ORIGINAL ARTICLE

Effects of reduced muscle glycogen on excitation–contraction coupling in rat fast-twitch muscle: a glycogen removal study

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Abstract

The aim of this study was to investigate the effects of an enzymatic removal of glycogen on excitation–contraction coupling in mechanically skinned fibres of rat fast-twitch muscles, with a focus on the changes in the function of Na^+–K^+-pump and ryanodine receptor (RyR). Glycogen present in the skinned fibres and binding to microsomes was removed using glucoamylase (GA). Exposure of whole muscle to 20 U mL^{-1} GA for 6 min resulted in a 72% decrease in the glycogen content. Six minutes of GA treatment led to an 18 and a 22% reduction in depolarization- and action potential-induced forces in the skinned fibres, respectively. There was a minor but statistically significant increase in the repriming period, most likely because of an impairment of the Na^+–K^+-pump function. GA treatment exerted no effect on the maximum Ca^{2+} release rate from the RyR in the microsomes and the myofibrillar Ca^{2+} sensitivity in the skinned fibres. These results indicate that reduced glycogen per se can decrease muscle performance due to the impairment of SR Ca^{2+} release and suggest that although Na^+–K^+-pump function is adversely affected by reduced glycogen, the extent of the impairment is not sufficient to reduce Ca^{2+} release from the sarcoplasmic reticulum. This study provides direct evidence that glycogen above a certain amount is required for the preservation of the functional events preceding Ca^{2+} release from the sarcoplasmic reticulum.

Keywords Muscle fatigue · Ryanodine receptor · Na^+–K^+-pump · Ca^{2+} release · Dihydropyridine receptor

Introduction

Repeated contraction of skeletal muscle leads to a functional impairment known as muscle fatigue. The cause of fatigue is multi-factorial and can involve various metabolic and ionic factors (Kent-Braun et al. 2012; Cheng et al. 2018). Among these factors, none have been more evident or universally agreed upon than the need for a reserve of glycogen in the working muscle cell (Green 1991). After a series of studies conducted by Hultman and colleagues (Bergström et al. 1967; Hermansen et al. 1967), a strong relationship between muscle glycogen depletion and fatigue during moderately intense exercise has been confirmed on multiple occasions (Karlsson and Saltin 1971; Pernow and Saltin 1971; Galbo et al. 1979; Duhamel et al. 2006a, b).

Despite the well-accepted role of glycogen, the link between glycogen depletion and reduced force production is not fully understood. Chin and Allen (1997) were the first to observe that glycogen-depleted muscle fibres exhibit reduced tetanic myoplasmic $[Ca^{2+}]$ transients in mouse intact single fibres, indicating that glycogen depletion results in decreased Ca^{2+} release of the sarcoplasmic reticulum (SR) contributing to reduced force production. Although subsequent studies supported these findings (Nielsen et al. 2014; Cheng et al. 2017), the precise mechanism(s) by which glycogen depletion leads to a decreased SR Ca^{2+} release is not yet known. Skinned fibre experiments have shown that glycogen depletion causes a loss of the fibre ability to respond to depolarization of the transverse tubular (T)-system, even in the presence of high levels of ATP (Stephenson et al. 1999; Barnes et al. 2001), suggesting that glycogen depletion-related decease in the SR Ca^{2+} release is not due to the function of glycogen as an energy store. On the other hand, it has been proposed that glycogen is essential for supplying ATP to triadic junction, which is a 12 nm wide diffusionally restricted

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microcompartment formed by the T-system and the terminal cisternae of the SR (Ørtenblad and Nielsen 2015).

The major proteins responsible for SR Ca^{2+} release are Na^{+}–K^{+}-pump, dihydropyridine receptor (DHPR), and ryanodine receptor (RyR) (Wada et al. 2013; Cheng et al. 2018). Considering that Na^{+}–K^{+}-pumps in the T-system preferentially use glycolytically-derived ATP (Dutka and Lamb 2007), it is reasonable to assume that glycogen depletion leads to impaired function of the Na^{+}–K^{+}-pumps. To date, no studies have provided direct evidence supporting this hypothesis. With regard to the RyR, Ørtenblad et al. (2011) reported that cross-country skiers showed a decrease in glycogen level to half of the normal level after completing approximately 20 km of a trial. Reduced glycogen content can lead to reductions in the maximum Ca^{2+} release rate from the SR. In this experiment, estimation of SR Ca^{2+} release rate was performed in the presence of sufficient ATP using SR vesicles and a direct activator of the RyR. Although this implied that reduced glycogen content leads to a structural alteration of the RyR, the content of glycogen associated with SR vesicles was not evaluated in that study (Ørtenblad et al. 2011).

Furthermore, in most of the earlier studies, the comparison between glycogen content and SR Ca^{2+} release before and/or after fatiguing muscle contraction was used to derive the conclusion that glycogen depletion inhibits Ca^{2+} release from the SR (Chin and Allen 1997; Stephenson et al. 1999; Barnes et al. 2001; Ørtenblad et al. 2011; Nielsen et al. 2014; Cheng et al. 2017). In this case, the possibility that the correlation between glycogen and SR Ca^{2+} release could not be causative cannot be precluded. To gain further insights into the role of muscle glycogen, it is needed to assess the muscle force and SR Ca^{2+} release in quiescent muscle fibres in which glycogen is removed.

These previous findings have inspired us to investigate the effects of an enzymatic removal of glycogen on excitation–contraction (E–C) coupling in the skinned fibres, with a focus on the changes in the function of Na^{+}–K^{+}-pump and RyR. In this study, the hypotheses were tested that glycogen removal causes a reduction in the action potential (AP)- and depolarization (depol)-induced force productions in quiescent skinned fibres and that impaired Na^{+}–K^{+}-pump function is responsible, at least in part, for the reduction in AP-induced force.

Materials and methods

Ethical approval and animal care

All procedures were approved by the Animal Care Committee of Hiroshima University. Forty-three Wistar rats, 9–10 weeks old, were used in this study (Charles River Laboratories, Yokohama, Japan). The animals were housed in a thermally controlled room at 20–24 °C with a 12-h light/dark cycle and provided with standard chow (CE-2, CLEA Japan, Tokyo, Japan) and water ad libitum. Before muscle sampling, the rats were sacrificed with an overdose of pentobarbital sodium (200 mg/kg), followed by cervical dislocation. All experiments (muscle sampling) were initiated at approximately the same time to avoid large diurnal variations in muscle glycogen content.

Effects of glucoamylase on glycogen content

In this study, muscle glycogen was removed using glucoamylase (GA). The time course of GA-induced changes in the glycogen content was examined using whole muscle. This is because of the technical limitations in measuring glycogen content in a single muscle fibre. The extensor digitorum longus (EDL) muscles were excised from both hindlimbs. The muscles obtained from both limbs were combined, minced on ice, and divided into four parts. Of these parts, one part was washed with K-hexamethylethylene-diaminetetraacetic acid (HDTA) solution (Table 1). The remaining three parts were exposed to K-HDTA (K-HDTA$_{GA}$) solution containing 20.0 U mL^{-1} GA for 2, 4, or 6 min at 25 °C, and then washed with K-HDTA solution for 10 s. Immediately after the wash, each part was exposed to 2.0 N perchloric acid at − 10 °C for 20 min. It is plausible that this exposure completely inactivated GA activity, because 2 N perchloric acid is a very strong acid (pH < 1). The samples were centrifuged at 2000×g for 10 min at 4 °C and the supernatant was removed. To hydrolyze glycogen, the pellets were boiled in 2.0 N HCl for 2 h at 100 °C and then neutralized with 2 N NaOH. The glycogen content was determined fluorometrically (excitation fluorescence, 340 nm; emission fluorescence, 460 nm) using the production of NADPH (Lowry and Passonneau 1972). The glycogen content was expressed in µmol glycosyl units per gram wet wt.

Effects of glycogen removal on the maximum SR Ca^{2+} release in microsome

The gastrocnemius and vastus lateralis muscles were excised from both limbs and all the muscle tissues were combined for the microsomal preparation. This is because a large volume of tissue is required for the microsomal preparation. The microsomes were prepared according to Mishima et al. (2006) and were used for measurement of the maximum SR Ca^{2+} release rate and the glycogen content. The muscles were homogenized in 5 vol (vol mass^{-1}) of ice-cold homogenizing buffer consisting of 20 mM N-2-hydroxyethylpiperazine-N''-2-ethanesulfonic (HEPES; pH 7.0), 0.2% (mass vol^{-1}) sodium azide, 0.2 mM phenylmethanesulphonyl fluoride, and 1 mM EDTA. The resultant

Table 1 Composition of solutions used to determine contractile properties in skinned fibers

Solutions	K$^+$ (mM)	Na$^+$ (mM)	Mg$^{2+}_{free}$ (mM)	EGTA$_{total}$ (mM)	HDTA$_{total}$ (mM)	Caffeine (mM)	GA (U mL^{-1})	Ca$^{2+}_{free}$(M)
K-HDTA	126	36	1.0	0.05	50.0	–	–	$10^{-6.9}$
Na-HDTA	–	162	1.0	0.05	50.0	–	–	$10^{-6.9}$
K-HDTA$_{GA}$	126	36	1.0	0.05	50.0	–	20	$10^{-6.9}$
K-HDTA$_{CAFF}$	126	36	1.0	0.45	49.8	2–10	–	$10^{-7.1}$
Low [Mg^{2+}]	126	36	0.05	0.05	50.0	–	–	$10^{-6.9}$
Maximum Ca^{2+}	126	36	1.0	50.0	–	–	–	$\sim 10^{-4.7}$
Relaxation	126	36	1.0	50.0	–	–	–	$< 10^{-9}$
Load	126	36	1.0	49.0	–	–	–	$10^{-6.7}$
Full release	126	36	0.015	0.5	49.5	30	–	$10^{-8.0}$

All solutions contained (in mM), 90 N-2-hydroxyethylpiperazine-N″-2-ethanesulfonic, 8 ATP$_{total}$ and 10 creatine phosphate and had a pH of 7.09–7.11 at 25 °C. The maximum Ca^{2+} solution additionally contained 49.5 mM Ca$^{2+}_{total}$

CAFF caffeine, *GA* glucoamylase, *HDTA* hexamethylethylene-diaminetetraacetic acid

homogenate was centrifuged at 8000×*g* for 15 min at 4 °C. The supernatant was collected and divided into a non-treated and a GA-treated part and KCl (600 mM) was added to solubilize remaining actomyosin. To remove glycogen, the buffer for the GA-treated part was supplemented with 20 U mL^{-1} GA and the samples were gently stirred on ice for 1 h. This treatment for glycogen removal [but not 6-min GA treatment at 25 °C (see below)] was necessary because of the following: (1) both SR Ca^{2+} release rate and the glycogen content associated with the SR membrane must be evaluated in the same individual microsomes and (2) our preliminary experiment showed that when the homogenates were placed at 25 °C for several min in the process of the microsomal preparation, it was difficult to obtain highly reproducible results with regard to SR Ca^{2+} release rate. After the GA treatment, the samples were centrifuged at 50,000×*g* for 2 h at 4 °C and the resultant pellets were resuspended in a storage buffer. The storage buffer for SR Ca^{2+} release estimation was similar to the homogenizing buffer, but contained 300 mM sucrose and 150 mM KCl, whereas the buffer for glycogen estimation did not contain any sucrose. The protein concentration was determined by the Bradford assay (1976) using bovine serum albumin as the standard.

The SR Ca^{2+} release rate was estimated using a Ca^{2+} fluorescent dye indo-1, as described previously (Kanzaki et al. 2016). Aliquots of the sample were incubated for 3 min at 37 °C in the assay buffer composed of 100 mM KCl, 20 mM HEPES, 10 mM NaN$_3$, 6.8 mM potassium oxalate, 0.5 mM MgCl$_2$, and 1 μM indo-1 (pH 7.0). SR Ca^{2+} uptake was initiated by adding 1 mM Mg-ATP and allowed to continue until little or no change in the [Ca^{2+}] was observed. Ca^{2+} release was initiated by adding 10 mM (final concentration) 4-chloro-*m*-cresol (CMC). CMC was used because of the following: (1) CMC is a potent releasing agent specific for the RyR and fully inhibits SR

Ca^{2+}-ATPase activity (Tupling and Green, 2002) and (2) previous studies regarding the effects of glycogen on Ca^{2+} release of microsome also utilized CMC (Ørtenblad et al. 2011). The use of CMC makes it possible to evaluate the net Ca^{2+} release function of the RyR and to compare the results of this study with the previous findings. The [Ca^{2+}] was monitored using a fluorometer (CAF-110; Nihon-Bunko, Kyoto, Japan). The excitation light was emitted by a high-pressure Xe lamp equipped with a monochromator and applying a filter at 349 nm. The emission fluorescence was determined by a pair of photomultipliers by applying 410 nm and 500 nm filters. The [Ca^{2+}] was computed using a ratiometric method as described by Grynkiewicz et al. (1985).

To hydrolyze microsomal glycogen, the sample was incubated with gentle shaking in the solution composed of 174 mM acetic acid (pH 4.8) and 20.0 U mL^{-1} GA for 2 h at 40 °C (Mishima et al. 2006). The glycogen content was determined in a manner similar as described above.

Skinned fibre preparation

Mechanically skinned fibres were prepared according to Lamb and Stephenson (1990, 1994). The EDL muscle was pinned out at resting length under paraffin oil and kept cool on an ice pack. The single muscle fibres were dissected under a stereo-microscope and mechanically skinned by rolling back the sarcolemma with fine forceps. One or two skinned fibres were obtained from a single whole muscle. A segment of the skinned fibre was then connected to a force transducer (Muscle tester, SI, Germany), stretched to 1.2 times resting length and transferred to a bath containing 2 ml of the K-HDTA solution. All skinned fibre experiments were carried out at 25 °C.

Basic solutions

K-HDTA solution used to polarize the T-system consisted of 37 mM Na^+, 126 mM K^+, 50 mM HDTA, 90 mM HEPES, 0.05 mM EGTA, 8 mM ATP_{total}, and 10 mM creatine phosphate, and had a pH of 7.09–7.11 at 25 °C (Table 1). The free Mg^{2+} concentration was set at 1.0 mM except the low $[Mg^{2+}]$ and the release solutions. The Na-HDTA solution was similar to the K-HDTA solution, except that K^+ was replaced by Na^+. The maximum Ca^{2+} solution was also similar to that in the K-HDTA solution, except that HDTA was replaced with 49.5 mM Ca-EGTA and 0.5 mM free EGTA. The relaxation solution contained 50 mM free EGTA.

Depolarization-induced force

The skinned fibres were depolarized by replacing K-HDTA with Na-HDTA solution for approximately 5–6 s, which typically resulted in Ca^{2+} release and production of substantial force. This procedure was repeated until the peak force response reached a stable state. Then, to explore the effect of glycogen removal on depol-induced force, the skinned fibre was treated as follows:

Step 1: The depol-induced force was measured
Step 2: The skinned fibre was exposed to K-HDTA$_{GA}$ solution for 2 min
Step 3: The fibre was washed with K-HDTA solution

The steps 1–3 were repeated thrice, and finally, the depol-induced force was measured again, i.e., the effect of GA exposure for up to 6 min was examined. At the end of experiment, the skinned fibre was exposed to a low $[Mg^{2+}]$ solution to verify that the SR was adequately loaded with Ca^{2+}, and then maximum Ca^{2+}-activated force was determined by exposing the fibre to the maximum activating Ca^{2+} solution. For variables that were changed by the GA-treatment, we performed control experiments, in which the fibres were exposed to K-HDTA solution for 6 min.

Caffeine threshold

To determine the effect of glycogen removal on a caffeine (CAFF) threshold, the skinned fibres were treated as follows:

Step 1: The SR of the skinned fibre was depleted of Ca^{2+} with full release solution
Step 2: After washing the fibre with K-HDTA solution, the SR was loaded to the maximum capacity by exposing the fibre to load solution for 3 min

Step 3: After washing the fibre with K-HDTA solution, the fibre was exposed to K-HDTA$_{CAFF}$ solution with a given CAFF concentration (2, 4, 6, 8, or 10 mM)
Step 4: The steps 1–3 were repeated. In step 3, the fibre were exposed to increasing CAFF concentrations every time the cycle progressed
Step. 5: After washing the fibre with K-HDTA solution, the fibre was exposed to K-HDTA$_{GA}$ solution for 6 min
Step 6: Steps 1–4 were repeated
Step 7: The fibre was exposed to maximum Ca^{2+} solution

In this study, the CAFF threshold was defined as a CAFF concentration that produces a force response corresponding to 10% of force induced by the full release solution.

Myofibrillar Ca^{2+} sensitivity

Force-$[Ca^{2+}]$ curves were established with solutions containing various $[Ca^{2+}]$ ($10^{-6.8}$, $10^{-6.7}$, $10^{-6.4}$, $10^{-6.6}$, $10^{-6.0}$, $10^{-5.8}$, $10^{-5.6}$, $10^{-5.4}$ and $\sim 10^{-4.7}$) produced by mixing the maximum Ca^{2+} solution and relaxation solution in appropriate proportions. The free $[Ca^{2+}]$ in the mixed solution was calculated according to the affinity constant given by Moisescu and Thieleczek (1978). The contractile apparatus was directly activated by exposing the skinned fibre to a series of solutions containing rising $[Ca^{2+}]$. This procedure was performed before and after 6-min GA treatment. The force response elicited by different $[Ca^{2+}]$ was expressed as a percentage of the maximum Ca^{2+}-activated force. $[Ca^{2+}]_{50}$ was defined as the $[Ca^{2+}]$ required to produce half-maximal force.

Action potential-induced force

The skinned fibre was placed in a small chamber containing ~ 600 μL of K-HDTA solution and centered between two platinum wire electrodes. The fibre length was adjusted to optimize the force developed at 50 Hz. The fibre was stimulated with 1 ms pulses at 80 V cm^{-1} with a single pulse (1 Hz), followed by 5 pulses at 5 Hz, 10 pulses at 10 Hz, and 20 pulses at 50 Hz. This procedure was performed before and after 6-min GA treatment.

In a separate experiment, to evaluate the ability of the T-system to generate a second closely spaced AP, pairs of identical pulse (duration 1 ms; 80 V cm^{-1}) were applied to the skinned fibre, with different time gaps (2–20 ms) between the end of the first pulse and the start of the second pulse (inter-pulse spacing) (Dutka and Lamb 2007). As described by Dutka and Lamb (2007), if the second pulse of a pair can generate the second AP (that is., after the first AP, the plasma membrane can be returned from

the depol to the polarization phase within the inter-pulse spacing), the force induced by a pair of pulses exceeds that induced by a single pulse, whereas if not, the force remains unchanged. In the study by Dutka and Lamb (2007) in which the T-system was partially depolarized by 66 mM K^+, the repriming period (RP) was defined as the time taken for $>50\%$ incremental increase in the force induced by a pair of pulse compared to the force elicited by a single pulse. On the other hand, we defined the RP as $>40\%$ increase in this study, because our preliminary experiments showed that approximately 1/4 of the skinned fibres did not exceed 50% under well-polarized conditions. The major determinant of the RP is the resting membrane potential of the sealed t-system, which critically depends on the Na^+–K^+-pump activity.

Statistical analyses

The data are presented as mean $\pm$ SD. Student's paired t test, one-way repeated measures ANOVA, or two-way repeated measures ANOVA were used to compare the data (Sigmaplot version 14, Systat Software Inc., San Jose, CA, USA). When significant differences were detected by ANOVA, Holm-Sidak post hoc analysis was performed. The acceptable level of significance was set at $P < 0.05$.

Results

Time course of GA-induced changes in glycogen content in the whole muscle and in depolarization-induced force response in the skinned fibre.

A rapid loss of the glycogen content during the first 2 min of GA treatment was observed (Fig. 1). At 2 min, the glycogen content declined to 48% of that at 0 min. Thereafter, the glycogen content further decreased progressively and was 28% at 6 min. As described above (see methods), immediately after GA treatment for a given period, GA activity was inactivated. It seems likely, therefore, that time-course changes in the glycogen content in the skinned fibres resemble those observed in the whole muscles. GA treatment resulted in a progressive reduction in the depol-induced forces (Fig. 2). At 2, 4, and 6 min, the force amounted to 95, 90, and 82%, respectively, of that at 0 min. There was a significant difference between the forces at 0 and 6 min. Considering that there were no changes in the myofibrillar Ca^{2+} sensitivity and the maximum Ca^{2+}-activated force (Fig. 3), it seems quite plausible that the decrease in depol-induced force was caused by a reduced SR Ca^{2+}-release. Low Mg^{2+}-induced force always exceeded 80% of the maximum Ca^{2+}-activated force (Fig. 2a), indicating that the GA-elicited decrease
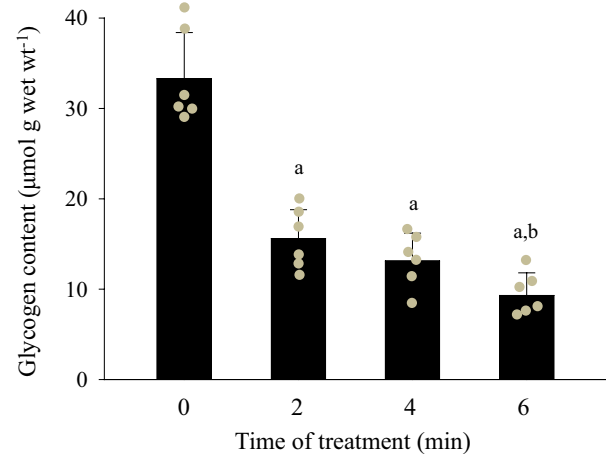

Fig. 1 Time course of glucoamylase-elicited changes in glycogen content in whole muscle. The EDL muscle was divided into four parts and each part was exposed to 20 U mL^{-1} GA for 0, 2, 4, or 6 min. The glycogen content is expressed in μmol glycosyl units per gram wet wt. Values are means + SD (n = 6 for each time point). Individual data are displayed as grey circles. [a]$P < 0.05$, versus 0 min; [b]$P < 0.05$, versus 2 min (one-way repeated measures ANOVA). *n* number of muscles

in force was not due to a reduction in the Ca^{2+} content of the SR lumen. On the basis of these results, the effects of 6-min GA treatment were only examined in the subsequent experiments. An exposure of the skinned fibre to K-HDTA solution for 6 min did not affect depol-induced forces (Fig. 2c).

Action potential-induced force response and repriming period in the skinned fibre

As with depol-induced force, 6-min GA treatment also resulted in the reduction of AP-induced force. More pronounced reductions were observed for the forces at lower stimulation frequencies; the forces at 1 and 50 Hz decreased to 46 and 78%, respectively, of that at pre-GA treatment (Fig. 4b). As a result, the ratio of force at 1 Hz to that at 50 Hz significantly declined to 51% of that at pre-GA treatment (Fig. 4d). This result indicates that decreased glycogen can lead to prolonged low-frequency force depression (Watanabe et al. 2015; Watanabe and Wada 2016) and supports the above-mentioned assumption that decreased glycogen may result in reduced SR Ca^{2+}-release (Allen et al. 2008; Cheng et al. 2017). As shown in Fig. 5c, 6-min GA treatment resulted in a minor but statistically significant increase (4.5 ms at pre-GA treatment vs. 5.3 ms at post-GA treatment) in the RP. An exposure of the skinned fibre to K-HDTA solution for
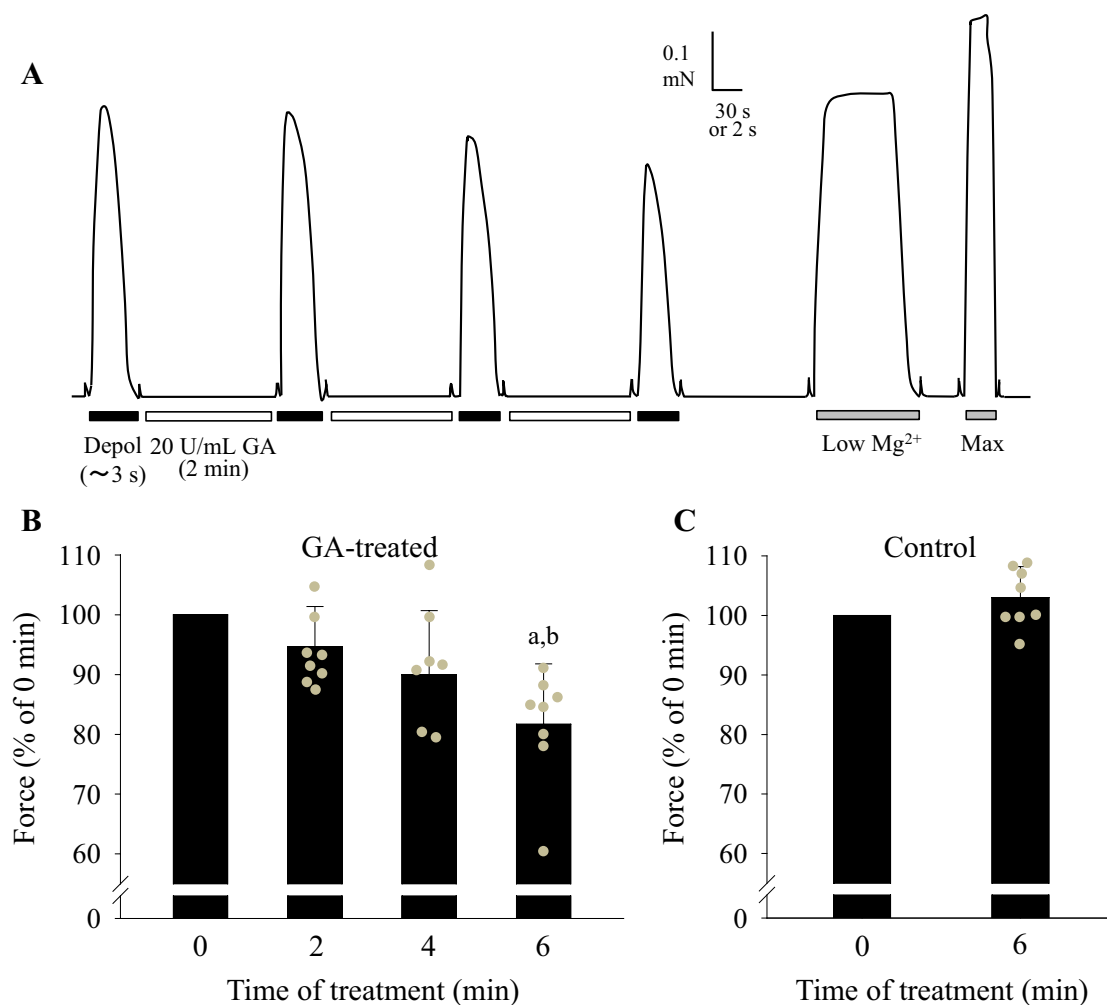
Fig. 2 Time course of GA-elicited changes in depolarization (depol)-induced force response in skinned fibre. **a** typical example of depol-induced force in GA-treated fibres. The EDL skinned fibre was depolarized by substituting K^+ in the solution with Na^+. Exposure to K-HDTA solution with 20 U mL^{-1} GA elicited a progressive depression in force. Time scale: 2 s during depolarization and low $[Mg^{2+}]$ and 30 s elsewhere. **b** and **c** mean + SD (n = 8 for each case) of depol-induced force in GA-treated and control fibres, respectively. The control fibers were exposed to K-HDTA solution for 6 min. The results are expressed as a percentage of the value at 0 min. Individual data are displayed as grey circles. [a]$P < 0.05$, versus 0 min; [b]$P < 0.05$, versus 2 min (one-way repeated measures ANOVA). *n* number of fibres, *Max* maximum Ca^{2+} solution

6 min did not affect the action potential-induced force responses and the RP (Figs. 4c, e, 5d).

Ca^{2+} release rate in the microsome and caffeine sensitivity in the skinned fibre

The maximal ability of the RyR to release Ca^{2+} was evaluated in most of previous studies, in which the effects of glycogen were examined using microsomes or muscle homogenates (Duhamel et al. 2006a, b; Ørtenblad et al. 2011). To compare the present results with the previous findings, we measured the maximum RyR Ca^{2+} release rate using 10 mM CMC. As shown in Fig. 6, despite a marked decrease (by 76%) of the glycogen content, GA treatment had no effect on the maximum Ca^{2+} release rate. The CAFF threshold of the SR has been considered as an indicator of open probability of the RyR (Bakker and Berg 2002; Watanabe et al. 2015). Although 6-min GA treatment tended to increase the CAFF threshold ($P = 0.102$), the difference between pre- and post-treatment did not reach statistical significance (Fig. 7).
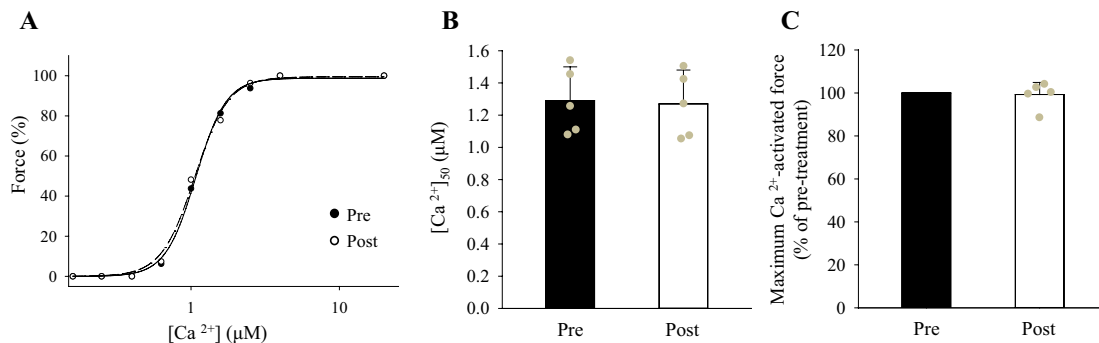
Fig. 3 Effects of glycogen depletion on myofibrillar Ca^{2+} sensitivity and maximum Ca^{2+}-activated force in skinned fibre. **a** Hill fits to force-$[Ca^{2+}]$ data before and after GA treatment. The EDL skinned fibre was exposed to a series of solutions containing ascending $[Ca^{2+}]$. This procedure was also performed after exposing the fibre to 20 U mL^{-1} GA for 6 min. The $[Ca^{2+}]$ was expressed on a 10 logarith-mic scale. **b** mean + SD (n = 5 for each case) of $[Ca^{2+}]_{50}$ for Hill fits in *A*. **c** mean + SD (n = 5 for each case) of maximum Ca^{2+}-activated force. The results are expressed as a percentage of the value at pre-GA treatment. Individual data are displayed as grey circles. *n* number of fibres, *Pre* pre-GA treatment, *Post* post-GA treatment, *$[Ca^{2+}]_{50}$* $[Ca^{2+}]$ required to produce half-maximum force

Discussion

To the best of our knowledge, this is the first study to investigate the effects of glycogen removal on E–C coupling in skinned fibres, providing direct evidence of a correlation between glycogen depletion and impaired muscle performance. Our results support the previous findings that a glycogen-related reduction in force is attributable to a reduction in SR Ca^{2+} release and indicate that a decrease in the glycogen content causes functional impairment of Na^+–K^+-pump.

The Na^+–K^+-pumps are critical in minimizing muscle Na^+ and K^+ disturbances, which are caused by repeated firings of the AP, and in preserving the membrane excitability (Green 2004; Cheng et al. 2018). A substantial amount of evidence suggests that impaired Na^+–K^+-pump function is one of the causes of muscle fatigue (Fowles et al. 2002; McKenna et al. 2006; Mishima et al. 2008). Reactive oxygen species play an important role in Na^+–K^+-pump dysfunction (Medved et al. 2004; McKenna et al. 2006). Dutka and Lamb (2007) observed in skinned fibres that even in the presence of bulk ATP and creatine phosphate, the RP was shortened by the addition of phospho(enol)pyruvate, implying that Na^+–K^+-pumps preferentially use ATP derived by glycolysis. This led to a proposal that not only increased reactive oxygen species but also glycogen depletion can jeopardise the function of the Na^+–K^+-pump, which in turn results in impaired muscle performance (Ørtenblad et al. 2013; Ørtenblad and Nielsen 2015).

The slight but significant increase (4.5 to 5.3 ms) in the RP observed in the glycogen-depleted fibres is in agreement with the finding of Dutka and Lamb (2007). Although this change reveals a loss of the Na^+–K^+-pump function, we suggest that the impaired Na^+–K^+-pump function, which is mediated via the reduced glycogen content, is unlikely to be directly related to deterioration of muscle performance during glycogen-depleting exercise. This suggestion is based on the following two reasons. First, in the fast-twitch muscles of the rats, the maximal force induced by electrical stimulation is achieved at a frequency of ~ 100 Hz. When the pulse duration is 1 ms at 100 Hz, the inter-pulse spacing is 9 ms. The increased RP (5.3 ms) is much less than the inter-pulse spacing of 9 ms. This implies that even when the fibres develop maximal force, each pulse is capable of generating AP in the glycogen-removed fibres. In human skeletal muscles, the inter-pulse spacing is even greater, as the motor unit discharge rates during voluntary contractions rarely exceed 30 Hz (Keeton and Binder-Macleod 2006). Secondly, in certain situations, repeated activation of muscles causes increased extracellular $[K^+]$ and decreased intracellular $[K^+]$ (Allen et al. 2008). Although the RP increases in such circumstances (i.e., increased extracellular $[K^+]$) (Dutka and Lamb 2007), a substantial increase in the extracellular $[K^+]$ occurs only during extremely high-intensity exercise and sustained electrical stimulation, but not during prolonged exercise (Allen et al. 2008; Cheng et al. 2018).

Our result of no change in the maximum SR Ca^{2+} release rate in microsomes is contradictory to the findings of previous studies performed in human skeletal muscles. Those studies utilized an SR vesicle and a direct activator (CMC) of the RyR for analysis and demonstrated a reduction in the maximum Ca^{2+} release rate in the muscles undergoing glycogen-depleting exercise (Duhamel et al. 2006a, b; Ørtenblad et al. 2011). Although the reason for this discrepancy is unclear, the present results strongly suggest that the maximum RyR function is not affected, at least, by a reduction
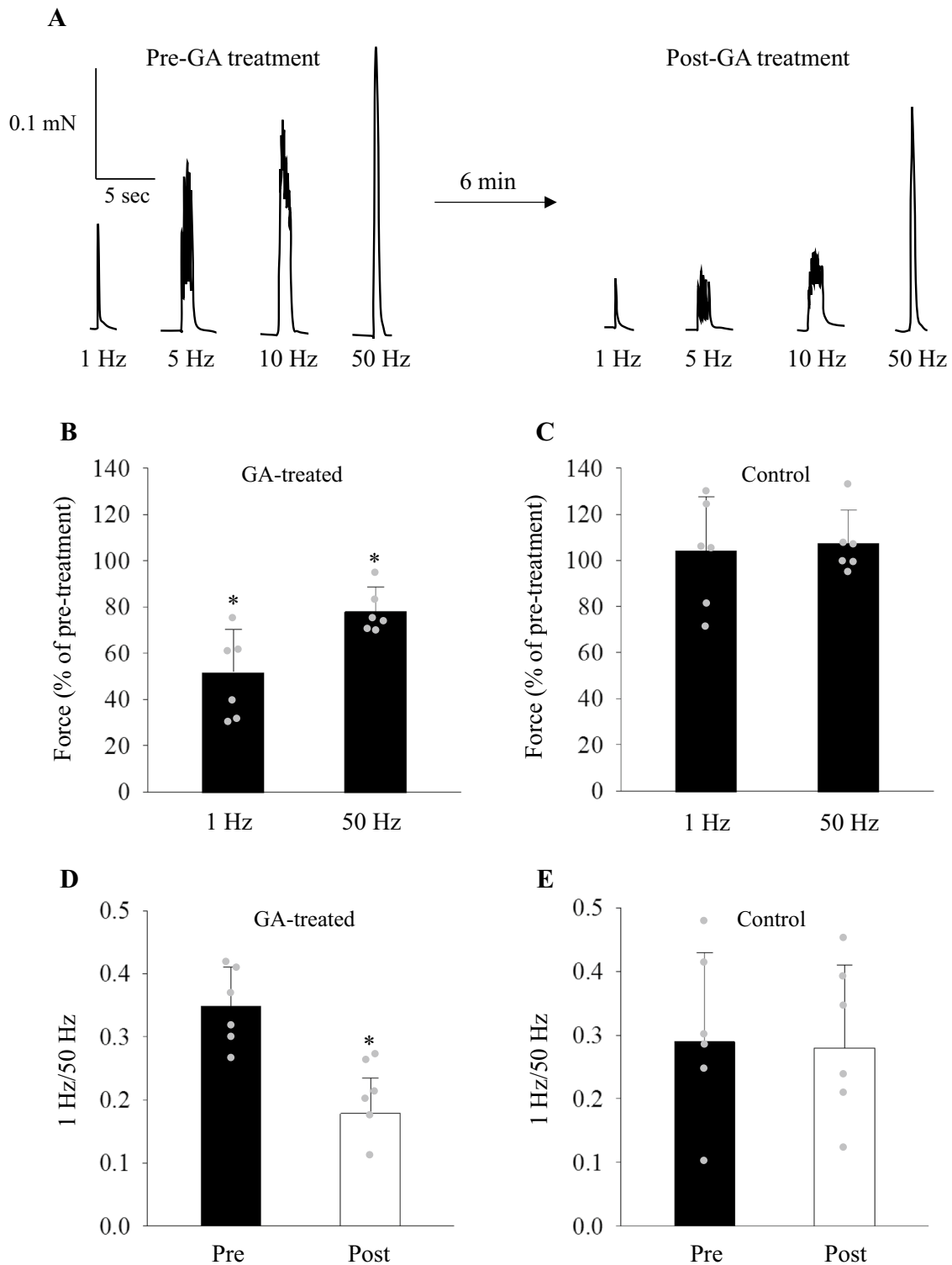
Fig. 4 Effects of glycogen depletion on action potential-induced force response in skinned fibre. **a** typical example of action potential-induced force before and after GA treatment. The EDL skinned fiber was stimulated with a single pulse (1 Hz), 5 pulses at 5 Hz, 10 pulses at 10 Hz, and 20 pulses at 50 Hz. This procedure was also performed after exposing the fiber to 20 U mL^{-1} GA for 6 min. **b** and **c** mean ± SD (n = 6 for each case) of force after treatment in GA-treated and control fibres, respectively. The control fibers were exposed to K-HDTA solution for 6 min. The results are expressed as a percentage of the pre-treatment value. **d** and **e** mean + SD (n = 6 for each case) of ratio of force at 1 Hz to that at 50 Hz in GA-treated and control fibres, respectively. Individual data are displayed as grey circles. *$P < 0.05$, versus Pre (Student's paired t test). n number of fibres, *Pre* pre-treatment, *Post* post-treatment
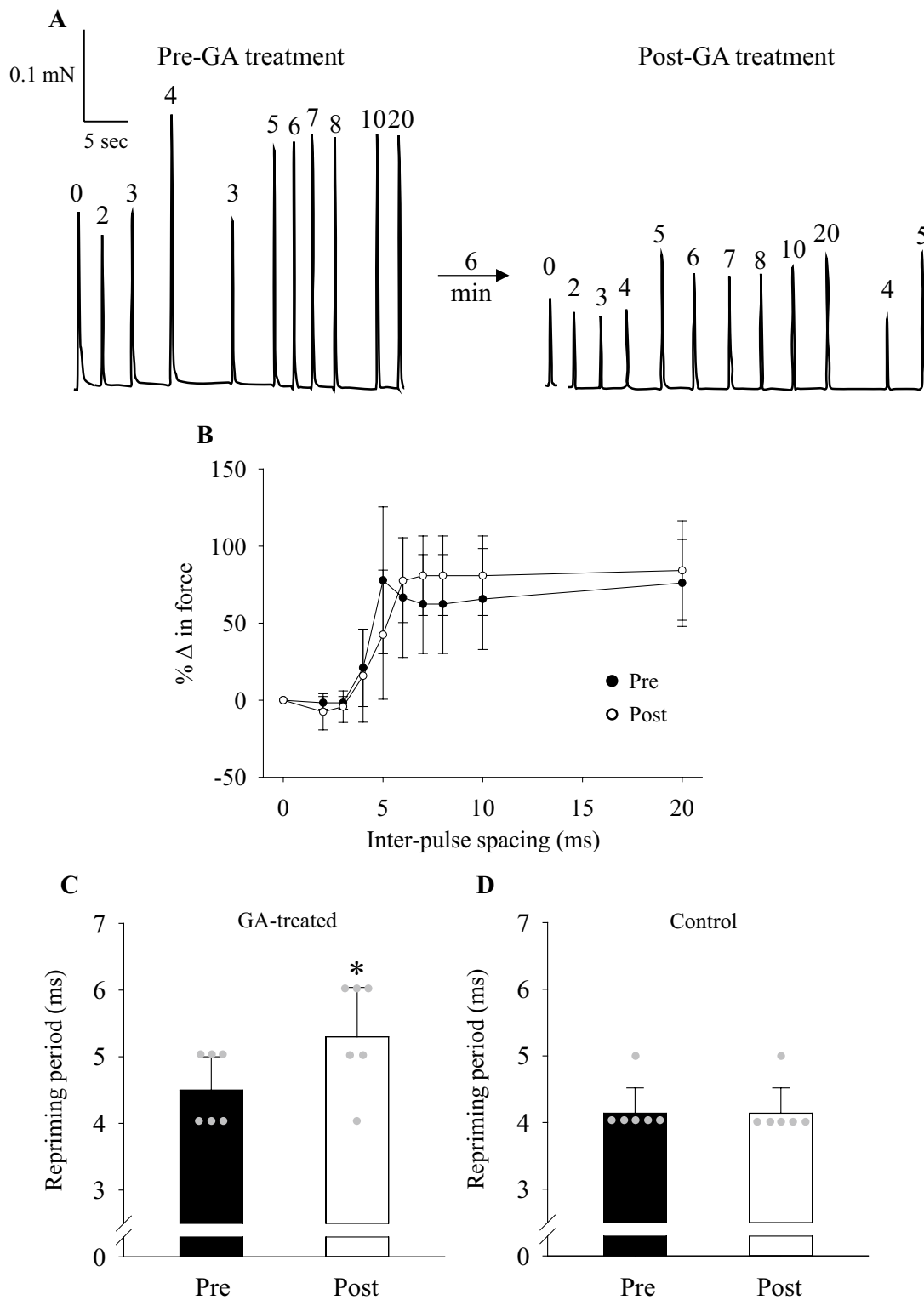
Fig. 5 Effects of glycogen depletion on action potential repriming in skinned fibre. **a** typical example of action potential-induced force before and after GA treatment. The EDL skinned fibre was stimulated with a pair of identical pulses separated by 2–20 ms (inter-pulse spacing, shown above each response) This procedure was also performed after exposing the fiber to 20 U mL^{-1} GA for 6 min. "0 ms" indicates a single pulse. **b** means + SD (n = 6) of relative changes in force plotted as relative force vs. inter-pulse spacing in GA-treated fibres. The relative changes in force are expressed as a percentage of the single pulse. **c** and **d** mean + SD (n = 6 for each case) of repriming period in GA-treated and control fibers, respectively. The control fibers were exposed to K-HDTA solution for 6 min. Individual data are displayed as grey circles. *$P < 0.05$, versus Pre (Student's paired t test). n number of fibres, *Pre* pre-GA treatment, *Post* post-GA treatment
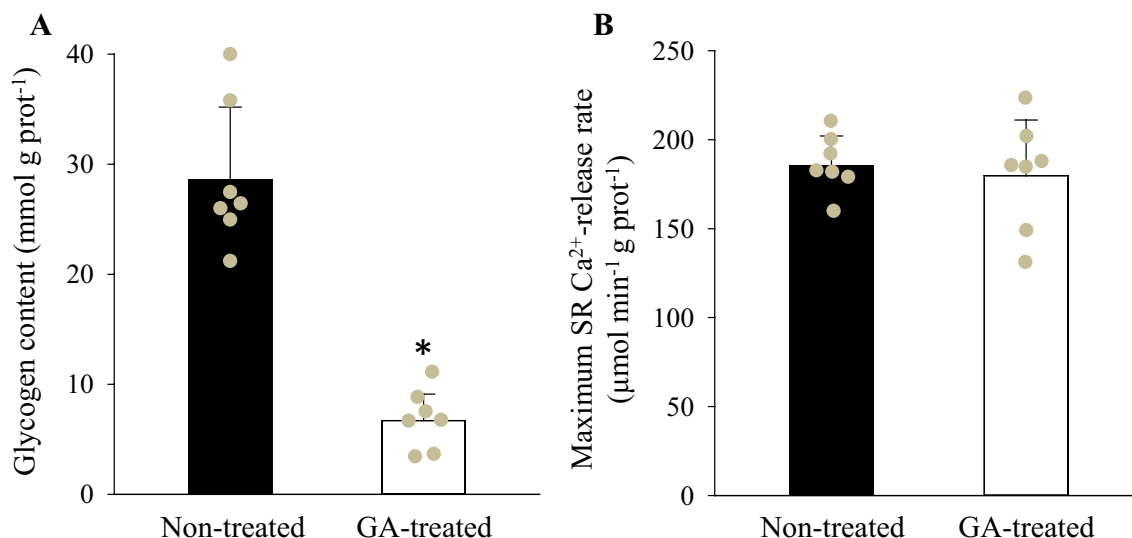
Fig. 6 Effects of glycogen depletion on glycogen content (**a**) and SR Ca^{2+}-release rate (**b**) in microsomes. Microsomes were prepared from the mixture of gastrocnemius and vastus lateralis muscles. Glycogen associated with the SR was removed by incubating muscle homogenates in a solution with 20 U mL^{-1} GA. The glycogen content is expressed in µmol glycosyl units per gram protein. The maximum SR Ca^{2+} release was initiated by 10 mM 4-chloro-*m*-cresol. Values are means + SD (n = 7 for each case). Individual data are displayed as grey circles. *$P < 0.05$, versus non-treated (Student's paired *t* test). *n* number of muscles, *GA* glucoamylase

in glycogen associated with the SR membrane. As with CMC, CAFF is also a direct activator of the RyR. The lack of change in the CAFF threshold with GA treatment makes it unlikely that reduced glycogen causes the decreased open probability that occurs in fatigued muscle fibres (Watanabe et al. 2015). It is possible, however, that the result of CAFF threshold might reflect the assay variability, small sample size, and consequently the likelihood of a Type II error. With the consideration that CAFF threshold increases to much higher levels in fatigued fibres than in GA-treated ones [7.4 mM in fatigued fibres (see Watanabe et al. 2015) vs. 5.3 mM in GA-treated fibres], if glycogen is involved in the open probability of the RyR, the decreased glycogen content would only make a small contribution to the reduced open probability.

This study was unable to draw an unequivocal conclusion on the mechanism(s) by which glycogen depletion can impair the ability of the SR to release Ca^{2+}. As a working hypothesis, Chin and Allen (1997) and Ørtenblad and Nielsen (2015) proposed that there is a functional coupling between ATP supplied by glycolysis and ATP utilized within the SR-T-tubule triadic gap. The α1- and β-subunits of the DHPR contain multiple sites for phosphorylation that is critical during muscle contraction (Favero 1999). It is possible

that ATP required for phosphorylation could be supplied by glycogen. In this regard, glycogen located in the intramyofibrillar space has been shown to be an essential role in skeletal muscle E–C coupling (Nielsen et al. 2009, 2014). Alternatively, as suggested by Stephenson et al. (1999), glycogen may anchor molecules which are locally important for the E–C coupling or fulfill a vital role in maintaining the normal structure of the triad junction.

In summary, we have shown that enzymatic glycogen removal results in the depression in AP- and depol-induced force production in quiescent muscle fibres resulting from a reduced Ca^{2+} release from the SR. It seems unlikely that structural alterations of the RyR are primarily responsible for this impairment of the SR. Although the Na^+–K^+-pump function is adversely affected by the glycogen removal, the extent of impairment is not enough to reduce SR Ca^{2+} release. This study provides direct evidence that glycogen above a certain amount is required for the preservation of the functional events preceding Ca^{2+} release from the SR. Additional studies using e.g., glycolytic enzyme inhibitors, are required to elucidate the precise mechanisms underlying glycogen depletion-related depressions in the capacity of the SR to release Ca^{2+}.

A

B
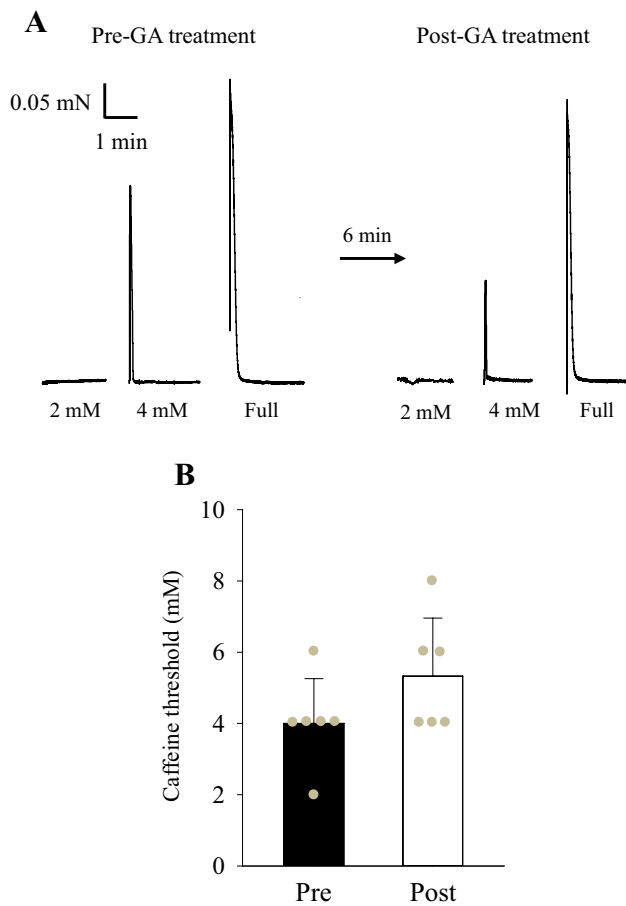
Fig. 7 Effects of glycogen depletion on caffeine threshold in skinned fibre. **a** typical example of caffeine-induced force before and after GA treatment. The skinned fibres were exposed to increasing concentrations of caffeine (2.0–10.0 mM) after the sarcoplasmic reticulum was maximally loaded with Ca^{2+}. This procedure was also performed after exposing the fiber to 20 U mL^{-1} GA for 6 min. **a** mean + SD (n = 6) of caffeine threshold. The caffeine threshold was defined as a caffeine concentration that produces a force response corresponding to 10% of force induced by full release solution. Individual data are displayed as grey circles. *n* number of fibres, *Pre* pre-GA treatment, *Post* post-GA treatment

Compliance with ethical standards

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