

# VU Research Portal

## Sensory pathways of muscle phenotypic plasticity: Calcium signalling through CaMKII

Eilers, W.

2013

### **document version**

Publisher's PDF, also known as Version of record

[Link to publication in VU Research Portal](#)

### **citation for published version (APA)**

Eilers, W. (2013). *Sensory pathways of muscle phenotypic plasticity: Calcium signalling through CaMKII*.

### **General rights**

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal ?

### **Take down policy**

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

### **E-mail address:**

[vuresearchportal.ub@vu.nl](mailto:vuresearchportal.ub@vu.nl)

Effect of CaMKII overexpression on  
skeletal alpha-actin transcription in rat  
skeletal muscle



Wouter Eilers, Richard T. Jaspers, Arnold de Haan & Martin Flueck

## Abstract

The effect of CaMKII overexpression on the transcriptional activity of one of its potential target genes, skeletal alpha-actin (SKAA) was investigated. The luciferase gene controlled by a SKAA promoter fragment was transfected into *m. soleus* (SOL) and *m. gastrocnemius* (GM) of both hindlimbs of three month-old female wistar rats (n=13). CaMKII was overexpressed in SOL and GM of the right leg only. Luciferase activity measured in protein extracts of the transfected muscles seven days after transfection was significantly lower in SOL (p=0.02) and GM (p=0.04) of the CaMKII-transfected leg. SKAA mRNA level tended to be lower in the CaMKII transfected leg, but this was not significant. We conclude that CaMKII overexpression has a negative effect on SKAA gene transcription, but the mechanisms underlying this effect require further investigation.

## Introduction

Repeated high mechanical loading of skeletal muscle typically results in hypertrophy, which requires an increased net synthesis of contractile proteins actin and myosin. Overloading of chicken muscle by attaching weights to the wing increases the activity of the skeletal alpha-actin (SKAA) gene promoter in the *m. anterior latissimus dorsi* (Carson et al., 1995). The serum response element SRE1 in the promoter of the SKAA gene, which is bound by the serum response factor (SRF), is necessary and sufficient to induce the loading-dependent increase in SKAA promoter activity (Carson et al., 1996). However, the upstream mechanisms regulating this promoter activity are not completely understood.

Calcium/calmodulin-dependent protein kinase II (CaMKII) is a candidate to transduce increased muscle recruitment into changes in SKAA expression.  $\text{Ca}^{2+}$ /calmodulin-independent CaMKII activity and SRF phosphorylation are both increased after chronic overload of chicken muscle and knee extension-endurance training in humans (Fluck et al., 2000b, Rose et al., 2007b). Phosphorylation of SRF at Ser103 increases its binding to serum response elements (Rivera et al., 1993), while phosphorylation at Thr160 inhibits this binding (Wheaton and Riabowol, 2004). CaMKII can phosphorylate SRF at both Ser103 and Thr160 (Fluck et al., 2000a). Because there is a positive association between CaMKII activity and SRF binding to the SKAA promoter after high muscle loading, we hypothesised that CaMKII overexpression would increase the activity of the SKAA promoter, and SKAA mRNA level in rat skeletal muscle. To test this hypothesis, we introduced a SKAA-promoter construct containing three functional SRE elements into muscles of the right and left hind limbs of rats. CaMKII was overexpressed in the right leg only. To test whether CaMKII-dependent regulation depends on fibre type, we used fast-twitch *m. gastrocnemius* and slow-twitch *m. soleus* for these experiments.

## Methods

### Ethical approval

A total of 13 female Wistar rats were used for the experiments described here. *In situ* contraction protocols and the majority of the transfections 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 and a skeletal alpha-actin reporter gene

#### *Plasmids*

PCDNA3 plasmids encoding full-length cDNA for CaMKII $\alpha$  and CaMKII $\beta$  were a gift from Dr. M Neal Waxham (University of Texas, Houston, USA). PGL2 plasmid encoding full-length luciferase under control of 424basepairs upstream of the transcription start site of the chicken SKAA gene (SKAA-424) (Marsh et al., 1998) was a gift from Dr. Frank W. Booth (University of Missouri, Columbia, USA). This promoter construct contains three serum response elements, two Sp1 binding elements, one E Box, one MCAT and one TATA Box as described previously (Carson et al., 1995). These regulatory elements are conserved across species (Ordahl and Cooper, 1983).

#### *Transfection*

Three month-old female Wistar rats (n=13; Harlan Laboratories and Charles River; body weight: 191-230 grams) were used to overexpress CaMKII in *m. gastrocnemius medialis* (GM) and *m. soleus* (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

plasmid mixture with a total volume of 90  $\mu\text{l}$  were administered intramuscularly using a 29-gauge insulin syringe. GM was administered four injections over the length of the muscle with a total volume of 180  $\mu\text{l}$ . A mix of expression plasmid for full-length CaMKII $\alpha$  (pCDNA3-CaMKII $\alpha$ ; 0.22  $\mu\text{g}/\mu\text{l}$ ) and full-length CaMKII $\beta$  (pCDNA3-CaMKII $\beta$ ; 0.22  $\mu\text{g}/\mu\text{l}$ ) in Tris-Borate-EDTA buffer was injected into muscles of the right leg together with the SKAA-424 reporter plasmid (0.55  $\mu\text{g}/\mu\text{l}$ ). Muscles of the left leg were injected with the reporter plasmid only (1  $\mu\text{g}/\mu\text{l}$ ). 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 individually afterwards, where they resumed normal activity within hours after surgery. After seven days, animals were anaesthetised with 12.5% urethane to measure muscle contractile parameters as described in chapter 3. Transfected muscles were harvested from both legs of anaesthetised rats and snap-frozen in liquid nitrogen. The rats were killed afterwards by an intra-cardiac injection of Euthasol® while under full anaesthesia.

### **Luciferase reporter assay**

Frozen muscle samples were homogenised in RIPA buffer as described for western blots in chapter 3. Homogenates were centrifuged at 10000 RPM for 10 minutes at 4°C. The supernatant was collected and stored at -80°C. Protein concentration was determined with the BCA method (Pierce, Rockford IL, USA). To assay luciferase activity, 20  $\mu\text{g}$  of protein was added to a reaction mix (tricine (2 mM, pH 7.8);  $\text{MgCl}_2$  (5 mM), EDTA (2 mM), dithiothreitol (33.3 mM), ATP (0.25 mM), Coenzyme A (0.27 mM and firefly luciferin (Promega; 0.47mM)) in a white 96-well plate (Eppendorf, Stevenage, UK) at a final volume of 100  $\mu\text{l}$ . Light production was measured with a Hidex Chameleon Plate reader (Hidex; Turku, Finland) for 10 minutes, and the maximum emission ( photon counts per second) was taken as a measure of luciferase activity. Measurements were performed in triplicates and intra-animal muscle pairs were

always assayed simultaneously. A reference protein sample from a luciferase-expressing muscle was always run alongside the experimental samples to check the reproducibility of the luciferase measurements.

### **RNA isolation and RT-PCR**

RNA extraction and RT-PCR analysis were carried out as described elsewhere (van Wessel et al., 2010). Total RNA was extracted from frozen 25  $\mu$ m 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). 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  $\mu$ l total reaction volume. Tubes were heated at 25°C for 5 min, followed by 42°C for 30 min. Finally, the tubes were heated to 85°C for 5 min to stop the reaction and stored at -80°C until used in the PCR reaction.

For each PCR target, 5  $\mu$ l of the RT reaction product was amplified in duplicate using Fast Sybr Green mastermix (Applied Biosystems). 18S ribosomal RNA (18S rRNA) and skeletal alpha-actin (SKAA) mRNA were amplified. Primer sequences are shown in table 1. Amplification efficiency of the primers used was 94.5-102.0%, 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  $2^{-Ct}$ . To quantify the SKAA transcript, 18S rRNA cycle threshold was subtracted from the mean cycle threshold value of the specific target to obtain  $\Delta C_t$  and converted into a relative concentration by  $2^{-\Delta C_t}$ .

### **Statistics**

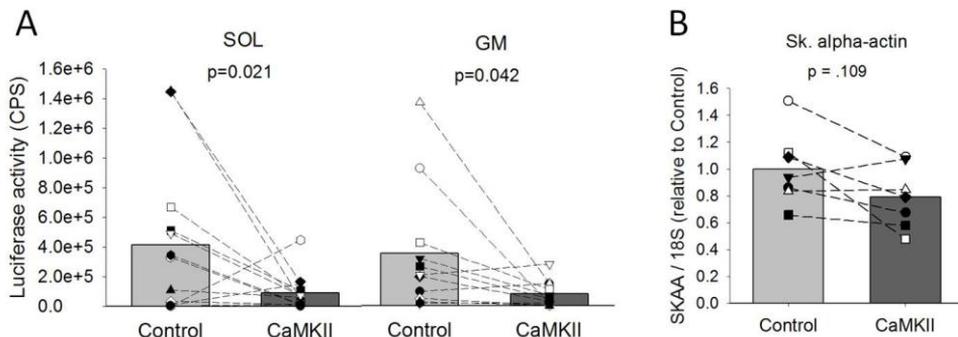
The differences in luciferase activity and SKAA transcript level between control- and CaMKII-transfected muscle pairs were tested with a two-sided wilcoxon signed-ranks test. Significance was accepted at  $p < 0.05$ .

Target mRNA	PCR primer sequence 5'→3'	GenBank accession nr
18S RNA	Forward: CGAACGTCTGCCCTATCAACTT	EU 139318.1
	Reverse: ACCCGTGGTCACCATGGTA	
SKAA	Forward: CGACATCGACATCAGGAAGGA	NM 019212.2
	Reverse: GGTAGTGCCCCCTGACATGA	

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

## Results

Luciferase activity was significantly lower in CaMKII-transfected SOL and GM (Figure 1A), indicating that SKAA promoter activity had been lower in these muscles. In accordance with the lower promoter activity, SKAA transcript levels tended to be lower in CaMKII-transfected *m. soleus* (Figure 1B), but this was not significant.



**Figure 1: Effects of CaMKII overexpression on SKAA promoter activity and mRNA level**

A). Luciferase activity (in photon counts per second, CPS) in 20  $\mu$ g of protein homogenate of CaMKII+SKAA-424-luciferase-transfected muscles (CaMKII) and SKAA-424-luciferase-transfected only (Control) muscles. SOL: *m. soleus*. GM: *m.gastrocnemius*. B). SKAA mRNA level normalized to 18S rRNA in CaMKII+SKAA-424-luciferase-transfected (CaMKII) and SKAA-424-luciferase-transfected only (Control) *m. soleus*. Dots indicate individual measurements, and dashed lines connect measurements on intra-animal muscle pairs. Statistical significance of the comparison between Control- and CaMKII-transfected muscles is indicated above the bars. SKAA: skeletal alpha-actin.

## Discussion

In contrast to our expectations, SKAA promoter activity in CaMKII-transfected muscles was decreased rather than increased compared to control-transfected muscles, and SKAA mRNA tended to be decreased in CaMKII-transfected muscles.

What might be the explanation for our observation that SKAA-424 promoter activity is decreased with CaMKII overexpression, regardless of fibre type? CaMKII can phosphorylate SRF at two sites at least, Ser103 and Thr160 (Fluck et al., 2000a).

Phosphorylation at Ser103 increases its binding to the serum response element (Rivera et al., 1993), while phosphorylation of SRF at Thr160 inhibits binding to the SRE (Wheaton and Riabowol, 2004). Therefore, a possible explanation for our results is that CaMKII overexpression increased SRF phosphorylation at Thr160, which reduced SRF binding to the SRE and the expression of the SKAA -424 promoter-dependent luciferase activity.

Is it possible that CaMKII activates another pathway with inhibitory effects on SKAA gene expression? CaMKII can affect the transcription of multiple genes through phosphorylation of histone deacetylase 4 (HDAC4) (Backs et al., 2006). However, phosphorylation of HDAC4 releases HDAC4-mediated inhibition of SRF-activated transcription of a SKAA-reporter gene (Davis et al., 2003), and would thus be expected to increase, rather than decrease, SKAA promoter activity and transcription. CaMKII also phosphorylates H1 histone *in vitro* (Woodgett et al., 1983), but phosphorylated H1 histone is also associated with increased, rather than decreased transcriptional activity (Zheng et al., 2010). In conclusion, no pathway is known through which CaMKII can inhibit SKAA gene expression.

If CaMKII only activates pathways that stimulate gene expression, as discussed above, perhaps overexpression of CaMKII somehow inhibited this positive effect on gene transcription. The intracellular localisation of overexpressed CaMKII has been shown to be important for the CaMKII-dependent effect on gene transcription in other cell types. In ventricular cardiomyocytes, nuclear-targeted CaMKII was required to activate transcription of the atrial natriuretic factor (ANF) (Ramirez et al., 1997). However,

nuclear CaMKII isoforms were depleted from the nucleus by overexpression of cytosolic isoforms which do not contain a nuclear localisation sequence (NLS), and this prevented ANF transcription. This phenomenon has also been observed in fibroblasts where nuclear or cytosolic targeting depended on the expression ratio of CaMKII isoforms containing an NLS and CaMKII isoforms that do not (Srinivasan et al., 1994). The  $\alpha/\beta$ -CaMKII isoforms used in the present study do not contain a NLS and were localised to the cytoplasm and excluded from the nucleus of transfected fibres (Chapter 3). In nuclear extracts of non-transfected chicken skeletal muscle, CaMKII activity has been measured (Fluck et al., 2000a) which raises the possibility that  $\alpha/\beta$ -CaMKII overexpression had an inhibitive effect on gene expression similar to that observed in cardiomyocytes. However, the CaMKII isoforms thought to be expressed in skeletal muscle do not contain a nuclear localisation sequence (Bayer et al., 1998). This suggests that nuclear targeting of these isoforms might depend on the anchoring protein  $\alpha$ -KAP (Nori et al., 2003), and that factors other than the ratio of nuclear and cytosolic CaMKII isoforms expressed are important in determining whether CaMKII is localised to the nucleus. In conclusion, it is unclear whether CaMKII can be depleted from the nuclei of skeletal muscle fibres by overexpression of cytosolic CaMKII isoforms.

We conclude that CaMKII affects the activity of the SKAA promoter in skeletal muscle, but the precise mechanisms underlying this regulation are yet unclear. Future research should identify whether manipulation of CaMKII affects SRF phosphorylation, and which promoter elements are involved in the CaMKII-dependent regulation in skeletal muscle *in vivo*.

### **Acknowledgements**

We thank Dr. M. Neal Waxham (The University of Texas Medical School, Houston, USA) for donating the  $\alpha/\beta$ -CaMKII plasmids and Dr. Frank W. Booth (University of Missouri, Columbia, USA) for donating the skeletal alpha-actin reporter plasmid.