Sensory pathways of muscle phenotypic plasticity: Calcium signalling through CaMKII

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Ca^{2+}/calmodulin-dependent protein kinase II affects contractile, but not fatigue-related phenotype in rat skeletal muscle

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Abstract

Calcium/calmodulin-dependent protein kinase II (CaMKII) is the main CaMK in skeletal muscle and its expression increases with endurance training. Because CaMKII has been implicated as a regulator of mitochondrial biogenesis and calcium handling, we investigated the effects of acute CaMKII overexpression in skeletal muscle *in vivo*. We overexpressed α/β-CaMKII in adult rat *m. gastrocnemius* (GM) and *m. soleus* (SOL) via gene electro-transfer. CaMKII overexpression did not alter cytochrome c oxidase IV (COXIV) protein levels in either GM or SOL. Likewise, mRNA levels of oxidative phosphorylation components COXIV, COXI and the transcriptional coactivator PGC-1α were not different between empty- and CaMKII-transfected *m. soleus*, whereas succinate dehydrogenase subunit b mRNA was decreased in CaMKII-transfected *m. soleus* (-26%, $p<0.05$). Force parameters of transfected muscles were measured *in situ* with intact innervations and perfusion. Strength and fatigue resistance of control- and CaMKII-transfected soleus and gastrocnemius did not differ. However, CaMKII overexpression decreased twitch time-to-peak force ($p<0.05$) and half-relaxation time ($p<0.05$). In addition, CaMKII-overexpressing *m. soleus* fibres displayed increased sarco/endoplasmic reticulum Ca^{2+}-ATPase 2 expression compared to non-transfected fibres ($p<0.001$). Our results suggest that CaMKII is not sufficient for mitochondrial biogenesis, but regulates the contractile muscle phenotype.

Introduction

Calcium/calmodulin-dependent protein kinase II (CaMKII) is a multimeric phosphotransferase which can decode calcium transients through autophosphorylation at threonine287 (De Koninck and Schulman, 1998). In heart muscle cells, CaMKII is known to control ion channels and gene expression (Anderson et al., 2011). In skeletal muscle, CaMKII has been shown to be the main multifunctional CaMK and its phosphorylation at threonine287 and activity are increased during acute exercise bouts in rats and humans (Rose et al., 2007a, Rose et al., 2006). CaMK activation has been implied to regulate mitochondrial biogenesis (Wu et al., 2002, Ojuka et al., 2003, Wright et al., 2007) and calcium re-uptake into the sarcoplasmic reticulum (SR) via a possible effect on SERCA2 (Hawkins et al., 1994). This, and the observation that endurance exercise training increases total CaMKII protein expression (Benziane et al., 2008, Rose et al., 2007b) suggest a role for CaMKII in the control of muscle plasticity.

Our current understanding of the physiological role of native CaMKII holoenzymes in whole muscle is limited, as it has been mainly derived from the characterisation of the effects of germline overexpression of a constitutively active CaMKIV mutant (Wu et al., 2002), which has lost its calcium-dependent regulation, and is not expressed in skeletal muscle (Akimoto et al., 2004). Furthermore, our current understanding of the role of CaMKII in calcium handling and fatigue in skeletal muscle is derived from *in vitro* studies (Hawkins et al., 1994) and short-term inhibition studies with pharmacological agents in single muscle fibres (Tavi et al., 2003). However, skeletal muscle fibres exist as a continuum of contractile phenotypes with distinct differences in calcium handling (Baylor and Hollingworth, 2003) and recruitment patterns (Hennig and Lomo, 1985). This indicates that pronounced differences in CaMKII-mediated calcium sensing and downstream effects may exist between functionally distinct muscle types.

We hypothesised that hetero-multimeric CaMKII controls the mitochondrial and contractile phenotype of skeletal muscle via regulation of gene expression, and that this would differ between a slow-twitch and fast-twitch muscle of the lower leg (i.e. *m. soleus* (SOL) and *m. gastrocnemius* (GM)), due to differences in fibre recruitment in

these muscles during self-initiated locomotion (Gorassini et al., 2000). We tested this by assessing the effects of somatic co-overexpression of native alpha- and beta-CaMKII isoforms, with similar substrate specificity and structure as the muscle isoforms (Woodgett et al., 1984, Gaertner et al., 2004), on selected molecular markers of the contractile and mitochondrial phenotype, and functional characteristics of the targeted soleus and gastrocnemius medialis muscles.

Methods

Ethical approval

A total of 23 female Wistar rats were used for the experiments described here. *In situ* contraction protocols and the majority of the transfection experiments were carried out at the MOVE Research Institute Amsterdam, VU University Amsterdam, The Netherlands and approved by the local Animal Experiments Committee. Two transfection experiments were carried out at the Department of Cardiovascular Surgery, University Hospital Bern, Switzerland and approved by the animal protection commission of the Canton of Berne, Switzerland.

Somatic overexpression of CaMKII

Plasmids

PCDNA3 vectors encoding full-length cDNA for CaMKIIα and CaMKIIβ were a gift from Dr. M Neal Waxham (University of Texas, Houston, USA). PGL2 plasmid encoding full-length luciferase under control of 424 basepairs upstream of the transcription start site of the chicken skeletal alpha-actin gene (Marsh et al., 1998) was a gift from Dr. Frank W. Booth (University of Missouri, Columbia, USA).

Transfection

Three month-old female Wistar rats (Harlan Laboratories/Charles River; 191-230 grams, n=13) were used to overexpress CaMKII in GM and SOL. The animals were

anaesthetised with 2-4% isoflurane through inhalation. Hind limbs were shaved, and skin was disinfected with 70% ethanol. An incision was made into the skin and fascia between GM and *m. tibialis anterior*. SOL was subsequently exposed and liberated, after which four injections of a plasmid mixture with a total volume of 90 µl were administered intramuscularly with a 29-gauge insulin syringe. GM was administered four injections over the length of the muscle with a total volume of 180 µl. A mix of expression plasmid for full-length CaMKIIα (pCDNA3-CaMKIIα; 0.22 µg/µl) and full-length CaMKIIβ (pCDNA3-CaMKIIβ; 0.22 µg/µl) in TBE buffer was injected into muscles of the right leg together with the -424 skeletal alpha-actin reporter plasmid (0.55 µg/µl). Data obtained from this reporter construct are beyond the scope of this chapter, and are therefore reported in a separate chapter. Muscles of the left leg were injected with the reporter plasmid only (1 µg/µl). Right and left transfected muscles will henceforth be referred to as 'CaMKII-transfected' and 'control-transfected', respectively. DNA injection was followed by electroporation with a GET42 electropulser (E.I.P. Electronique et Informatique du Pilat, Jonzieux, France). Needle electrodes were placed along SOL or into GM and pulse protocols were applied as described previously (Durieux et al., 2009).

After electroporation, the skin wound was closed with sutures, and the animal was allowed to recover from anaesthesia. Animals were kept in cages afterwards, where they resumed normal activity within hours after surgery. After seven days, animals were anaesthetised for measurement of muscle contraction parameters (see below), sacrificed by intra-cardiac injection of Euthasol® (VU University Amsterdam) or anaesthetised with 3% isoflurane and euthanised by dislocation of the cervical vertebrae and rapid exsanguination (University Hospital Bern). Transfected muscles were harvested from both legs and snap-frozen in liquid nitrogen.

Muscle-tendon complex preparation

For measurement of isometric muscle contraction parameters, rats were anaesthetised by intra-peritoneal injections of 1.2 ml/100 gram body weight of 12.5% urethane (De Haan et al., 2003). 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. Experiments

were carried out at room temperature (24ºC). Rats were kept on a heated pad to prevent hypothermia. Hind limbs were shaved and skin was removed, after which GM and 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, both muscles were kept intact and tendons of GM and SOL were attached to a force transducer via Kevlar wires. The sciatic nerve was severed proximally and received stimulation with rectangular pulses through an external electrode controlled by a computer. Force data were sampled at a frequency of 1000 Hz.

Activation of exogenous CaMKII in transfected skeletal muscle

A two-minute stimulation protocol consisting of either intermittent isometric tetanic contractions at 100Hz stimulation frequency (Rose et al., 2007a), or continuous stimulation at 10Hz was applied to α/β-CaMKII overexpressing GM and SOL *in situ*. The muscles (n=6) were freeze-clamped between liquid nitrogen-cooled aluminium grips during stimulation after two minutes. Non-stimulated, α/β-CaMKII overexpressing contra-lateral muscles were subsequently dissected and frozen in liquid nitrogen. Proteins were extracted from the muscle and subjected to SDS-PAGE followed by immunoblotting as described below. The phosphorylated form of exogenous β-CaMKII was identified based on its molecular weight (60.4 kDa), which is very similar to that of δ_a (60.1 kDa), the second largest CaMKII isoform in skeletal muscle (Bayer et al., 1998).

***In situ* contraction protocol**

Optimum length of the muscle-tendon complex (the length of the muscle-tendon complex at which maximum force was produced) for isometric contractions was first estimated using twitches, then determined using a protocol consisting of two twitches and one tetanic contraction (pulse duration 100 μsec, tetanic stimulation frequency: 100 Hz, train duration: 400 ms (De Haan et al., 1993)). Muscles were kept below slack length between contractions and rest duration between maximal contractions was approximately one minute. After determination of optimum length, muscles rested for five minutes. A high-intensity contraction protocol (stimulation frequency: 100Hz; train

duration: 300 ms, one train every 800 ms) was then applied to induce muscle fatigue (50 tetanic contractions, duration: 300 ms, rest interval: 500 ms) with muscles set to optimum length. GM and SOL were dissected after the end of stimulation 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 blot, immunohistochemical and RT-PCR analysis as described below.

Force traces collected during muscle stimulation were analysed using custom written software based on Matlab (v 7.5.0., The Mathworks Inc., MA, USA). Time to peak twitch force (TTP), half-relaxation time (HRT), maximum active twitch force (F_{tw}) and active maximum tetanic force (F_{max}) (total force minus passive force) were determined. The same contraction parameters were determined for a group of non-transfected muscles (NTm; SOL/GM n=8). Values obtained for the two twitch values in each trace were averaged.

Protein biochemistry

Western blotting

To analyse protein expression, frozen 25 µm thick cross-sections taken from the centre portion of the muscle were homogenised in ice-cold RIPA buffer (50mM TRIS-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% v/v Nonidet 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, 1 µg/ml leupeptin, 0.2 µg/ml pepstatin, 0.1 µg/ml aprotinin, all from Sigma-Aldrich, Dorset, UK) using a Polytron homogeniser (Kinematica, 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 and phospho^{Thr287}-CaMKII were analysed by western blotting followed by immunodetection. Homogenates were denatured by addition of SDS-PAGE buffer (final concentration: 50 mM TRIS-HCl (pH 6.8), 2% w/v SDS, 2% w/v bromophenol blue, 10% v/v glycerol, 2% β-mercaptoethanol) and five minutes heating at 95ºC. 20-40 µg of protein was separated by SDS-PAGE and

transferred overnight onto a nitrocellulose membrane (GE Healthcare, Little Chalfont, UK). Membranes were stained with Ponceau S solution to confirm equal protein loading and transfer. The membrane was blocked in 5% skimmed milk in TRIS-buffered saline (pH 7.4) with 0.05% tween-20 (TBS-T), 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 2 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 and COXIV). Finally, membranes were incubated with species-specific horseradish peroxidase-conjugated secondary antibodies (Millipore, Watford, UK). Membranes were washed in TBS-T for 4 x 5 minutes after both antibody incubations. Antibodies were detected with an enhanced chemiluminescence kit (Pierce, Rockford IL, USA). Light signals were captured with a ChemiDoc XRS system (Biorad, Hemel Hempstead, UK).

Transfected muscle pairs from the same animal were run on the same blot. Measures were limited to animals whose CaMKII-transfected muscles showed increased expression of the exogenous CaMKII isoforms on a western blot. Protein bands were quantified with Quantity One version 4.6.8 (Biorad). For the analysis of COXIV protein, a reference muscle sample was run on every blot, and COXIV protein bands were standardised to the intensity of this sample. These values were subsequently expressed as relative to the mean of the empty-transfected muscles.

In vitro CaMKII phosphorylation

To detect endogenous and exogenous calcium/calmodulin-sensitive protein bands, CaMKII was phosphorylated *in vitro* by addition of a calcium/calmodulin mixture to protein extracts of non-transfected and transfected muscle as described previously (Fluck et al., 2000b). Phosphorylated CaMKII was subjected to SDS-PAGE and immunoblotted as described.

Immunofluorescence & confocal microscopy

To investigate differences in protein expression at the single fibre level, immunofluorescence stains were performed on cryosections of transfected muscles. Sections (12 µm thickness) were cut on a cryostat and dried for 30 minutes on glass slides. Sections were then fixed with ice-cold acetone and blocked with 5% normal goat serum in phosphate buffered saline, pH 7.5 (PBS). CaMKII and COXIV or CaMKII and SERCA2 were detected simultaneously using commercially available primary antibodies (dilutions: anti-CaMKII: 1/250; anti-COXIV: 1/250, anti-SERCA2 (Abcam #Ab3625 or Abcam #2A7-A1): 1/200) as described and species-specific Alexa 488/555 secondary antibodies (Invitrogen, Paisley, UK). Sections were washed with PBS for 4 x 5 minutes after both antibody incubations. To detect nuclei, sections were incubated for 10 minutes with TO-PRO-3 iodide (Invitrogen). Immunolabelled sections were embedded in fluorescence compatible mounting medium (DAKO, Ely, UK).

Protein expression in transfected fibres was analysed on a TCS SP5 confocal microscope (Leica, Milton Keynes, UK). A 10x objective was used in combination with 4x optical zoom. The fluorescent labels were excited with an Argon laser at 488nm and HeNe lasers at 543 nm and 633 nm. The pinhole was set to match the thickness of the stained section and the focus plane was adjusted to maximise signal detection. Dyes were excited separately using a sequential scanning mode. Detected light spectra were set to maximise signal detection, but care was taken to prevent cross-excitation of dyes. Laser intensity was set to produce images with few under- or overexposed pixels, and low levels of non-specific staining, as indicated by light emission from sections that had been labelled with the secondary antibodies only. 8-Bit images were captured at 2048 x 2048 pixels, using 100 Hz scanning speed and 5 times line averaging.

COXIV staining in CaMKII-transfected muscle fibres (identified based on CaMKII staining intensity by visual inspection) was quantified with ImageJ (*rsbweb.nih.gov/ij/)*. Fibres were circumscribed manually and the average pixel intensity within the fibre was measured. An approximately equal number of non-transfected fibres in the same image was measured as well, and acted as the control group of fibres to which the transfected fibres were compared.

RT-PCR

RNA extraction from muscles and RT-PCR analysis were carried out as described elsewhere (van Wessel et al., 2010). Total RNA was extracted from frozen 25 µm sections of transfected muscles using the RiboPure kit (Applied Biosystems). RNA concentration and purity (260/280 nm ratio; mean: 2.06, range: 1.92-2.09) were determined using a spectrophotometer (Nanodrop Technologies, Wilmington, DE). Total RNA concentration in muscle tissue was expressed as RNA (ng) per weight of the analysed sample (mg). Five hundred nanogram of total RNA per muscle was reverse transcribed using the high capacity RNA-to-cDNA kit (Applied Biosystems) containing random primers in a 20 µl total reaction volume. Tubes were heated at 25 ºC for 5 min, followed by 42 ºC for 30 min. Finally, the tubes were heated to 85ºC for 5 min to stop the reaction and stored at -80 ºC until used in the PCR reaction.

For each PCR target, 5 µl of the RT reaction product was amplified in duplicate using Fast Sybr Green mastermix (Applied Biosystems). The following transcripts were targeted: 18S ribosomal RNA (18S rRNA), cytochrome-c oxidase subunit 1 (COXI), cytochrome-c oxidase 4 (COXIV), succinate dehydrogenase subunit b (SDHb), peroxisome proliferator-activated receptor gamma-coactivator 1 alpha (PGC-1α) and SERCA2a. PCR primers were designed using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Primer sequences and Genbank accession numbers for the transcripts are shown in table 1. Amplification efficiency of the primers used was 92.7-102.0%, and melting curve analysis demonstrated specific amplification. The range of cycle threshold values was 13-25. For 18S rRNA, mean cycle threshold values were converted into relative concentrations by 2^{-Ct}. For all other transcripts, 18S rRNA cycle threshold was subtracted from the mean cycle threshold value of the specific target to obtain ΔC_t and converted into relative concentrations by $2^{-\Delta Ct}$.

Target mRNA	PCR primer sequence 5'→ 3'	Genbank Accession nr.
18S RNA	Forward: CGAACGTCTGCCCTATCAACTT	EU 139318.1
	Reverse: ACCCGTGGTCACCATGGTA	
COXI	Forward: TGCCAGTATTAGCAGCAGGT	X14848.1
	Reverse: GAATTGGGTCTCCACCTCCA	
COXIV (isoform 1)	Forward: AGTCCAATTGTACCGCATCC	NM 017202.1
	Reverse: ACTCATTGGTGCCCTTGTTC	
SDH (subunit b)	Forward: CAGAGAAGGGATCTGTGGCT	NM 001100539.1
	Reverse: TGTTGCCTCCGTTGATGTTC	
PGC-1α	Forward: ATGAGAAGCGGGAGTCTGAA	NM 031347.1
	Reverse: GCGCTCTTCAATTGCTTTCT	
SERCA2a	Forward: GGCCCGAAACTACCTGGAGCC	NM 001110139.2
	Reverse: CAACGCACATGCACGCACCC	

Table 1: Primers sequences used for RT-PCR analysis of mRNA targets

Statistics

Statistical analyses were carried out with SPSS16 (SPSS Inc, IL, USA). Immunofluorescence data from non-transfected fibres and CaMKII-transfected fibres in the same muscle were analysed with two-tailed paired t-tests. Western blot and RNA data from control- and CaMKII-transfected muscles were analysed with two-tailed Wilcoxon signed ranks tests. RNA data from non-transfected muscles and control-transfected muscles were analysed with a Mann-Whitney U-test. Effects of transfection on twitch and tetanic force parameters from non-transfected and control-transfected muscles were tested with a factorial ANOVA (muscle x transfection). Correlations between the increase in CaMKII protein level following transfection and changes in COXIV protein, all transcripts and force parameters were tested using Spearman's rho. Non-parametric tests were run with exact significance. Significance level was set at $p < 0.05$.

Results

Endogenous and exogenous CaMKII expression and phosphorylation in skeletal muscle

In vitro phosphorylation of CaMKII allowed the identification of four Ca^{2+}/CaM-dependent isoforms in rat skeletal muscle. Based on previously published results (Bayer et al., 1998), we identified these bands as the β_M, δ_A, δ_D and γ_B CaMKII isoforms (Fig. 1A). When CaMKII was overexpressed, we detected increased protein levels of α- and β-CaMKII isoforms (which have molecular weights of 50 and 60 kDa, respectively) compared to control-transfected muscle (Fig. 1B). When homogenate of CaMKII-transfected muscle was incubated with calcium and calmodulin, increased phosphorylation of CaMKII was detected (Fig. 1C). The overexpressed CaMKII was localized in the cytoplasm, but not in the myonuclei (Fig. 1D)

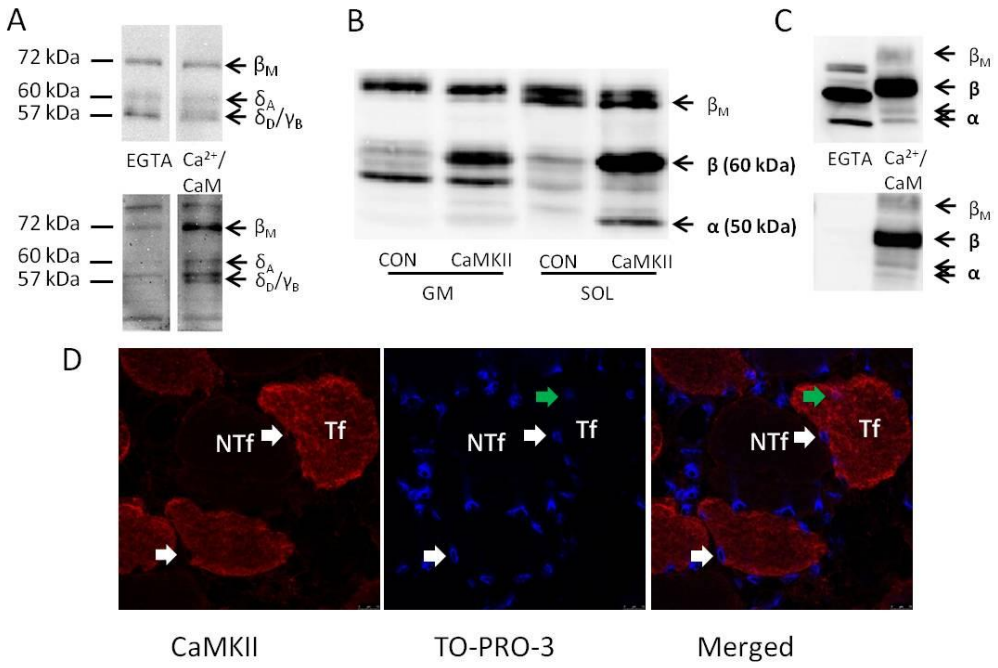

Figure 1: Expression of endogenous and exogenous CaMKII in rat skeletal muscle

A: Endogenous total CaMKII (top panel) and phospho^{Thr287}-CaMKII (bottom panel) of rat GM homogenate after incubation with EGTA (left) or Ca^{2+}/Calmodulin (Ca^{2+}/CaM). CaMKII isoforms were identified based on data from (Bayer et al., 1998). B: Representative western blot image demonstrating overexpression of the exogenous α (50kDa) and β (60kDa) CaMKII isoforms in *m. gastrocnemius medialis* (GM) and *m. soleus* (SOL). Note that skeletal muscle expresses endogenous isoforms with molecular weights of 72 and 57-60 kDa (Bayer et al., 1998). CON: Control-transfected muscle; CaMKII: CaMKII-transfected muscle. C: Total CaMKII (top panel) and phospho^{Thr287}-CaMKII (bottom panel) of α/β-CaMKII-transfected *m. soleus* homogenate after incubation with EGTA or Ca^{2+}/CaM. D: Immunofluorescence image demonstrating non-nuclear localisation of overexpressed CaMKII (red) in skeletal muscle fibres. Example CaMKII-transfected (Tf) and non-transfected fibres in the same muscle (NTf) are marked in the images by white letters. Nuclei are stained in blue. White arrows point to representative nuclei of transfected fibres. Green arrows point to central nucleus in transfected fibre, indicating regeneration.

We assessed phosphorylation of endogenous and exogenous CaMKII after a two minute isometric contraction protocol. In contrast to a previous study (Rose et al., 2007a), we did not observe a consistent increase in phosphorylation of the endogenous CaMKII isoforms after the two minute isometric protocol (GM: n=6; SOL: n=6; Fig. 2). However, in a number of stimulated muscles, we observed increased phospho-CaMKII with the molecular weight of exogenous β-CaMKII (see arrows in Fig. 2).

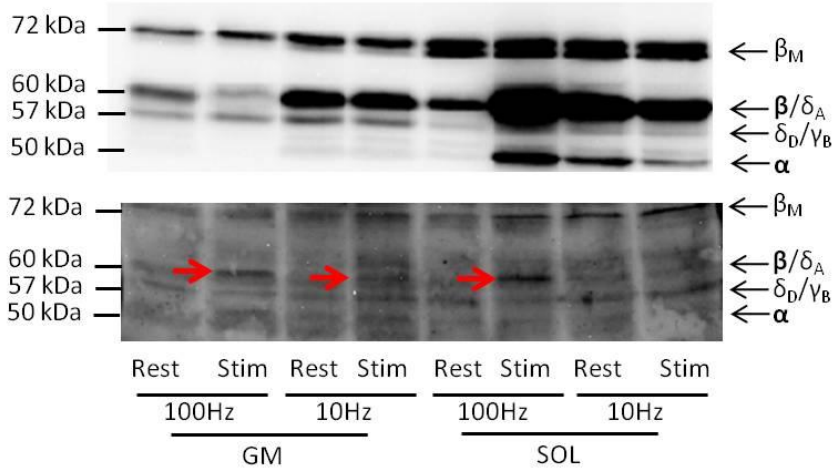

Figure 2: Phosphorylation of exogenous CaMKII in rat skeletal muscle *in situ*

Total CaMKII (top panel) and phospho^{Thr287}-CaMKII in transfected *m. gastrocnemius medialis* (GM) and *m. soleus* (SOL). Endogenous and exogenous (bold) CaMKII isoforms are labelled. Red arrows indicate phosphoylated exogenous β-CaMKII in Stim-labelled lanes. Rest: non-

stimulated muscle. Stim: muscle subjected to *in situ* stimulation (with stimulation frequency as indicated below the blots).

Gene transfer-induced regeneration

The presence of small fibres, fibres with central nuclei, and the accumulation of nuclei outside the fibres in transfected muscles indicated that these muscles were undergoing regeneration after gene electrotransfer (Fig. 1D). The gene transfer procedure also increased the concentration of total RNA in muscle tissue (non-transfected muscle: 284 ng/mg tissue; control-transfected muscle: 708 ng/mg, $p<0.01$; Fig. 3). However, there was no difference in RNA concentration between control- and CaMKII-transfected intra-animal pairs (708 ng/mg vs. 735 ng/mg, $p=0.81$; Fig. 3A).

CaMKII is not sufficient to increase mitochondrial gene expression

RT-PCR was used to investigate whether CaMKII overexpression increased transcript levels of components of the oxidative phosphorylation chain encoded by either nuclear (SDHb, COXIV) or mitochondrial (COXI) DNA, and the transcriptional co-factor PGC-1α which, when overexpressed, increases mitochondrial biogenesis (Wu et al., 1999). This analysis was limited to SOL, because of the higher transfection efficiency achieved in this muscle. Seven muscle samples were selected for analysis based on exogenous CaMKII expression level. 18S rRNA content did not differ between control- and CaMKII-transfected muscles (Fig. 3B), and was used as an internal reference for the other transcripts. SDHb mRNA level was lower in CaMKII-overexpressing muscles (-26%, $p=0.04$), with no significant differences in transcript levels of COXIV, COXI or PGC-1α (Fig. 3C-F). No correlation was observed between the degree of CaMKII overexpression and the difference in transcript level for any of the transcripts.

To test whether overexpression of α/β CaMKII was sufficient to increase COXIV protein expression in GM and SOL, cryosections of transfected muscles were co-stained for CaMKII and COXIV (Fig. 4A). COXIV staining intensity as measured by immunofluorescence in individual fibres of transfected muscles did not differ between CaMKII-overexpressing and non-transfected fibres of both GM (non-transfected: n=51,

transfected: n=54; p=0.91) and SOL muscle (non-transfected: n=35, transfected: n=38; p=0.38) (Fig. 4A/B).

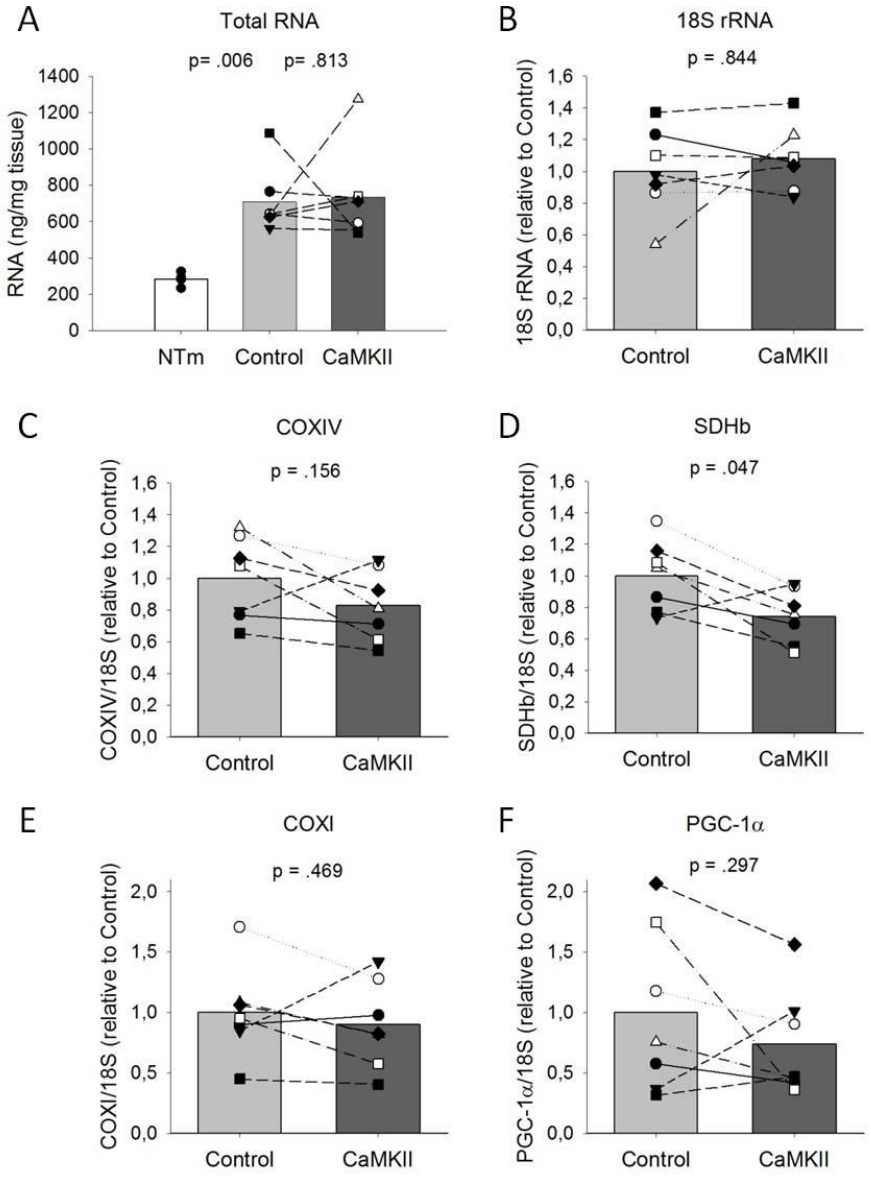

Figure 3: Effects of CaMKII transfection on mitochondrial gene transcript levels

A: Concentration of total RNA in transfected and non-transfected muscles. B-F: relative concentrations of indicated transcripts in control and CaMKII-transfected *m. soleus*. Bars indicate mean data. Raw data are plotted as symbols and lines indicate intra-animal pairs. Same symbols

indicate measurements on the same muscle. Significance of control- vs. CaMKII-transfected differences is indicated. NTm: Non-transfected muscle; Control: reporter only transfected; CaMKII: reporter + α/β-CaMKII transfected.

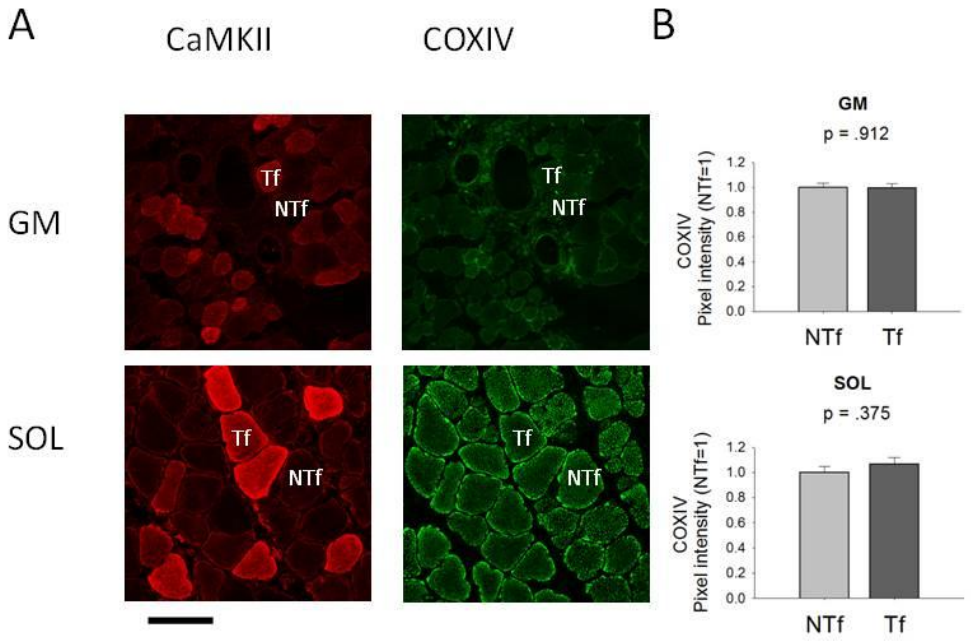

Figure 4: COXIV protein expression in CaMKII-transfected fibres

A: Immunofluorescence images of transfected muscle fibres of *m. gastrocnemius medialis* (GM) and *m. soleus* (SOL) stained for different proteins as indicated. Black bar indicates 100 µm. B: Graphs displaying mean ± S.E.M. pixel intensities of transfected (Tf) and non-transfected (NTf) fibres in GM and SOL. Example Tf and NTf are marked in the images by white letters. Significance of NTf vs. Tf differences is indicated. Data are relative to NTf.

CaMKII overexpression increases the speed of twitch contraction and relaxation

CaMKII has been thought to affect calcium release from, and/or calcium re-uptake into, the SR. Because twitch contraction and relaxation speed during isometric contractions depend mainly on the properties of the SR (see for review Schiaffino, 2010), we investigated whether these parameters differed between control- and CaMKII-

transfected muscles. Both TTP and HRT were higher in control-transfected muscle compared to non-transfected muscle (TTP: +22%, p=0.05, main effect; HRT: +32%, p=0.03, main effect; Fig. 5). We observed a significant decrease in TTP and HRT in CaMKII-transfected muscles compared to control-transfected muscles (TTP: -16%, p=0.02, main effect; HRT: -15%, p=0.04, main effect; Fig. 5).

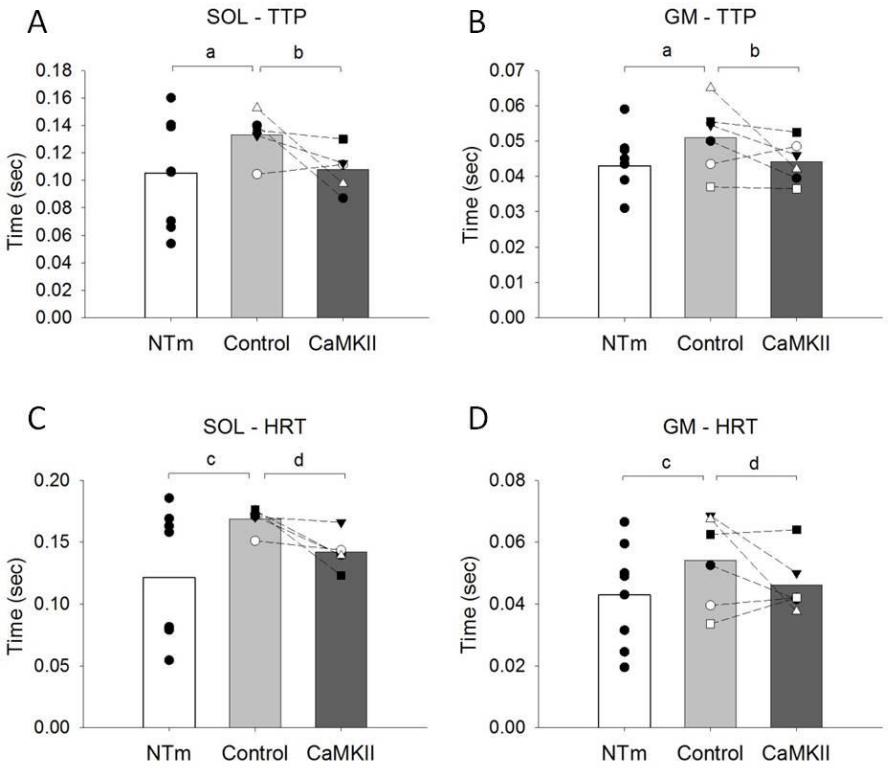

Figure 5: Effects of CaMKII overexpression on twitch contraction/relaxation time

A: *m. soleus* (SOL) time-to-peak twitch force (TTP); B: *m. gastrocnemius medialis* (GM) TTP; C: SOL twitch half relaxation time (HRT); D: GM twitch HRT. Bars indicate mean data. Raw data are plotted as black dots for non-transfected muscles and as symbols for transfected muscle pairs. Lines indicate intra-animal pairs and symbols indicate measurements on the same muscle. NTm: Non-transfected muscles; Control: reporter only-transfected muscles; CaMKII: reporter + α/β-CaMKII-transfected muscles. [a] Main effect of gene transfer on TTP ($p<0.05$); [b] Main effect of CaMKII overexpression in TTP ($p<0.05$); [c] Main effect of gene transfer on HRT ($p<0.05$); [d] Main effect of CaMKII overexpression on HRT ($p<0.05$).

SERCA2 protein expression increases in CaMKII overexpressing *m. soleus* fibres

We investigated whether the observed reduction in HRT might be due to increased expression of the SERCA2 isoform. Examples of immunofluorescence CaMKII/SERCA2 co-stained *m. soleus* fibres are shown in figure 6A. SERCA2 staining intensity in CaMKII-overexpressing *m. soleus* fibres was significantly higher compared to that in non-transfected fibres of the same muscle (non-transfected: n=221, transfected: n=276; $p<0.001$; Fig. 6B). Identical results were obtained when the staining was repeated with a different anti-SERCA2 antibody.

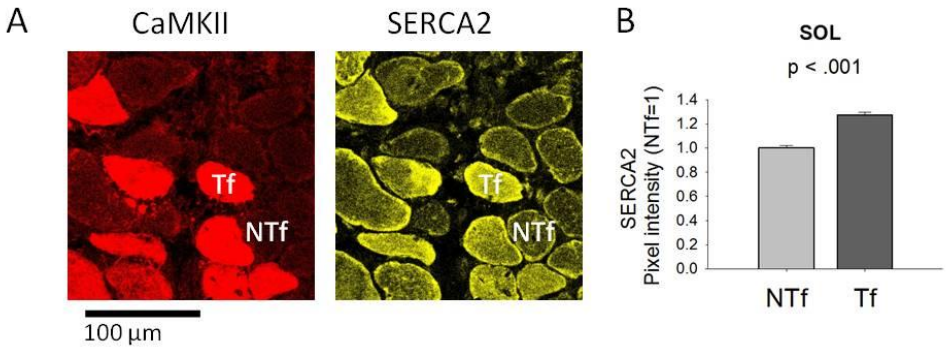

Figure 6: CaMKII overexpression increases SERCA2 expression in *m. soleus* fibres

A: Example immunofluorescence images of *m. soleus* fibres stained for different proteins as indicated above the image. Bar indicates 100 µm. B: Graphs displaying mean ± S.E.M. pixel intensities of transfected (Tf) and non-transfected (NTf) fibres in GM and SOL. Example Tf and NTf fibres are marked in the images by white letters. Significance of NTf vs. Tf differences is indicated. Data are relative to NTf.

We used RT-PCR to investigate whether CaMKII overexpression increased SERCA2 transcript levels. Primer sequences for primers targeting the SERCA2a isoform, which is the predominant SERCA isoform in normal rat *m. soleus* (Zador et al., 1996), are shown in table 1. We did not observe an increase in SERCA2a mRNA in CaMKII-transfected compared to control-transfected *m. soleus* (Fig. 7). Rather, a trend for a down-regulation was apparent.

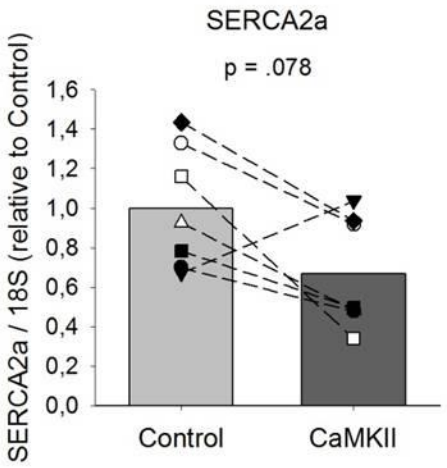

Figure 7: Effect of CaMKII-overexpression on SERCA2a transcript level in *m. soleus* fibres.
Graph displays the relative transcript concentration in control and CaMKII-transfected *m. soleus*. Bars indicate mean data. Raw data are plotted as symbols (the same as Fig. 4) and lines indicate intra-animal pairs. Same symbols indicate measurements on the same muscle. Significance of control- vs. CaMKII-transfected differences is indicated.

CaMKII overexpression does not increase maximum force and fatigue resistance

Gene electro-transfer caused a decrease in maximal tetanic force ($p<0.001$, main effect), which was larger in SOL (-49%) than in GM (-14%) (Fig.8). Maximum twitch force was also reduced ($p=0.03$, main effect), but this reduction was of a lower magnitude compared to the reduction in maximal tetanic force (SOL: -18%; GM: -7%) (Fig.8). Both maximum tetanic and maximum twitch force did not differ between control- and CaMKII-transfected muscles. Finally, we observed no difference in fatigue resistance between control- and CaMKII-transfected GM during 50 repeated tetanic contractions (Fig. 9).

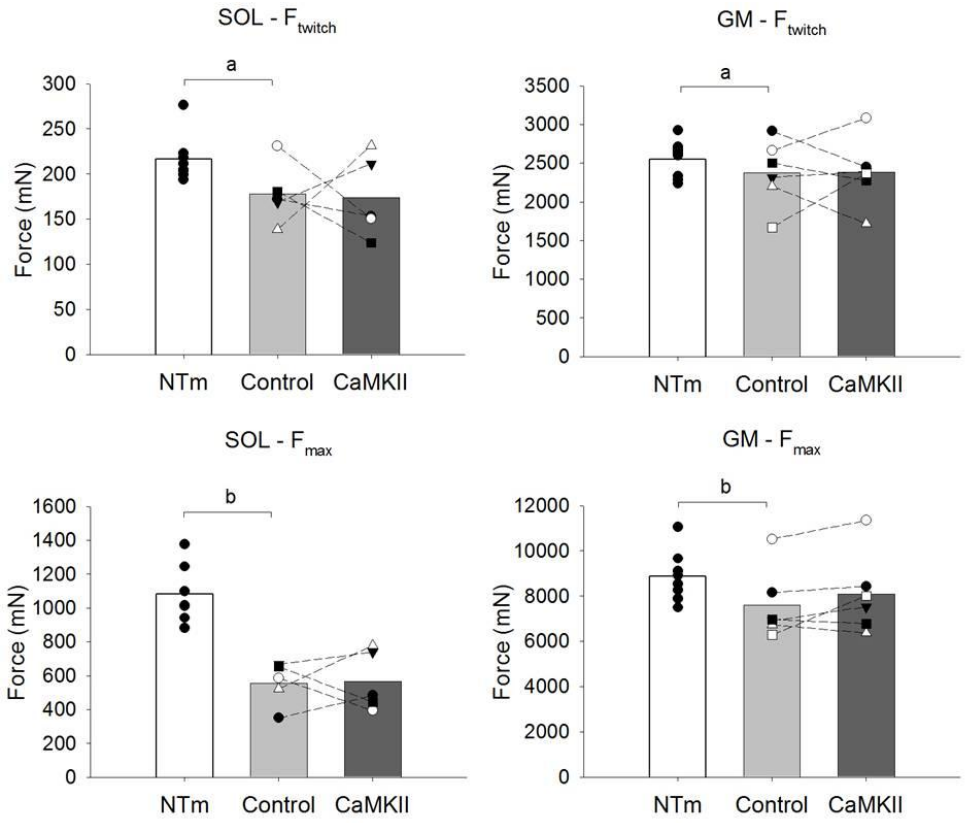

Figure 8: Maximum twitch and tetanic force of transfected and non-transfected muscles

Top left: *m. soleus* (SOL) twitch force (F_{twitch}); Top right: *m. gastrocnemius medialis* (GM) twitch force; Bottom left: SOL tetanic force (F_{max}); Bottom right: GM tetanic force. Bars indicate mean data. Raw data are plotted as black dots for non-transfected muscles and as symbols for transfected muscle pairs. Lines indicate intra-animal pairs and symbols indicate measurements on the same animal. NTm: Non-transfected muscles; Control: reporter only-transfected muscles; CaMKII: reporter + α/β-CaMKII-transfected muscles. [a] Main effect of gene transfer on F_{twitch} ($p<0.05$); [b] Main effect of gene transfer on F_{max} ($p<0.001$).

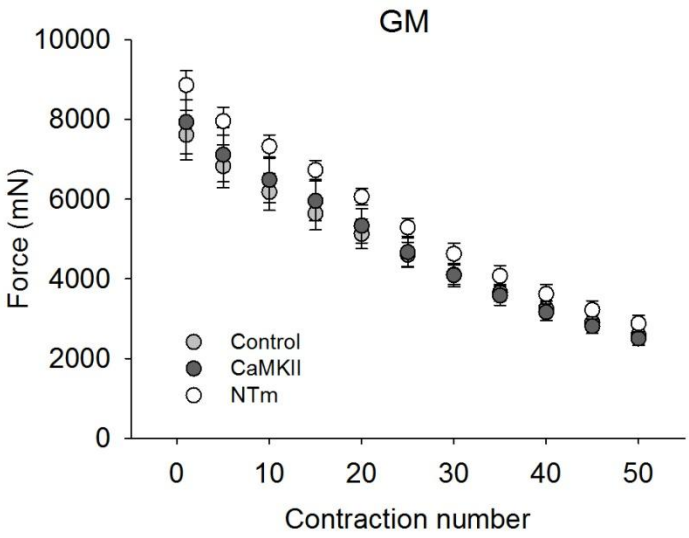

Figure 9: Effects of CaMKII overexpression on force reduction during repeated maximal tetanic contractions of GM

Graph displays tetanic force of *m. gastrocnemius medialis* (GM) during a contraction protocol consisting of 50 repeated tetanic contractions. Maximal tetanic force (mean ± S.E.M.) of every fifth contraction is shown. NTm: Non-transfected muscles; Control: reporter only-transfected muscles; CaMKII: reporter + α/β-CaMKII-transfected muscles.

Discussion

This is the first study that investigates whether manipulation of CaMKII expression affects skeletal muscle phenotype *in vivo*. In contrast to our hypothesis, we did not observe an increase in expression of genes related to mitochondrial biogenesis. However, twitch contraction and relaxation times were reduced in CaMKII-transfected muscles while SERCA2 protein levels in CaMKII-transfected muscle fibres were increased. Our results suggest the involvement of CaMKII in the regulation of the contraction-related phenotype, rather than mitochondrial biogenesis.

Technical considerations

Muscle damage and subsequent regeneration are inherent to gene electro-transfer, and this enhances the plasticity of the muscle. In myotoxin-induced muscle regeneration mitochondrial enzyme expression is decreased, and recovers from day 3 onwards (Duguez et al., 2002). The increase in total RNA concentration in control-transfected muscles compared to non-transfected muscles in the present investigation (Fig. 3) was at least in part due to an increase in 18S rRNA (relative 18S rRNA levels, corrected for RNA concentration: NTm: 1.00±0.13; Control-transfected: 3.19±0.62; $p<0.01$), suggesting a higher ribosomal content and translational capacity in these muscles. Therefore, we propose this model is sensitised to stimuli that affect the muscle phenotype.

The influence of the gene electro-transfer procedure *per se* does not affect the results of the comparison between transfected and non-transfected fibres, as these were located in the same region of the muscle (Fig. 4A & 6A), and both groups consisted of relatively small, regenerating fibres which were size-matched between the groups and displayed central nuclei.

Increasing total protein level of native CaMKII in skeletal muscle results in potentially higher CaMKII activity which is still amenable to physiological regulation. In cardiac myocytes, overexpressing native CaMKII has effects on both functional characteristics and the expression of genes involved in excitation-contraction coupling and cardiac hypertrophy (Ramirez et al., 1997, Ronkainen et al., 2011). Our results suggest that exogenous CaMKII can be activated in skeletal muscle during muscle recruitment *in vivo* (Fig. 2). It is possible that increasing the physical activity of the rats in the period after the transfection procedure would have enhanced the effects of the overexpressed wild-type CaMKII because of increased recruitment of GM and SOL. It has been estimated that motor units in the soleus muscles of caged, but freely moving rats are active for 25-30% of a 24- hour period (Hennig and Lomo, 1985). Furthermore, rat SOL is recruited during postural and slow running activity (Gorassini et al., 2000). Therefore, we expected transfected fibres in this muscle to be frequently recruited. GM has been shown to be moderately activated during postural and slow running tasks and to be increasingly recruited during running at higher speeds (Gorassini et al., 2000). It is therefore possible that the exogenous CaMKII in transfected GM fibres was not

sufficiently activated during normal cage activity for CaMKII activity-dependent effects on mitochondrial protein expression to manifest.

CaMKII overexpression affects twitch contraction and relaxation speed

Twitch contraction and relaxation times of CaMKII-overexpressing muscles were reduced compared to control-transfected muscles (Fig.5). This is consistent with CaMKII-dependent phosphorylation-induced increases in SERCA2 and RyR activities *in vitro* (Hawkins et al., 1994, Xu and Narayanan, 1999, Dulhunty et al., 2001), and with the finding that CaMKII inhibition reduced calcium release in single mouse muscle fibres (Tavi et al., 2003). CaMKII is targeted to the SR by binding to membrane-anchoring protein α-KAP, an α-CaMKII splice variant (Bayer et al., 1998). In skeletal muscle fibres, overexpressed α-KAP localises mostly to the longitudinal SR (Nori et al., 2003), where the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) is situated. Furthermore, CaMKII is targeted to the junctional SR (Leddy et al., 1993), Therefore, CaMKII possibly has access to SERCA and RyR and could increase their activities through phosphorylation. However, GM expresses mainly the SERCA1 isoform that cannot be phosphorylated by CaMKII (Hawkins et al., 1994), and inhibitory effects of CaMKII on RyR activity have also been reported (Wang and Best, 1992). Therefore, it is unclear if CaMKII-dependent phosphorylation of SERCA and RyR played a role in decreasing twitch contraction and relaxation times in our study.

Because the contraction experiments were carried out seven days after transfection, it is possible that structural adaptations occurred that decreased TTP and HRT. As mentioned, transfected fibres appear in a regenerating region of the muscle. If we assume that the observed increases in relaxation/contraction times in control-transfected muscles compared to those in non-transfected muscles are due to changes in muscle properties in this region, it becomes apparent that most of the effect of the gene transfer procedure is reversed or mitigated in the CaMKII overexpressing muscles (Fig. 5). Such adaptations might include increased densities of the RyR and SERCA on the SR membrane. Indeed, our data suggested that SERCA2 protein expression was increased in CaMKII-transfected *m. soleus* fibres (Fig. 6), a process which possibly contributed to the observed decrease in relaxation time.

We observed no significant increase in maximal force production (Fig. 8). This might be explained by specific effects of the electroporation-induced damage on muscle force characteristics. The gene transfer procedure alone reduced maximal twitch force, but maximal tetanic force was more strongly reduced (Fig. 8). Specific force was likely also decreased in electroporated muscles. A decreased specific force and an increased ratio of twitch force to tetanic force in regenerating rat *m. soleus* have been shown to be associated with the expression of developmental isoforms of EC-coupling proteins (Esposito et al., 2007). Therefore, it may be that any increase in F_{max} was masked by negative effects of muscle damage on EC-coupling.

CaMKII overexpression reduces, rather than increases, mitochondrial gene expression

In contrast to our expectations, we observed no increase in mitochondrial protein COXIV (Fig. 4) and fatigue resistance (Fig. 9) in CaMKII-overexpressing muscles compared to control-transfected muscles. To the best of our knowledge, this is the first study investigating whether CaMKII increases the expression of a mitochondrial protein in any tissue type.

Constitutively active CaMKIV increases mitochondrial biogenesis when overexpressed from an embryonic stage onward (Wu et al., 2002), but this CaMK is not endogenously expressed in skeletal muscle (Akimoto et al., 2004, Rose et al., 2006) and CaMKIV knock-out mice do not display altered muscle adaptation in response to training (Akimoto et al., 2004). Whether CaMKIV would have a similar function in skeletal muscle compared to CaMKII is questionable, since the two proteins have different substrate specificity and intracellular localisation (Sun et al., 1996, Srinivasan et al., 1994, Sun et al., 1994).

The absence of a difference in fatigue resistance between control- and CaMKII-transfected muscles might be due to the muscle oxidative capacity not being the limiting factor in the synthesis of ATP under full anaesthesia. Our repeated contraction protocol almost certainly required a significant contribution of oxidative metabolism to ATP synthesis (Westra et al., 1988), but if blood flow to the working muscle was insufficient to meet the increased demand for oxygen, differences in oxidative capacity of the muscles may be masked.

Our finding that CaMKII overexpression did not increase COXIV protein expression was confirmed at the mRNA level. Furthermore, transcript levels of other key genes involved in mitochondrial biogenesis were either not significantly changed or decreased (Fig. 3). This absence of increased gene expression is in accordance with experiments demonstrating that acute inhibition of CaMKII *in vivo* in mice did not affect GLUT4 protein expression (Witczak et al., 2010). The results demonstrate that CaMKII is not sufficient to increase mitochondrial gene expression, but do not rule out the possibility that it is required for the response to exercise. The idea that CaMKII signalling acts in conjunction with other signalling pathways is supported by the observation that the activity of a GLUT4-enhancer in mouse tibialis anterior muscle is only decreased when CaMKII is co-inhibited with either AMPK or calcineurin (Murgia et al., 2009).

Conclusion

Our results support a role for CaMKII in the regulation of muscle contraction and SERCA2 expression, but not in increasing mitochondrial biogenesis. The fact that these observations were made after an *in vivo* intervention in whole muscle is significant in the context of current literature on CaMKII function in *ex vivo* or *in vitro* systems.

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