Sensory pathways of muscle phenotypic plasticity: Calcium signalling through CaMKII

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Regulation of CaMKII autophosphorylation by recruitment and muscle type

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Abstract

Calcium/calmodulin-dependent protein kinase II (CaMKII) is activated in skeletal muscle during exercise, but how this activity is regulated by the pattern of muscle recruitment is unknown. As this likely has implications for its function, we investigated if CaMKII activation depends on recruitment frequency and what the time course of its activity is after muscle stimulation, using *in situ* stimulation of adult rat skeletal muscle. Protein levels of total CaMKII and phospho^{Thr287}-CaMKII were measured for the β_M, δ_A & δ_D/γ_B isoforms. In the first experiment, *m. soleus* (SOL) and *m. gastrocnemius medialis* (GM) were stimulated at active slack length with 100 electrical impulses at 10 or 150 Hz. This protocol increased phospho^{Thr287}-CaMKII of δ_A ($p=0.05$) and δ_D/γ_B ($p=0.05$) isoforms in red GM (GMR), δ_A isoform ($p=0.043$) in white GM (GMW), but none of the isoforms in SOL. There was no effect of frequency on the level of phospho^{Thr287}-CaMKII. In GMW, we observed a decrease in total CaMKII after 150 Hz stimulation in two isoforms (β_M: $p=0.03$, δ_D/γ_B: $p=0.03$). In the second experiment, we sampled GM at rest, after determination of muscle optimum length and at 2, 10 or 60 minutes following a two-minute isometric contraction protocol. After the contractile activity required to determine the optimum length, the level of phospho^{Thr287}-CaMKII for the δ_A isoform tended to be increased compared to rest in red GM. Levels of phospho^{Thr287}-CaMKII were higher in GMW compared to GMR regardless of stimulation (β_M: $p<0.01$, δ_A: $p<0.01$, δ_D/γ_B: $p=0.01$). In white GM, the level of total CaMKII decreased over time after stimulation (β_M: $p<0.01$, δ_A: $p=0.04$, δ_D/γ_B: $p=0.04$). Our results suggest that phospho^{Thr287}-CaMKII is increased after stimulation with 100 twitches, but that this phosphorylation is only detectable within 15 minutes after contraction.

Introduction

Calcium/calmodulin-dependent protein kinase II (CaMKII) has been suggested to regulate gene expression, calcium channels and glucose transport in skeletal muscle (Tavi et al., 2003, Wright et al., 2007, Witczak et al., 2010). The regulation of its activation likely determines in which context these functions are relevant. For example, if CaMKII is more strongly activated at high, compared to low, recruitment frequencies, it is likely to be more important for muscle function during high frequency muscle recruitment and muscle adaptation to such recruitment. CaMKII is activated by binding of calcium/calmodulin (Ca^{2+}/CaM) (Hudmon and Schulman, 2002), and therefore, availability of Ca^{2+}/CaM determines whether CaMKII is activated. It has been shown that CaMKII can be activated/phosphorylated in human skeletal muscle by exercise (Rose et al., 2006) and in rat fast-twitch muscle by *in situ* electrical stimulation (Rose et al., 2007a). However, if and how this activation of CaMKII is regulated by the specific recruitment pattern is unknown.

Following binding of Ca^{2+}/CaM, CaMKII can undergo autophosphorylation at Thr287 and gain Ca^{2+}/CaM-independent activity. *In vitro*, it has been shown that the generation of Ca^{2+}/CaM-independent, or autonomous, CaMKII activity depends on the frequency, amplitude and duration of Ca^{2+}/CaM pulses (De Koninck and Schulman, 1998). Motor neuron firing rate is modulated during locomotor tasks *in vivo*, with slow-twitch motor neurons firing between 5-40 Hz and fast-twitch motor neurons firing at 60-300 Hz, depending on the specific locomotor task (Hennig and Lomo, 1985, Gorassini et al., 2000). These motor unit recruitment frequencies are coupled to different frequencies of calcium release from the sarcoplasmic reticulum and/or differences in cytoplasmic calcium concentration changes (Westerblad and Allen, 1993), and it has been suggested that there is a positive relation between the recruitment frequency of muscle fibres and the amount of CaMKII activity developed during the period of muscle recruitement (Chin, 2005).

This idea is supported by the finding that high (80% $VO2_{max}$), but not low (40% $VO2_{max}$) intensity cycling exercise increased phosphorylation of CaMKII at Thr287 immediately after exercise (Egan et al., 2010). Presumably, the higher muscle power output required for cycling at 80% $VO2_{max}$ was at least in part due to increased motor unit firing frequencies. However, when higher power outputs are required, it is

likely that a larger number of motor units is recruited (MacIntosh et al., 2000, Beltman et al., 2004) and therefore a different percentage of muscle fibres may be active at the site of the biopsy compared to the low intensity condition, which could affect the outcome of such studies.

Of further importance is whether CaMKII activity remains elevated after exercise or is turned off. Autonomous CaMKII activity could theoretically outlast the calcium signal, but even during continued electrical stimulation this activity can be reduced within seven minutes in skeletal muscle (Rose et al., 2007a). A high-intensity cycling protocol resulted in increased levels of phospho^{Thr287}-CaMKII in *m. vastus lateralis* "immediately" after exercise, but these levels had returned to the pre-exercise values at three hours post-exercise (Egan et al., 2010, Benziane et al., 2008). It is unknown how long CaMKII phosphorylation remained elevated after exercise in these studies.

Finally, the consequences of the differences in calcium kinetics and concentration changes between slow- and fast-twitch muscles are unknown. Calcium transients in fast-twitch muscle rise faster, have a higher amplitude (20 µM vs. 10 µM) and decay faster (Baylor and Hollingworth, 2003). It has been hypothesised that, due to the slow kinetics of CaMKII activation relative to those of calcium concentration changes, the difference between slow- and fast-twitch fibres would not have significant effects on CaMKII signalling (Tavi and Westerblad, 2011). However, this has not been tested experimentally.

To investigate whether CaMKII acts as a 'decoder' of calcium signals in skeletal muscle, we kept *in situ* stimulated muscles below slack length (i.e. left of the ascending limb of the force-length relationship) to uncouple the calcium influx from force production. We investigated whether CaMKII was differentially phosphorylated at Thr287 after high and low frequency stimulation. Because it is not known if the different CaMKII isoforms are differently activated by muscle stimulation, we analysed their phosphorylation separately. We stimulated three separate muscle compartments, slow-twitch *m. soleus* (SOL) and fast-twitch oxidative (GMR) and glycolytic (GMW) *m.gastrocnemius* in our experiments to assess whether effects of stimulation differ between different fibre types. To investigate whether phosphorylation of the CaMKII isoforms remains elevated after muscle stimulation, we measured its time course after

repeated tetanic contractions, which is thought to further activate CaMKII in rat GM (Rose et al., 2007a)

We hypothesized that
1) Phospho^{Thr287}-CaMKII levels are higher after stimulation with pulses at 150 Hz compared to stimulation with the same number of pulses at 10 Hz.
2) Phospho^{Thr287}-CaMKII levels would increase more in the low oxidative GMW compared to high-oxidative GMR and SOL.
3) Phospho^{Thr287}-CaMKII levels are increased in stimulated muscle compared to non-stimulated muscle at 10 minutes, but not at 60 minutes after the end of stimulation.

Methods

Animals

Three month-old female Wistar rats (Harlan Laboratories; two-minute isometric contraction experiment: 191-230 grams, n=20; slack contraction experiment: 205-220 grams, n=12) were anaesthetised initially by intraperitoneal injections of 1.2 ml/100 gram body weight of 12.5% urethane. Ear and foot reflexes were tested to check whether the animal was sufficiently anaesthetised. Subsequent injections of 0.3-0.5 ml, up to a maximum of 1.5 ml, were given every 10 minutes afterwards until reflexes had disappeared. Rats were kept on a heated pad (±37 ºC) to prevent hypothermia.

Muscle-tendon preparation

Hind limbs were shaved and skin was removed, after which *m. gastrocnemius medialis* (GM) and *m. soleus* (SOL) were exposed and mechanically isolated by removing as much as possible the myofascial connections to surrounding muscles. Blood supply to and nerve innervations of SOL and GM were kept intact. The calcaneus was cut from the talus, while still attached to the Achilles tendon. The sciatic nerve was dissected free, proximally severed and electrically stimulated through an external electrode controlled by a computer (50µs rectangular pulses).

Contraction protocols

Effect of low and high frequency stimulation at short muscle length on phospho^{Thr287}-CaMKII levels

Experiments were performed at room temperature (23ºC). Muscles were kept below slack length because the used different stimulation frequencies would result in different muscle forces and energy consumption patterns if the muscles were kept at optimum length. Rats were divided into two groups: 1) GM and SOL stimulated at 10 Hz. 2) GM and SOL stimulated at 150 Hz stimulation. Muscles of the right leg were stimulated. As CaMKII is phosphorylated within seconds after the start of contraction (Rose et al., 2007a) both groups received a total of 100 pulses (stimulation current: 3mA; pulse duration: 50 µs). The total duration of stimulation was 10 seconds for the 10 Hz group and 0.67 seconds for the 150 Hz group). After stimulation, GM and SOL were dissected as rapidly as possible and snap-frozen in liquid nitrogen. The first muscle was typically sampled after 1 minute, the second after 2 minutes. In half of the experiments, SOL was sampled first, and in the other half GM was sampled first. Subsequently, the non-stimulated left muscles were dissected and snap-frozen. Rats were killed by intracardial injection of Euthasol® while fully anaesthetised. Muscles were stored at -80°C until used for western blot analysis.

Time course of CaMKII phosphorylation after muscle contractions

The temperature of GM was kept at approximately 35°C using an envelope into which warm water vapour was sprayed. The Achilles tendon was tied to a Kevlar thread, which was subsequently attached to a force transducer. Force data were sampled at a frequency of 1000 Hz. Optimum muscle length (the length of the muscle-tendon complex at which maximum tetanic force was produced) was estimated using twitches, and subsequently determined using 3-4 tetanic contractions. GM rested for two minutes between each tetanic contraction and for 15 minutes after determination of optimum length. A stimulation protocol consisting of a series of 24 pulse trains was then applied (stimulation current: 3mA; pulse duration: 50 µs), resulting in tetanic maximal isometric contractions (stimulation frequency: 150Hz; 1 train every 5 s; train duration: 200 ms).

This protocol resulted in a decrease in maximal tetanic force of approximately 30% (Fig. 1)

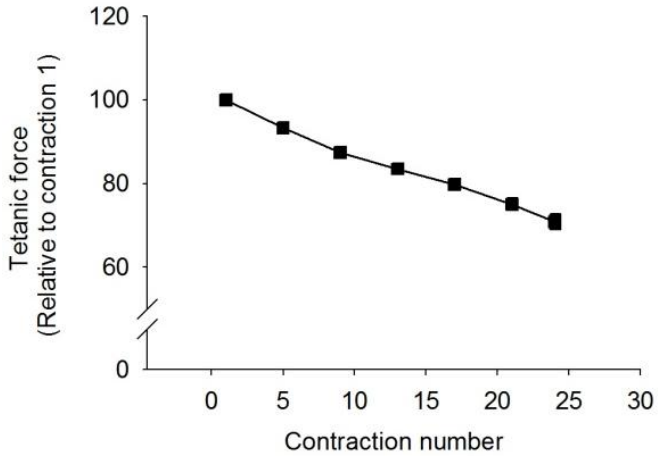

Figure 1: Decrease in tetanic force during the 'two-minute isometric' contraction protocol
Data are displayed as means ±SEM. Note that the error bars are barely visible because they are often smaller than the symbols indicating the mean relative titanic force.

Five groups of muscles were defined by the time point of sampling: 1) resting; 2) optimum length determination (L_{OPT}; 3) two minutes after the contraction protocol; 4) 10 minutes after the contraction protocol; 5) 60 minutes after the contraction protocol (Fig. 2). At the defined time points, GM was dissected and snap-frozen in liquid nitrogen. Animals were killed by intra-cardiac injection of Euthasol®, while still fully anaesthetised. Frozen muscles were stored at -80ºC until used for western blotting.

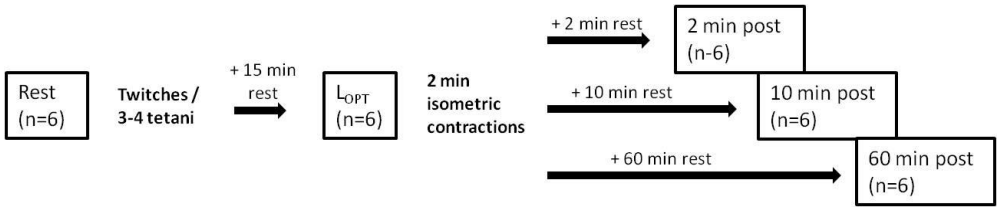

Figure 2: Experimental design of the 'two-minute isometric' contraction protocol.
Left and right *m. gastrocnemius medialis* of the animals were randomly assigned to one of five groups: rest, L_{OPT}, 2 min post, 10 min post or 60 min post. The imposed stimulation and periods of rest following stimulation are described in this figure. Details of the '2 min isometric contractions' protocol can be found in the methods section. At the end of the prescribed stimulation protocol/resting period, the muscles were dissected and frozen in liquid nitrogen.

Protein analysis

Frozen muscle was cut into 25 µm thick cross-sections in a cryostat, which were collected in a 2 ml tube and kept at -20ºC. Chemicals were obtained from Sigma-Aldrich (Poole, United Kingdom) unless stated otherwise. Ice-cold RIPA buffer (50mM TRIS-HCl (pH 7.5), 150mM NaCl, 1mM EDTA, 1% v/v Non-Idet P40 substitute, 0.25% w/v sodium deoxycholate plus freshly added protease/phosphatase inhibitors: 1 mM NaF, 1 mM Na_3VO_4, 0.1 mM PMSF, 1µg/ml leupeptin, 0.2 µg /ml pepstatin, 0.1 µg/ml aprotinin) was then added and sections were homogenised on ice using a Polytron homogeniser (Kinematica AG, Luzern, Switzerland). Crude homogenates were aspirated 5-10 times through a 0.8 mm syringe needle, and stored at -80ºC until use for analysis. An aliquot of the aspirated homogenate was taken for determination of protein concentration with the bicinchoninic acid protein assay (Pierce, Rockford IL, USA).

Protein levels of total CaMKII, phospho^{Thr287}-CaMKII and cytochrome-c oxidase subunit IV (COXIV) were analysed by western blotting followed by immunodetection. Homogenates were denatured by addition of sample buffer (final concentration: 50mM TRIS-HCl (pH 6.8), 2% w/v SDS, 10% v/v glycerol, 2% β-mercaptoethanol) and 5 minutes heating at 95ºC. 20 µg of protein was separated by SDS-PAGE and transferred overnight onto a nitrocellulose membrane (GE Healthcare Life Sciences, Little Chalfont, United Kingdom), while transfer buffer was cooled in ice. Membranes were stained with Ponceau S solution to confirm equal protein loading and transfer. The stain was washed off with TRIS-buffered saline with tween-20 (TBS-T; 20mM TRIS-HCl (pH 7.5), 0.9% w/v NaCl, 0.05% Tween-20) and the membrane was blocked in antibody incubation solution, followed by incubation with a primary antibody for either pan-CaMKII (BD Bioscience, #611292, dilution: 1/2500), phospho^{Thr287}-CaMKII (Cell Signalling Technology #3361, dilution: 1/1000) or COXIV (Cell Signalling Technology, #4850, dilution 1/2000) for two hours. Antibody incubation solutions were 5% milk in TBS-T (for pan-CaMKII) or 5% bovine serum albumin (BSA) in TBS-T (for phospho^{Thr287}-CaMKII). Residual antibody was washed away with 4 washes of 5 minutes in TBS-T, followed by incubation with species-specific horseradish peroxidase-conjugated secondary antibodies. Antibodies were detected with an enhanced chemiluminescence kit (Pierce, Rockford IL, USA). Light signals were captured with a ChemiDoc XRS system (Biorad, Hemel Hempstead, United Kingdom).

Samples from all experimental groups from the *in situ* contraction experiments were analysed on the same blot, so direct comparisons of protein expression could be made. Protein bands were quantified with Quantity One version 4.6.8 (Biorad). Background-corrected band intensities on each blot were normalized to the sum of the intensities of the same band in all lanes to correct for differences in overall staining intensity of the blots. The obtained values were related to the mean of the 'rest' group.

In vitro CaMKII phosphorylation

To ensure specificity of the phospho^{Thr287}-CaMKII antibody in our setup and to detect calcium/calmodulin-sensitive protein bands, CaMKII was phosphorylated *in vitro* by incubating 5 µl of crude muscle homogenate with a reaction mix containing calcium/calmodulin (Enzo Life Sciences, Exeter, United Kingdom) (total volume: 50 µl; 0.1 mM ATP, 10 mM HEPES (pH 7.4), 5 mM $MgCl_2$, 0.1% Tween-20 and 0.5 mM $CaCl_2$ + 1 µM calmodulin or 5 mM EGTA) for 30 minutes at 30ºC. The reaction was stopped by the addition of 16.7 µl 4x sample buffer. Samples were heated to 95 ºC and proteins were separated with SDS-PAGE on a 15% acrylamide (Biorad) gel and subjected to western blotting as described. Based on data from (Bayer et al., 1998), we identified the detected CaMKII bands as β_M (72 kDa), δ_A (60 kDa) and a combination of δ_D and γ_B (58 kDa).

Muscle compartments

Red and white portions of GM (GMR and GMW, respectively), which contains compartmentalised fibres with high or low oxidative capacity (De Ruiter et al., 1995), were analysed separately. Tissue from GMR or GMW was obtained by cutting cryosections from the proximal or distal end of the frozen muscle, respectively.

Statistics

Slack contraction protocol

The effect of stimulation on total and phospho^{Thr287}-CaMKII levels was tested with factorial ANOVAs (stimulation x frequency), with repeated measures on the factor

stimulation. The effect of stimulation frequency was tested by assessing the interaction between stimulation and frequency. These tests were performed separately for GMR, GMW and SOL. We expected phospho^{Thr287}-CaMKII to increase in stimulated muscles (Rose et al., 2007a). Therefore, one-sided post-hoc tests were carried out to analyse the effect of stimulation on phospho^{Thr287}-CaMKII. Comparisons between muscles stimulated at a specific frequency and their intra-animal resting controls were made with Wilcoxon signed ranks tests

Two-minute isometric contraction protocol.
The effect of stimulation on total and phospho^{Thr287}-CaMKII levels was tested with Kruskal-Wallis ANOVAs for GMR and GMW. Mann-Whitney U-tests were used to test differences between individual time points within one compartment and Wilcoxon signed ranks tests were used to test differences between compartments at one specific time point. Correlations between COXIV and phospho^{Thr287}-CaMKII were tested with Spearmon's Rho. Significance level was set at $p \leq 0.05$.

Results

The acute effects of stimulation frequency on total and phospho^{Thr287}-CaMKII in GM and SOL

We investigated CaMKII phosphorylation in GMR, GMW and SOL after stimulation with 100 pulses at 10 or 150 Hz. In GMR, we observed a significant increase in phospho^{Thr287}-CaMKII for the δ_A and δ_D/γ_B, but not for the β_M isoform (β_M: p=0.066; δ_A: p=0.048; δ_D/γ_B: p= 0.050). In GMW, we observed a significant increase in δ_A only (β_M: p=0.114; δ_A: p=0.043; δ_D/γ_B: p= 0.447), whereas in SOL none of the phospho^{Thr287}-CaMKII isoforms was significantly increased (Fig. 3). We did not observe an interaction effect between stimulation and frequency on phospho^{Thr287}-CaMKII in GMR, GMW or SOL.

Surprisingly, we observed a decrease in total CaMKII in GMW after 150 Hz stimulation (Wilcoxon signed ranks test; β_M: p=0.03, δ_A: p=0.44, δ_D/γ_B: p=0.03), but not after 10 Hz

stimulation (stimulation x frequency interaction: β_M: p=0.03, δ_A: p=0.22, δ_D/γ_B: p=0.03) (Fig. 4). Such a decrease was not observed in GMR or SOL (Fig. 4).

For all muscles, there was no difference in actin levels on the blots between resting and stimulated samples (GMR: p=0.21; GMW: p=0.22; SOL: p=0.40) and no statistical interaction effect between stimulation and frequency on actin levels (GMR: p=0.47; GMW: p=0.72; SOL: p=0.52) (Appendix, Fig. A1).

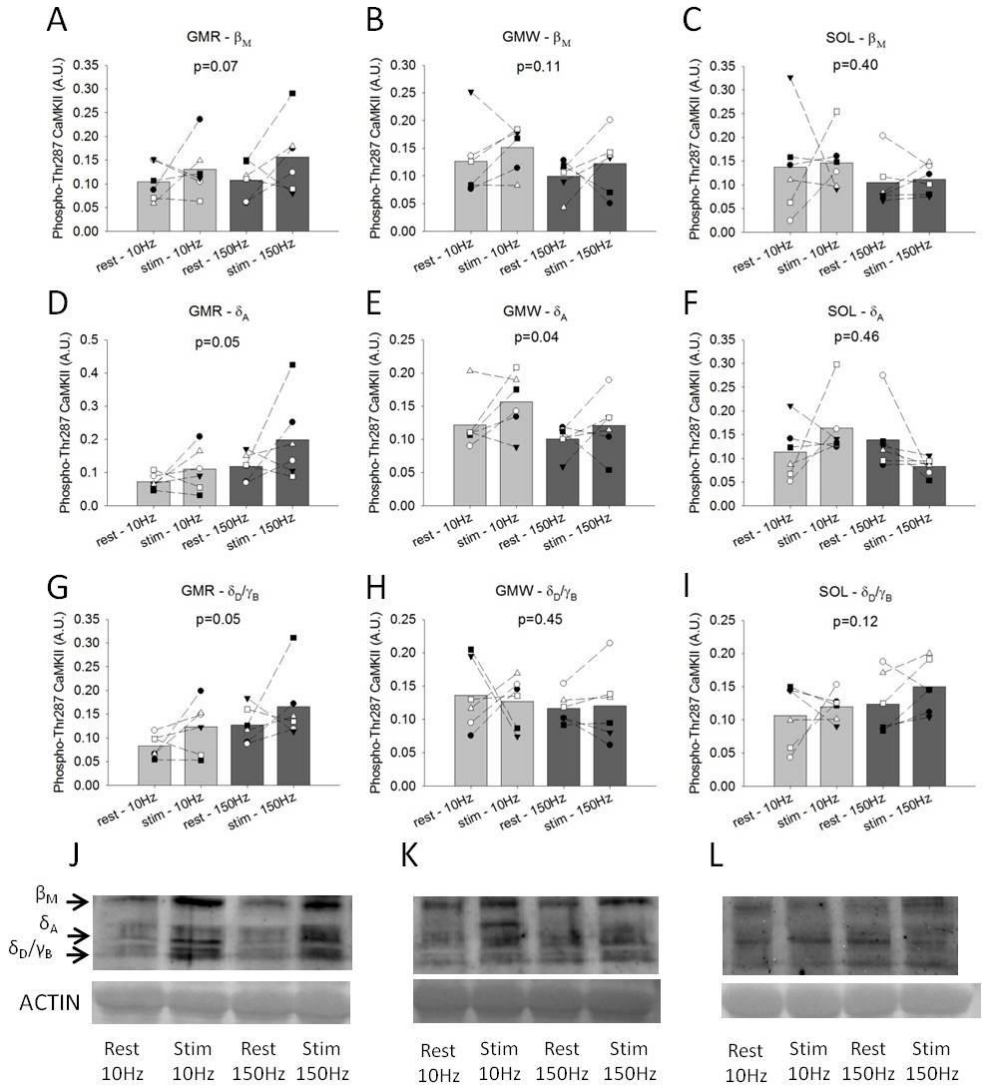

Figure 3: Phospho^{Thr287}-CaMKII levels in rat *m. gastrocnemius medialis* and *m. soleus* samples after slack contractions.

Graphs display phospho^{Thr287}-CaMKII levels for β_M, δ_A and δ_D/γ_B as determined by western blotting followed by immunodetection. A: GMR, β_M-CaMKII; B: GMW, β_M-CaMKII; C: SOL, β_M-CaMKII; D: GMR, δ_A-CaMKII; E: GMW, δ_A-CaMKII; F: SOL, δ_A-CaMKII; G: GMR, δ_D/γ_B-CaMKII; H: GMW, δ_D/γ_B-CaMKII; I: SOL, δ_D/γ_B-CaMKII. Bars represent mean phospho^{Thr287}-CaMKII levels and symbols represent the amount of phospho^{Thr287}-CaMKII in individual muscles. Same symbols represent muscles from the same animal within one frequency group. Muscles connected by a dashed line are intra-animal pairs. Note the different scales of different graphs. GMR = High oxidative, (red) *m. gastrocnemius medialis*. GMW = low oxidative (white) *m. gastrocnemius medialis*. SOL = *m. soleus*. Rest- xxHz denotes non-stimulated muscle, contra-

lateral to the muscle stimulated at xx Hz which is denoted by Stim – xxHz. Significance of stimulation effect is indicated in each graph. A.U. = arbitrary units. Examples of phospho^{Thr287}-CaMKII immunoblots and poncesu s-stained actin bands are shown below the graphs. J: GMR; K: GMW; L: SOL.

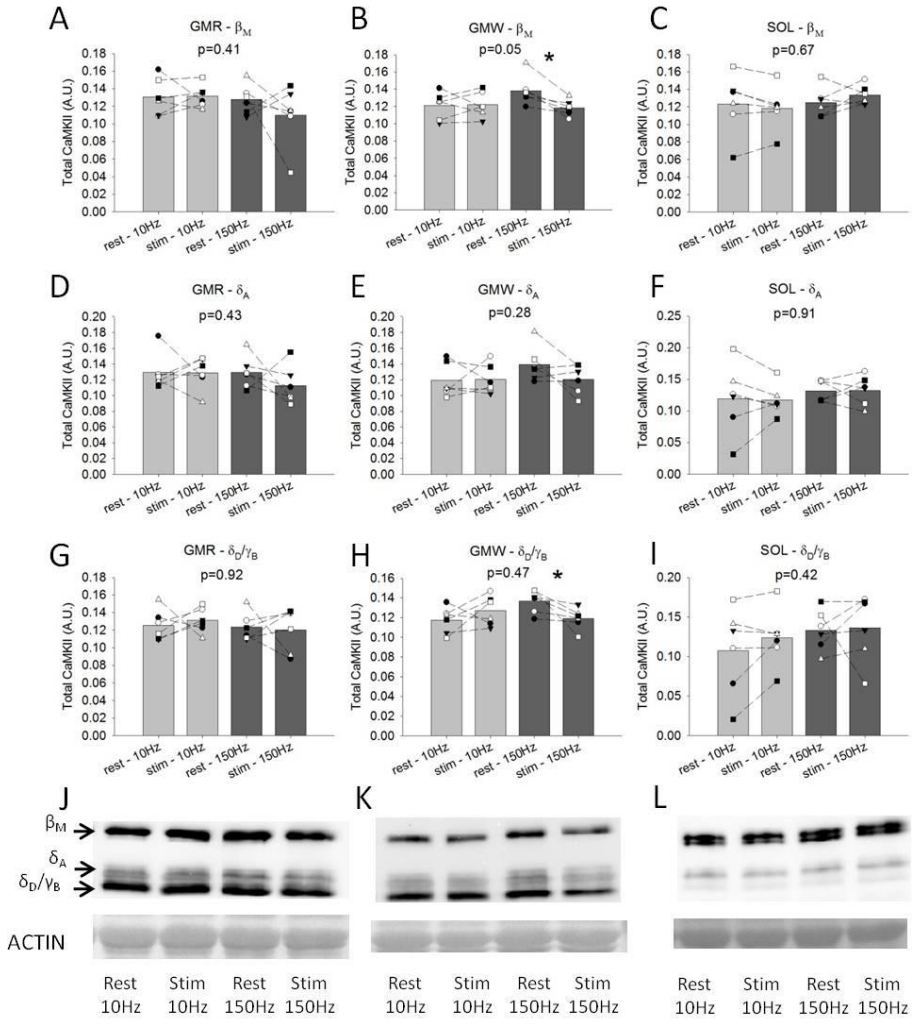

Figure 4: Decrease in total CaMKI in rat white *m. gastrocnemius medialis* after slack contractions.

Graphs display total CaMKII levels for β_M, δ_A and δ_D/γ_B as determined by western blotting followed by immunodetection. A: GMR, β_M-CaMKII; B: GMW, β_M-CaMKII; C: SOL, β_M-CaMKII; D: GMR, δ_A-CaMKII; E: GMW, δ_A-CaMKII; F: SOL, δ_A-CaMKII; G: GMR, δ_D/γ_B-CaMKII; H: GMW, δ_D/γ_B-CaMKII; I: SOL, δ_D/γ_B-CaMKII. Bars represent mean total CaMKII

levels and symbols represent the amount of total CaMKII in individual muscles. Same symbols represent muscles from the same animal within one frequency group. Muscles connected by a dashed line are intra-animal pairs. Note the different scales of different graphs. GMR = High oxidative, (red) *m. gastrocnemius medialis*. GMW = low oxidative (white) *m. gastrocnemius medialis*. SOL = *m. soleus*. Rest- xxHz denotes non-stimulated muscle, contra-lateral to the muscle stimulated at xx Hz which is denoted by Stim – xxHz. Significance of stimulation effect is indicated in each graph. A.U. = arbitrary units. Examples of total CaMKII immunoblots and ponceau s-stained actin bands are shown below the graphs. J: GMR; K: GMW; L: SOL.

The effect of two-minute isometric contractions on total and phospho^{Thr287}-CaMKII in red and white GM compartments

We investigated CaMKII phosphorylation in the two compartments of GM after a two-minute isometric protocol. For both compartments, we did not observe a significant main effect of stimulation on phospho^{Thr287}-CaMKII for any of the isoforms (GMR: β_M: p=0.33, δ_A: p=0.09, δ_D/γ_B: p=0.157; GMW: β_M: p=0.91, δ_A: p=0.75, δ_D/γ_B: p=0.27). When the mean phospho^{Thr287}-CaMKII levels at the time points at which these were highest for each CaMKII isoform were compared to phospho^{Thr287}-CaMKII levels in the resting muscles, we only observed a trend for an increase in the δ_A isoform in GMR after the contractile activity necessary to determine the optimum length (Fig. 5). 60 Minutes after stimulation, phospho^{Thr287}-CaMKII levels tended to be decreased for β_M and δ_A isoforms compared to the time point at which mean phospho^{Thr287}-CaMKII levels were highest (Fig. 5). No significant differences in phospho^{Thr287}-CaMKII between individual time points were observed for any of the isoforms in GMW.

All isoforms showed higher phospho^{Thr287}-CaMKII levels in rested GMW compared to rested GMR (Main effect of compartment: β_M: p<0.01, δ_A: p<0.01, δ_D/γ_B: p=0.01) (Fig. 5). This difference was significant in the resting muscles for the β_M (p=0.02) and δ_A (p=0.03) isoforms, and was associated with a higher aerobic capacity in GMR, as reflected by a higher COXIV protein expression in GMR (p=0.03; Fig. 6). There was no difference in total CaMKII in rested muscles (Fig. 7).

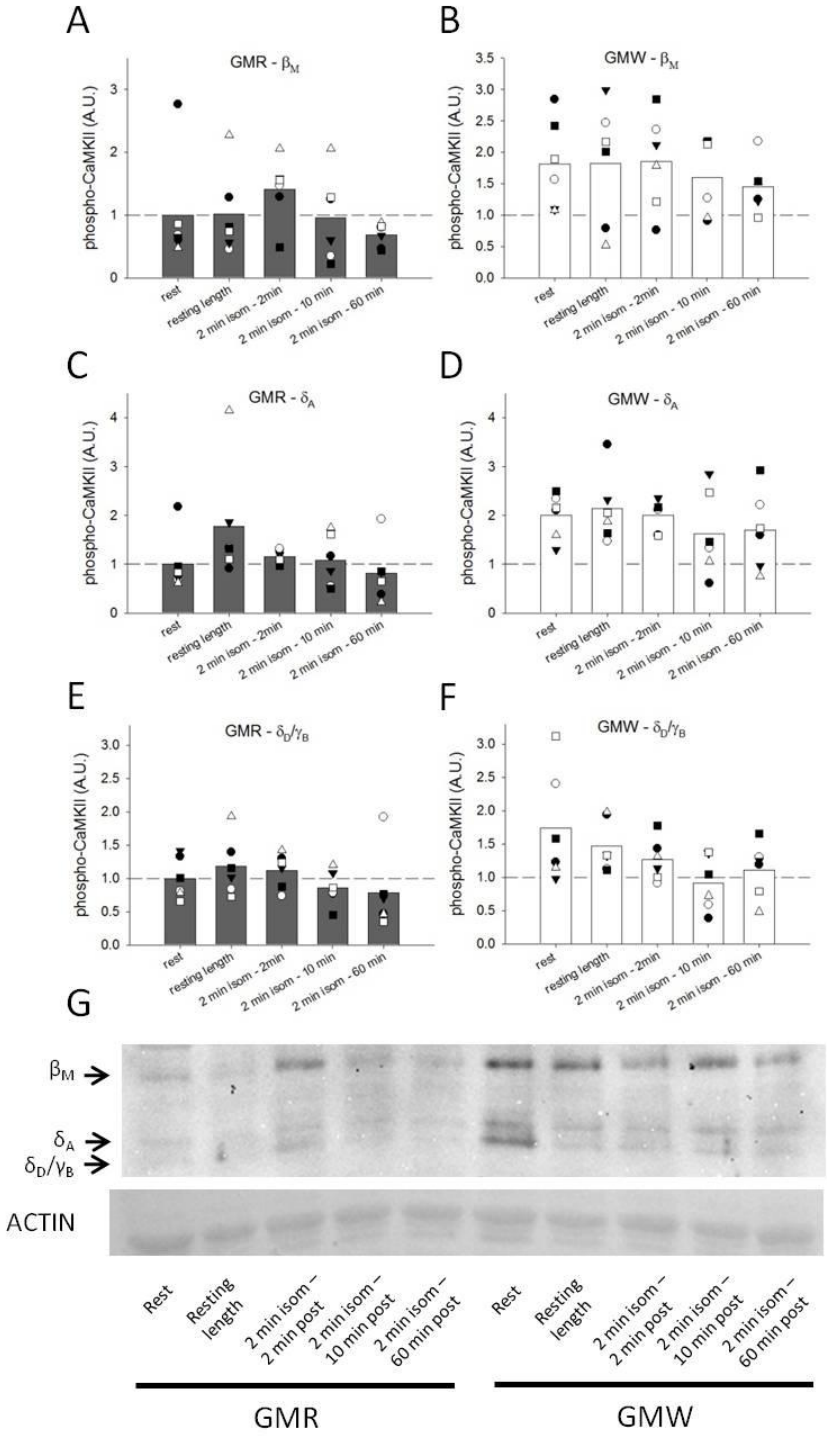
A
GMR - β_M
phospho-CaMKII (A.U.)
B
GMW - β_M
C
GMR - δ_A
D
GMW - δ_A
E
GMR - δ_D/γ_B
F
GMW - δ_D/γ_B
rest
resting length
2 min isom - 2min
2 min isom - 10 min
2 min isom - 60 min
G
β_M
δ_A
δ_D/γ_B
ACTIN
Rest
Resting length
2 min isom – 2 min post
2 min isom – 10 min post
2 min isom – 60 min post
GMR
GMW

Figure 5: Phospho^{Thr287}-CaMKII in rat *m. gastrocnemius medialis* samples before and after a two-minute isometric contraction protocol.

Graphs display phospho^{Thr287}-CaMKII levels as determined by western blotting followed by immunodetection. A: GMR, β_M-CaMKII; B: GMW, β_M-CaMKII; C: GMR, δ_A-CaMKII; D: GMW, δ_A-CaMKII. E: GMR, δ_D/γ_B-CaMKII; F: GMW, δ_D/γ_B-CaMKII. Bars represent mean phospho^{Thr287}-CaMKII levels and symbols represent the amount of phospho^{Thr287}-CaMKII in individual samples. Amounts are relative to the mean of the resting GMR samples. The dashed line represents the mean level of phospho^{Thr287}-CaMKII in the resting GMR samples.G: Examples of immunoblot for phospho^{Thr287}-CaMKII in GMR and GMW, and ponceau s-stained actin bands. A.U. = arbitrary units. GMR = High oxidative (red) *m. gastrocnemius medialis*. GMW = low oxidative (white) *m. gastrocnemius medialis*.

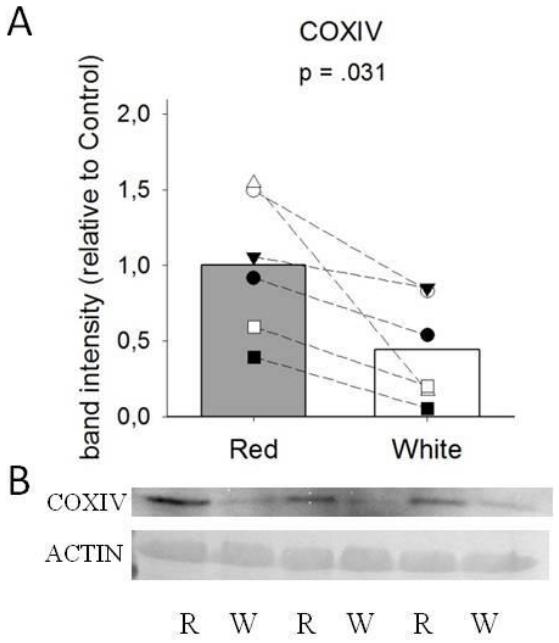

Figure 6: COXIV protein expression in oxidative and glycolytic *m. gastrocnemius*

A: Graph displays COXIV levels as determined by western blotting followed by immunodetection. Bars represent mean COXIV levels and symbols represent the amount of COXIV in individual samples. Muscles connected by a dashed line are intra-animal pairs. Significance of the difference between red and white GM is indicated. B: Example COXIV immunoblot is shown below the graph. R = red GM; W = white GM.

Total CaMKII was significantly decreased after stimulation in GMW (Main effect of stimulation: β_M: $p<0.01$, δ_A: $p=0.04$, δ_D/γ_B: $p=0.04$), but not in GMR (β_M: $p=0.13$, δ_A: $p=0.89$, δ_D/γ_B: $p=0.49$) (Fig. 7).

There were no differences in actin levels on the blots (GMR phospho^{Thr287}-CaMKII blots: $p=0.80$; GMR, total CaMKII blots: $p=0.96$; GMW, phospho^{Thr287}-CaMKII blots: $p=0.99$; GMW, total CaMKII blots: $p=0.94$; Appendix, Fig. A2) indicating equal protein loading in all conditions.

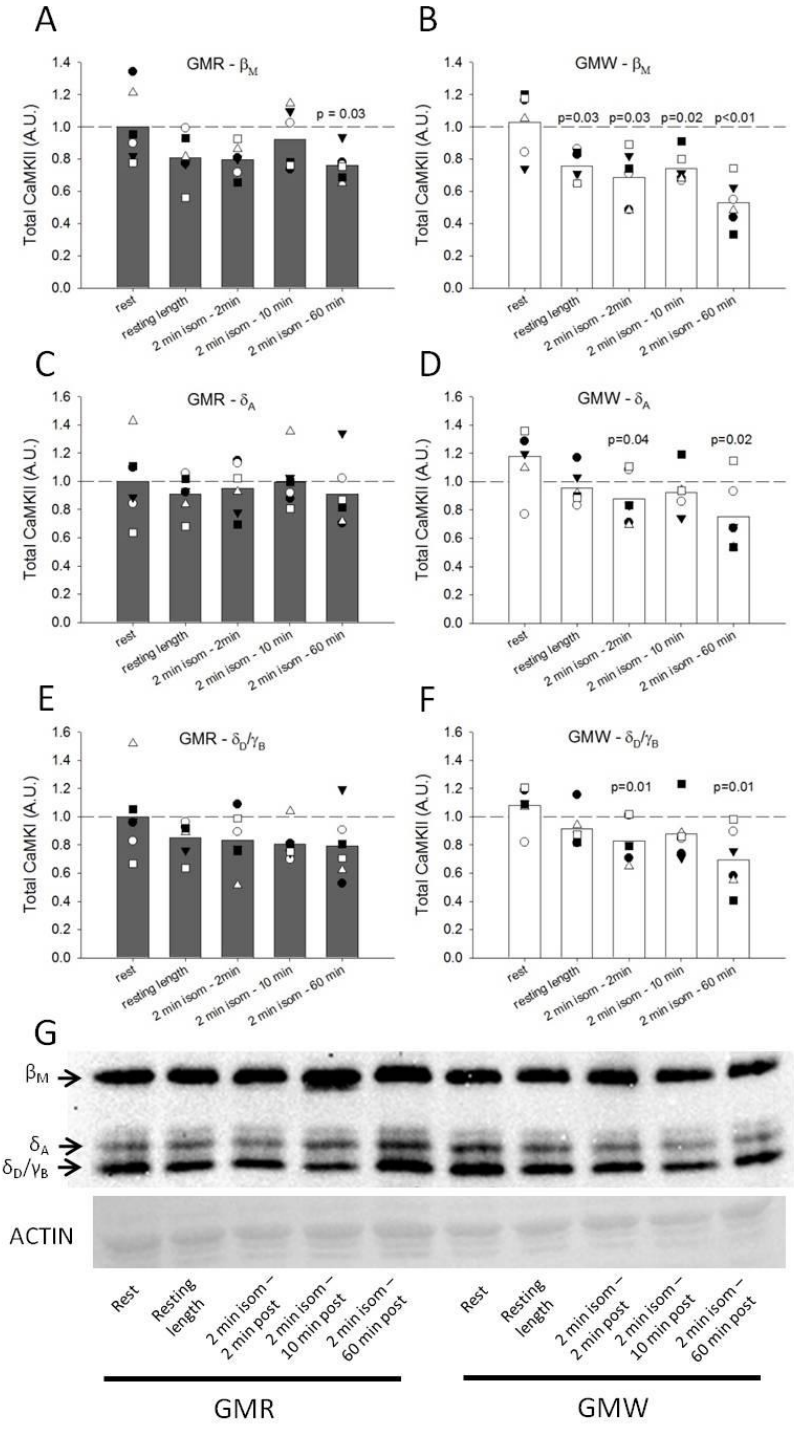
A
GMR - β_M
p = 0.03
B
GMW - β_M
p=0.03 p=0.03 p=0.02 p<0.01
C
GMR - δ_A
D
GMW - δ_A
p=0.04 p=0.02
E
GMR - δ_D/γ_B
F
GMW - δ_D/γ_B
p=0.01 p=0.01
Total CaMKII (A.U.)
rest
resting length
2 min isom - 2min
2 min isom - 10 min
2 min isom - 60 min
G
β_M
δ_A
δ_D/γ_B
ACTIN
Rest
Resting length
2 min isom – 2 min post
2 min isom – 10 min post
2 min isom – 60 min post
GMR
GMW

Figure 7: Total CaMKII in rat *m. gastrocnemius medialis* samples before and after a two-minute isometric contraction protocol.

Graphs display total CaMKII levels as determined by western blotting followed by immunodetection. A: GMR, β_M-CaMKII; B: GMW, β_M-CaMKII; C: GMR, δ_A-CaMKII; D: GMW, δ_A-CaMKII. E: GMR, δ_D/γ_B-CaMKII; F: GMW, δ_D/γ_B-CaMKII. Bars represent mean total CaMKII levels and symbols represent the amount of total CaMKII in individual samples. Amounts are relative to the mean of the resting GMR samples. The dashed line represents the mean level of total CaMKII in the resting GMR samples. P-values in the figure indicate significance of the difference compared to the 'rest' group. G: Examples of immunoblot for total CaMKII in GMR and GMW and ponceau s-stained actin bands. A.U. = arbitrary units. GMR = High oxidative (red) *m. gastrocnemius medialis*. GMW = low oxidative (white) *m. gastrocnemius medialis*.

Discussion

Main results

Our results suggest that stimulation of GM with 100 twitch pulses can increase autophosphorylation of CaMKII (Fig. 3). However, in contrast to our hypothesis, we did not observe significant differences in phospho^{Thr287}-CaMKII after low and high frequency stimulation (Fig. 3). We observed higher basal phospho-CaMKII levels in GMW compared to GMR (Fig. 5). Finally, we observed an unexpected decrease in total CaMKII in GMW after high-frequency stimulation (Fig. 4 & Fig. 7).

Technical considerations

After both the 'two minute isometric contraction' protocol and 'slack contraction' protocol we observed a large variability in phospho^{Thr287}-CaMKII levels in SOL and GM compartments (Fig. 3 & Fig. 5). Autophosphorylation of CaMKII at Thr287 occurs only when two Ca^{2+}/CaM-bound subunits interact (Hanson et al., 1994, Rich and Schulman, 1998). Autophosphorylation increases Ca^{2+}/CaM affinity many fold (Meyer et al., 1992) and therefore, small differences in the fraction of autophosphorylated CaMKII subunit could lead to large differences in the rate of autophosphorylation. *In vitro*, it has been demonstrated that the development of autonomous CaMKII activity displays very high sensitivity to changes in [Ca^{2+}] when interacting with protein phosphatase 1 (PP1) (Bradshaw et al., 2003). It may be that variability in intracellular

[Ca^{2+}] in our experiments contributed to the observed variation in phospho^{Thr287}-CaMKII.

Some non-stimulated samples had higher phospho^{Thr287}-CaMKII levels compared to the average level in stimulated samples (Fig. 3 & Fig. 5). This indicates that factors other than muscle stimulation affect phospho^{Thr287}-CaMKII levels in skeletal muscle. Our observation that resting phospho^{Thr287}-CaMKII was higher in GMW compared to that in GMW suggests that the level of phospho^{Thr287}-CaMKII is related to the oxidative capacity of the muscle. However, we did not observe a significant correlation between phospho^{Thr287}-CaMKII and COXIV protein levels within these compartments (GMR: Rho=0.27; GMW: Rho=0.33), suggesting that the variation in phospho^{Thr287}-CaMKII levels cannot be explained by differences in oxidative capacity within the GM compartments.

Auto-phosphorylation of CaMKII at Thr287 is not the only mechanism that regulates the activity of the kinase. Ca^{2+}/CaM-dependent activity may play an important role in intracellular signalling. Furthermore, kinase activation is also regulated by autophosphorylation of Thr306/307. These residues are in the CaM-binding domain and their phosphorylation inhibits CaM binding to this domain (Hanson and Schulman, 1992, Colbran, 1993). Finally, Met281/282 of CaMKII can be oxidized, which is a parallel mechanism of activation to autophoshorylation at Thr287 (Erickson et al., 2008). Although a previous study has found that levels of phospho^{Thr287}-CaMKII and autonomous CaMKII activity correlated quite well in rat skeletal muscle (Rose et al., 2007a), it is possible that the levels of phospho^{Thr287}-CaMKII in our study do not perfectly reflect CaMKII activity.

Implications for CaMKII-signalling in skeletal muscle

We observed lower phospho^{Thr287}-CaMKII levels in the red compared to the white compartment of GM (Fig. 5). Basal phosphorylation levels of mitogen-activated protein kinases p38, p42 and p44, and AMP-activated protein kinase (AMPK) have also been shown to be lower in the ‘red‘, compared to the ‘white’ rat *m. tibialis anterior* compartment (Ljubicic and Hood, 2008). Autophosphorylation at Thr287 increases the affinity of CaMKII for Ca^{2+}/CaM manyfold (Meyer et al., 1992). Therefore, Ca^{2+}-CaM-dependent CaMKII activity in GMW may be sensitised to changes in $[Ca^{2+}]_i$.

CaMKII has been implicated as a regulator of the 'slow oxidative' muscle phenotype (Mu et al., 2007) and mitochondrial biogenesis (Wright et al., 2007). However, slow-twitch *m. soleus* was the only muscle compartment in which none of the CaMKII isoforms displayed increased autophosphorylation in response to the slack contraction protocol. Furthermore, GMW has a higher level of phospho^{Thr287}-CaMKII than GMR (Fig. 5). These results suggest a negative association of CaMKII-signalling with features of the slow-oxidative phenotype. Possibly, the lower amplitude of calcium concentration changes in *m. soleus* compared to *m. gastrocnemius medialis* (Baylor and Hollingworth, 2003) can explain the absence of an increase in CaMKII autophosphorylation in SOL, which could be reflective of the effect of training on acute CaMKII signalling. Similarly, it has been shown that AMPK activation is attenuated in trained skeletal muscle, and that this is associated with a reduction in the activating signal (i.e. the AMP: ATP ratio) after training (McConell et al., 2005). Training-induced changes in muscle phenotype may have a similar effect on CaMKII signalling by altering calcium cycling in skeletal muscle (Green et al., 2003). In contrast to this hypothesis, CaMKII autophosphorylation in human skeletal muscle was not attenuated after a 10 day exercise training program (Benziane et al., 2008). However, this training protocol was possibly not sufficient to induce changes in the expression of calcium-regulatory proteins.

We found no evidence for an effect of motor unit recruitment frequency on phospho^{Thr287}-CaMKII levels (Fig. 3). It is possible that the recruitment frequencies used in this study and those occurring in skeletal muscle *in vivo* are too high to be converted into differing amounts of autonomous kinase activity. *In vitro*, frequency-dependence of CaMKII autophosphorylation was shown for frequencies between 1 and 4 Hz (De Koninck and Schulman, 1998). However, the duration of these pulses was much longer than the duration of a Ca^{2+} pulse in fast- and slow-twitch skeletal muscle fibres (200 ms vs. 50-100 ms (Baylor and Hollingworth, 2003)), and decreasing the duration of the Ca2+-pulse shifted the frequency-autonomous activity relation towards higher frequencies (De Koninck and Schulman, 1998). Thus, although CaMKII might be differentially activated by *in vivo*-relevant muscle recruitment frequencies, this is not exhibited in skeletal muscle after electrical stimulation.

Our data indicate that phospho^{Thr287}-CaMKII decreases during a one-hour time course after cessation of stimulation (Fig. 5). Combining results from the slack

experiments (Fig. 3) and the two-minute isometric contraction protocol (Fig. 5) suggests that increases in phospho^{Thr287}-CaMKII are detectable within the first two minutes after contraction but not after 15 minutes. Different protein phosphatases (PP1, PP2A, PP2C) can de-phosphorylate Thr286 (the alpha-CaMKII homologue of Thr287) in rat forebrain neurons (Strack et al., 1997), and as these are also expressed in skeletal muscle (Cohen, 1989), they may play a role in regulating CaMKII phosphorylation in skeletal muscle.

Muscle phenotype-specific decrease in total CaMKII

In both the slack contraction experiment and the two-minute isometric contractions protocol, total CaMKII was decreased in GMW after stimulation with pulses at 150 Hz, but not with 10 Hz stimulation (Fig. 4 & Fig. 7). The stimulated samples in the slack experiment were always sampled before the resting controls. This suggests the decrease in total CaMKII was due to factors related to stimulation, and were not an artifact of the experimental setup.

This raises the question as to what caused the decrease in total CaMKII in white GM. Given the time frame in which this effect occurred, there are three potential explanations: 1) A post-translational modification of the protein reduced the antigenicity of the CaMKII antibody for its epitope, or binding to the membrane during blotting. 2) Translocation of CaMKII to a non-soluble subcellular fraction, which prevented it from entering the gel during SDS-PAGE. 3) Degradation of CaMKII. The last option cannot explain the acute decrease in total CaMKII observed in GMW during the slack experiment, but degradation of CaMKII could be partially involved in explaining the difference between total CaMKII levels 60 minutes post-stimulation compared to that of resting muscle (Fig. 7).

Currently, no posttranslational modifications of CaMKII are known in the association domain of the protein, which is where our CaMKII antibody binds according the specifications given by the manufacturer (immunogen: rat alpha CaMKII aminoacids 448-460). In neurons, CaMKII has been shown to be able to translocate to different compartments of the cell upon stimulation by Ca^{2+}/CaM (Bayer and Schulman, 2001). However, whether this occurs in muscle, what the target binding sites are, and whether these fractions would not be solubilised in our protein extraction procedure, is unknown. It should be noted that our protocol did solubilise and separate the β_M

isoform, which is targeted to the SR membrane via anchoring protein αKAP (Bayer et al., 1998).

Finally, degradation of CaMKII protein might have occurred. The Ca^{2+}-dependent protease μ-calpain can degrade CaMKII into 30-35 kDa fragments (Hajimohammadreza et al., 1997, Yoshimura et al., 1996), and mice overexpressing the calpain-inhibitor calpastatin have increased CaMKII protein levels (Otani et al., 2006). These studies indicate that CaMKII is subject to degradation by calpains in skeletal muscle. We have found no evidence of immunoreactive bands on our western blots with an approximate molecular weight of 30-35 kDa. It is therefore unclear what caused the observed decrease in total CaMKII in GMW.

Note that in previous experiments in which rat GM was stimulated *in situ* a decrease in total CaMKII was not detected (Rose et al., 2007a). However, in this study red and white GM were not separately analysed, whereas our data suggest that muscle phenotype has a significant effect on the decrease in detected total CaMKII.

Conclusion

Our results suggest that CaMKII can be autophosphorylated by a short contraction protocol, but that this phosphorylation is only detectable within 15 minutes after contraction. Furthermore, other factors than stimulation appear to strongly affect CaMKII phosphorylation. We detected a decrease in total CaMKII after high-frequency stimulation in fast glycolytic muscle. Therefore, investigations into interactions of other factors than Ca^{2+}/CaM with CaMKII signalling in skeletal muscle are warranted.

Acknowledgements

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Appendix: Western blot loading controls

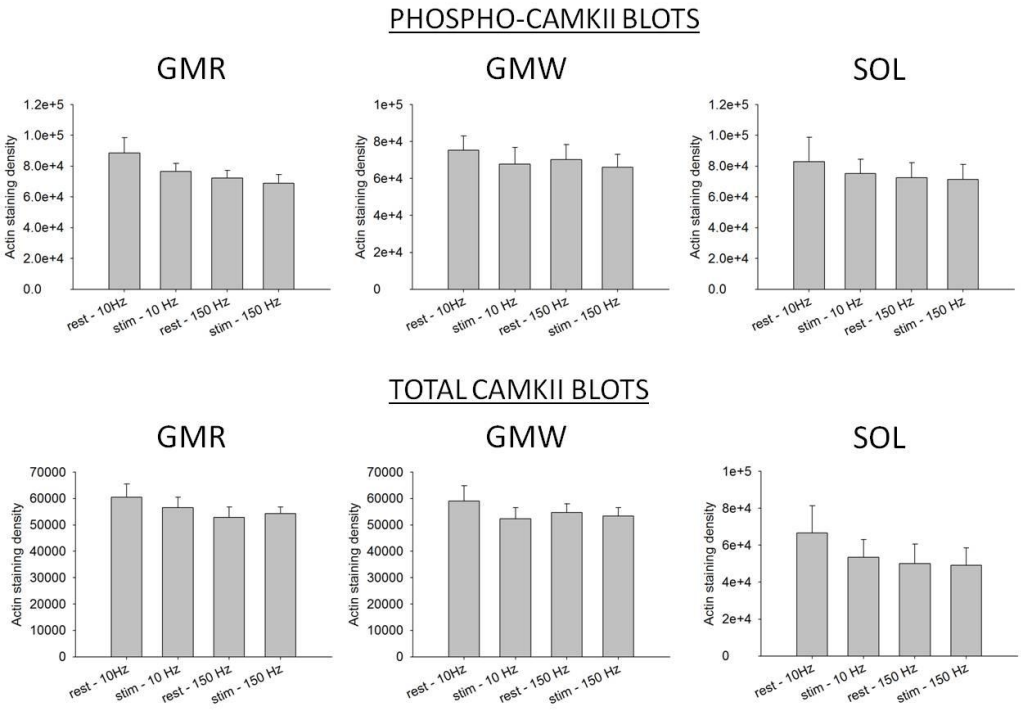

Figure A1: Western blot loading controls for 'slack contraction' experiments
Graphs displaying quantifications of ponceau s-stained actin on blots of muscle samples from the 'slack contraction' experiment. Data are displayed as means ± S.E.M. Note that the graphs have different scales. GMR = High oxidative (red) *m. gastrocnemius medialis*. GMW = Low oxidative (white) *m. gastrocnemius medialis*. SOL = *m.soleus*.

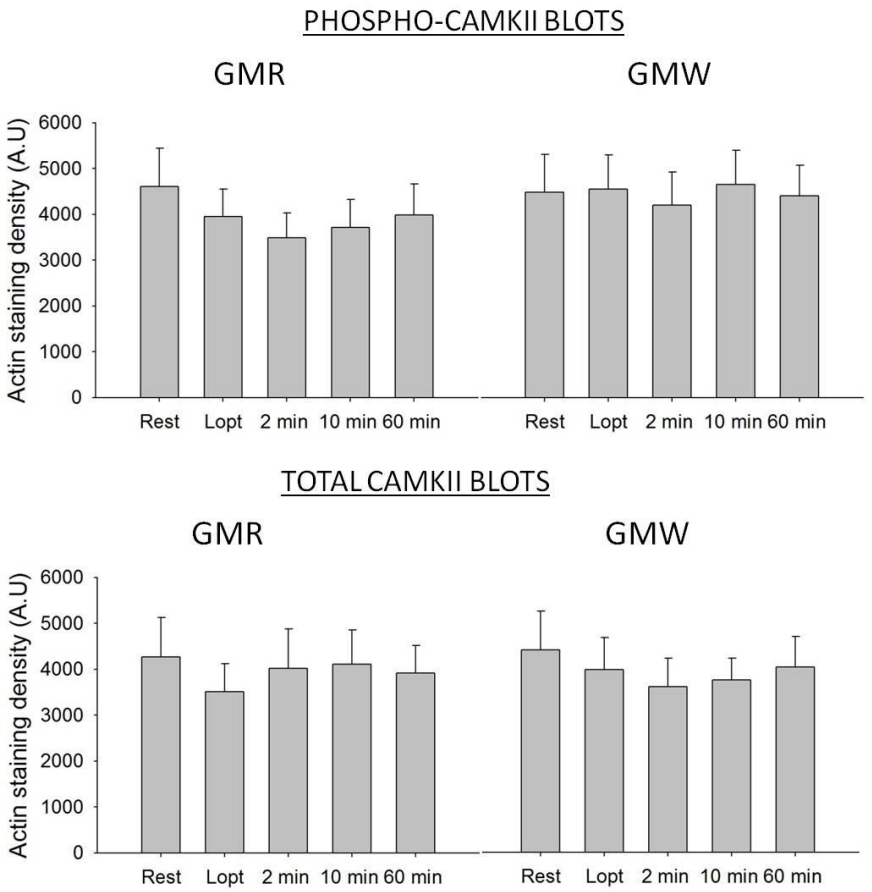

Figure A2: Western blot loading controls for '2 minutes isometric contraction' experiments Graphs displaying quantifications of ponceau s-stained actin on blots of muscle samples from the '2 minutes isometric contraction' experiment. Data are displayed as means ± S.E.M. GMR = High oxidative (red) *m. gastrocnemius medialis*. GMW = Low oxidative (white) *m. gastrocnemius medialis*.