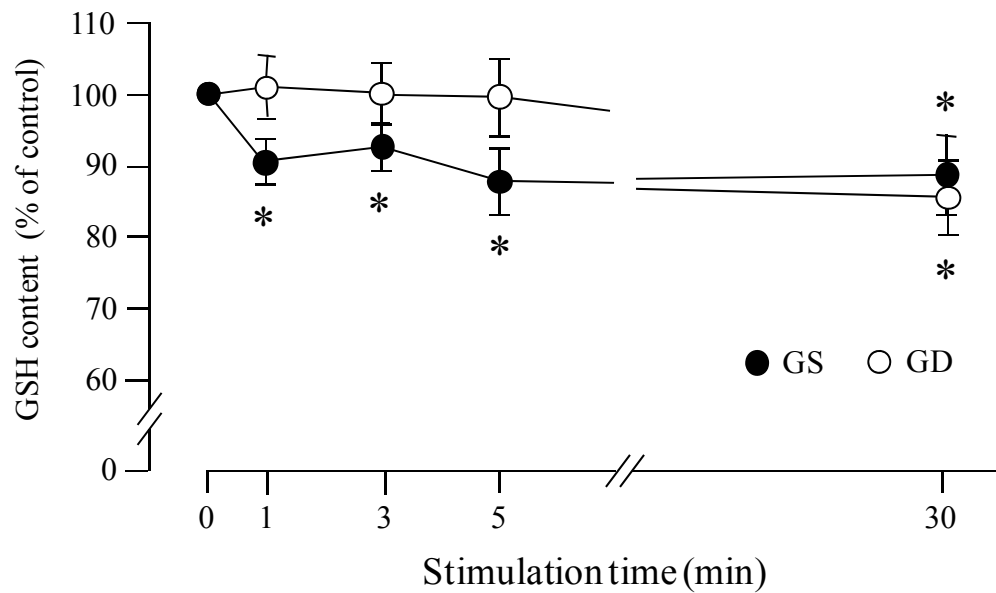
Fig. 8. Changes in reduced glutathione (GSH) content in superficial (GS) and deep (GD) regions of stimulated gastrocnemius muscles. For the stimulation protocol, see legend to Fig. 1. Values are means ± SE (n = 8 per each time point), as expressed as percentages of the values in unstimulated contralateral (control) muscles. For control GS from 1-, 3-, 5- and 30-min stimulated rats, the absolute values (means ± SE) were 1.19 ± 0.08, 1.26 ± 0.10, 1.25 ± 0.04 and 1.26 ± 0.08 μmol g^{-1} wet wt, respectively, and for control GD, they were 1.31 ± 0.10, 1.30 ± 0.09, 1.40 ± 0.11 and 1.32 ± 0.11 μmol g^{-1} wet wt, respectively. *$P < 0.05$, vs control.