Title: Inhibition of glycogenolysis prolongs action potential repriming period and impairs muscle function in rat skeletal muscle

Running title: Glycogenolysis, action potential repriming period, and muscle function

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Key points summary

- Muscle glycogen content is associated with muscle function, but the physiological link between the two is poorly understood.
- This study investigated the effects of inhibiting glycogenolysis, while maintaining high overall energy status, on different aspects of muscle function.
- We demonstrate here that Na\(^+\),K\(^+\)-ATPase activity depends on glycogenolytically derived ATP regardless of high global ATP, with a decrease in activity leading to reduced force production and accelerated fatigue development.
- The results support the concept of compartmentalised energy transfer with glycogen metabolism playing a crucial role in intramuscular ATP resynthesis and ion regulation.
- This study gives specific insights into muscular function and may help towards a better understanding of glycogen storage diseases and muscle fatigue.

Abstract

Skeletal muscle glycogen content is associated with muscle function and fatigability. However, little is known about the physiological link between glycogen content and muscle function. Here we aimed to investigate the importance of glycogenolytically derived ATP *per se* on muscle force and action potential (AP) repriming period i.e. time before a second AP can be produced (indicative of Na\(^+\),K\(^+\)-ATPase activity). Single fibres from rat extensor digitorum longus muscles were isolated and mechanically skinned in order to investigate force production and AP repriming period while global ATP and PCr concentrations was kept high. Importance of glycogenolytically derived ATP was studied by inhibition of glycogen phosphorylase (DAB (1,4-dideoxy-1,4-imino-D-arabinitol, 2mM) or CP (CP-316,819, 10µM)) or glycogen removal (amyloglucosidase, 20U/ml). Tetanic force decreased by 21(15)% (P<0.001) and 76(28)% (DAB) or 94(6)% (CP, P<0.001) in well-polarised and partially depolarised fibres, respectively. In depolarised fibres, twitch force decreased by 16(10)% and 55(26)% with DAB and CP, respectively, with no effect in well-polarised fibres (84(10)%, P=0.14). There was no effect of glycogen phosphorylase inhibition on repriming period in well-polarised fibres (5 (4:5) vs. 4 (4:5) ms, P=0.26), while the repriming period was prolonged from 6 (5:7) to 8 (7:10) ms (P=0.01) in partially depolarised fibres. In line with this, glycogen removal increased repriming period from 5 (5:6) to 6 (5:7) ms (P=0.003) in depolarised fibres. Together, these data strongly indicate that blocking glycogenolysis attenuates Na\(^+\),K\(^+\)-ATPase activity, which in turn increases the repriming period and reduces force, demonstrating a functional link between glycogenolytically derived ATP and force production.
First author profile
For Rasmus Jensen

I am a PhD Fellow at Department of Sports Science and Clinical Biomechanics, University of Southern Denmark, where I also obtained my master’s degree after a short visit to the Department of Kinesiology, California State University. The initial experiments in the present study was initiated as part of my master thesis and convinced me to pursue a career in research. My primary interest is how muscle function and metabolism interact, and I am currently expanding my focus to include in-vivo studies in humans and hope to continue to combine in-vivo and in-vitro experiments in the future.
Introduction

Skeletal muscle glycogen content is associated with muscle function and fatigability. However, the connection between glycogen depletion and the development of fatigue, as well as the precise mechanism whereby muscle glycogen affects the series of events that ultimately result in fatigue, is not yet fully understood. Chin and Allen (1997) elegantly demonstrated a clear connection between muscle glycogen content and steps in the excitation-contraction (E-C) coupling leading to reduced tetanic cytosolic Ca\(^{2+}\) levels and force production. This has subsequently been confirmed and refined by observations of an association of impaired sarcoplasmic reticulum (SR) release rate with low glycogen both in rodents and humans (Barnes et al., 2001; Tupling, 2004; Ørtenblad et al., 2011). Furthermore, by using mechanically skinned fibres, enabling a constant [ATP], it has been demonstrated that the reduced force production with low glycogen is independent of bulk cellular [ATP] (Barnes et al., 2001; Nielsen et al., 2009), suggesting that glycogen depletion-related decrease in force and impairments in E-C coupling preceding cross-bridge cycling is due to compartmentalised energy production.

While there is a clear effect of glycogen availability on SR Ca\(^{2+}\) release, measured in isolated intact and skinned fibres as well as in SR vesicle preparations, less is known about the steps in E-C coupling preceding SR Ca\(^{2+}\) release, i.e. excitability (Tupling, 2004; Ørtenblad et al., 2013). In an early study, Clausen (1965) showed that when muscle Na\(^+\),K\(^-\)-ATPase activity is blocked with ouabain at rest, glycogen accumulates, probably due to a lower utilisation rate. In line with the direct coupling between glycogen content and Na\(^+\),K\(^-\)-ATPase activity, intracellular Na\(^+\) decreases if glycogen breakdown is stimulated with epinephrine at rest, however, ouabain significantly attenuates this decrease (James et al., 1999). In addition, inhibiting glycolysis with iodoacetate leads to the accumulation of intracellular Na\(^+\) at rest (Okamoto et al., 2001). A direct link between energy state and excitability of the muscle was demonstrated by blocking cross-bridge cycling and SR Ca\(^{2+}\) release with BTS and dantrolene, respectively, thereby conserving energy during repeated electrical stimulations, which in turn improved the ability of muscles to maintain excitability during high-frequency stimulation (Macdonald et al., 2007). This indicates a strong link between metabolites, energetic state, and excitability of the muscle. The effects of glycogenolytically derived ATP on muscle excitability are further substantiated in single fibres, where there is a pronounced role of glycogen content in force production mediated by repeated action potentials (APs) (Nielsen et al., 2009). Also, glycogen content is related to force production when directly stimulating the voltage sensors by Na\(^+\) depolarisations in some studies (Stephenson et al., 1999; Barnes et al., 2001), but not all (Goodman et al., 2005). A relationship between glycogen and fatigue with AP-induced depolarisations, but not when directly stimulating the voltage sensors, would be expected if low muscle glycogen affects t-system polarisation and/or excitability (Overgaard et al., 1999; Ørtenblad & Stephenson, 2003; Nielsen et al., 2004; Dutka & Lamb, 2007b, a).

In experiments using isolated mechanically skinned single fibres, where the intracellular environment can be readily changed and global [ATP] can be maintained at a high and constant level, the addition of phosphoenolpyruvate, which increases glycolytic ATP resynthesis, decreases t-system repriming period (indicative of increased Na\(^+\),K\(^-\)-ATPase activity) when the t-system is already...
partly depolarised (Dutka & Lamb, 2007a). Similarly, enzymatically removing glycogen granules using glucoamylase leads to small increases in repriming period (Watanabe & Wada, 2019). In both studies, the effects on twitch and tetanic forces were as expected, i.e. force loss due to partial depolarisation was attenuated with phosphoenolpyruvate (Dutka & Lamb, 2007a), and force decreased with glucoamylase (Watanabe & Wada, 2019) despite no changes in global energy levels. To further understand the connection between Na⁺,K⁺-ATPase activity and glycogenolytically derived ATP per se, studies utilising enzymatic inhibition of glycogen phosphorylase are needed in order to avoid possible structural perturbations from the physical removal of glycogen when using glucoamylase.

The aim of the present study was to investigate the effects of glycogenolytically derived ATP per se on action potential repriming period (i.e. estimated time before a second AP can be produced, indicative of Na⁺,K⁺-ATPase activity) and muscle function including whole muscle fatigue development, and force production in whole muscles and single fibres. We hypothesised that blocking glycogenolytically derived ATP production leads to increased action potential repriming period, reduced twitch and tetanic force, and accelerated fatigue development.

A novel approach is used here, studying glycogenolytically derived ATP on muscle function by using two distinct glycogen phosphorylase inhibitors in combination with mechanically skinned single fibres and in isolated whole extensor digitorum longus (EDL) muscles. Mechanically skinned fibres enable direct access to the intracellular environment and steps in the E-C coupling, while at the same time maintaining high and constant global energy levels. This experimental setup uniquely allows a paired design where glycogen phosphorylase can be acutely inhibited without affecting global energy status or glycogen content, thereby enabling an unequivocal study of the effects of glycogenolytically derived ATP per se.
Methods

Ethical approval
All handling and use of rats complied with Danish animal welfare regulations. All animals were kept in a thermostated environment at 21 °C with a 12 h / 12 h light - dark cycle and fed ad libitum at the Biomedical Laboratory, University of Southern Denmark.

Study design and preparations
To study the importance of glycogenolytically derived ATP on muscle function, we acutely inhibited glycogen phosphorylase using two distinct enzymatic inhibitors. DAB (1,4-dideoxy-1,4-imino-D-arabinitol) phosphorylates glycogen phosphorylase (forming glycogen phosphorylase a) while simultaneously inhibiting the activity of glycogen phosphorylase (Fosgerau et al., 2000; Andersen & Westergaard, 2002; Latsis et al., 2002; Walls et al., 2008). Conversely, CP (CP-316,819 or [R-R*,S*]-5-chloro-N-[2-hydroxy-3-(methoxyethylamino)-3-oxo-1-(phenylmethyl)propyl]-1H-indole-2-carboxamide) or closely related indole carboxamide compounds dephosphorylates glycogen phosphorylase (forming glycogen phosphorylase b) ultimately reducing glycogen phosphorylase activity (Andersen & Westergaard, 2002; Latsis et al., 2002; Suh et al., 2007). The inhibitors were used in whole muscle homogenates, single fibres either mechanically or chemically skinned, and in isolated whole muscles (only CP). The homogenates were used to evaluate the inhibitors (i.e. inhibitor potency), while the single fibre preparations were used to investigate the effect of glycogen phosphorylase inhibitors on muscle function. Specifically, mechanically skinned fibres were used to examine electrically evoked contractions and action potential repriming period, and chemically skinned fibres were used to examine possible adverse side effects from the inhibitors on contractile function. Experiments on isolated whole muscles were done to show possible translation to more complex tissues.

On experiment days, the animals were either stunned followed by cervical dislocation (smaller animals used for whole muscle experiments and chemical skinning) or sacrificed by an isoflurane (4 % vol./vol.) overdose followed by cervical dislocation (larger animals used for mechanical skinning and homogenate). A total of 63 rats were used for this study.

Homogenate and coupled assay
After sacrificing the rats (Sprague-Dawley, N = 3, ~550 g), the soleus, EDL and white parts of the quadriceps muscles were isolated, blotted dry on filter paper, and tendons and all visible connective tissue were removed. The muscles were then mixed with a homogenisation buffer in a 1:10 (w/v) concentration. The buffer contained (in mM): imidazole (50), NaCl (100), NaF (20), and dithiothreitol (DTT, 0.5). pH was adjusted to 7.00 using 1 M HCl. The muscles were then homogenised with a Potter-Elvehjem homogeniser (Kontes Glass Co. Vineland, New Jersey, USA) until the homogenate appeared homogenous. The homogenate was kept on ice whenever possible to reduce metabolic changes while the homogenization was processed, then quick-frozen in liquid nitrogen, and stored below -80°C until used to determine glycogen phosphorylase activity on a separate day. Glycogen phosphorylase activity was measured using a coupled assay preheated to 35°C as previously described (Passonneau & Lowry, 1993) using absorbance change over 12 min following basal activity.
measurement over 3 min. 50 µL homogenate was placed in a pre-made imidazole-acetate buffer with concentrations of DAB in the range of 0.1 to 3000 µM (n = 112 samples including controls), or CP in the range of 0.1 to 250 µM (n = 73 samples including controls) (total final volume = 1040 µL). The buffer contained (in mM): imidazole (35), imidazole-acetate (15), K2HPO4 (20), MgCl2 (0.5), EDTA (1.0), DTT (0.5) and 0.025 % BSA. pH was adjusted to 7.00 using 1 M HCl. On experiment days, glycogen (20 mg/ml), NADP+ (100 mM), muscle P-glucosumase (60 U/ml), leuconostoc G-6-P dehydrogenase (100 U/ml), 5’AMP (100 mM) and G-1,6-biP (0.2 mM), were all added to and pre-diluted in the assay buffer, before the assay was initiated by the addition of NADP+. Until then, the diluted enzymes and substrates were stored at -20°C. The inhibition-concentration relationship was described using a sigmoidal curve with a variable Hill slope.

Mechanically skinned fibres
After sacrificing the rats (male Sprague-Dawley (N = 19) or male Wistar rats (N = 1), ≥5 months old, weight ~600 g), EDL muscles were rapidly excised and pinned at their resting length under paraffin oil in a petri dish. The muscles were kept cool (~10°C) on an icepack. Individual fibre segments were mechanically skinned with jeweller’s forceps as described in detail elsewhere (Ørtenblad & Stephenson, 2003; Lamb & Stephenson, 2018). In brief, single fibres were isolated under a dissecting microscope using jeweller’s forceps and, following this, the sarcolemma was mechanically rolled back, which effectively skins the fibre. Consequently, the transverse tubular (t-) system reseals (Lamb et al., 1995), resulting in a functioning fibre with access to the intracellular environment, while still maintaining a membrane potential (due to the sealed t-tubuli). Thus, AP-induced contractions can be induced in the intact fibre (Posterino et al., 2000). After skinning, the fibre was mounted on a sensitive force transducer (AME875, SensoNor, Norway), stretched to 120% of resting length and immersed in solution mimicking the intracellular environment (see Solutions for skinned fibres). With this preparation, it is possible to measure a multitude of parameters including fibre excitability and force production while at the same time having direct access to the intracellular environment and maintaining the intracellular environment constant in terms of energy status ([ATP] and [PCr]).

Single-fibre AP-induced force
Using the mechanically skinned fibre preparation, we investigated the effect of glycogen phosphorylase inhibition on twitch and tetanic force production while maintaining high and constant global [ATP], and [PCr], in fibres that were well-polarised and partly depolarised (by changing [K⁺]) fibres (n = 37). In a paired design, where all force recordings were normalised to the most recent control stimulation, a twitch contraction (2 ms at 30 V corresponding to ~75 V/cm) followed by a tetanic contraction (0.8 s train at 50 Hz) and a second twitch contraction (all separated by 15 seconds) were elicited. This was done with the t-system well-polarised, then partly depolarized, and then again well-polarised, with the whole sequence firstly performed without glycogen phosphorylase inhibition, then with glycogen phosphorylase inhibition, and finally after a washout period to reverse the inhibition (Figure 2A).
Single fibre excitability

Membrane excitability was investigated in mechanically skinned fibres, using a functional approach proposed by Dutka and Lamb (2007a). When two consecutive electrical pulses (double pulse (DP)) are given, the first within a few milliseconds of the second, the second pulse will only induce a second AP if enough membrane Na+ channels have had sufficient time to recover from the fast inactivation caused by the first pulse. If a second AP is elicited, this will lead to additional Ca2+ release from the SR, which in turn results in a marked increase in force output. By eliciting DP stimulations in mechanically skinned single fibres, the direct effect of glycogen phosphorylase inhibition on action potential repriming period was evaluated while maintaining high and constant global [ATP], and [PCr], with the t-system well-polarised or partly depolarised. After partial depolarisation, the fibres were again repolarised in the control solution before inhibiting glycogen phosphorylase and re-examining the responses with the t-system well-polarised and then partly depolarized. Glycogen phosphorylase was inhibited using 2 mM DAB (n = 22) or 10 µM CP (n = 27). In a separate experiment, the sensitivity of the protocol to detect changes in Na+,K+-ATPase activity was evaluated by reducing [Na+]i to below-optimal concentrations (Km = 11 mM (Kristensen & Juel, 2010)), thus reducing Na+,K+-ATPase activity.

When eliciting DP stimulations, these were given with increasing interspacing periods between pulses (in increments of 1 ms up to 15 ms and 20 ms). The minimum interspacing period leading to an incremental increase in force response >50 % of the maximum incremental increase was defined as the repriming period (as suggested in Dutka and Lamb (2007a)). When calculating the incremental increase, the mean twitch force and DPs with interspacing periods of 1 and 2 ms were used as baseline because it was considered physiologically impossible to reprime the membrane within this time span. When glycogen phosphorylase was inhibited in partly depolarised fibres, some fibres produced very little or no force. In such cases, data from this treatment was excluded. Thus, 11 fibres completed the inhibition and the depolarisation+inhibition parts only. In the latter, some fibres showed a general decrease in force over time. Therefore, a linear regression based on the first three stimulations during this treatment was used to adjust for the decrease. Following regression, the estimated repriming period for each individual fibre was evaluated in order to ensure that the regressions yielded reasonable results.

In a separate series of experiments, we investigated the effects of glycogen removal on repriming period. Glycogen was removed from the mechanically skinned fibres by incubating in the stimulation solution with 20 U/ml amyloglucosidase for 6 minutes (Watanabe and Wada, 2018). To test the effects of amyloglucosidase treatment on glycogen content in mechanically skinned fibres, two fibres were divided into two segments and one segment of each pair was fixed for transmission electron microscopy analyses of glycogen content (Nielsen et al., 2009) before or after amyloglucosidase treatment, respectively. The repriming period was measured in well-polarised fibres, and partly depolarised fibres before and after amyloglucosidase treatment. Further, we tested possible effect of GP inhibition using 2 mM DAB in fibres pre-treated with amyloglucosidase. To reduce the force decline over time observed in some fibres, each block of double pulse stimulation (from 1-15 ms) was stopped when the interspacing period for an apparent increase in
force to be observed was exceeded by 2 ms. Finally, a double pulse stimulation with 20 ms interspacing period was elicited.

**Contractile apparatus function in chemically skinned fibres**

After sacrificing the rats (Wistar rats (N = 7), weight ~300 g), they were treated as described for the mechanically skinned fibre preparation until single fibres were isolated. Isolated fibres were mounted and stretched to 120% of resting length before chemically skinning by immersion in a resting solution (see Solutions for skinned fibres) with 1 % Triton-X100 for 2 minutes. Chemical skinning removes membranes but retains the contractile apparatus function similar to mechanical skinning (Lamb & Stephenson, 2018). Thus, this preparation allows investigation of adverse side effects of the GP inhibitors on the contractile function. After skinning the fibres were washed in resting solution and activated using different Ca\(^{2+}\) concentrations (from pCa=6.5 to pCa=4.7) while the force response was recorded to produce a force-pCa relation (Figure 4A). This was done in an initial run-through followed by a similar run-through without (vehicle only, n = 34), then again after the addition of 2 mM DAB (DAB, n = 30), or 10 µM CP (CP, n= 57). After a 10-minute wash-out period without activation, a third run-through was conducted without glycogen phosphorylase inhibitors.

During all single fibre experiments, force was continuously measured and recorded at 1000 Hz using a custom-made LabVIEW programme (LabView 8.0, National Instruments, Austin, TX, USA). All single fibre experiments were conducted at room temperature (23.1 (0.1) °C), with N and n denoting the number of rats and fibres examined, respectively.

**Solutions for skinned fibres**

The solutions were made to mimic the intracellular environment (Table 1) with constant high [ATP] and [PCr] (8 and 10 mM, respectively). In the experiments using the mechanically skinned fibre preparation, the t-system was partly depolarised by lowering intracellular [K\(^+\)]. This solution was made by mixing the standard stimulation solution (126 mM K\(^+\)) with a solution containing 0 K\(^+\) so that the final solution consisted of 59.5 % standard solution with a final K\(^+\) concentration at 75 mM (membrane potential app. -74 mV compared to app. -87 mV in controls (Ørtenblad & Stephenson, 2003)). When investigating contractile function in chemically skinned fibres, contractions were stimulated using solutions with different tightly buffered intracellular [Ca\(^{2+}\)]. To achieve this, a resting solution with virtually no free calcium and an activation solution with supramaximal [Ca\(^{2+}\)] were mixed in appropriate ratios. DAB was diluted in resting or stimulation solution depending on the experiment (stock = 100 mM), while CP was diluted in DMSO (stock = 1 mM). Therefore, an equal amount of DMSO was added under control settings.

**Glycogen content in mechanically skinned single fibres**

Skinned fibres segments were analysed for glycogen content using transmission electron microscopy as described by (Nielsen et al., 2009). 12 images were sampled at 10.000x magnification in a systematic, uniform, but random order, and volumetric glycogen content was quantified stereologically (point counting). Estimated coefficient of error for each fibre was in the range of 0.12-0.19.
Whole muscle fatigue

After sacrificing the rats (4-5-week-old Wistar rats (N = 37), weight ~115 g), intact EDL muscles (weight ~20–25 mg) were excised and mounted in thermostated chambers containing a standard Krebs-Ringer bicarbonate buffer (KRB) containing (in mM): NaCl (122.1), NaHCO₃ (25.1), KCl (2.8), KH₂PO₄ (1.2), MgSO₄ (1.2), CaCl₂ (1.3), and D-glucose (5.0), pH 7.4. All incubations took place at 30°C under continuous gassing with a mixture of 95% O₂ and 5% CO₂. The lengths of the muscles were adjusted for optimal force production by inducing electrical stimulations through platinum electrodes using twitches (0.2ms supramaximal pulses at 12V) and left for 30 min. The muscles were then allocated into one of two groups each incubating for 180 min. in a standard KRB, without (DMSO as vehicle) or with CP (0.1 mM, added from a 100 mM stock solution). Muscles then underwent fatiguing stimulation (400-ms trains of 60 Hz every 5 s) or rested for 15 min. Immediately afterwards, the muscles were rapidly detached, snap frozen in liquid N₂ and stored at -80°C until assessment of glycogen content. Samples (2.0–2.6 mg) were later freeze-dried, dissected free of non-muscle tissue, and powdered before glycogen content was estimated according to Pansonneau and Lowry (1993) and modified as previously described (Ørtenblad et al., 2000). Briefly, samples were boiled in 1 M HCl (0.5 ml) for 150 min. and rapidly cooled, mixed and centrifuged (3500 g) for 10 min. at 4°C. Glycogen content was estimated spectrophotometrically (DU 650, Beckman Coulter Inc., Brea, CA, USA) in a coupled assay and the absorbance change over 60 min. was used to estimate glycogen content in the sample.

Chemicals

All chemicals were purchased from Sigma-Aldrich Denmark A/S, Copenhagen, Denmark, except for HDTA, which was purchased from Fluka, Buchs, Switzerland, and isoflurane, which was purchased from the Biomedical Laboratory, Odense University Hospital, Odense, Denmark.

Statistics

All measures of central tendency, variance and statistical interference were conducted in StataIC 15.1 for Windows (32-bit) (StataCorp LP, College Station, TX, USA). The evaluation of the inhibitors was merely descriptive (no p-values calculated). In other experiments, except those related to excitability, mixed model linear regression analyses were conducted with time*group interactions, and rat or fibre ID as random effects. We tested for equal variance and normal distribution of the residuals. In one case (Hill slopes in force-pCa relation), data was not normally distributed, and this could not be solved with data transformation. Therefore, we used a non-parametric Kruskal-Wallis test to obtain p-values. The presented values are all arithmetic means (SD) except in experiments on fibre excitability. Here we report the median (25th percentile:75th percentile). In this experiment, we used a time-to-event analysis with interspacing period and repriming period as time and event, respectively, in a Cox proportional hazards model. Before analysis, we tested the proportional hazards assumption. In all experiments, an alpha-level of 0.05 was used.

GraphPad Prism 7 for Windows (GraphPad Software, Inc., La Jolla, CA, USA) was used to create plots and graphs, and to fit the data using sigmoid curves when examining the concentration-inhibition relation of the inhibitors and when describing the force-pCa relation. For the force-pCa relation, all force recordings were normalised to force when pCa = 4.7. Because some analyses yielded
ambiguous Hill slopes, these values were replaced with the mean Hill slope under control settings. Because this was mostly done in CP fibres, these were not included when analysing the changes in Hill slopes.

**Results**

**Glycogen phosphorylase inhibition in skeletal muscle homogenate**

Glycogen phosphorylase activity was investigated in a coupled assay using skeletal muscle homogenate with various glycogen phosphorylase inhibitor concentrations. Both inhibitors reduced glycogen phosphorylase activity, with CP being markedly more potent than DAB. Glycogen phosphorylase activity was completely blocked with 250 µM CP. Based on concentration-inhibition curves (Figure 1), 3800 µM DAB and 3.4 µM CP is needed to reduce glycogen phosphorylase activity 95 % (n = 112 and 73, respectively). The concentrations needed for 50 % inhibition were 2.0 and 0.7 µM for DAB and CP, respectively. Hill slopes were estimated to be 0.4 and 1.9 for DAB and CP, respectively.

**Effects of glycogen phosphorylase inhibition on force in single electrically-evoked contractions in mechanically skinned fibres**

Next, we investigated the effects of glycogen phosphorylase inhibition on electrically elicited force production in isolated mechanically skinned single fibres. In well-polarised fibres, tetanic force dropped to 79 (15)% (overall P < 0.001, no time*group interaction (P = 0.12), Figure 2B), and twitch force was maintained at 84 (10)% (P = 0.14) or decreased to 45 (26)% (P < 0.001) with DAB and CP, respectively (time*group interaction P < 0.001, Figure 2D). When partially depolarising t-system membrane potential, tetanic force decreased 72 (15)% of control force without inhibitors and to 25 (29)% and 14 (12)% with DAB and CP, respectively (P <0.001 for both inhibitors vs. depolarisation alone, time*group interaction P < 0.001, Figure 2C). Similarly, twitch force was reduced to 67 (21)% control force with depolarisation alone with an additional decrease to 20(26)% and 6 (6)% for DAB and CP, respectively (P < 0.001 for both inhibitors, time*group interaction P < 0.001, Figure 2E). After wash-out, we observed no recovery of twitch force in well-polarised DAB fibres (P = 0.75), but CP fibres partially recovered (P < 0.001). For tetanic force, a tendency towards an effect was seen (P for main effect = 0.10). In partially depolarised fibres, twitch and tetanic force did not recover after wash-out regardless of the inhibitor used (main effects: P = 0.61 and P = 0.07 (further decrease)).

**Effect of glycogen phosphorylase inhibition on action potential repriming period**

We validated the relationship between action potential repriming period and Na⁺,K⁺-ATPase activity by measuring the repriming period with varying [Na⁺] concentration. Indeed, the median repriming period increased from 4 (4:5, 25th percentile:75th percentile) ms with 36 and 12 mM Na⁺ to 6 (5:6) ms with 4 mM Na⁺ (P = 0.006 and 0.043, respectively) and 8 (6:9) ms with 2 mM Na⁺ (P = 0.001 and 0.004, respectively) (Figure 3B) with no statistical differences between 36 and 12 mM Na⁺ (P = 0.718), or 4 and 2 mM (P = 0.158).

When investigating the effect of glycogen phosphorylase inhibition on the repriming period, we saw no effect in well-polarised fibres (5 (4:5) and 4 (4:5) ms in controls and with GP inhibition,
respectively ($P = 0.26$, Figure 3C). However, in partially depolarised fibres the repriming period increased to 6 (5:7) ms ($P < 0.001$, Figure 3D), and further increased to 8 (7:10) ms when glycogen phosphorylase was inhibited ($P = 0.01$, Figure 3E) with no interaction between inhibitors ($P = 0.63$). This pattern was further supported when examining the mean change in repriming period, i.e. there was no change with glycogen phosphorylase inhibition in well-polarised fibres (0.3 (1.0) ms), but a relatively high change (3.3 (3.8) ms) in the repriming period in depolarised fibres.

A similar effect on repriming period in partly depolarised fibres was observed with glycogen removal using amylloglucosidase. This treatment reduced volumetric glycogen content in two fibres from 3.7 to 0.6 and 2.2 to 0.6 $\mu$m$^3$-$\mu$m$^{-1}$-$10^3$, respectively, corresponding to 83 % and 73 % reduction, respectively. In other fibres, median repriming period increased from 5 (5:6) ms before treatment to 6 (5:7) ms after treatment ($P = 0.003$, Figure 3F). Importantly, glycogen inhibition using DAB showed no additive effect (repriming period = 7 (6:8) ms, Figure 3G) in fibres already pre-treated with amylloglucosidase ($P = 0.61$ compared to amylloglucosidase treatment alone in partly depolarised fibres). Thus, blocking of glycogen utilisation, both by GP inhibition or glycogen removal, increases repriming period in fibres with partially depolarised t-systems.

Effect of glycogen phosphorylase inhibition on contractile apparatus function

In order to estimate possible effects of glycogen phosphorylase inhibitors on the contractile apparatus, the effects on maximal Ca$^{2+}$ induced force ($F_{\text{max}}$) and Ca$^{2+}$ sensitivity were evaluated. Compared to vehicle fibres, fibres inhibited with CP produced 11 (9) % less force ($P < 0.001$), while DAB fibres produced similar force (101 (3) % of vehicles, $P = 0.85$, time*treatment interaction $P < 0.001$, Figure 4B). Similarly, the [Ca$^{2+}$] needed to induce 50 % of the maximal Ca$^{2+}$ induced force ($pC_{50}$) was 5.83 (0.12) in vehicles (time effect $P = 0.50$, Figure 4C) with no difference in DAB fibres (time*group interaction for DAB $P = 0.65$), but a reduction in Ca$^{2+}$ sensitivity with CP ($pC_{50}$=5.65 (0.03), $P < 0.001$, time*group interaction $P < 0.001$). After a wash-out period, there was 5 (2) % recovery in maximal force in CP fibres ($P < 0.01$), consequently producing higher force than vehicles at this time point ($P < 0.001$) due to a 14 (4) % drop in vehicles. This was accompanied by a significant increase ($P < 0.001$) in $pC_{50}$ in CP fibres to 5.88 (0.12) which was higher than the $pC_{50}$ in vehicles (5.84 (0.08), $P < 0.01$). In general, the Hill slope (steepness) of the force-pCa relation curve was -8.9 (3.7) (pooled data) with no difference between groups ($P = 0.12$, $P = 0.64$ and $P = 0.79$ for DAB vs. vehicle in control, inhibition and after wash-out, respectively). In summary, because we saw no effects of DAB, glycogen phosphorylase inhibition does not lead to changes in contractile apparatus function, but CP has adverse side effects on maximal Ca$^{2+}$ induced force and Ca$^{2+}$ sensitivity. Thus, when examining force response with the use of CP, the results should preferably be corrected for this. In the present study, the data presented on twitch and tetanic force has been adjusted to account for the effect on maximal Ca$^{2+}$ induced force, but we were not able to adjust for the changes in Ca$^{2+}$ sensitivity, because this would require force-pCa relations for each fibre at each time point.
Effects of glycogen phosphorylase inhibition on glycogen utilisation and muscle function in isolated whole muscle

The effects of glycogen phosphorylase inhibition were estimated in whole muscles during repeated tetanic contractions. Following incubation with or without glycogen phosphorylase inhibition (0.1 mM CP and vehicle, respectively) in the presence of extracellular glucose, muscles underwent fatiguing stimulations or stayed at rest. Glycogen content was similar in both groups without contractions (114 (13) and 115 (19) mmol/kg dry weight in vehicle (n = 12) and CP muscles (n = 6), respectively, P = 0.83, (Figure 5B), while only the vehicle muscles showed lower glycogen content with contractions (48 (23) and 101 (27) mmol/kg dry weight in vehicle (n = 21) and CP (n = 19) muscles, respectively. Group*treatment interaction P < 0.001). This corresponds to a 58 % reduction in vehicle with contractions in vehicle only (P < 0.001) with no significant reductions in CP muscles (P = 0.11). In other words, based on the differences in mean glycogen pre and post stimulations, ~80 % of the glycogen breakdown during contractions was blocked by pre-incubation with CP.

Initially, muscles pre-incubated in CP produced lower force than vehicle (P < 0.001, (Figure 5A). During repeated contractions in both groups, force declined significantly during the stimulations beginning from the 10th contraction, with CP muscles declining more rapidly than vehicles (time*group interaction P < 0.001). In addition, an increase in force during the initial contraction phase in CP muscles was observed, while vehicle muscles remained stable until the 10th contraction. In summary, pre-incubation of whole muscles for 180 min with 0.1 mM CP led to lower glycogen utilisation and had clear effects on force production over time with markedly lower initial force response and an accelerated fatigue development (i.e. faster force decline).
Discussion

In this study we used two distinct glycogen phosphorylase inhibitors that do not share pharmacokinetic features in order to investigate the importance of glycogenolytically derived ATP for muscle function and Na⁺,K⁺-ATPase activity estimated from action potential repriming period. We demonstrate that glycogen phosphorylase inhibition leads to 1) reduced twitch and tetanic force in single fibres, and 2) prolonged repriming period (i.e. decreased fibre excitability) which we show is sensitive to changes in Na⁺,K⁺-ATPase activity. The latter was also confirmed when glycogen was removed with amyloglucosidase with no additive effects of GP inhibition after glycogen removal suggesting the results are not due to adverse side effects. Furthermore, GP inhibition in isolated whole muscles reduced initial tetanic force and accelerated fatigue development with repeated contractions. Importantly, the effects of GP inhibition are observed in the presence of glucose (whole muscle) and during high and constant intracellular [ATP] (skinned fibres), thereby demonstrating a direct role of glycogenolytically derived ATP. The findings suggest a tight coupling between glycogenolytic-glycolytic ATP production, Na⁺-K⁺-pump function, t-system excitability, and ultimately force production, and offer a possible explanation for the long-standing observation of force depression with low muscle glycogen availability (Allen et al., 2008; Ørtenblad et al., 2013).

A coupling between glycolysis and the Na⁺,K⁺-ATPases at rest has been suggested by stimulating the glycolytic rate hormonally (James et al., 1999) or by inhibiting the glycolytic rate (Okamoto et al., 2001) which leads to increased and reduced Na⁺,K⁺-ATPase activity estimated from intracellular Na⁺ concentrations, respectively. In order to investigate this coupling in contracting fibres without affecting global energy levels, we used the mechanically skinned fibre preparation where the extensive t-system representing the greater part of the plasma membrane reseals and becomes normally polarised when placed in a medium mimicking the cytosolic environment of the intact cell with high and constant [ATP]. By using this technique, it has previously been suggested that the repriming period is indicative of Na⁺,K⁺-ATPase activity with a longer repriming period indicating reduced activity (Dutka & Lamb, 2007a). This method was evaluated in the present study by manipulating Na⁺,K⁺-ATPase activity by decreasing intracellular [Na⁺], which led, as expected, to an increased repriming period. Thus, the results underpin the proposed link between Na⁺,K⁺-ATPase activity and repriming period. The finding here of a similar increase in the repriming period with glycogen phosphorylase inhibition or glycogen removal in combination with partial t-system depolarisation clearly demonstrates the importance of glycogenolytically derived ATP for Na⁺,K⁺-ATPase activity. In depolarised muscle fibres, Na⁺,K⁺-ATPase energy demand is expected to be high (Clausen, 2003; Pirkmajer & Chibal, 2016), further stressing the importance of readily available ATP production for the Na⁺,K⁺-ATPases. In support of this, Dutka and Lamb (2007a) found that stimulation of glycolytic ATP production by the addition of phosphoenerolpyruvate decreases the repriming period in partly depolarised, but not well-polarised, fibres (Dutka & Lamb, 2007a). They also showed that glycolytic ATP production per se is the cause of the effect, because the addition of pyruvate, which can be utilised in mitochondria, did not change the repriming period (Dutka and Lamb, 2007a). In line with this, it has recently been demonstrated that, by using the same preparation and methodology as in the present study, the repriming period in well-polarised fibres was increased when enzymatically removing glycogen using glucoamylase (Watanabe & Wada,
2019). Hence, data from previous and present studies using the mechanically skinned fibre preparation is in line with the idea that the Na⁺,K⁺-ATPase preferably uses endogenously derived energy, e.g. glycogenolytically derived ATP.

Macdonald and colleagues (Macdonald et al., 2007) demonstrated a possible link between the Na⁺,K⁺-ATPases and the metabolism and, in turn, force output by showing that energy conservation through inhibition of cross-bridge cycling and Ca²⁺ release during repeated electrical stimulations leads to attenuated force decline due to conserved membrane excitability. Similar connections are shown when increasing glycolytic ATP production by the addition of phosphoenolpyruvate, which increases force production (Dutka & Lamb, 2007a), or when reducing the glycogenolytic rate by removing glycogen using glucoamylase (Watanabe & Wada, 2019) or inhibiting glycogen phosphorylase (present study) which in both studies reduced force. Appreciating that in-vivo stimulations are typically in the form of trains often initiated with a high-frequency doublet discharge (Van Cutsem et al., 1998) with frequencies above 250 Hz in fast muscles from mice (Gorassini et al., 2000), the importance of well-functioning Na⁺,K⁺-ATPases becomes apparent. Indeed, the reduced Na⁺,K⁺-ATPase activity with glycogen phosphorylase inhibition demonstrated in the present study precludes AP firing at rates close to this. This is probably more pronounced during train stimulations, as the fibre membrane becomes partially depolarised between stimulations (Fraser et al., 2011) which further increases the repriming period.

Due to the high surface-to-volume ratio in the t-system and limited diffusion of K⁺ from the transverse tubuli, optimal Na⁺,K⁺-ATPase function is particularly important in order to maintain fibre excitability (Nielsen & de Paoli, 2007). 90% of the t-system is surrounded by the sarcoplasmic reticulum forming narrow triadic junctions (Dulhunty, 1984) with limited ATP diffusion from the global (i.e. cytosolic) pool (Han et al., 1992). Due to the heterogenic distribution in the fibre, glycogen may serve as a substrate pool for local ATP production close to the Na⁺,K⁺-ATPases (Ørtenblad et al., 2013). The present study supports this, as the effects of global glycogen phosphorylase inhibition were observed despite high and constant global intracellular [ATP] and [PCr].

The above discussion of a tight coupling between glycanolytic-glycolytic ATP production and repriming period is in line with previous results in other cell systems, where increases in the glycolytic rate lead to increases in Na⁺,K⁺-ATPase activity in inside-out vesicles from erythrocytes (Mercer & Dunham, 1981), renal cell culture (Sanders et al., 1983) and toad urinary bladders (Handler et al., 1969), but are attenuated or completely countered with the inhibition of Na⁺,K⁺-ATPase activity by incubation in ouabain in toad urinary bladders (Handler et al., 1969). Similarly, the effects of ouabain in rat cardiomyocytes are blunted when glucose is omitted (Sepp et al., 2014). A coupling between Na⁺,K⁺-ATPase activity and glycolysis and lactate production is also evident during sepsis in humans, where associated increased K⁺ pumping is caused by the epinephrine surge present in such patients (Levy et al., 2005). Indeed, increased glycolysis and glycogenolysis during experimental haemorrhagic shock or experimental sepsis are closely linked to stimulation of Na⁺,K⁺-ATPase activity due to epinephrine release, suggesting a tight coupling between Na⁺,K⁺-ATPase activity and glycogenolysis (Bundgaard et al., 2003). Furthermore, patients suffering from McArdle’s
disease (glycogen storage disease type V) are unable to utilise muscle glycogen, due to an inherited deficiency of glycogen phosphorylase, leading to an inability to use stored muscle glycogen and marked exercise intolerance (Santalla et al., 2014). Interestingly, a higher surface electromyography signal is measured during submaximal contractions in McArdle patients, which is indicative of a need to activate larger muscle mass for a given force output, suggesting reduced muscle excitability (Santalla et al., 2014). Also, McArdle patients have less Na⁺,K⁺-ATPases (Haller et al., 1998). Taken together, observations from both sepsis and McArdle patients are in line with the idea of a tight coupling between glycogenolytic-glycolytic rate and Na⁺,K⁺-ATPase activity.

Overall, previous and the presented results strongly support the model proposed by Epstein et al. (2014). In this model, glycolysis acts as a fast response to rapid transitions in energy demand despite high oxygenation. Glycogen is particularly important for this, because glucose may not substitute glycogen utilisation as indicated in the present (whole muscle experiment) and previous (Smith et al., 2010) studies. In short, it is clearly shown that a high global energy status is insufficient to maintain muscle function during glycogen phosphorylase inhibition or glycogen removal when ATP turnover is expected to increase. Thus, our inability to measure an effect of GP inhibition in well-polarised fibres (except during tetanic stimulations) perfectly fits a cellular model with restricted (but not blocked) diffusion from cytosol to compartments with ATP utilisation (Han et al., 1992; Vendelin et al., 2004). In this, ATP diffusion from the cytosol only becomes insufficient when ATP utilisation exceeds the ATP diffusion rate e.g. during partial depolarisation or repeated elicitations of APs, which increase the ATP turnover rate by the Na⁺,K⁺-ATPases. Overall, our results support the concept of compartmentalised ATP resynthesis within the fibre (Ovadi & Saks, 2004; Dharchowdhury et al., 2007) and that glycogen plays a crucial role in supporting key compartments (Ørtenblad & Nielsen, 2015).

In conclusion, a functional link between glycogenolytically derived ATP and force production via t-system repriming period, which is coupled to Na⁺,K⁺-ATPase activity, has been demonstrated. This strongly supports compartmentalised energy systems within the muscle fibre, with glycogen playing a key role supporting Na⁺,K⁺-ATPases via the glycogenolytic-glycolytic pathways.

Perspectives

There is a tight coupling between ATP utilisation and ATP resynthesis in a feedback system with increases in ATP utilisation, often stimulating an increase in ATP resynthesis. However, we have recently proposed that energy consumption and production are coupled with a feedforward mechanism, i.e. changes in ATP resynthesis lead to changes in ATP utilisation (Ørtenblad & Nielsen, 2015), as demonstrated by a tight coupling between muscle glycogen content and SR Ca²⁺ release rate. This is thought to be a protective mechanism to avoid significant ATP depletion followed by irreversible deficits in muscle function. In this study we demonstrate that the Na⁺,K⁺-ATPases are well-suited to be part of this mechanism, because of their reliance on ATP being readily available, which makes them sensitive to changes in energy status. In addition, because the Na⁺,K⁺-ATPases regulate membrane potential, they essentially regulate Ca²⁺ release from the sarcoplasmic reticulum and in turn ATP utilisation by the myosin and Ca²⁺ ATPases. Therefore, the tight coupling between this process is important for maintaining muscle function during glycolytic inhibition.
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**Additional information**

**Competing interests**

The authors declare that they have no competing interests.

**Author contributions**

The experiments were performed at the Department of Sports Science and Clinical Biomechanics, University of Southern Denmark. All authors contributed to the conception and design of the experiments, and analysis of data. RJ and NØ did the data collection. All authors contributed to the interpretation of data, drafting and/or revision of the manuscript, and approval of the final version of the manuscript. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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### Tables

**Table 1: Chemicals used in the solutions for the single fibre experiments.**

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Stimulation</th>
<th>0 K⁺</th>
<th>0 Na⁺</th>
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<th>Activation</th>
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<td>90</td>
<td>90</td>
<td>90</td>
<td>90</td>
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<tr>
<td>HDTA</td>
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<td>50</td>
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<tr>
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<td>0</td>
<td>142</td>
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</table>

All values are in mM. [ATP]=8 mM, [PCr]=10 mM, [Mg²⁺]_{free}=1 mM, and pH=7.10 in all solutions. *0.050 mM was used for the repriming protocol.
Figure 1: Concentration-inhibition relation using DAB (n = 30) and CP (n = 30). Values are normalised to the mean glycogen phosphorylase activity in homogenate without glycogen phosphorylase inhibitor.
Figure 2: The effect of glycogen phosphorylase inhibition (2 mM DAB or 10 µM CP) and partial depolarisation on tetanic and twitch forces A) Representative recorded force trace. Blocks of twitches before two tetanic contractions followed by a twitch are shown in well-polarised, depolarised and well-polarised (again) conditions: Stimulations without glycogen phosphorylase inhibition, with glycogen phosphorylase inhibition (DAB or CP), 10-minute wash-out period with twitch stimulations every 30 seconds, and after wash-out, B-E) Mean (SD) relative force following electrical stimulation. B+D) Effect of DAB (n = 8) or CP (n = 18) on (B) tetanic or (D) twitch force. C+E) Effect of partial depolarisation (Dep., n = 31) and the combined effect of partial depolarisation and DAB (n = 11) or CP (n = 19) on (C) tetanic or (E) twitch force. *P < 0.001 vs. vehicle; # P < 0.001 vs. Dep.; †P = 0.03 vs. DAB; ‡P = 0.02 vs. DAB, Dep.
Figure 3: Repriming period in well- and partly depolarised fibres with and without glycogen phosphorylase inhibition (2 mM DAB or 10 µM CP). A) Representative recorded force trace. When the interspacing period between two electrical stimulations is sufficient, force increases markedly (in B, another, but similar, protocol was used). B-E) Normal distribution curves of repriming periods with different treatments. Vertical dashed lines illustrate the apex of the normal distribution on the x-axes. B) repriming period in solutions with various [Na⁺]. Reduced [Na⁺] reduces Na⁺,K⁺-ATPase activity. Numbers indicate the [Na⁺] (in mM) used (n = 12 for [Na⁺] = 36 and 12, n = 6 for [Na⁺] = 4 and 2), C) comparison of control fibres (Con, n = 35) and fibres with glycogen phosphorylase inhibition (Inh) (DAB (n = 15) and CP (n = 22) pooled), D) Comparison of Con and partly depolarised fibres (Dep, n = 26), E) comparison of Dep and Dep with glycogen phosphorylase inhibition (Dep+Inh) (DAB (n = 5) and CP (n = 12) pooled). *P < 0.05 vs. the other treatment in the figure; †p <0.05 vs. 36 and 12 mM [Na⁺]; ‡p < 0.01 vs. 36 and 12 mM [Na⁺].

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Figure 4: Effect of glycogen phosphorylase inhibition (2 mM DAB (n = 11) or 10 µM CP (n = 19)) compared to vehicle (n = 12) on contractile properties. A) The activation protocol used. The labels on the x-axis indicate the pCa used. Force increases with lower pCa (i.e. higher [Ca$^{2+}$]). The protocol was repeated three times (without inhibitor (control), with inhibitor/vehicle, and after wash-out (wash)) for each fibre. B+C) Effect of glycogen phosphorylase inhibition on B) maximum Ca$^{2+}$ induced force, and C) Ca$^{2+}$ sensitivity (pCa50). *P < 0.001 vs. all other time points; #P < 0.01 vs. all other time points; †P < 0.001 vs. vehicle; ‡P < 0.01 vs. vehicle.
Figure 5: In-vitro whole-muscle stimulation protocol with and without the glycogen phosphorylase inhibitor CP (0.1 mM). A) Mean (SD) force production during tetanic stimulations (400 ms trains at 60 Hz) every 5 seconds (every 5th contraction shown) in vehicle (veh.) and CP muscles (n = 9 in each). B) Muscle glycogen content without stimulations (but otherwise same procedure) pre-incubated without and with CP (Veh. rest and CP rest, respectively), and after electrically elicited tetanic contractions every 5 seconds for 15 minutes (as presented in A) (Veh. stim and CP stim, respectively). *P < 0.001 vs. all other groups.
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