

SENSORY PATHWAYS OF MUSCLE PHENOTYPIC PLASTICITY: CALCIUM SIGNALLING THROUGH CAMKII

WOUTER EILERS

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Abstract

Skeletal muscle can adapt its structure to cope with the mechanical and metabolic stresses placed on it by various amounts and patterns of human movement. The release of calcium into the cytoplasm of muscle fibres is thought to have an important role in these adaptations, yet the calcium-dependent signalling pathways involved haven't been fully defined. Calcium/calmodulin-dependent protein kinase II (CaMKII) has been presumed to drive mitochondrial biogenesis in skeletal muscle, but this has not been investigated *in vivo*. The experiments in this thesis aimed to address how CaMKII is activated in response to electrical stimulation of skeletal muscle and how CaMKII affects the muscle phenotype. A rat model was used for two main reasons: 1) it allowed for imposing well-defined stimulation patterns onto phenotypically homogenous muscle fibre populations under controlled conditions *in situ*, and investigating the molecular response to these stimulation patterns, and 2) it allowed for manipulation of CaMKII signalling in muscle fibres *in vivo* through the use of electro-assisted somatic gene transfer. It was hypothesised that CaMKII would be activated in a muscle and recruitment pattern specific manner. Furthermore, it was hypothesised that CaMKII overexpression would increase the expression of mitochondrial markers. In chapter 2, the effect of recruitment frequency on CaMKII phosphorylation in slow-twitch *m. soleus* and fast-twitch *m. gastrocnemius medialis* is investigated. Furthermore, the time course of CaMKII phosphorylation after muscle stimulation is studied. Chapter 3 presents a study into the effects of *in vivo* CaMKII overexpression in *m. soleus* and *m. gastrocnemius* on mitochondrial gene expression and muscle contractile function. The effects of CaMKII overexpression on skeletal alpha-actin transcription are presented in chapter 4. In chapter 5, a mathematical model of CaMKII activation in sarcomeres is described, and used to investigate the effects of CaMKII overexpression on calcium handling and on contractile properties of a muscle fibre. It was concluded that CaMKII is activated by very brief stimulation in a recruitment frequency-independent manner, and that increased CaMKII protein levels increase SERCA expression, but not mitochondrial gene expression.

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Chapter 1: General introduction

Movement of the human body occurs by activating skeletal muscles, which exert force that is transmitted to the skeleton via tendons and other connective tissue structures. Sufficient muscle mass is needed to maintain mobility throughout the human life span, but is also important for whole body metabolic function as muscle provides a reservoir of amino acids, acts as an important glucose storage site, and is important in controlling resting metabolic rates of the body (Wolfe, 2006). Mitochondrial abundance in muscle is important for fatigue resistance and is essential for the body's capacity to use fatty acids as a source of energy (Hood, 2001). Maintaining good muscle health is therefore critical to maintaining overall health.

Skeletal muscle is a very plastic tissue which can adapt its structure to cope with the mechanical and metabolic stresses placed on it by various amounts and patterns of human movement (Fluck and Hoppeler, 2003). Important factors that regulate adaptations of muscle must be present in its muscle fibres, as it has been demonstrated that while active muscles adapt to a training program, non-active muscles in the same body do not adapt or adapt to a much lesser extent (Saltin et al., 1976). Knowledge of these factors underlying muscle adaptation might allow for the characterisation of the intramuscular response to a single exercise bout, and subsequent tailoring of training protocols to the needs of the individual, the monitoring of training by using the regulatory molecules as 'progress markers', and the development of gene and/or pharmacological therapies that selectively activate the signalling pathways which are important for skeletal muscle adaptation. This would provide the means to mimic some of the health benefits of exercise while eliminating the need for the actual exercise session. This could be of particular relevance to those who cannot exercise due to illnesses such as cardiovascular diseases and muscular dystrophies.

The aim of this thesis is to investigate the role of calcium/calmodulin-dependent protein kinase II (CaMKII), which is hypothesised to regulate adaptation of skeletal muscle to exercise. This introductory chapter will discuss muscle plasticity and why it is of interest to investigate the role of CaMKII in skeletal muscle.

Skeletal muscle phenotype and function

Skeletal muscle fibres are elongated multi-nucleated cells which consist of myofibrils containing highly arranged contractile protein filaments of which actin and myosin are the important ones. Contraction is initiated upon depolarisation of the plasma membrane of the muscle fibre which triggers a rise in cytoplasmic calcium concentration. This is mediated by the release of calcium from the sarcoplasmic reticulum (SR) through the ryanodine receptor (RyR). The SR is an intracellular calcium store that envelops the myofibrils. Calcium is continuously removed from the cytoplasm into the SR by the sarco/endoplasmic reticulum Ca^{2+} -ATPase (SERCA). This ensures that the intracellular levels of calcium are only significantly elevated when the muscle is recruited to contract. Cytoplasmic calcium binds to troponin C, which is part of a protein complex that blocks binding sites for myosin heads on the actin filaments. Upon binding of calcium to troponin C, the binding sites on actin are exposed and myosin heads can undergo cycles of attachment to actin, bending – thereby producing force – and detachment. The maximum force a muscle fibre can produce is determined by the number of contractile proteins arranged in parallel. All other things being equal, more parallel contractile filaments means a greater cross-sectional area of the fibre and greater maximal force.

As detachment of the myosin heads from the actin filaments costs energy in the form of ATP, it is essential that a sufficient supply of ATP to the myofilaments is maintained in order for muscle contraction to continue. During prolonged contraction, this energy is provided through anaerobic glycolysis and aerobic metabolism of carbohydrates and lipids. Key to this process is the generation of reduction equivalents NADH and FADH in the citric acid cycle, and the subsequent generation of ATP via oxidative phosphorylation, in the mitochondria. Mitochondrial density therefore determines the capacity for oxidative metabolism, and is an important factor in determining the fatigue resistance of skeletal muscles.

Although there is a large heterogeneity in the skeletal muscle phenotype, a key distinction can be made between slow-twitch and fast-twitch fibres, which express type I and type II myosin heavy chain, respectively (Schiaffino and Reggiani, 2011). Fast-twitch fibres display higher myosin ATPase activity, which allows for a higher maximum contractile speed (Bottinelli et al., 1996). Human skeletal muscles are made up of a mixture of these two main fibre types, and a phenotypic switch from slow- to fast-twitch or *vice versa* is thought to require extreme changes in muscle activity in humans (Harridge, 2007).

Therefore, when studying the mechanisms underlying skeletal muscle adaptation to exercise, it will be important to consider the influence of fibre type on the adaptation process.

Phenotypic adaptations of skeletal muscle

Phenotypic adaptations in response to altered muscle recruitment allow the muscle to optimise its functional capacity to the demands that are being placed on it. Cross-innervation experiments suggested that the nerve supplying the muscle affects muscle phenotype (Buller et al., 1960). It was later shown that specific nerve activity patterns could transform fast-twitch fibres into slow-twitch fibres and *vice versa* (Salmons and Vrbova, 1969) and that muscle activity, rather than substances released from the nerve ending, was important for changes in muscle fibre type (Lomo et al., 1974). Similarly, mitochondrial enzyme activity was shown to increase in skeletal muscle after training in rats (Holloszy, 1967) and humans (Gollnick et al., 1973), and the changes in enzyme activities were dependent on the nerve activity pattern (Golisch et al., 1970). These studies suggest the existence of local mechanisms present within skeletal muscles that connect recruitment-related stimuli with cellular adaptation. Experiments which demonstrate that isolated single fibres subjected to chronic electrical stimulation in culture display changes in expression of contractile proteins support that these mechanisms are, at least in part, present in the skeletal muscle fibres themselves (Liu and Schneider, 1998, Mu et al., 2007).

The mechanisms by which muscle recruitment controls phenotypic changes in skeletal muscle are still unclear. Trained athletes have higher baseline levels of metabolic gene transcripts in skeletal muscle (Puntschart et al., 1995, Schmitt et al., 2003), which might underlie their superior muscle endurance. Expression of *fos* and *jun* genes, which are typically expressed very rapidly in response to various extra- and intracellular stimuli, is transiently increased after a bout of exercise (Puntschart et al., 1998), and the transcription of genes involved in fat/glucose metabolism is also activated within the first four hours after an exercise bout (Pilegaard et al., 2000, Hildebrandt et al., 2003). It has been demonstrated that after an exercise session changes in the expression of many genes in muscle occur during the first hours of recovery (Pilegaard et al., 2000, Tunstall et al., 2002, Pilegaard et al., 2003, Mahoney et al., 2005, Schmutz et al., 2006, Mahoney et al., 2008). Increased mRNA levels provide increased template for protein synthesis by translation.

Therefore, the cumulative effects of these changes in transcript levels might underlie structural changes in muscle protein expression and improved muscle function (Fluck, 2006).

It has become clear that the intracellular homeostatic disturbances that occur during muscle contraction are signals that activate signalling pathways which regulate gene expression (Fluck and Hoppeler, 2003). Figure 1 provides an overview of some of these pathways in skeletal muscle, and shows how CaMKII is involved. The increase in intracellular calcium concentration during muscle contraction has been linked to multiple adaptive processes, which will be discussed in the next section.

The role of calcium in muscle adaptations

Intracellular calcium release is directly related to the degree of activation of a muscle fibre. This makes calcium an excellent candidate to relay muscle use-related information to signalling molecules and downstream activation of pathways that coordinate changes in gene expression and regulate muscle remodelling. Indeed, it has been shown that changes in intracellular calcium concentration can induce various adaptations of skeletal muscle. This was first demonstrated in cultures of muscle precursor cells, where an increase in the intracellular cytoplasmic calcium concentration by addition of a calcium ionophore (a compound that facilitates the transport of calcium across the plasma membrane) resulted in increased expression of type I myosin heavy chain and mitochondrial enzyme citrate synthase, and decreased expression of type II myosin heavy chain and glycolytic enzyme lactate dehydrogenase (Kubis et al., 1997). Also, in whole rat muscles *ex vivo*, chronically increasing calcium levels increased transcript levels of genes encoding mitochondrial enzymes (Wright et al., 2007). Finally, mice exposed to cold displayed increased basal levels of calcium and increased citrate synthase activity in skeletal muscles (Bruton et al., 2010). However, in all of these models the calcium concentration was chronically increased, which is very dissimilar to the transient increases in calcium concentration induced by muscle recruitment *in vivo*. In mice that lacked the gene for parvalbumin, an important calcium buffer in fast-twitch muscle, the duration of electrical stimulation-induced calcium transients, but not basal calcium concentration, was increased, as was the mitochondrial density (Chen et al., 2001, Racay et al., 2006). Conversely, slow-twitch muscles of parvalbumin-overexpressing mice had lower mitochondrial enzyme activity

(Chin et al., 2003). Thus, the levels of mitochondrial enzymes can also be affected by more subtle perturbations of calcium levels. However, the amplitude of calcium transients in mice which lack calsequestrin, a calcium buffer located in the SR, in their skeletal muscles were reduced, and yet the fast-twitch *m.extensor digitorum longus* of these animals had a higher mitochondrial density compared to wild-type controls (Paolini et al., 2007). Note that conclusions drawn from studies using transgenic mice models may be influenced by compensatory adaptations during development. Taken together, these results suggest that the contraction-induced increase in calcium concentration in muscle fibres leads to an increase in mitochondrial biogenesis.

This raises the question of how the increased calcium concentration is transduced into changes in gene expression. Various transcription factors that have been demonstrated to activate the expression of genes involved in muscle remodelling are sensitive to calcium-dependent signalling pathways (Bassel-Duby and Olson, 2006). The two most studied calcium transducers in skeletal muscle are CaMKII and calcineurin, a calcium/calmodulin-dependent protein phosphatase. Current literature suggests that calcineurin is sufficient and necessary to induce expression of slow fibre type-related myofibrillar proteins (Chin et al., 1998, Naya et al., 2000, Serrano et al., 2001, Parsons et al., 2004). In addition, lifelong expression of constitutively active calcineurin is sufficient to increase mitochondrial gene expression and maximal mitochondrial respiration (Long et al., 2007, Jiang et al., 2010). However, inhibiting calcineurin *in vivo* with cyclosporin did not prevent increased mitochondrial gene expression in response to swimming exercise in rats (Garcia-Roves et al., 2006). This suggests that calcineurin is not required for increased mitochondrial biogenesis in response to exercise, and other pathways must be involved.

CaMKII as a calcium-dependent regulator of skeletal muscle phenotype

A possible candidate to relay calcium concentration changes to multiple adaptation processes is calcium/calmodulin-dependent protein kinase II (CaMKII), which is the focus of investigation in this thesis. CaMKII is a protein serine/threonine kinase, which has first been discovered in neuronal membranes (Schulman and Greengard, 1978), and is thought to be involved in the formation of memory (Lisman et al., 2002). Four CaMKII genes are present in the human genome, encoding alpha, beta, gamma and delta isoforms of CaMKII. All isoforms have multiple splice variants, which results in at least 38 different variants

being expressed in a cell-specific pattern (Tombes et al., 2003). All four CaMKII genes contain a N-terminal kinase domain, a C-terminal association domain and a variable autoregulatory region (Hudmon and Schulman, 2002) and form dodecamers with very similar structures, but slightly different sensitivities for calcium-bound calmodulin (Gaertner et al., 2004).

CaMKII is part of a larger protein family of calcium/calmodulin-dependent protein kinases, of which CaMKI, CaMKIV and calcium/calmodulin-dependent kinase kinase (CaMKK) have multiple substrates. The CaMK family of genes also includes glycogen phosphorylase kinase, myosin light chain kinase and eukaryotic elongation factor 2 kinase (also known as CaMKIII), which have single substrates.

CaMKI seems to be expressed in mouse skeletal muscle, but not in human skeletal muscle (Rose et al., 2006, Witczak et al., 2007). CaMKII is expressed in rodent and human skeletal muscle (Woodgett et al., 1982, Rose et al., 2006). CaMKIV is expressed in neither human, nor rodent skeletal muscle (Akimoto et al., 2004, Rose et al., 2006). This is relevant, as inferences have been made about the function of CaMKII based on studies into the effects of other members of the CaMK family. However, CaMKII is the only multifunctional CaMK expressed in human skeletal muscle (Rose et al., 2006). CaMKK alpha, which acts upstream of CaMK's, is also expressed in human skeletal muscle (Rose et al., 2006). This kinase phosphorylates CaMKI and IV, but not CaMKII (Tokumitsu et al., 1995), and it is therefore possible that CaMKK alpha is a part of a separate calcium/calmodulin-dependent pathway in skeletal muscle.

The role of CaMKII in structural adaptation of skeletal muscle to exercise is currently unclear. CaMKII is activated during cycling exercise (Rose et al., 2006), and this activation depends on cycling intensity (Egan et al., 2010). However, it is not known which intramuscular factors related to cycling intensity are important for this regulation. Basal CaMKII activity was increased in rooster skeletal muscle by overloading (Fluck et al., 2000b) and in human *m. vastus lateralis* by cycling exercise (Rose et al., 2007b), suggesting it may be involved in the adaptation to these protocols.

CaMK's have been proposed to regulate a number of biological processes in skeletal muscle (Fig. 1). Transgenic mice overexpressing a constitutively active mutant of CaMKIV have increased mitochondrial density, which was thought to be caused by

increased expression of transcription co-factor peroxisome proliferator-activated receptor γ coactivator-1 (PGC-1) expression (Wu et al., 2002). Overexpression of PGC-1 increased mitochondrial biogenesis in cultured myotubes (Wu et al., 1999). Many studies have demonstrated that CaMK's can regulate the activity of various transcription factors involved in the regulation of PGC-1 expression, usually in cultured myoblasts (Lu et al., 2000b, McKinsey et al., 2000a, McKinsey et al., 2000b, Wu et al., 2000, Handschin et al., 2003). The picture emerging from these studies is that CaMK's regulate gene expression in skeletal muscle cells by a general mechanism involving the phosphorylation of histone deacetylases (HDACs; Fig. 1). HDACs bind to transcription factor, myocyte enhancer factor 2 (MEF2), and inhibit its transcriptional activity. Upon phosphorylation by CaMK, HDAC is removed from the cell nucleus and MEF2 can bind to the PGC1 promoter and activate PGC1 transcription. These studies suggest that CaMK activates mitochondrial biogenesis in skeletal muscle. However, these studies have used overexpression of constitutively active mutants of CaMKI or CaMKIV, neither of which is expressed in human skeletal muscle (Rose et al., 2006). The question remains whether CaMKII also regulates these factors. It has been demonstrated in cardiac myocytes that CaMKII can phosphorylate HDAC4, thereby inducing its export from the nucleus (Backs et al., 2006), and activating a MEF2-dependent reporter gene (Zhang et al., 2007), but in skeletal muscle this information is still missing.

CaMKII isolated from chicken skeletal muscle phosphorylates serum response factor (SRF) (Fluck et al., 2000a), a transcription factor which is essential for skeletal alpha-actin gene transcription during skeletal muscle development (Croissant et al., 1996). This indicates that CaMKII may also be involved in the regulation of skeletal alpha-actin expression (Fig. 1), which is increased in response to stretch overloading of chicken skeletal muscle (Gregory et al., 1990). Furthermore, CaMKIV activates SRF-dependent transcriptional activity by removing HDAC4-dependent SRF inhibition in cardiomyocytes (Davis et al., 2003), indicating there may be another mechanism whereby CaMK's regulate gene transcription in skeletal muscle through HDACs.

The involvement of CaMKII in regulating gene expression in skeletal muscle and adaptation of its phenotype has also been suggested by studies using pharmacological CaMK inhibitors. Electrical stimulation of cultured adult mouse muscle fibres results in export of HDAC4 from the nucleus and activation of a MEF2-dependent reporter gene, and

these effects are blocked by the calmodulin-competitive inhibitor KN62 (Liu et al., 2005). KN93, another calmodulin-competitive CaMK inhibitor, inhibits the expression of slow-fibre type genes in cultured adult mouse muscle fibres (Mu et al., 2007). In rats, KN93 blocked the swimming exercise-induced binding of MEF2 to the glucose transporter 4 (GLUT4) promoter (Smith et al., 2008). These studies suggest that CaMK's are involved in the regulation of MEF2 activity and gene expression in whole muscle. However, inhibition of calcium-induced expression of mitochondrial genes by KN93 has only been demonstrated in cultured myotubes (Ojuka et al., 2003). Furthermore, the KN62/KN93 inhibitors suppress the activity of all CaMK's (Wayman et al., 2008) and have effects on calcium/calmodulin-independent enzymes and ion channels as well (Davies et al., 2000, Gao et al., 2006, Ledoux et al., 1999). Therefore, it is not certain whether the observed effects in these studies can be ascribed to inhibition of CaMKII only.

Finally, there is evidence that CaMKII has acute effects on muscle function. In mouse skeletal muscle, its inhibition has been shown to decrease calcium release from the SR (Tavi et al., 2003) and glucose uptake during muscle contraction (Witczak et al., 2010).

In conclusion, although it has recently been claimed that "*there is overwhelming experimental support for CaN and CaMKII being capable of inducing major changes in muscle fibre properties*" (Tavi and Westerblad, 2011), the above review of the current literature indicates that although there are suggestions that CaMKII is a key regulator of the expression of genes involved in muscle fibre adaptations *in vivo*, this has not been tested yet by modifying CaMKII isoforms *in vivo*.

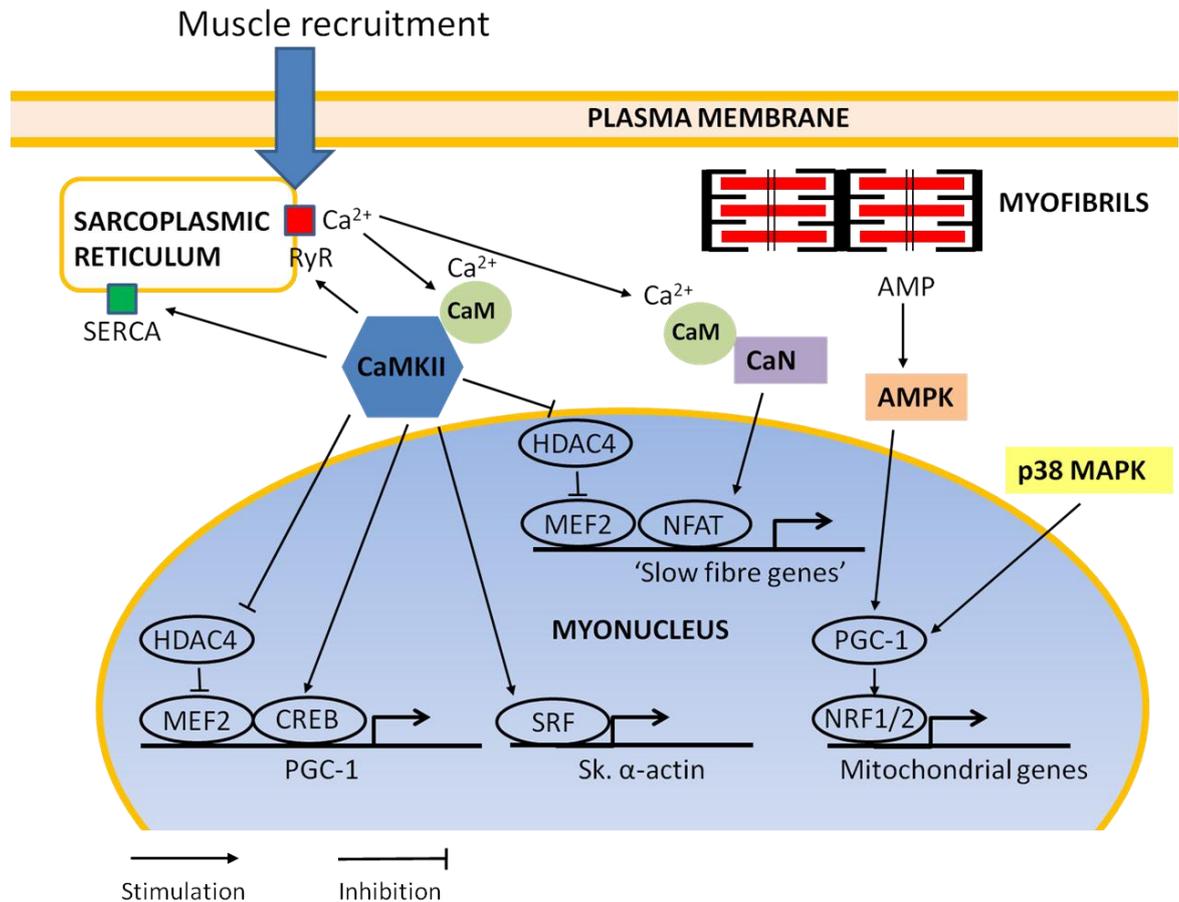


Figure 1: Potential intracellular signalling pathways involved in skeletal muscle adaptation to exercise
 Diagram showing how CaMKII may affect muscle phenotype by altering gene expression. In addition, CaMKII is thought to stimulate calcium release from, and uptake into, the sarcoplasmic reticulum. A few other pathways thought to be important for skeletal muscle adaptation are also shown. Note that not all factors currently believed to regulate muscle adaptation are included in this overview, and that the scientific evidence for the pathways shown here is incomplete. Recent reviews on the topic are available (Gundersen, 2011). Abbreviations: RyR: ryanodine receptor; SERCA: sarco/endoplasmic reticulum Ca²⁺-ATPase; CaMKII: calcium/calmodulin-dependent protein kinase II; CaM: calmodulin; CaN: calcineurin; AMPK: 5'-AMP-activated protein kinase; p38 MAPK: p38 Mitogen-activated protein kinase; HDAC: histone deacetylase; MEF: myocyte enhancer factor; CREB: cAMP-reactive element binding protein; SRF: serum response factor; Sk. α-actin: skeletal alpha-actin; NFAT: nuclear factor of activated t-cells; PGC-1: peroxisome proliferator-activated receptor gamma coactivator 1; NRF1/2: nuclear respiratory factor 1 & nuclear respiratory factor 2.

Aims and thesis outline

The gap in our current understanding of CaMKII function in skeletal muscle is how its activity is regulated in skeletal muscle by the recruitment pattern, and what the effects of

CaMKII are on skeletal muscle function *in vivo*, in slow- and fast-twitch muscle. The experiments in this thesis aim to address this gap in knowledge. A rat model is used, because it allows for imposing well-defined stimulation patterns onto phenotypically homogenous muscle fibre populations under controlled conditions *in situ*, and investigating the molecular response to these stimulation patterns. Furthermore, it allows for manipulation of CaMKII signalling in muscle fibres *in vivo* through the use of electro-assisted somatic gene transfer.

In **chapter 2**, the effect of recruitment frequency on CaMKII phosphorylation in slow-twitch *m. soleus* and fast-twitch *m. gastrocnemius medialis* is investigated. Furthermore, the time course of CaMKII phosphorylation after muscle stimulation is studied. **Chapter 3** presents a study into the effects of *in vivo* CaMKII overexpression in *m. soleus* and *m. gastrocnemius* on mitochondrial gene expression and muscle contractile function. The effects of CaMKII overexpression on skeletal alpha-actin transcription are presented in **chapter 4**. Experimental control is inherently limited in biological experiments and therefore it can be difficult to identify cause and effect. Mathematical modelling provides perfect control of system parameters, which allows dissecting biological mechanisms and generating new hypotheses. In **chapter 5**, a mathematical model of CaMKII activation in sarcomeres is described, and used to investigate the effects of CaMKII overexpression on calcium handling and on contractile properties of a muscle fibre. The thesis is concluded with a general discussion in **chapter 6** of the implications of the results for our understanding of CaMKII function in skeletal muscle.

Chapter 2: Regulation of CaMKII autophosphorylation by recruitment and muscle type

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 was increased compared to rest in red GM ($p=0.03$). 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 ($\text{Ca}^{2+}/\text{CaM}$) (Hudmon and Schulman, 2002), and therefore, availability of $\text{Ca}^{2+}/\text{CaM}$ 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 $\text{Ca}^{2+}/\text{CaM}$, CaMKII can undergo autophosphorylation at Thr287 and gain $\text{Ca}^{2+}/\text{CaM}$ -independent activity. *In vitro*, it has been shown that the generation of $\text{Ca}^{2+}/\text{CaM}$ -independent, or autonomous, CaMKII activity depends on the frequency, amplitude and duration of $\text{Ca}^{2+}/\text{CaM}$ 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 recruitment (Chin, 2005).

This idea is supported by the finding that high (80% $\text{VO}_{2\text{max}}$), but not low (40% $\text{VO}_{2\text{max}}$) 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% $\text{VO}_{2\text{max}}$ 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, Benziene 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 μ M vs. 10 μ M) 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 hypothesised 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 ($\pm 37^\circ\text{C}$) 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 (50 μs 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 (23°C). 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 μ s). 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 μ s), 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)

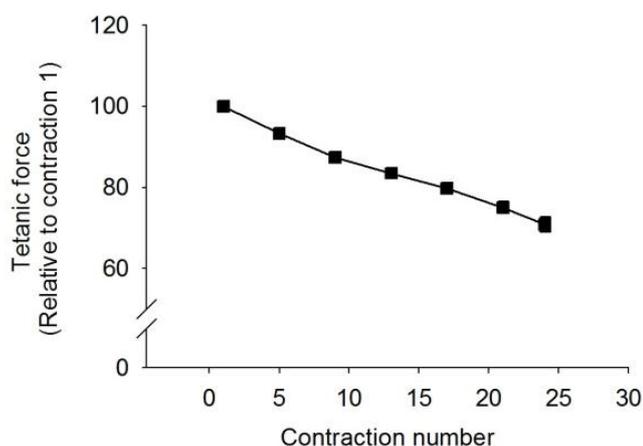


Figure 1: Decrease in tetanic force during the ‘two-minute isometric’ contraction protocol

Data are displayed as means \pm SEM. Note that the error bars are barely visible because they are often smaller than the symbols indicating the mean relative tetanic 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 -80°C until used for western blotting.

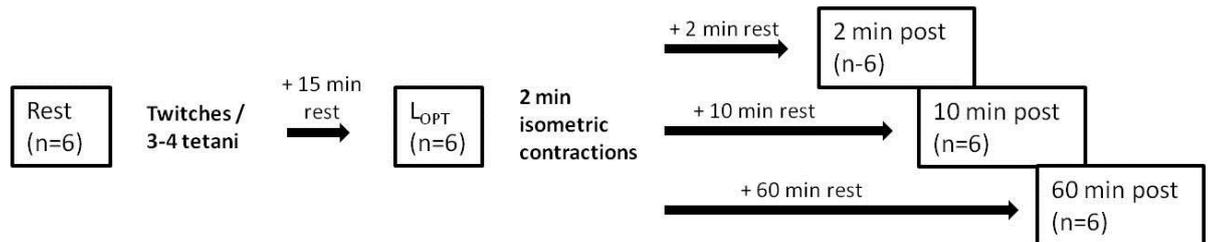


Figure 2: Experimental design of the ‘two-minute isometric’ contraction protocol.

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 -20°C . 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, 1 $\mu\text{g}/\text{ml}$ leupeptin, 0.2 $\mu\text{g}/\text{ml}$ pepstatin, 0.1 $\mu\text{g}/\text{ml}$ 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 -80°C 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 95°C. 20 μ g 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 μ l of crude muscle homogenate with a reaction mix containing calcium/calmodulin (Enzo Life Sciences, Exeter, United Kingdom) (total volume: 50 μ l; 0.1 mM ATP, 10 mM HEPES (pH 7.4), 5 mM MgCl₂, 0.1% Tween-20 and 0.5 mM CaCl₂

+ 1 μ M calmodulin or 5 mM EGTA) for 30 minutes at 30°C. The reaction was stopped by the addition of 16.7 μ l 4x sample buffer. Samples were heated to 95 °C 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 Spearman'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).

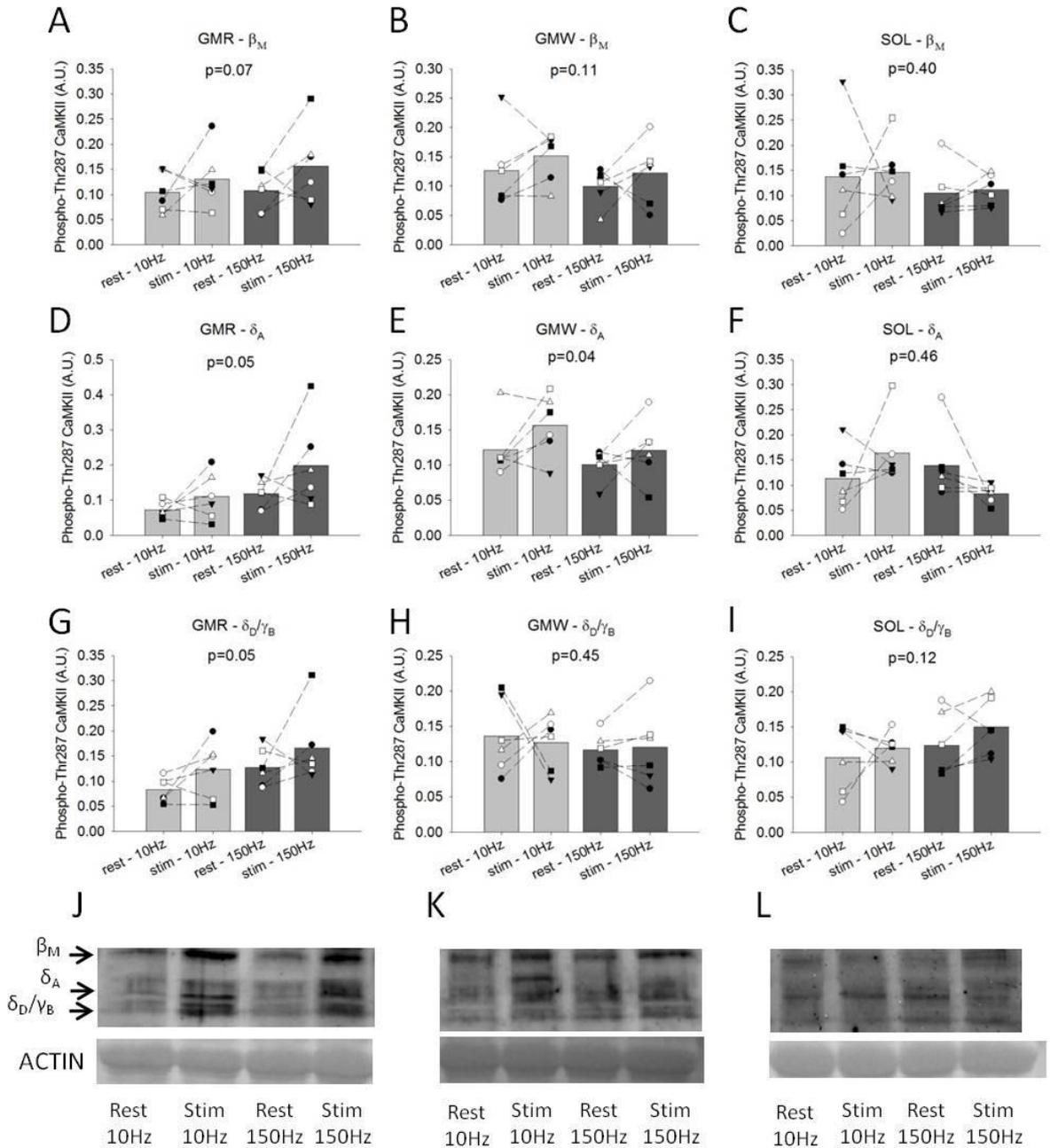


Figure 3: Phospho^{Thr287}-CaMKII levels in rat *m. gastrocnemius medialis* and *m. soleus* samples after slack contractions.

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 ponceu s-stained actin bands are shown below the graphs. J: GMR; K: GMW; L: SOL.

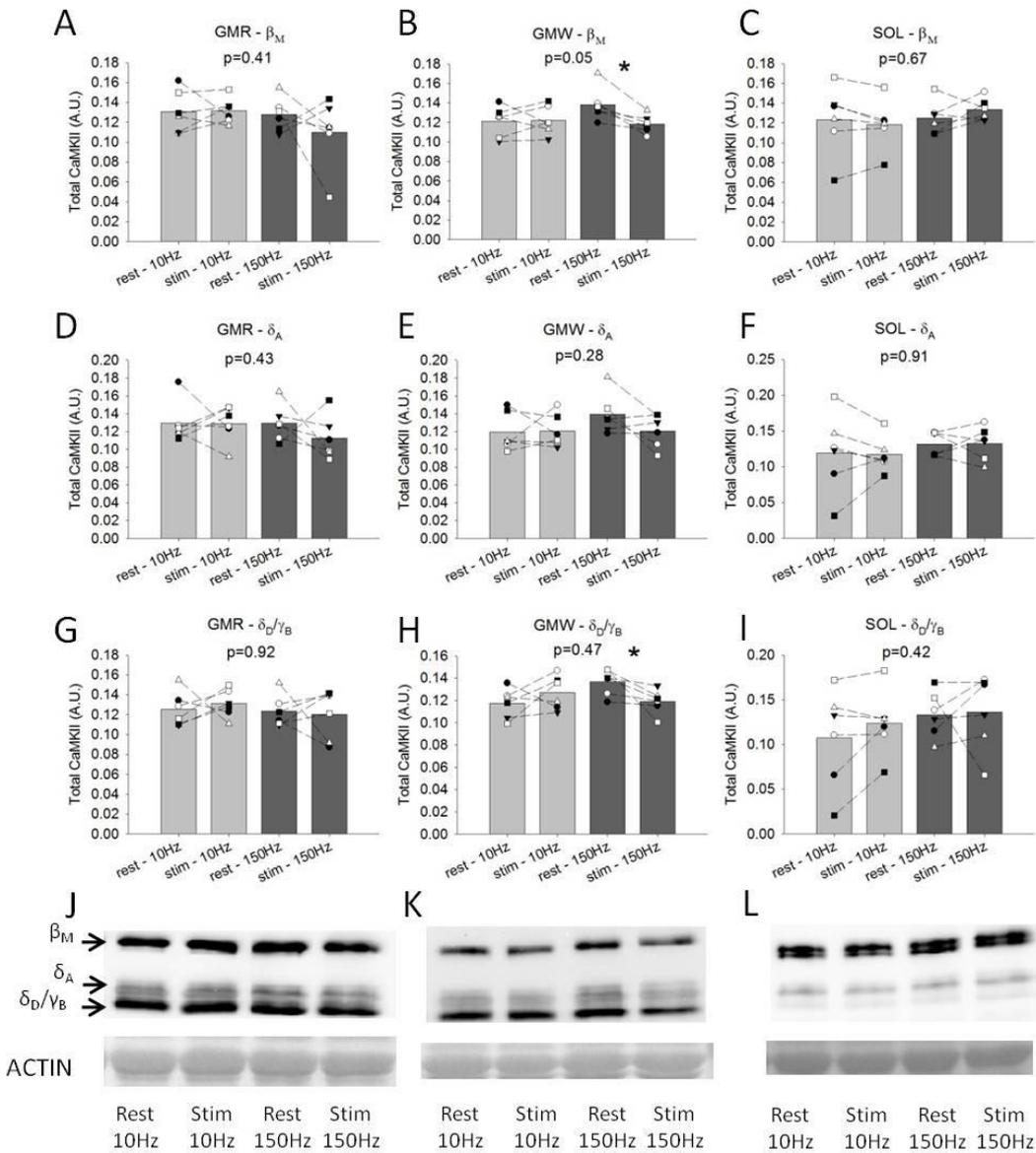


Figure 4: Decrease in total CaMKII in rat white *m. gastrocnemius medialis* after slack contractions. Graphs display total CaMKII levels for β_M , δ_A and δ_D/γ_B as determined by western blotting followed by immunodetection. A: GMR, β_M -CaMKII; B: GMW, β_M -CaMKII; C: SOL, β_M -CaMKII; D: GMR, δ_A -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).

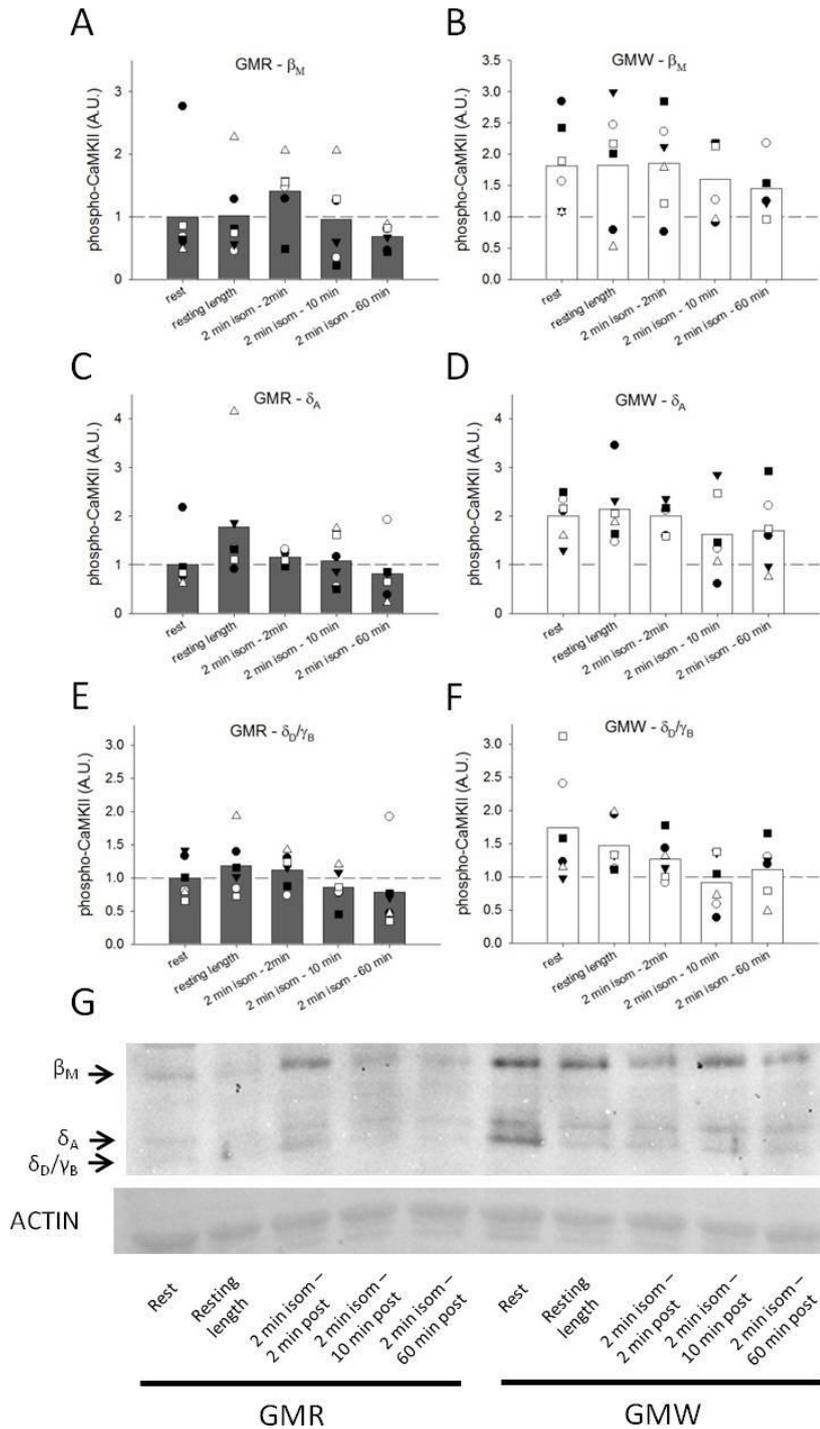


Figure 5: Phospho^{Thr287}-CaMKII in rat *m. gastrocnemius medialis* samples before and after a two-minute isometric contraction protocol.

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*.

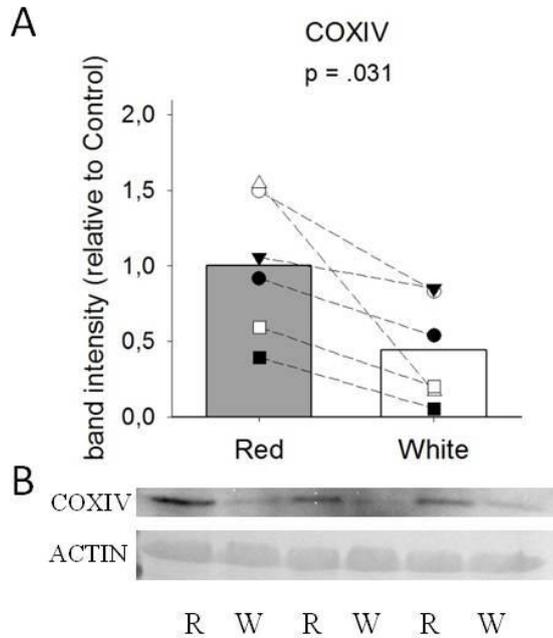


Figure 6: COXIV protein expression in oxidative and glycolytic *m. gastrocnemius*

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.

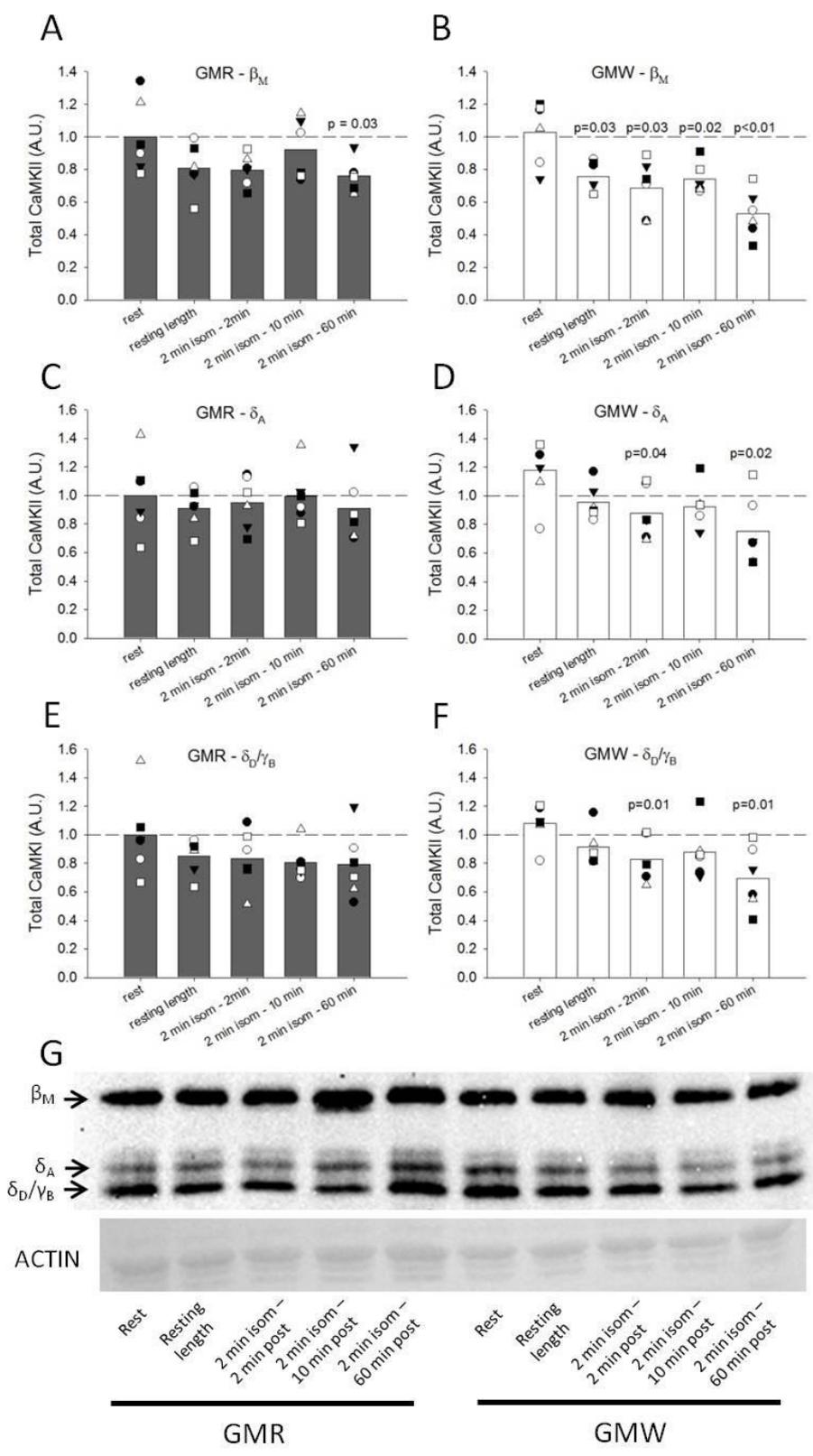


Figure 7: Total CaMKII in rat *m. gastrocnemius medialis* samples before and after a two-minute isometric contraction protocol.

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 GMV 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. Ca²⁺/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 autophosphorylation 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 (Ljubcic and Hood, 2008). Autophosphorylation at Thr287 increases the affinity of CaMKII for Ca²⁺/CaM manyfold (Meyer et al., 1992). Therefore, Ca²⁺-CaM-dependent CaMKII activity in GMW may be sensitised to changes in [Ca²⁺]_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 (McConnell 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 Ca²⁺ 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 α KAP (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

We thank Daniëlle van Overbeek for carrying out the ‘two-minute isometric contraction’ *in situ* experiment and Tinelies Busé-Pot for assistance during all *in situ* contraction experiments.

Appendix: Western blot loading controls

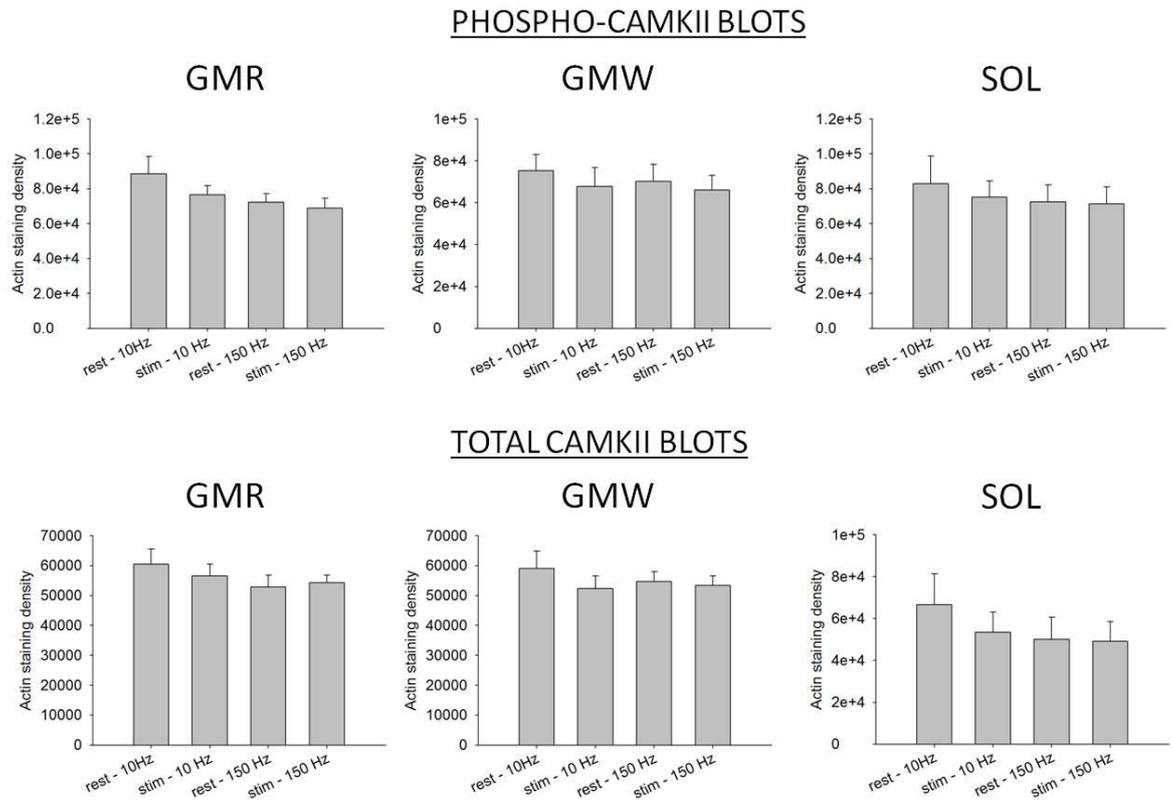


Figure A1: Western blot loading controls for ‘slack contraction’ experiments

Graphs displaying quantifications of ponceau s-stained actin on blots of muscle samples from the ‘slack contraction’ experiment. Data are displayed as means \pm 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*.

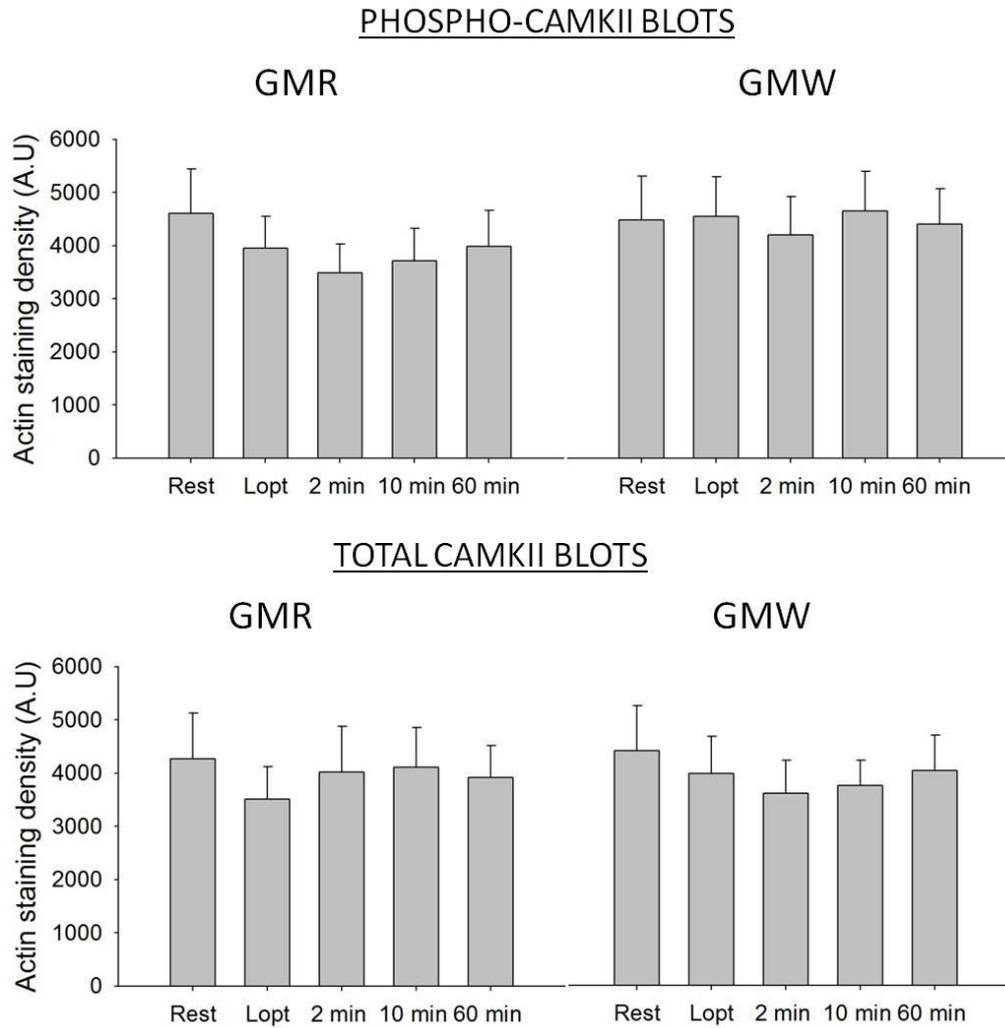


Figure A2: Western blot loading controls for ‘2 minutes isometric contraction’ experiments

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 \pm S.E.M. GMR = High oxidative (red) *m. gastrocnemius medialis*. GMW = Low oxidative (white) *m. gastrocnemius medialis*.

Chapter 3: Ca²⁺/calmodulin-dependent protein kinase II affects contractile, but not fatigue-related phenotype in rat skeletal muscle

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-1 α 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 Ca²⁺-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 CaMKII α and CaMKII β 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 CaMKII α (pCDNA3-CaMKII α ; 0.22 $\mu\text{g}/\mu\text{l}$) and full-length CaMKII β (pCDNA3-CaMKII β ; 0.22 $\mu\text{g}/\mu\text{l}$) in TBE buffer was injected into muscles of the right leg together with the -424 skeletal alpha-actin reporter plasmid (0.55 $\mu\text{g}/\mu\text{l}$). 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 $\mu\text{g}/\mu\text{l}$). 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 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 (24°C). 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 μ sec, 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 -80°C 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 μ m 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 μ g/ml leupeptin, 0.2 μ g/ml pepstatin, 0.1 μ g/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 -80°C 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 95°C . 20-40 μ g 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 °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 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-1 α) 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 $2^{-\Delta C_t}$.

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: ACTCATTGGTGCCCTTGTTT	
SDH (subunit b)	Forward: CAGAGAAGGGATCTGTGGCT	NM 001100539.1
	Reverse: TGTTGCCTCCGTTGATGTTC	
PGC-1 α	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)

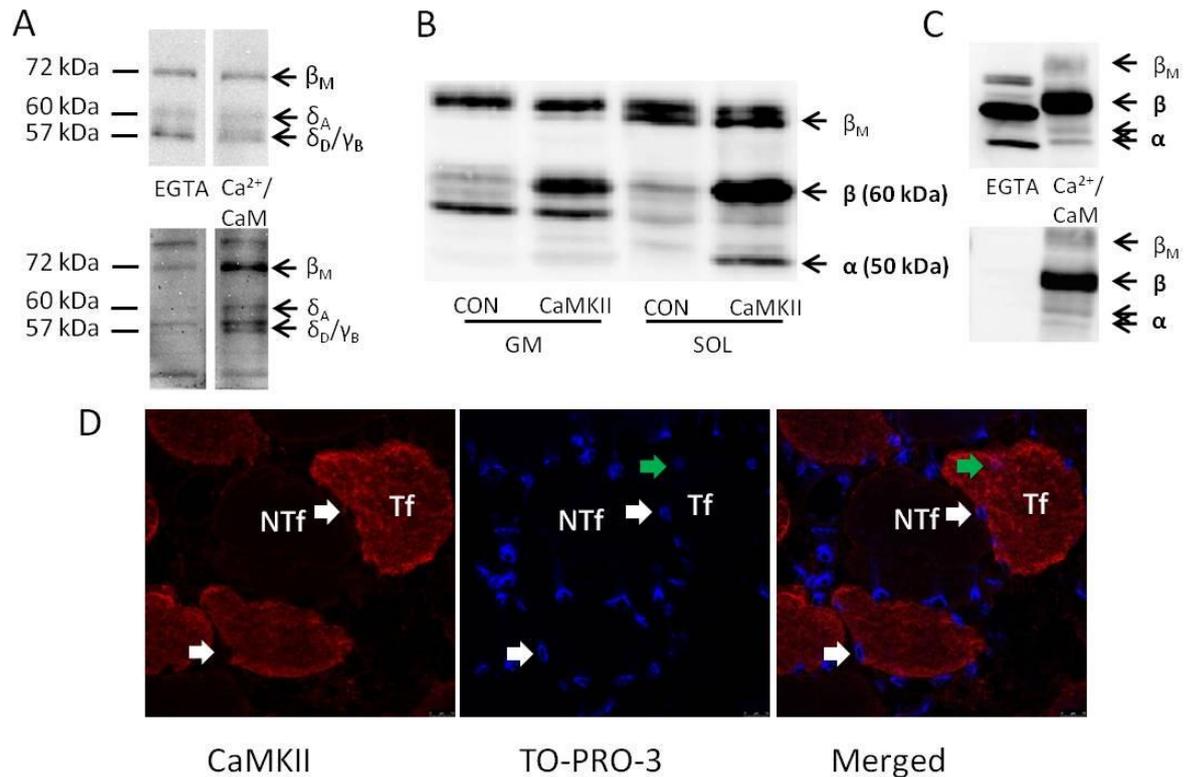


Figure 1: Expression of endogenous and exogenous CaMKII in rat skeletal muscle

A: Endogenous total CaMKII (top panel) and phospho^{Thr287}-CaMKII (bottom panel) of rat GM homogenate after incubation with EGTA (left) or Ca²⁺/Calmodulin (Ca²⁺/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 Ca²⁺/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).

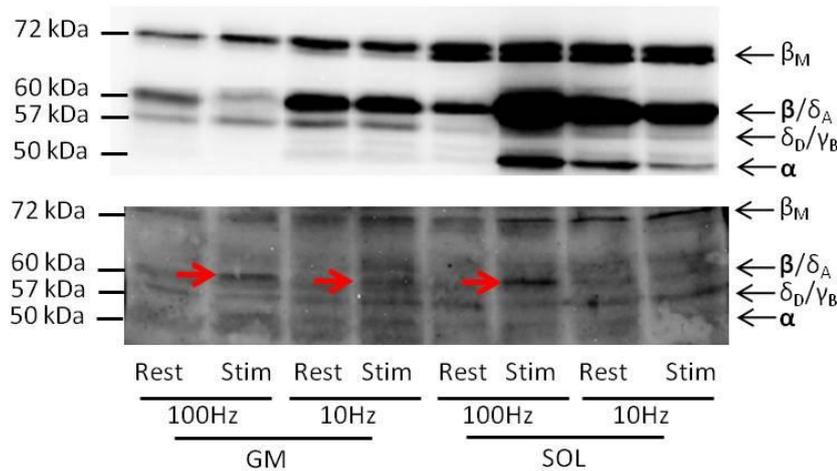


Figure 2: Phosphorylation of exogenous CaMKII in rat skeletal muscle *in situ*

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 phosphorylated 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-1 α 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-1 α (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).

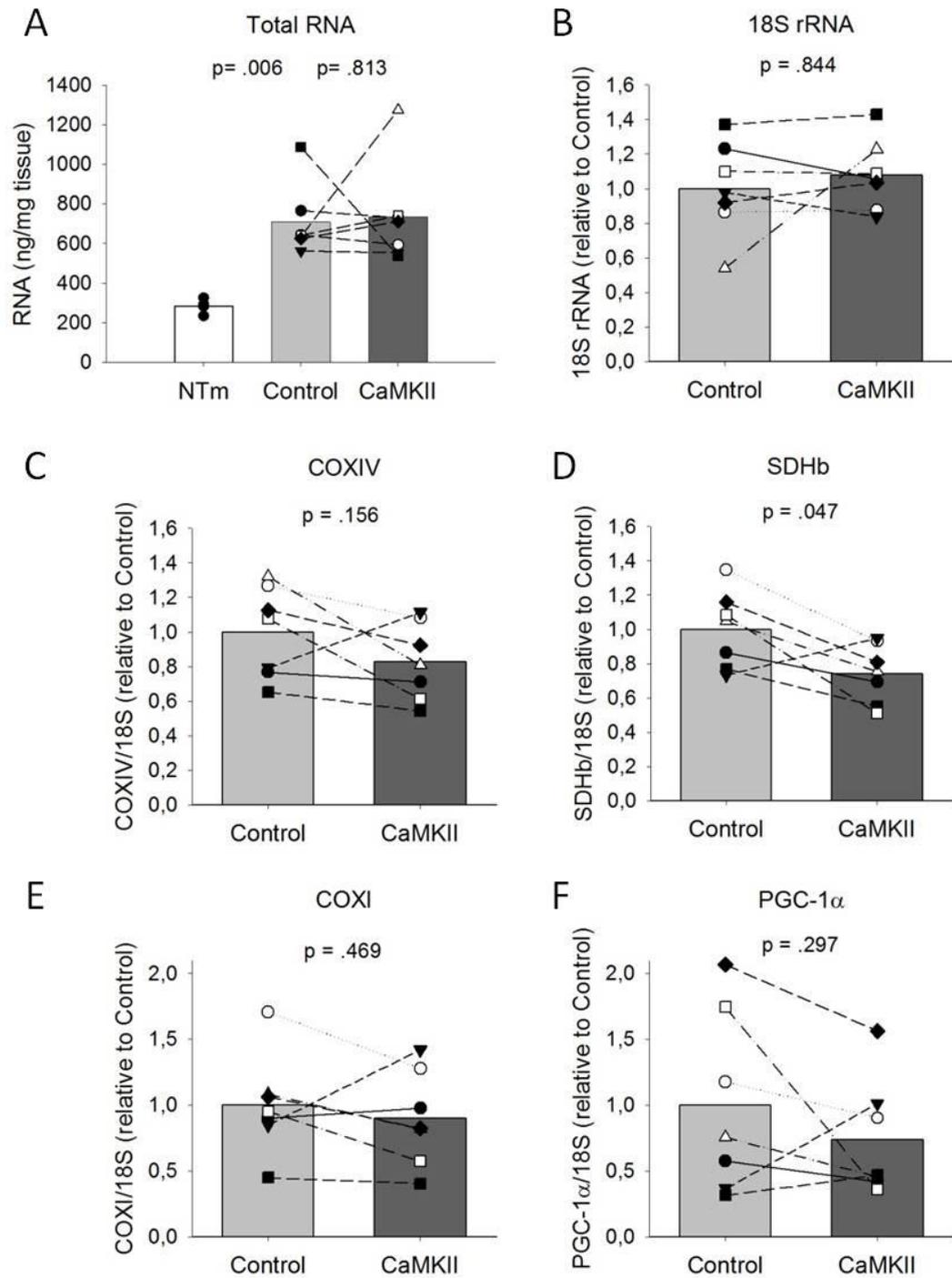


Figure 3: Effects of CaMKII transfection on mitochondrial gene transcript levels

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.

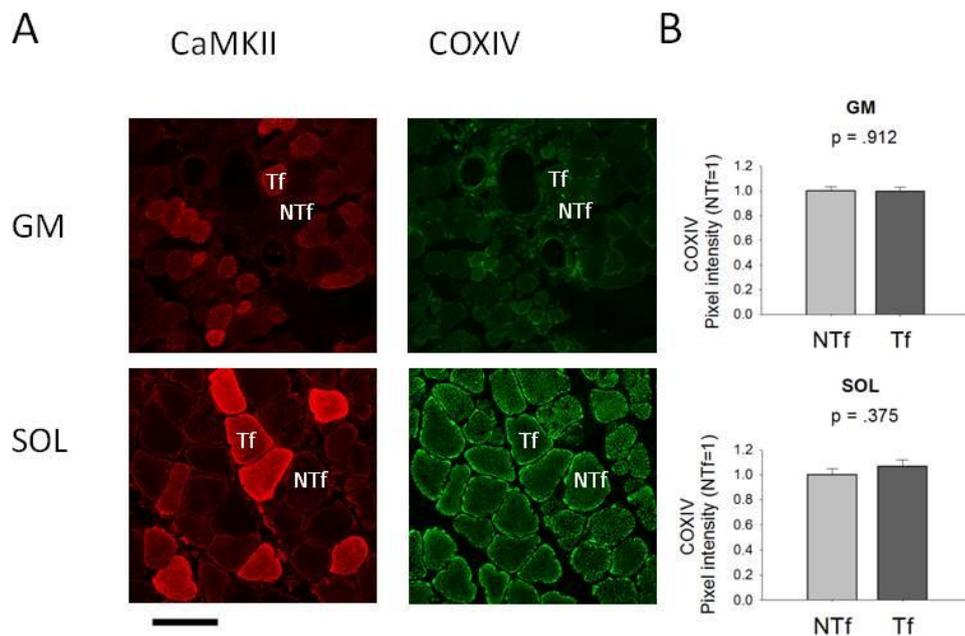


Figure 4: COXIV protein expression in CaMKII-transfected fibres

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 μ m. B: Graphs displaying mean \pm 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).

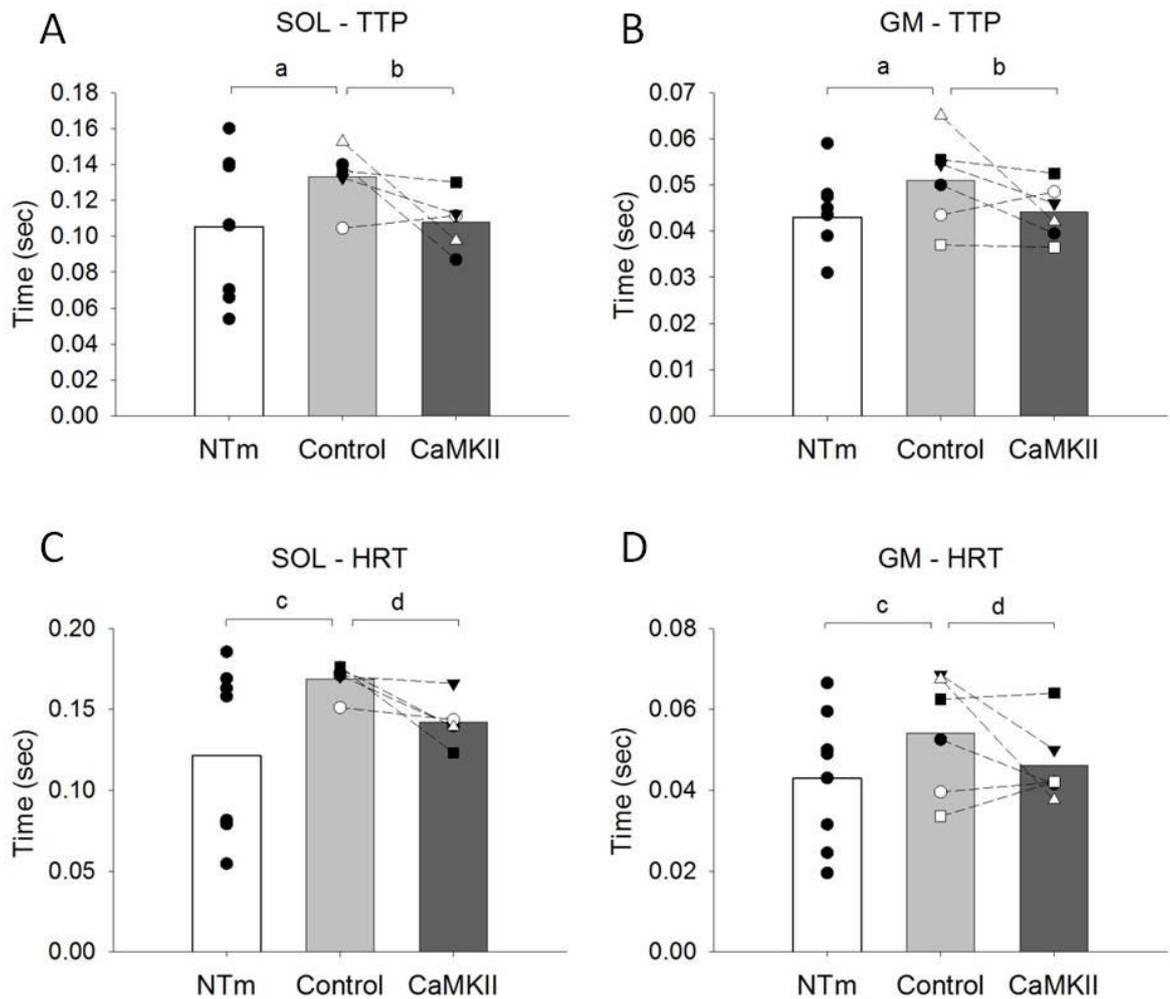


Figure 5: Effects of CaMKII overexpression on twitch contraction/relaxation time

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.

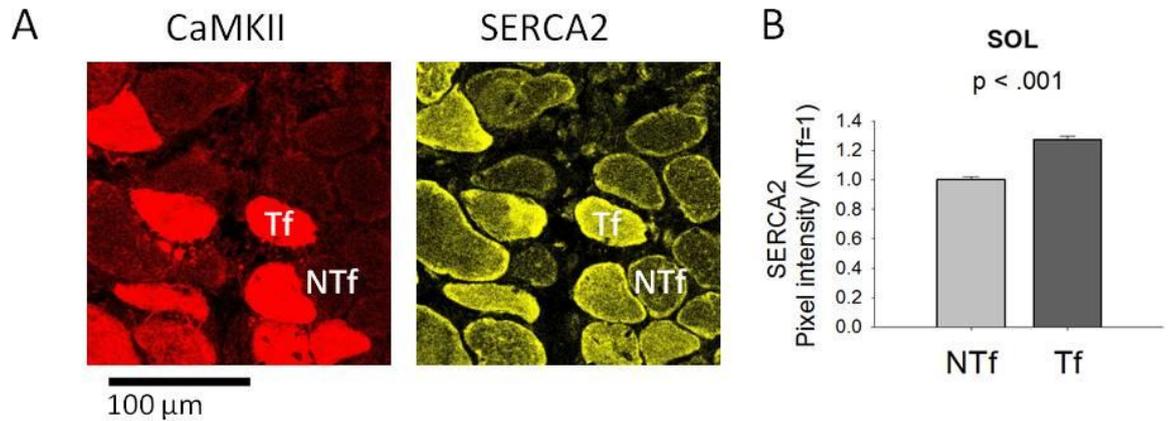


Figure 6: CaMKII overexpression increases SERCA2 expression in *m. soleus* fibres

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 \pm 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.

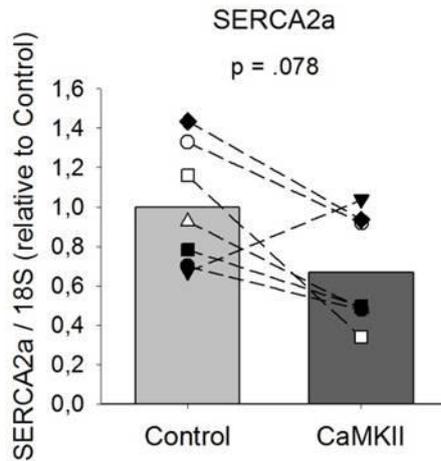


Figure 7: Effect of CaMKII-overexpression on SERCA2a transcript level in *m. soleus* fibres.

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).

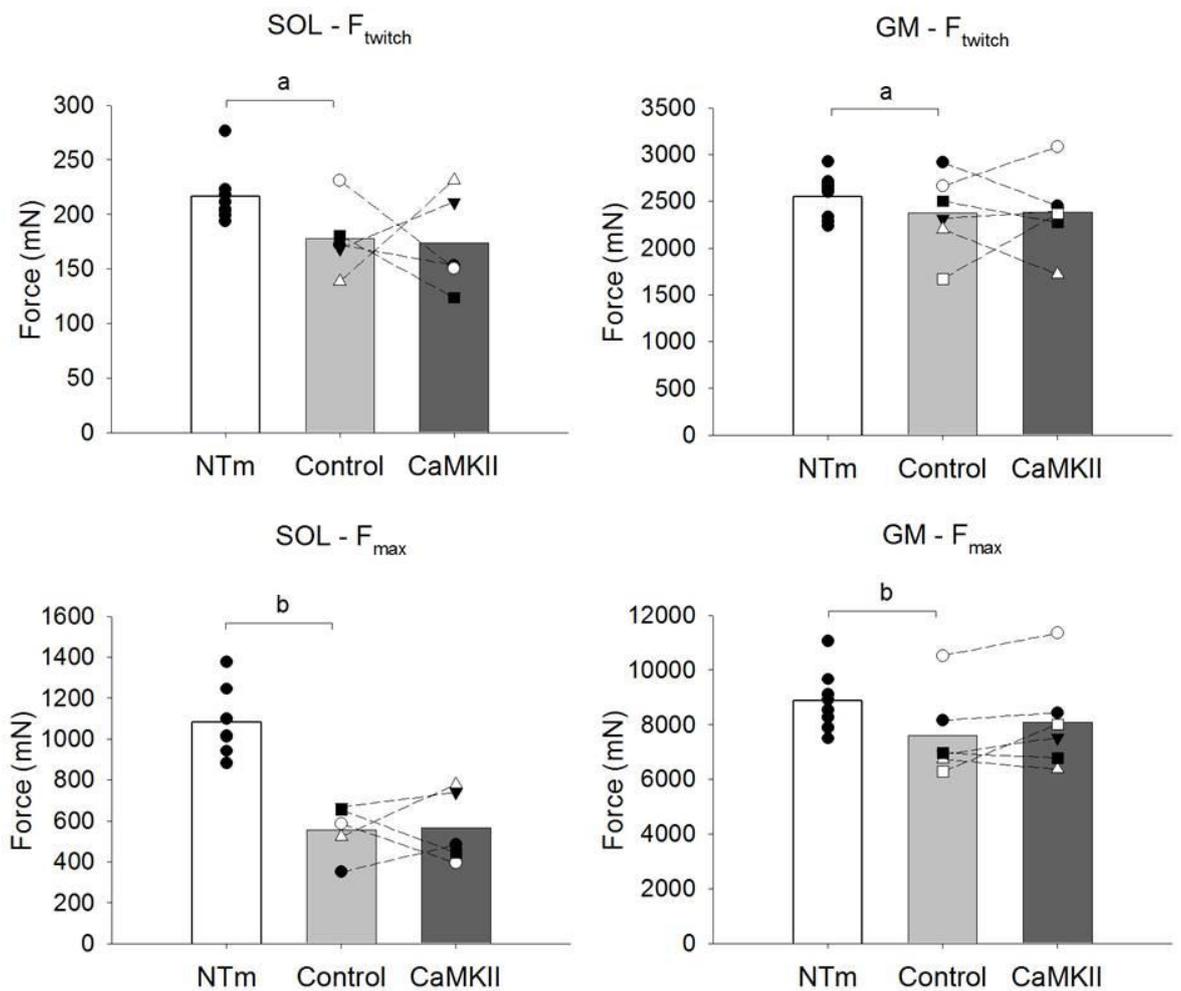


Figure 8: Maximum twitch and tetanic force of transfected and non-transfected muscles

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} ($p < 0.001$).

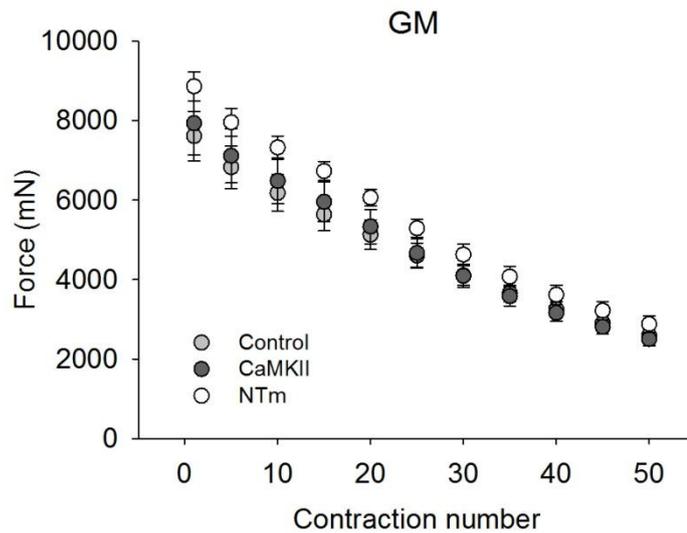


Figure 9: Effects of CaMKII overexpression on force reduction during repeated maximal tetanic contractions of GM

Graph displays tetanic force of *m. gastrocnemius medialis* (GM) during a contraction protocol consisting of 50 repeated tetanic contractions. Maximal tetanic force (mean \pm 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.

Acknowledgements

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Chapter 4: Effect of CaMKII overexpression on skeletal alpha-actin transcription in rat skeletal muscle

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. 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 α and CaMKII β 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 μ 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 μ l. A mix of expression plasmid for full-length CaMKII α (pCDNA3-CaMKII α ; 0.22 μ g/ μ l) and full-length CaMKII β (pCDNA3-CaMKII β ; 0.22 μ g/ μ l) in Tris-Borate-EDTA buffer was injected into muscles of the right leg together with the SKAA-424 reporter plasmid (0.55 μ g/ μ l). Muscles of the left leg were injected with the reporter plasmid only (1 μ g/ μ 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 µg of protein was added to a reaction mix (tricine (2 mM, pH 7.8); MgCl₂ (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 µl. 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 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). 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°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 µl 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 ΔC_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.

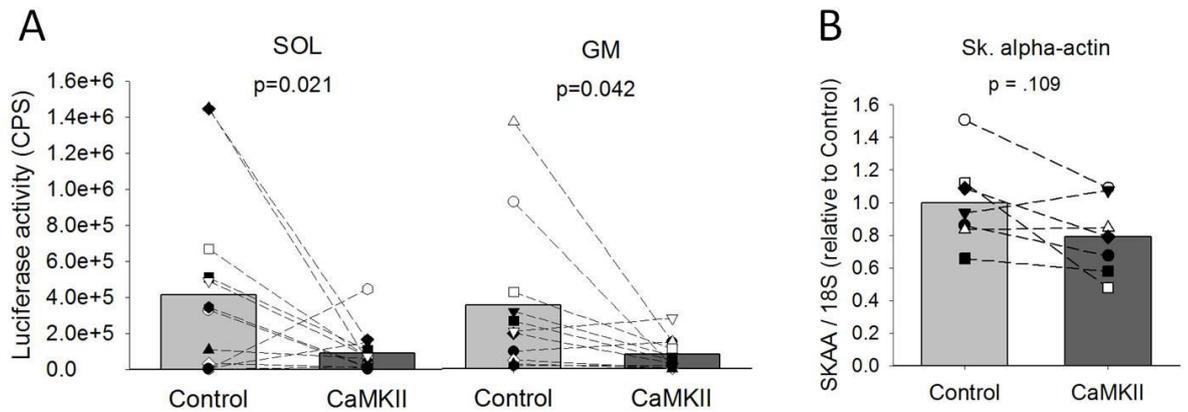


Figure 1: Effects of CaMKII overexpression on SKAA promoter activity and mRNA level

A). Luciferase activity (in photon counts per second, CPS) in 20 μ 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 α/β -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 α/β -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 α -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 α/β -CaMKII plasmids and Dr. Frank W. Booth (University of Missouri, Columbia, USA) for donating the skeletal alpha-actin reporter plasmid.

Chapter 5: Computational modelling of CaMKII activity and its effects on calcium dynamics in sarcomeres

Abstract

Calcium/calmodulin-dependent protein kinase II (CaMKII) has been demonstrated to modulate calcium release from the sarcoplasmic reticulum and phosphorylate the sarco/endoplasmic reticulum Ca^{2+} -ATPase *in vitro*. CaMKII overexpression in skeletal muscle *in vivo* increased twitch contraction and relaxation speed (chapter 3). However, it is unclear if CaMKII-dependent modification of RyR and SERCA activity is sufficient to explain the increase twitch speed. In addition, it is unclear if CaMKII activation is similar throughout the subsarcomeric space or if activation is higher near the calcium release sites. To address these questions, we used a mathematical model of spatiotemporal sarcomeric $[\text{Ca}^{2+}]$ dynamics coupled to a biochemical model of CaMKII activation. Modelling results were compared to experimental results. The model predicted substantial spatial gradients in CaMKII activity in sarcomeres of fast- and slow-twitch muscle fibres during single and repeated RyR openings. Increasing the CaMKII concentration in the model did not produce faster twitch speeds, which could be explained by minimal CaMKII activation during twitch contraction. Experimental overexpression of CaMKII increased SERCA2 expression in rat *m. soleus* muscle fibres and this could at least partially explain the observed decrease in twitch relaxation time (chapter 3). We conclude that not an increased CaMKII-dependent SERCA activity, but increased SERCA protein level underlies the increase in twitch relaxation speed after CaMKII overexpression.

Introduction

In skeletal muscle, calcium/calmodulin-dependent protein kinase II (CaMKII) is presumed to regulate calcium release from, and re-uptake into, the sarcoplasmic reticulum (SR) of skeletal muscle fibres through modification of the ryanodine receptor (RyR) and the sarco/endoplasmic reticulum Ca^{2+} -ATPase (SERCA), respectively. Acute pharmacological inhibition of CaMKII has an inhibitory effect on calcium release during tetanic contractions of single fast-twitch mouse fibres (Tavi et al., 2003), suggesting that CaMKII stimulates opening of the RyR. Furthermore, SERCA phosphorylation by CaMKII increases maximal activity of the slow-twitch SERCA isoform in isolated SR membranes (Hawkins et al., 1994, Xu and Narayanan, 1999). In addition, CaMKII increases SERCA activity by phosphorylating regulatory proteins sarcolipin (Bhupathy et al., 2009, Tupling et al., 2011) and phospholamban (MacLennan and Kranias, 2003). Finally, CaMKII overexpression in rat skeletal muscle *in vivo* decreased twitch contraction and relaxation times (chapter 3). These findings support the idea that the speed of calcium release and re-uptake in muscle fibres can be altered via modification of RyR and SERCA by CaMKII.

These targets, however, have a specific spatial distribution within a sarcomere. RyR is located at the terminal cisternae of the SR (Inui et al., 1987), whereas SERCA is located on the longitudinal SR (Jorgensen et al., 1982). It has been demonstrated in mouse *m. flexor digitorum brevis* fibres that the increase in cytoplasmic $[\text{Ca}^{2+}]$ after a single depolarisation depends on the location within a sarcomere, with the largest change occurring near the calcium release site, and the smallest changes near the m-line (Gomez et al., 2006). As the increase in cytoplasmic $[\text{Ca}^{2+}]$ is probably essential for contraction-induced CaMKII activation, there may also be a substantial gradient in CaMKII activity in sarcomeres. This raises the question whether CaMKII can affect SERCA, a target which is not in the immediate vicinity of the calcium release site, in skeletal muscle fibres.

We investigated whether, upon calcium release from the SR, CaMKII is activated to a greater extent when it's located in the vicinity of the RyR and whether increasing sarcomeric CaMKII concentration decreases the speed of calcium release from and re-uptake into the SR. The complexity of the required measures means that an *in vivo* approach is unfeasible. We therefore coupled computational models of spatiotemporal sarcomeric $[\text{Ca}^{2+}]$ dynamics in slow-twitch and fast-twitch fibres to models of CaMKII activation and RyR/SERCA modulation.

Methods

Description of spatiotemporal calcium dynamics model

A computational model describing spatiotemporal $[Ca^{2+}]$ dynamics in a half sarcomere of a fast-twitch mouse muscle (Groenendaal et al., 2008) was extended with a detailed biochemical model describing CaMKII activation (Saucerman 2008). Both models consist of coupled differential equations and contain no stochastic elements. The code for the sarcomeric model was kindly provided by Dr. Willemijn Groenendaal (Eindhoven University of Technology, The Netherlands). The sarcomeric model describes a cylinder consisting of four radial layers, of which the inner three form the myoplasm and the outer layer forms the sarcoplasmic reticulum (SR). Longitudinally, the layers are divided into six parts of equal volume, to form 18 myoplasmic elements and six SR elements (Fig. 1A). The number of elements in the model was limited to 24 to prevent excessive computational times and remain within computer memory limits. The elements include buffering of calcium by troponin-C, parvalbumin (in fast-twitch muscle) and ATP in the myoplasm and calsequestrin in the SR. In the model, calcium is able to diffuse within the myoplasm and the SR, and is transported between these two compartments through the RyR and SERCA, which have distinct locations on the modelled SR (Fig. 1A).

Sarcomeres of both fast-twitch (FT) and slow twitch (ST) muscle fibres were modelled. Clear structural and biochemical differences exist between these fibre types and these were implemented as previously described (Groenendaal, 2011). In comparison with the FT model, the ST model has:

- no parvalbumin
- a 50% lower SR volume, relative to the volume of the myoplasm.
- 2.4 fold lower maximal RyR activity
- 3 fold lower maximal SERCA activity
- fewer Ca^{2+} binding sites on troponin
- 1.5 fold higher resting myoplasmic $[Ca^{2+}]$
- lower ATP concentration

Extension of calcium model with CaMKII reaction scheme

To each myoplasmic element in the model, we added a CaMKII reaction scheme describing Ca^{2+} binding to calmodulin (CaM) (Fig. 1B) and subsequent binding of Ca^{2+} -CaM to CaMKII (Saucerman and Bers, 2008). Six different fractions of CaMKII exist in the model (Fig. 1C): Pi (inactive), Pb2 (Ca_2CaM -bound), Pb (Ca_4CaM -bound), Pt2 (Ca_2CaM -bound and phosphorylated at Thr287), Pt (Ca_4CaM -bound and phosphorylated at Thr287) and Pa (unbound and phosphorylated at Thr287). Of these fractions, Pb, Pt2, Pt and Pa were assumed to have 100% activity. In the model, CaMKII phosphorylation is reversed by protein phosphatase 1 (PP1) (Fig. 1C).

Some modifications were made to the CaMKII reaction scheme described by Saucerman & Bers. We removed CaM-buffering from the model and modelled only free CaM. Furthermore, we used different reaction kinetic parameter values for a number of reactions, which we found to be more in agreement with available literature (Appendix). The absolute concentrations of Pi, Pb, Pb2, Pt, Pt2 and Pa, instead of concentrations normalized to total CaMKII concentration, were used to calculate reaction rates. This allowed us to investigate the effect of changes in CaMKII concentration on calcium dynamics. Isoform-specific parameters for the β -CaMKII isoform were used to describe CaM-affinity for CaMKII (Gaertner et al., 2004).

CaMKII-dependent modification of SERCA activity was implemented in the ST model as previously described for modelled cardiomyocytes (Koivumaki et al., 2009), while in the FT model regulation of SERCA activity was absent (Hawkins et al., 1994). In both the FT and the ST model, the RyR parameter 'Power1' was made dependent on the level of active CaMKII to prolong opening of the RyR in a similar way to that used for a model of cardiomyocytes (Hund and Rudy, 2004) (see Appendix). The values of KmMax and DPowermax, which describe how strongly CaMKII activity affects the activity of RyR and SERCA (Appendix), were set so that the model could reproduce the CaMKII inhibition-induced decrease in calcium release during a 70Hz contraction, which has been previously demonstrated experimentally in single mouse fast-twitch muscle fibres (Tavi et al., 2003). The experiment by Tavi et al, which involved pharmacological inhibition of CaMKII, was modelled by removing the CaMKII-dependent modifications of RyR from our FT model. Although it was not explicitly demonstrated by Tavi et al. (2003), it was assumed that the pharmacological inhibition of CaMKII was absolute and specific. The FT

values of K_{mMax} and $D_{Powermax}$ were subsequently incorporated into the ST model as well, as there is no similar experimental evidence available for this muscle type.

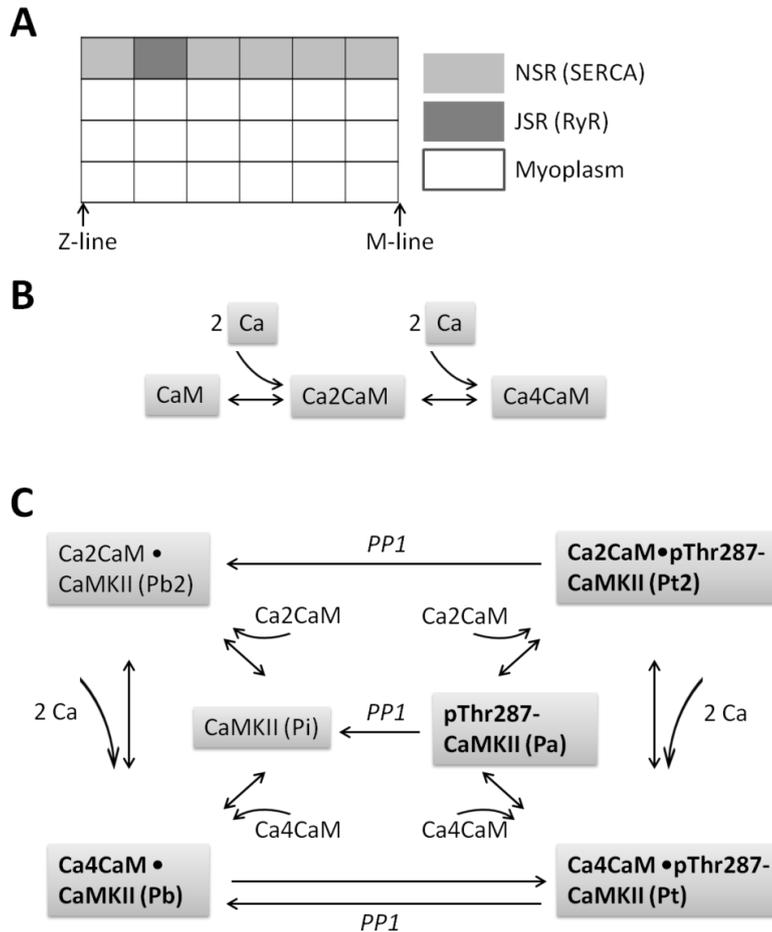


Figure 1: Schematized calcium model and CaM-CaMKII reaction schemes.

A: Simplified visualisation of the different spatial elements in the computational model of calcium dynamics in a half-sarcomere, adapted from Groenendaal et al. (2008). The model is bordered by a z-line on one side and an m-line on the other side. The grey elements (top row) make up the SR and the white elements (bottom two rows) make up the myoplasm. Note that the actual model is a cylinder, and the elements are actually rings, with the lower border of the bottom elements as their centre.

B: Reaction scheme for sequential binding of calcium to the C-terminal and then the N-terminal EF-hand of calmodulin.

C: Reaction scheme for binding of calcium and calmodulin to CaMKII, and conversion of the CaMKII fractions to a phosphorylated state. pThr287 indicates that CaMKII is phosphorylated at threonine287. Phosphorylation of CaMKII at threonine 287 in the model is reversed by the action of protein phosphatase 1 (PP1). CaMKII fractions in bold have 100% activity and the CaMKII fractions in normal font have no activity. Both CaM and CaMKII reaction schemes were adapted from Saucerman & Bers (2008). Two-way arrows indicate reversibility of the reaction. PP1: Protein phosphatase 1. Ca, CaM, Ca₂CaM, Ca₄CaM, Pi, Pb, Pb₂, Pt, Pt₂ and Pa, but not PP1, can change over time in the model. Values of reaction parameters for this reaction scheme can be found in the Appendix.

Assumptions/estimated parameter values

To the best of our knowledge, the concentration of CaMKII in skeletal muscle is unknown. We assumed a concentration of 1 μM , which has previously been used for simulations of CaMKII in skeletal muscle fibres (Tavi et al., 2003). CaMKII activity in non-stimulated muscle is 10% of maximal Ca^{2+} /CaM-stimulated activity (Rose et al., 2007a). Therefore, the initial concentration of Pa was set to 0.1 μM (see Appendix).

Total [CaM] in skeletal muscle has been estimated to be approximately 10-20 μM (Chiesi and Carafoli, 1983). However, in many cell types, CaM is presumed to be a limiting factor for calcium signalling (Persechini and Stemmer, 2002). In cardiomyocytes, most (99%) of CaM is buffered (Wu and Bers, 2007) and assumed to be unavailable for binding to CaMKII. Assuming similar CaM buffering in skeletal muscle, we set the concentration of free CaM to 0.15 μM (1% of total). [PP1] was set so CaMKII activity lasted for several tens of seconds after a simulation of 100 RyR openings, in agreement with data from chapter 2. In the model, no diffusion of any of the CaM or CaMKII fractions takes place. [CaM] and [CaMKII] were equal in all myoplasmic elements of the model, and in the FT and ST models.

Simulations were performed with Matlab (The Mathworks, v7.5.0) on an ASUS K53S laptop (2.2GHz processor, 8 GB RAM) and the ordinary differential equation (ODE) solver 15s was used. Conservation of mass during the simulations was checked.

Results

Model validation

The calcium dynamics model predicts a substantial spatial gradient in $[\text{Ca}^{2+}]$ in sarcomeres during twitch contraction (Groenendaal et al., 2008). We compared spatially averaged $[\text{Ca}^{2+}]$ during a simulated twitch of the FT and ST versions of the model with experimental data obtained from electrically stimulated mouse skeletal muscle fibres (Baylor and Hollingworth, 2003). The model describes rise time and peak $[\text{Ca}^{2+}]$ amplitude of the twitch calcium peak quite well (Table 1), but the slow second half of the decrease in $[\text{Ca}^{2+}]$ in ST fibres reported by Baylor and Hollingworth (2003) was absent in the model.

Therefore, our modelled ST relaxation time was shorter than the experimentally determined relaxation time (Table 1). Adjusting the RyR opening parameters (Appendix) did not improve the modelled $[Ca^{2+}]$ decrease time without substantial changes in the peak $[Ca^{2+}]$. As we judged the peak $[Ca^{2+}]$ to be the most important parameter determining CaMKII activation, we considered the model to match the experimental data of Baylor & Hollingworth sufficiently well to model $[Ca^{2+}]$ -dependent CaMKII activation.

Parameter	<i>Baylor and Hollingworth (2003)</i>		<i>Model</i>	
	FT (EDL)	ST (SOL)	FT	ST
TTP (ms)	2.0	2.5	2.0	2.1
Peak $[Ca^{2+}]$ (μ M)	21.7	8.6	20.2	8.9
HRT (ms)	2.0	4.4	1.7	1.9

Table 1: Comparison of modelled and experimental $[Ca^{2+}]$ during one pulse stimulation

Experimental measures were carried out on single mouse muscle fibres within small bundles of fibres at 28°C (Baylor and Hollingworth, 2003). Model results were obtained from simulations of a single RyR opening. TTP: Time-to-peak $[Ca^{2+}]$ during a twitch. HRT: Half-relaxation time of the calcium transient during a twitch. FT: fast-twitch. ST: slow-twitch. EDL: mouse *m. extensor digitorum longus*. SOL: mouse *m. soleus*.

To validate the CaMKII reaction scheme, we first compared modelled Ca^{2+} -sensitivity of CaMKII autophosphorylation and the effect of phosphatase concentration on this relationship to that of an independent experimental dataset (Bradshaw et al., 2003).

Reagent and ion concentrations, and reaction times described by Bradshaw et al. were used for these simulations. The model reproduced the concentration for half-maximal activation and steepness of the relationship between $[Ca^{2+}]$ and CaMKII autophosphorylation quite well (Fig. 2A). Furthermore, we compared the modelled relation between $[CaM]$ and CaMKII activity, and between $[CaM]$ and CaMKII autophosphorylation, with another independent experimental dataset (Gaertner et al., 2004). The model properly described the experimentally determined $[Ca^{2+}]$ concentrations for half-maximal CaMKII activity and the steepness of the curve (Fig. 2B&C).

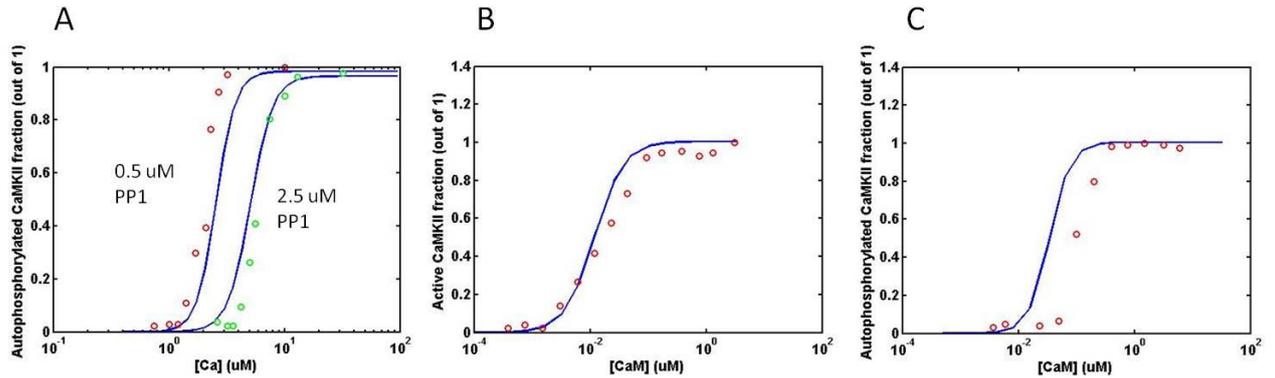


Figure 2: Validation of CaM-CaMKII reaction scheme

A: Comparison of modelled $[Ca^{2+}]$ - phospho^{Thr287}-CaMKII relation (blue lines) with published data (red open circles: 0.5 μ M PP1; green circles: 2.5 μ M PP1) (Bradshaw et al., 2003). B: Comparison of modelled $[CaM]$ - CaMKII activity relation (blue line) with independent experimental data (Gaertner et al., 2004). C: Comparison of modelled $[CaM]$ - CaMKII autophosphorylation relation (blue lines) with independent experimental data (Gaertner et al., 2004). Experimental data points are estimated from graphs in cited papers. PP1: Protein phosphatase 1.

Simulation of prolonged repeated opening of the RyR (i.e. >100 openings) resulted in a maximum autophosphorylated (Pt, Pt2 & Pa) CaMKII fraction of 14% of total CaMKII. This is somewhat lower than the experimentally determined maximal autonomous activity of 22-23% of maximal CaMKII activity in *in situ* stimulated rat *m. gatrocnemius* muscle (Rose et al., 2007a). However, it is in accordance with the experimentally supported idea that autonomous CaMKII activity does not exceed a ceiling of approximately 20% of maximal activity.

Effect of $[Ca^{2+}]$ distribution on CaMKII activation

We determined maximal total CaMKII activity (defined as the sum of Pb4, Pt2, Pt4 and Pa) during simulations of single and repeated RyR openings in the FT and ST model. Furthermore, we determined whether there was an effect of RyR opening frequency on CaMKII activity, as the frequency of exposure of CaMKII to calcium/calmodulin determines the development of CaMKII activity *in vitro* (De Koninck and Schulman, 1998). CaMKII activity was determined for each myoplasmic compartment. An overview

of the range of increase in CaMKII activity in the myoplasmic elements of the FT and ST models can be found in Table 2.

	FT			ST		
	Twitch	Tetanus	Train	Twitch	Tetanus	Train
10 Hz	0 - 6.5%	0.2 - 59%	3.6 - 84%	0 - 1.2%	0.2 - 29%	3.6 - 44%
100 Hz		0.5 - 95%	1.9 - 81%		0.7 - 64%	2.3 - 58%

Table 2: Increase in total CaMKII activity during single or repeated RyR openings

The range of increase in CaMKII activity in myoplasmic elements of FT and ST models from the element with the lowest increase to the element with the highest increase. Activity was determined at time points during simulations as indicated in Figures 3, 4 & 5.

As expected, the highest CaMKII activity after one simulated RyR opening occurred near the calcium release site, and no increase in activity occurred in the elements close to the m-line (Fig. 3).

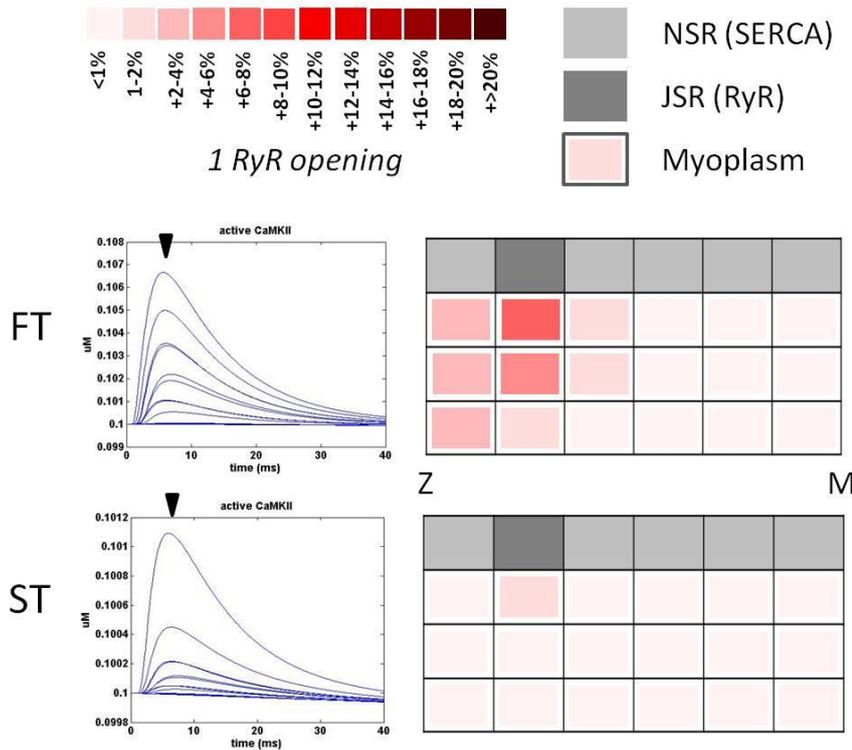


Figure 3: CaMKII activity during a single RyR opening

Total simulated CaMKII activity (the sum of Pb4, Pt2, Pt4 and Pa) during a simulated single RyR opening in the fast-twitch (FT) and slow-twitch (ST) model (see text for model details). Graphs on the left display development of total CaMKII activity over time (in milliseconds) during a single RyR opening in the FT and ST model as indicated. Blue lines represent CaMKII activity in different myoplasmic compartments. Black inverted triangles indicate the approximate time point at which the CaMKII activity gradient was analysed, and visualised in the grids on the right. Note that the scale of the y-axis differs in the FT and ST graph. Grids on the right visualise the spatial distribution of the maximal increase in total CaMKII activity during a single simulated RyR opening. Colours indicate the percentage increase compared to the initial total activity, and the scale is indicated above the figure. Grid layout is the same as in Fig. 1A. The locations of the z-line and m-line of the half-sarcomeric model are indicated by Z and M, respectively. NSR: non-junctional sarcoplasmic reticulum. JSR: junctional sarcoplasmic reticulum. SERCA: sarco/endoplasmic reticulum Ca^{2+} -ATPase. RyR: Ryanodine receptor.

We investigated whether the CaMKII activity gradient persisted when repeated RyR openings were simulated. After ten RyR openings, the spatial gradient in activity increase was stronger than after one RyR opening (Table 2, Fig. 4). However, when simulating ten repeated trains of ten RyR openings with one-second intervals between the trains, the spatial distribution of CaMKII activity during the final ten RyR openings of the protocol is remarkably similar to that during the first ten openings. The main difference between the

first and last train is an increase in baseline CaMKII activity of the most activated elements after the tenth train (Fig. 5), which is mainly due to the increase in fraction Pa, the autonomous CaMKII activity. Nevertheless, in all cases CaMKII activation was highest near the calcium release site and higher in the elements closest to the z-line than in the elements closest to the m-line (Fig. 3). These data suggest that CaMKII activity is highest in the vicinity of the calcium release site during muscle contractions.

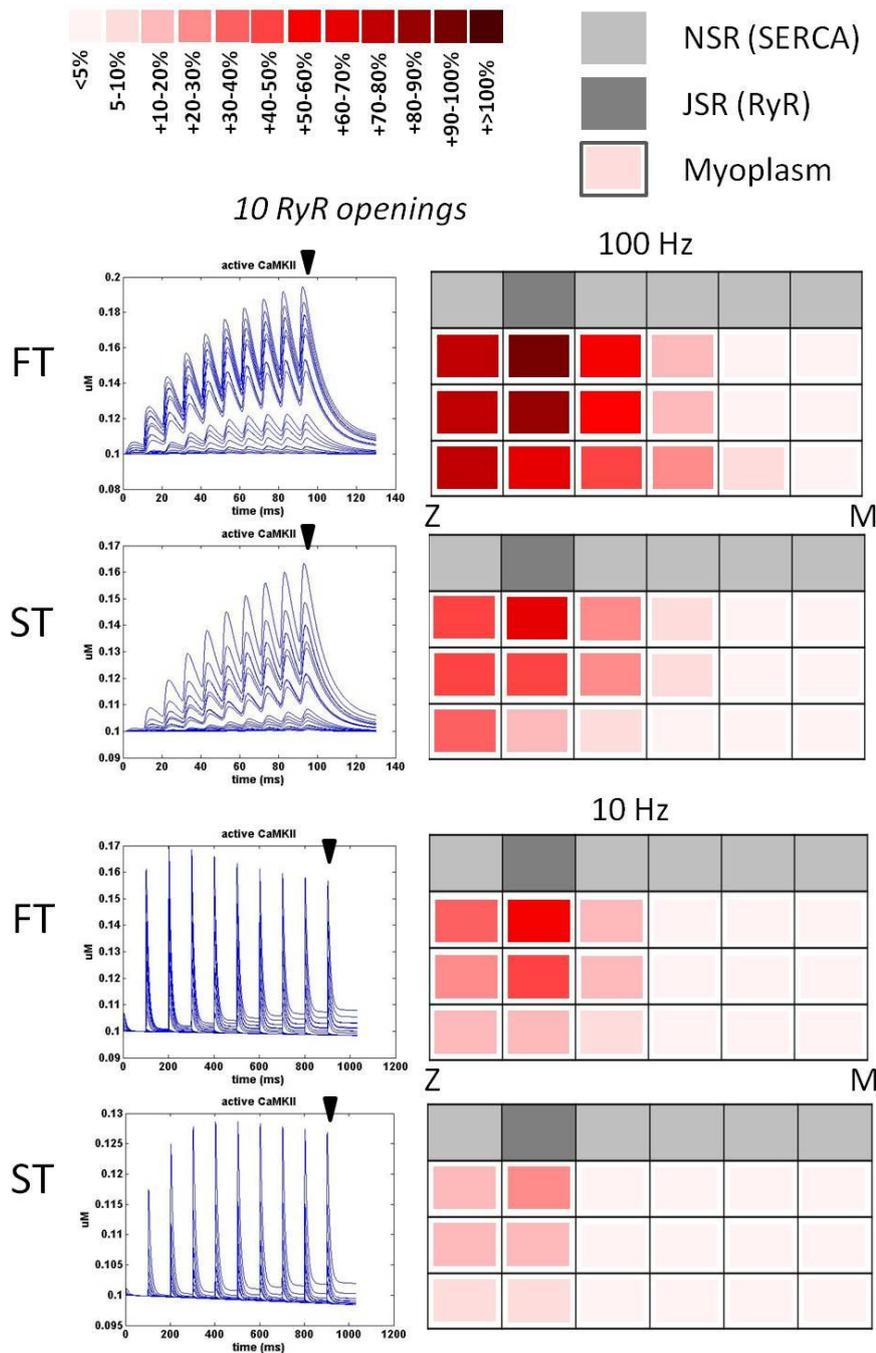


Figure 4: CaMKII activity during repeated RyR openings

Total simulated CaMKII activity (the sum of Pb4, Pt2, Pt4 and Pa) during a simulation of ten RyR openings in the fast-twitch (FT) and slow-twitch (ST) model (see text for model details). Graphs on the left display development of total CaMKII activity over time (in milliseconds) during ten simulated RyR openings in FT and ST model as indicated, and at a RyR opening frequency of 100Hz (upper two graphs) or 10Hz (lower two graphs). Blue lines represent CaMKII activity in different myoplasmic compartments. In order to optimise visualisation of the graphs, the scale of the y-axis differs in each graph. Black inverted triangles indicate the approximate time point at which the CaMKII activity gradient was analysed, and visualised in the grids on the right. Grids on the right visualise the spatial distribution of maximal total CaMKII activation during the simulation of ten RyR openings. Colours indicate the percentage increase compared to the initial total activity, and the scale is indicated above the figure. Note that colour scale in this figure is of larger magnitude compared to Fig. 3. Grid layout is the same as in Fig. 1A. The locations of the z-line and m-line of the half-sarcomeric model are indicated by Z and M, respectively. NSR: non-junctional sarcoplasmic reticulum. JSR: junctional sarcoplasmic reticulum. SERCA: sarco/endoplasmic reticulum Ca²⁺-ATPase. RyR: Ryanodine receptor.

Models of FT and ST sarcomeres were used to investigate whether CaMKII activation differs between these muscle types. CaMKII activation was higher in the FT model compared to the ST model after single and repeated RyR openings (Table 2, Fig. 3, 4 & 5). This is associated with a higher peak [Ca²⁺] amplitude in the FT model (Table 1), and in partial agreement with experimental data demonstrating that phospho^{Thr287}-CaMKII levels increase in fast-twitch, but not slow-twitch rat muscle after electrical stimulation with 100 electrical pulses *in situ* (chapter 2).

To investigate whether RyR opening frequency affects CaMKII activation, we simulated repeated RyR openings at both 10 Hz and 100 Hz. After ten RyR openings, CaMKII activation was substantially higher when RyR opening occurred at 100 Hz compared to 10 Hz (Table 2, Fig. 4). However, after ten trains of RyR openings, this difference between the frequencies had disappeared in the FT model and was reduced in the ST model (Table 2, Fig. 5). This was in agreement with experimental data demonstrating no effect of frequency on phospho^{Thr287}-CaMKII levels after *in situ* stimulation of rat skeletal muscle with 100 electrical pulses at 10 or 150 Hz (Chapter 2). These data suggest that a low frequency of RyR opening results in lower CaMKII activation during the initial openings, but that as RyR opening continues, the CaMKII activation during low frequency RyR

opening eventually matches that during high-frequency RyR opening, and is not likely to be a factor during repeated muscle contractions.

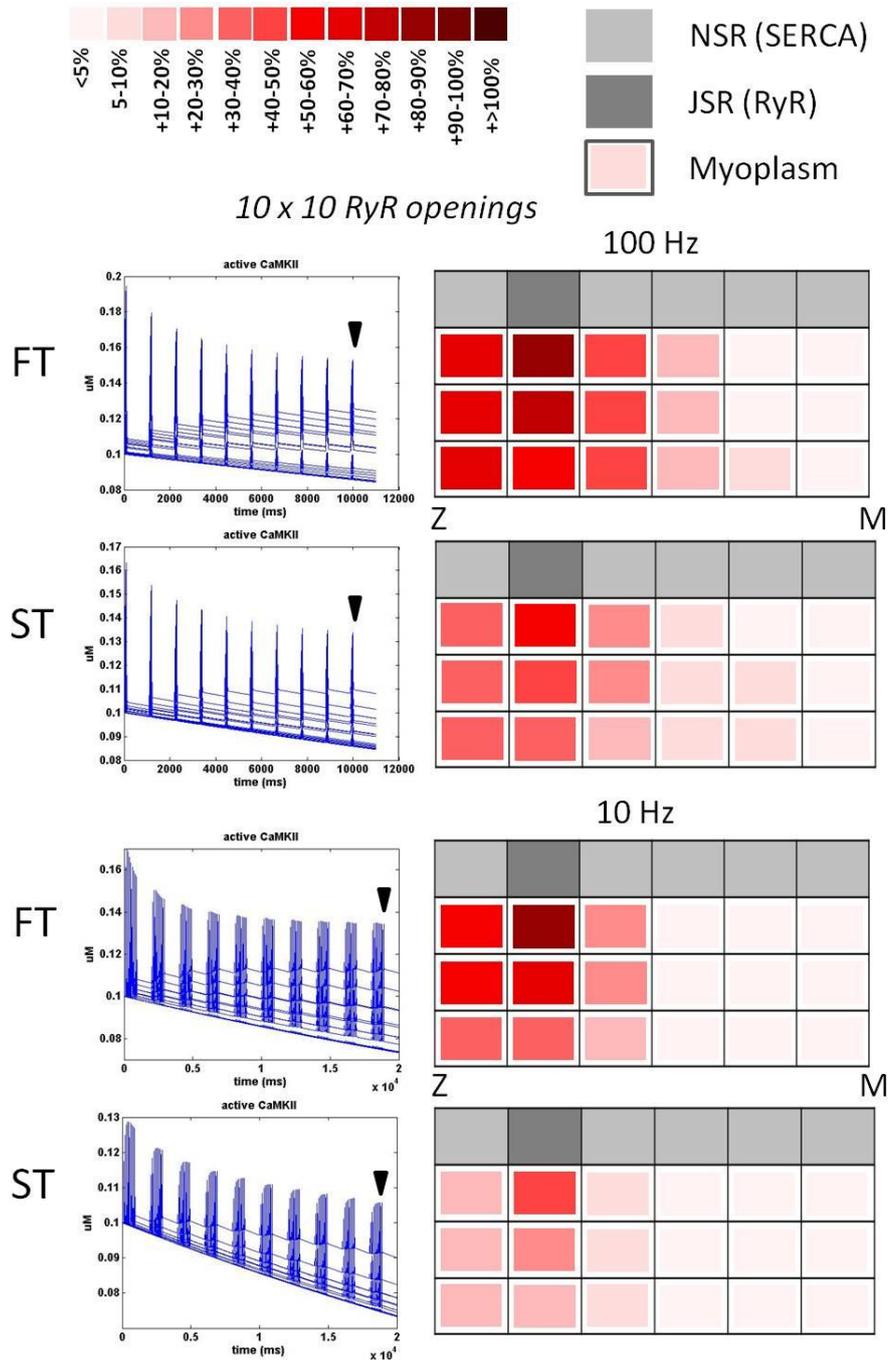


Fig. 5: CaMKII activity during repeated trains of RyR openings

Total CaMKII activity (the sum of Pb4, Pt2, Pt4 and Pa) during a simulation of 10 x 10 RyR openings in the fast-twitch (FT) and slow-twitch (ST) model (see text for model details). Graphs on the left display development of total CaMKII activity over time (in milliseconds) during the 10 x 10 RyR openings protocol in FT and ST model as indicated, and at a RyR opening frequency of 100Hz (upper two graphs) or 10Hz (lower two graphs). The interval between each train of ten RyR openings was one second. Blue lines represent CaMKII activity in different myoplasmic compartments. In order to optimise visualisation of the graphs, the scale of the y-axis differs in each graph. Black inverted triangles indicate the approximate time point at which the CaMKII activity gradient was analysed, and visualised in the grids on the right. The decreasing baseline CaMKII activity can be attributed to PP1 activity, but is adjusted for when calculating the increase in activity in each myoplasmic element. Grids on the right visualise the spatial distribution of maximal total CaMKII activation during the final 10 pulses of the simulation protocol. Colours indicate the percentage increase compared to the initial total activity, and the scale is indicated above the figure. Note that the colour scale in this figure is the same as that in Fig. 4, but of a larger magnitude compared to Fig. 3. Grid layout is the same as in Fig. 1A. The locations of the z-line and m-line of the half-sarcomeric model are indicated by Z and M, respectively. NSR: non-junctional sarcoplasmic reticulum. JSR: junctional sarcoplasmic reticulum. SERCA: sarco/endoplasmic reticulum Ca^{2+} -ATPase. RyR: Ryanodine receptor.

Effect of CaMKII concentration on calcium dynamics in FT and ST fibres

We used the model to investigate the effect of increased CaMKII concentration, which has been shown to increase relaxation speed in rat skeletal muscle (chapter 3), on calcium dynamics. CaMKII concentration was increased 50-fold in each myoplasmic element. This increased the local concentrations of total active CaMKII during a simulation of a single RyR opening compared to the same simulation with 'normal' CaMKII concentration (Fig. 6A/C). This increase occurred mainly in the elements closest to the RyR. However, it had no effect on the development of the $[\text{Ca}^{2+}]$ or calcium-bound troponin-C concentration ($[\text{Ca}^{2+}\text{-TropC}]$) over time in the FT and ST model (Fig. 6B/D).

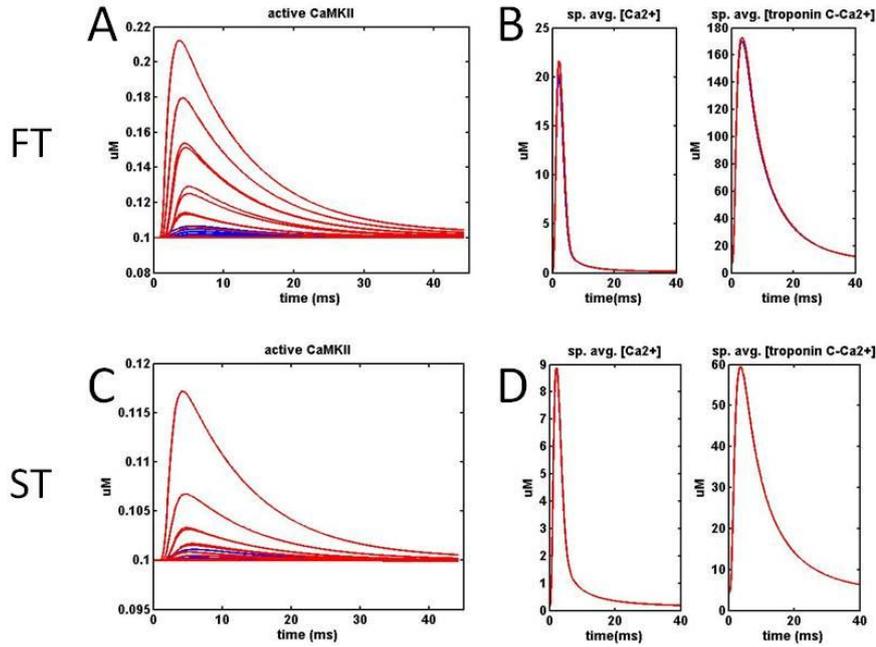


Figure 6: The effect of increased CaMKII levels on twitch characteristics

A/C: Total CaMKII activity (the sum of Pb, Pt, Pt2 and Pa) during a simulation of a single RyR opening in the fast-twitch (FT; A) and slow-twitch (ST; C) model in which CaMKII concentration is increased. Lines indicate CaMKII activity in different myoplasmic compartments. Blue lines: $[CaMKII] = 1 \mu M$; Red lines: $[CaMKII] = 50 \mu M$. Note that the scales on the y-axes are different. B/D: Spatially averaged $[Ca^{2+}]$ (left graphs) and $[Ca^{2+}-TropC]$ (right graphs) during a simulation of one RyR opening in the FT (B) and ST (D) model. Blue lines: $[CaMKII] = 1 \mu M$; Red lines: $[CaMKII] = 50 \mu M$. Note that scales on the y-axes are different.

An alternative explanation for the experimentally observed increase in relaxation speed is an increase in SERCA expression (chapter 3). When increasing maximal SERCA activity in the model by two-fold to reflect increased SERCA protein level, $[Ca^{2+}]$ and $[Ca^{2+}-TropC]$ were reduced during the relaxation phase of the twitch (Fig. 7 B/D). These data suggest that the decrease in relaxation time when CaMKII is overexpressed in rat skeletal muscle cannot be explained by CaMKII-induced increases in SERCA activity, but can be at least partially explained by increased SERCA expression levels.

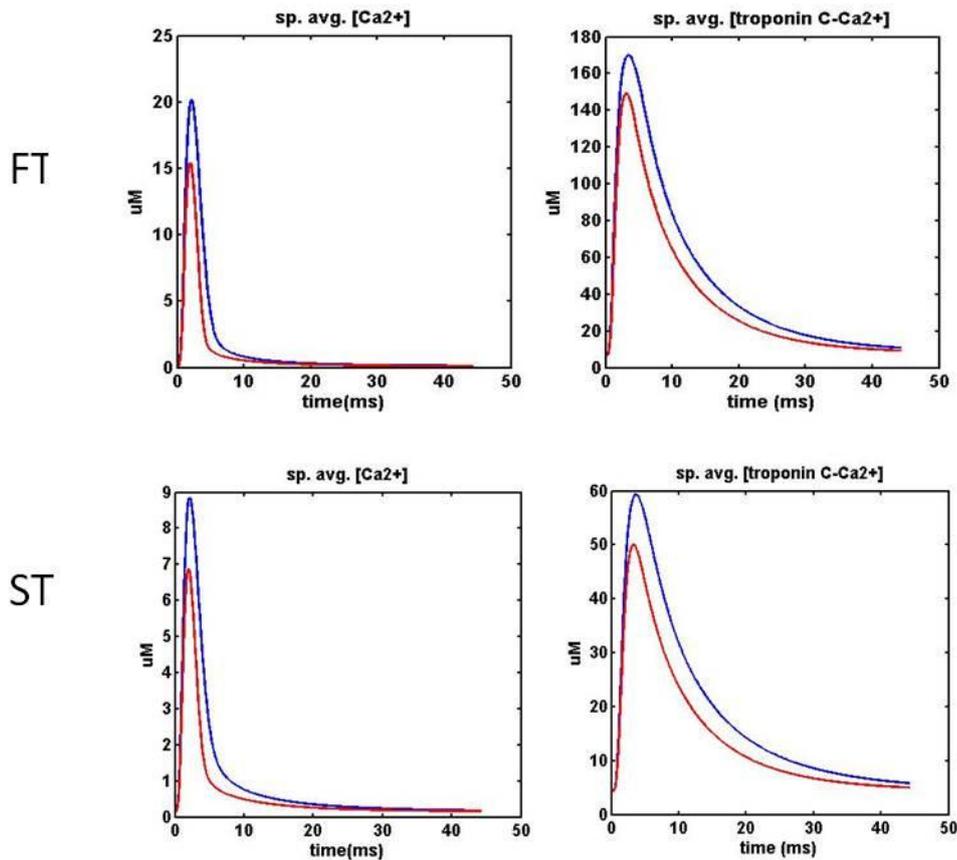


Figure 7: The effect of increasing SERCA levels on twitch characteristics

Spatially averaged $[Ca^{2+}]$ (left graphs) and $[Ca^{2+}$ -TropC] (right graphs) during a simulation of a single RyR opening in the fast-twitch (FT) and slow-twitch (ST) model. Blue lines: SERCA activity = normal; Red lines: SERCA activity = 2 x normal. Note that scales on the y-axes are different.

Discussion

Main findings

We aimed to investigate whether CaMKII might be differentially activated within a sarcomere depending on its subsarcolemmal location and whether decreases in twitch contraction and relaxation times after CaMKII overexpression can be explained by CaMKII-dependent modification of RyR and SERCA. We found that the modelled spatial gradient in cytoplasmic $[Ca^{2+}]$ results in a two-fold decrease in CaMKII activity between the Ca^{2+} release site (i.e. near the z-line) and the part of the sarcomere close to the m-line. This gradient existed during simulations of a single RyR opening and simulations of

repeated trains of RyR opening (Fig. 3, 4 & 5). Furthermore, simulated CaMKII activity increased more in the FT model than in the ST model (Fig. 3, 4 & 5). Finally, we found that CaMKII-dependent modulation of RyR and SERCA cannot explain CaMKII overexpression-induced decreases in twitch contraction and relaxation times (Fig. 6).

Model limitations

RyR and SERCA are both influenced by a plethora of interacting factors (Zalk et al., 2007, Vangheluwe et al., 2005), which were not modelled because of the lack of quantitative descriptions of their effects. It is unknown whether these factors might mediate or influence CaMKII-dependent effects on RyR or SERCA.

Direct modification of RyR by a calcium/calmodulin-dependent protein kinase-dependent phosphorylation has been shown to inhibit RyR opening in isolated SR membrane patches of frog skeletal muscle (Wang and Best, 1992). In contrast, pharmacological inhibition of CaMKII in single mouse skeletal muscle fibres resulted in decreased calcium release during tetanic stimulation (Tavi et al., 2003), which suggests the effect of CaMKII on calcium release is stimulatory. We valued the latter study more, as the data were obtained from intact fibres with intact SR which may include CaMKII-RyR interactions which are no longer present in isolated membrane patches. Therefore, we modelled a stimulatory effect of CaMKII on RyR.

To the best of our knowledge, the exact concentrations of CaM, CaMKII and PP1 in skeletal muscle fibres are unknown. PP1 concentration was set based on experimental results demonstrating that CaMKII phosphorylation can persist for up to two minutes after stimulation of rat skeletal muscle with 100 pulses (chapter 2). The effects of CaM concentration on global CaMKII activity in the model indicated that this is an important determinant of CaMKII activity (Fig. 8). Although increasing the concentration of CaMKII and CaM in the model increased the absolute levels of active CaMKII, a spatial gradient in CaMKII activity still existed (see Fig. 6A/C for the effect of increased CaMKII concentration). In addition, increasing the CaM concentration two-fold in the model did not affect the conclusion that CaMKII-dependent modification of RyR and SERCA was not sufficient to affect the speed of calcium release and re-uptake (Fig. 8).

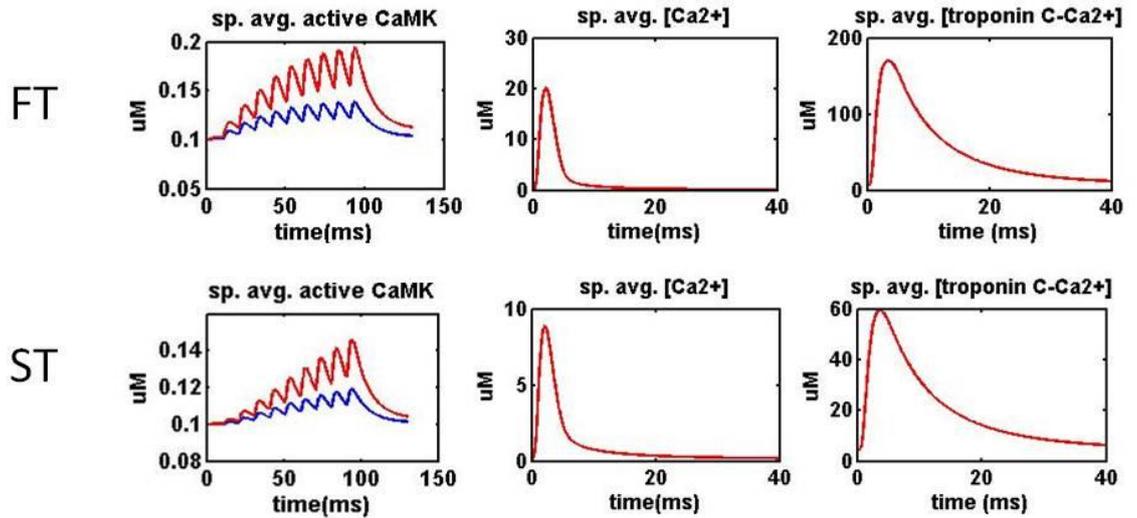


Figure 8: Effect of increased calmodulin concentration on CaMKII activity and calcium release

Spatially averaged [active CaMKII] during a simulation of 10 repeated pulses at 100 Hz, and spatially averaged $[Ca^{2+}]$ and $[Ca^{2+}$ -TropC] during a simulation of a single RyR opening in the fast-twitch (FT) and slow-twitch (ST) models. Blue lines: $[CaM] = 0.15 \mu M$. Red lines: $[CaM] = 0.30 \mu M$. Note that scales on the y-axes are different between graphs for FT and ST models.

The experimentally determined effect of CaMKII overexpression on muscle twitch relaxation was derived from muscle force traces (chapter 3). Although our model predicted that a CaMKII-dependent increase in SERCA expression (chapter 3) leads to a decrease in calcium twitch time, the development of force by cross-bridges was not modelled. Therefore, it is unclear to what extent the modelled change in calcium dynamics in the model translate into a change in force dynamics. However, as differences in calcium dynamics are presumed to underlie the difference in twitch time between FT and ST muscle (Schiaffino, 2010), the modelled decrease in calcium twitch time is likely to have an effect on muscle force dynamics.

Implications of modelling results for the function of CaMKII in skeletal muscle

CaMKII has many substrates and one mechanism to achieve signalling specificity is physical targeting of the kinase to its substrates (Bayer and Schulman, 2001). As our model predicted that the spatial gradients in CaMKII activity persist during repeated RyR openings, CaMKII substrates in skeletal muscle fibres will be differentially regulated by CaMKII, dependent on their subsarcolemmal location (Table 2; Fig. 3, 4, & 5).

RyR is located at the terminal cisternae of the SR (Inui et al., 1987), whereas SERCA is located on the longitudinal SR (Jorgensen et al., 1982). CaMKII is targeted to the SR by a non-kinase splice variant of the α -CaMKII isoform, α KAP (Bayer et al., 1998). α KAP co-localises with SERCA2 to the longitudinal SR in mouse *m. soleus* fibres (Nori et al., 2003), suggesting that CaMKII may be physically targeted to SERCA. Furthermore, CaMKII is located at the terminal SR in the vicinity of the RyR (Chu et al., 1990). Thus, CaMKII appears to be spatially targeted to these targets. As CaMKII activity is highest at the site of RyR, and barely activated at a fraction of the elements that contain SERCA, CaMKII-dependent regulation of RyR in muscle fibres may be stronger compared to that of SERCA, or the SERCA-associated regulatory proteins phospholamban and sarcolipin.

Similarly, local CaMKII activation could have implications for CaMKII-dependent regulation of other substrates. CaMKII was found to both complex with, and phosphorylate, glyceraldehyde 3-phosphate dehydrogenase, glycogen phosphorylase, glycogen debranching enzyme and creatine kinase in SR-membranes, (Singh et al., 2004). It is unclear whether this complex is located in the immediate vicinity of RyR, where it would be most strongly activated.

Interestingly, β -CaMKII, including the muscle specific β_m isoform, binds to f-actin in biochemical essays as well as in cultured neuronal cells, an interaction which has been shown to be inhibited by Ca^{2+} /CaM-binding (Shen et al., 1998, O'Leary et al., 2006, Sanabria et al., 2009). As our simulations suggest that the actin filaments of sarcomeres are located in the region where Ca^{2+} /CaM-binding to CaMKII takes place (Figs 3, 4 & 5), this raises the question whether CaMKII might regulate actin assembly or bundling in skeletal muscle. However, CaMKII binding to actin in skeletal muscle has not yet been demonstrated, and this could be an interesting area for future research.

Overexpression of CaMKII in rat skeletal muscle increases the speed of twitch contraction and relaxation (chapter 3). When we increased the CaMKII concentration in the model, the calcium and calcium-bound troponin-C concentration dynamics during a single RyR opening did not differ from that predicted by the simulation with 'normal' CaMKII concentration (Fig. 6). The results of our simulation suggest that CaMKII is barely activated during one pulse (Fig. 3), which explains the absence of differences between the simulations with different CaMKII concentrations. CaMKII overexpression increased SERCA2 expression in rat skeletal muscle (chapter 3). In contrast to only increasing

[CaMKII], additionally increasing maximal SERCA activity in the model caused an earlier decrease in $[Ca^{2+}]$ and $[Ca^{2+}\text{-TropC}]$ (Fig. 7B/D). Therefore, increased SERCA expression may partially explain the increased relaxation speed observed in CaMKII-overexpressing rat muscles (chapter 3).

Although CaMKII activation was highest near the RyR, increasing $[CaMKII]$ in the model did not result in a faster rise in $[Ca^{2+}]$ in the model (Fig. 6B/D). This is unlikely to be related to insufficient CaMKII activity, as increasing baseline CaMKII activity by 50-fold in the model only resulted in an approximate doubling of the peak $[Ca^{2+}]$ amplitude, but not a decrease in time to peak $[Ca^{2+}]$ or $[Ca^{2+}\text{-TropC}]$ (Fig. 9). More likely, the absence of an effect of increased $[CaMKII]$ on $[Ca^{2+}]$ rise time is related to the fact that the description of RyR in the model and its modification by CaMKII (Appendix) lacks the relevant components required to model the physiological effect of CaMKII on the RyR.

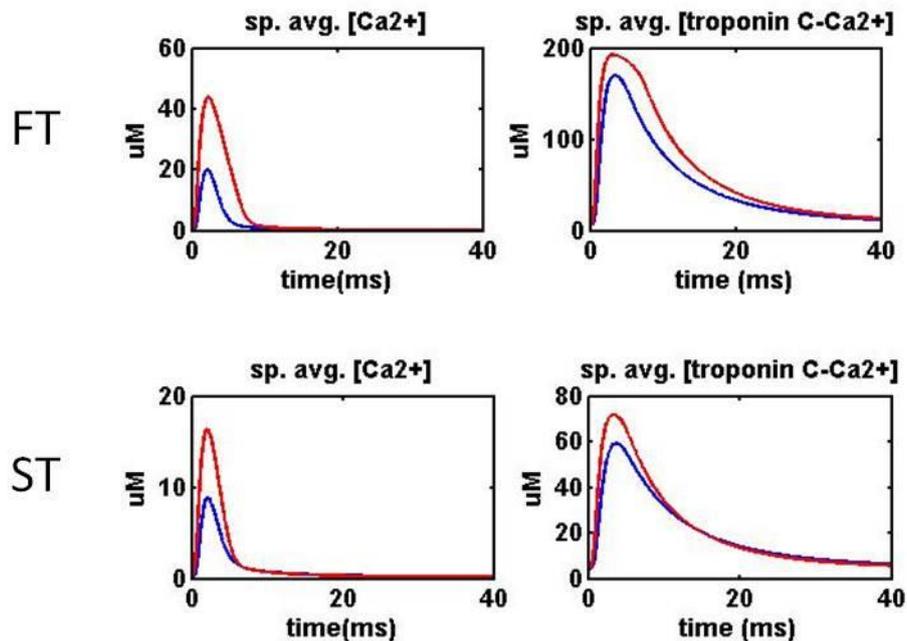


Figure 9: Effect of increased basal CaMKII activity on sarcomeric calcium release

Spatially averaged $[Ca^{2+}]$ (left graphs) and $[Ca^{2+}\text{-TropC}]$ (right graphs) during a simulation of a single RyR opening in the fast-twitch (FT) and slow-twitch (ST) model. Blue lines: basal $[active\ CaMKII] = 0.1\ \mu M$. Red lines: basal $[active\ CaMKII] = 5.0\ \mu M$. Note that scales on the y-axes are different.

Finally, we argue that the existence of a spatial gradient in CaMKII activity also has implications for the use of constitutively active mutants when using transgenic/overexpression approaches to investigate the function of CaMKII, as these introduce CaMKII activity distributed throughout the entire cell, and may therefore also introduce phosphotransfer activity to substrates that would normally not receive this.

Conclusion

The model is a useful tool to investigate CaMKII signalling towards its targets in skeletal muscle sarcomeres when reaction kinetic information regarding their activation becomes available. Our modelling of CaMKII activity in skeletal muscle sarcomeres and its effects show CaMKII is locally activated, and its spatial activity gradient is maintained during repeated stimulation. Therefore, the location of CaMKII in a sarcomere is likely to be of importance for its function and should be considered when evaluating potential CaMKII targets for physiological relevance.

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Appendix: Reaction parameter values

Reaction rate calculations

Reaction rates for the CaM and CaMKII reaction schemes were calculated as in Saucerman & Bers (2008), with the modification of using absolute concentrations of Pi, Pb, Pb2, Pt, Pt2 and Pa. Some reactions were excluded as described in the methods section. Values of the reaction parameters can be found in the tables below.

Calcium-calmodulin binding

Parameter	Description	Value	Reference
CaMFreeR	Free CaM concentration	0.15 μM	(Chiesi and Carafoli, 1983)
Mg	Myoplasmic Mg ²⁺ concentration	1 μM	(Jones et al., 2004)
K	Myoplasmic K ⁺ concentration	160 μM	(Sejersted and Sjogaard, 2000)
Kd02	-	14.546	(Saucerman and Bers, 2008)
Kd24	-	836.69	(Saucerman and Bers, 2008)
k20	2 Ca dissociation from CaM (C-terminal)	10 s^{-1}	(Saucerman and Bers, 2008)
k02	2 Ca binding to CaM (C-terminal)	$k20/Kd02 \mu\text{M}^{-2} \text{s}^{-1}$	(Saucerman and Bers, 2008)
k42	2 Ca dissociation from CaM (N-terminal)	500 s^{-1}	(Saucerman and Bers, 2008)
k24	2 Ca binding to CaM (N-terminal)	$k42/Kd24 \mu\text{M}^{-2} \text{s}^{-1}$	(Saucerman and Bers, 2008)

Table 2: Reaction parameters for calcium-calmodulin binding

CaMKII reactions

Parameter	Description	Value (units)	Reference
CaMKII _{tot}	Total CaMKII concentration	1 μM or 50 μM	-
PiR	Initial inactive CaMKII fraction	0.9	-
PbR	Initial Ca ₄ CaM-bound CaMKII	0	-

	fraction		
Pb2R	Initial Ca ₂ CaM-bound CaMKII fraction	0	-
PtR	Initial Ca ₄ CaM-bound pThr287-CaMKII fraction	0	-
Pt2R	Initial Ca ₂ CaM-bound pThr287-CaMKII fraction	0	-
PaR	Initial unbound pThr287-CaMKII fraction	0.1	(Rose et al., 2007a)
PP1tot	Total PP1 concentration	0.1 μM	-
Kbi	Ca ₄ CaM dissociation from Pb	0.54 s ⁻¹	(Gaertner et al., 2004)
Kib	Ca ₄ CaM binding to Pb	kbi/25.7e-3 μM ⁻¹ s ⁻¹	(Gaertner et al., 2004)
kb2i	Ca ₂ CaM dissociation from Pb ₂	14*kbi s ⁻¹	(Shifman et al., 2006)
kib2	Ca ₂ CaM binding to Pb ₂	Kbi μM ⁻¹ s ⁻¹	-
kb42	2 Ca dissociation from Pb	k42/5 s ⁻¹	(Shifman et al., 2006)
kb24	2 Ca binding to Pb ₂	k24 μM ⁻² s ⁻¹	-
Kta	Ca ₄ CaM dissociation from Pt	kbi/1000 s ⁻¹	(Meyer et al., 1992)
Kat	Ca ₄ CaM binding to Pt	kib μM ⁻¹ s ⁻¹	(Meyer et al., 1992)
kt2a	Ca ₂ CaM dissociation from Pt	14*kt2a s ⁻¹	(Shifman et al., 2006)
kat2	Ca ₂ CaM binding to Pt	kib μM ⁻¹ s ⁻¹	-
kt42	2 Ca dissociation from Pt	k42/5 s ⁻¹	-
kt24	2 Ca binding to Pt ₂	k24 μM ⁻² s ⁻¹	-
kPP1	Thr287 dephosphorylation	1.72 s ⁻¹	(Saucerman and Bers, 2008)
KmPP1	Km for Thr287 dephosphorylation	11 μM	(Bradshaw et al., 2003)

Table 3: Reaction parameters for the CaMKII reaction scheme

CaMKII-dependent modification of RyR and SERCA

FT model

$$\text{RyR} = ([\text{Ca}]_{\text{JSR}} - [\text{Ca}]_{\text{MYO}}) * \text{CaMax} * (1 - e^{-(t/\text{tau1})})^{(\text{Power1} - D\text{Powermax})} * (e^{-(t/\text{tau2})})^{\text{Power2}}$$

$$\text{SERCA} = \text{CaPump} * [\text{Ca}]^2 / ([\text{Ca}]^2 + \text{KdPump}^2)$$

ST model

RyR: as in FT model

$$\text{CaMKIIreg} = \text{CaMKactive}^3 / (\text{KmCaMK}^3 + \text{CaMKactive}^3)$$

$$\text{SERCA} = (\text{Jmax_up} * \text{CaMKIIreg} + 1) * \text{CaPump} * [\text{Ca}]^2 / ([\text{Ca}]^2 / \text{KdPump}^2)$$

Parameter	Description	Value (units)	Reference
CaMax	Maximum RyR activity	FT: 6.981*10 ⁵ ST: 2.9088* 10 ⁵	- -
Tau1	RyR opening parameter	FT: 1.11 ST:1.1	(Groenendaal et al., 2008) -
Tau2	RyR opening parameter	FT: 1.4074 ST:1.5	(Groenendaal et al., 2008) -
Power1	RyR opening parameter	FT: 5 ST:5.2	(Groenendaal et al., 2008) -
Power2	RyR opening parameter	FT: 3 ST:3	(Groenendaal et al., 2008) -
CaPump	Maximum SERCA activity	FT: 170.2749 ST: 56.7583	(Groenendaal et al., 2008) (Groenendaal et al., 2008)
KdPump	[Ca ²⁺] at which SERCA activity is half-maximal	FT: 1 μM ST: 1 μM	(Groenendaal et al., 2008) (Groenendaal et al., 2008)
CaMKactive	Total concentration of active CaMKII	Pb+Pt2+Pt+Pa μM	
KmCaMK	Concentration of active CaMKII at which effect on substrates is 50% of maximum effect	0.2 μM	-
Jmax_up	Maximal % increase in KdPump	70%	(Hund and Rudy, 2004)
DPower1max	Maximal % increase in Power 1	30%	-

Table 4: Parameters related to RyR and SERCA activity and their CaMKII-dependent modification

Chapter 6: Summary and general discussion

In this chapter, the results of the experiments described in this thesis are summarised and suggestions for experiments are made to further elucidate the role of Ca^{2+} -calmodulin-dependent protein kinase II (CaMKII) and calcium-dependent signalling in regulating skeletal muscle adaptation.

Summary

Skeletal muscle is able to adapt its phenotype in response to increased usage, and improve its strength and fatigue resistance. Calcium-dependent signalling is presumed to increase mitochondrial biogenesis, an important adaptation to improve fatigue-resistance. CaMKII has been suggested to promote a slow-twitch, high oxidative phenotype in skeletal muscle, but its physiological role in muscle adaptation has so far not been investigated *in vivo*. The work described in this thesis aimed to elucidate the role of CaMKII in skeletal muscle *in vivo*. For this purpose, a rat model which allows for imposing defined stimulation protocols on skeletal muscle *in situ* was combined with the manipulation of CaMKII signalling by somatic gene transfer and a computational model of CaMKII signalling.

In chapter 2, the question was addressed whether CaMKII activation depends on recruitment frequency and whether its activity remains after muscle stimulation ceases. Adult rat skeletal muscle was stimulated *in situ* by different stimulation protocols after which protein levels of total CaMKII and phospho^{Thr287}-CaMKII were measured for the β_M , δ_A & δ_D/γ_B isoforms at different time points. This study consisted of two experiments. In the first experiment, *m. soleus* (SOL) and *m. gastrocnemius medialis* (GM) were stimulated with 100 electrical impulses at 10 or 150 Hz at their active slack length (i.e. a length at which no active force is produced). This protocol increased phospho^{Thr287}-CaMKII of δ_A and δ_D/γ_B isoforms in red GM, δ_A isoform in white GM, but none of the isoforms in SOL. There was no effect of frequency on the level of phospho^{Thr287}-CaMKII. A decrease in total β_m and δ_D/γ_B CaMKII was observed in GMW after 150 Hz stimulation. These results suggest that CaMKII is more strongly activated in fast-twitch muscle, and that motor neuron firing frequency is not an important factor in determining CaMKII activation.

In the second experiment, we sampled GM at rest, after determination of optimum length and at 2, 10 or 60 minutes after a two-minute isometric contraction protocol at optimum length. The following observations were made: 1) After the contractile activity required to determine the optimum length of the muscle, phospho^{Thr287}-CaMKII for the δ_A isoform was increased in red GM compared to rest. 2) Phospho^{Thr287}-CaMKII was higher in white GM compared to red GM regardless of stimulation. 3) Total CaMKII decreased over time in white GM. These results suggest that after a short contraction protocol, phospho^{Thr287}-CaMKII is transiently increased in an isoform- and muscle-dependent way. The results of the first and second experiment together suggest that CaMKII remains activated for 1 to 15 minutes after stimulation.

In chapter 3, the effects of acute CaMKII overexpression in skeletal muscle *in vivo* were investigated. α/β -CaMKII was overexpressed in adult rat *m. gastrocnemius* (GM) and *m. soleus* (SOL) by electroporation-mediated gene transfer. We hypothesised that CaMKII overexpression would increase the expression of mitochondrial genes. However, our experiments showed that CaMKII overexpression did not increase protein levels of cytochrome c oxidase IV (COXIV) in GM or SOL. The mRNA levels of oxidative phosphorylation components COXIV, COXI and transcriptional co-activator peroxisome proliferator-activated receptor gamma co-activator 1 α were not different in empty- and CaMKII-transfected SOL, whereas succinate dehydrogenase subunit b mRNA was decreased in CaMKII-transfected SOL. Force parameters of transfected muscles were measured *in situ* with intact innervations and perfusion. Strength and fatigue resistance of control- and CaMKII-transfected SOL and GM did not differ. However, we observed that CaMKII overexpression caused a decrease in twitch time-to-peak force and half-relaxation time. In addition, CaMKII-overexpressing SOL fibres displayed increased sarco/endoplasmic reticulum Ca²⁺-ATPase 2 expression compared to non-transfected fibres. Our results therefore suggest that increased CaMKII levels alone are not sufficient to increase mitochondrial biogenesis, but may be involved in calcium handling and the regulation of contractile force characteristics.

In addition to its role in mitochondrial biosynthesis, CaMKII is presumed to be involved in the regulation of a signalling pathway that controls skeletal alpha-actin expression. In chapter 4, we investigated the effect of CaMKII overexpression on the transcription of

skeletal alpha-actin (SKAA). We hypothesised that CaMKII overexpression would increase SKAA promoter activity and mRNA levels. The luciferase gene controlled by a SKAA promoter fragment was electro-transferred into *m. soleus* (SOL) and *m. gastrocnemius* (GM) of both legs. CaMKII was overexpressed in SOL and GM of the right leg only. In contrast to our hypothesis, luciferase activity measured in protein extracts of the CaMKII-transfected muscles SOL and GM was significantly lower compared to that in muscles transfected with the reporter only. SKAA mRNA level tended to be lower in SOL of the CaMKII transfected leg, but this was not significant. We concluded that CaMKII overexpression had a negative effect on SKAA gene transcription, but that the mechanisms underlying this effect require further investigation.

The results described in chapter 3 demonstrated that CaMKII overexpression *in vivo* decreased twitch contraction and relaxation time. However, it was unclear if direct CaMKII-dependent modification of the calcium release and re-uptake channels could have contributed to this effect. In chapter 5, we investigated whether CaMKII is activated more strongly when it's located in the vicinity of the RyR, and if increasing CaMKII concentration in sarcomeres is sufficient to explain decreased twitch contraction and relaxation times following CaMKII overexpression in rat skeletal muscle. A mathematical model of spatiotemporal sarcomeric $[Ca^{2+}]$ dynamics coupled to a biochemical model of CaMKII activation was used. The model predicted substantial spatial gradients in CaMKII activity in sarcomeres of fast- and slow-twitch muscle fibres during single and repeated RyR openings. Increasing CaMKII concentration in the model to mimic CaMKII overexpression did not produce decreased twitch contraction and relaxation times as observed *in vivo* (chapter 3). It was suggested that this may be due to the fact that CaMKII is minimally activated during a twitch. As CaMKII overexpression was shown to increase SERCA2 expression in *m. soleus* muscle fibres (chapter 3), the effects of increased SERCA levels were also modelled. This resulted in a decreased relaxation time in the model. We concluded that a CaMKII overexpression-induced increase in SERCA expression, but not modification of SERCA, can at least partly explain the observed decrease in twitch contraction and relaxation times.

Considerations regarding overexpression of wild-type CaMKII by electro-assisted gene transfer

A few technical issues related to the approach used to overexpress CaMKII in rat skeletal muscle have not been explicitly addressed in the individual chapters, and will be discussed here.

The results of the transfection experiments described in this thesis demonstrate that CaMKII overexpression has effects on the expression of SERCA2 protein and skeletal alpha-actin promoter activity. This raises the question as to what the mechanisms are via which these effects were achieved. It is generally presumed that CaMKII affects cellular functions through its kinase activity. Because a wild-type CaMKII was overexpressed, the activation of the exogenous CaMKII likely depended on muscle recruitment-induced increases in calcium/calmodulin concentration. We attempted to determine whether exogenous CaMKII was activated after *in situ* stimulation of muscle, by comparing phospho^{Thr287} levels in stimulated and non-stimulated CaMKII-transfected muscles (chapter 3). Although increased phospho^{Thr287} levels of the exogenous β -CaMKII isoform was observed in some of these experiments, this was not consistent. It is also possible that Ca^{2+} /CaM-dependent, rather than Ca^{2+} /CaM-independent activity (which is reflected by phospho^{Thr287}-CaMKII) is increased in CaMKII overexpressing muscles. However, as Ca^{2+} /CaM-dependent activity cannot be measured, it cannot be concluded with certainty that any observed effects of CaMKII overexpression are due to increased (contraction-induced) kinase activity.

COXIV was measured as a marker of mitochondrial density, as its expression in skeletal muscle is increased by CaMKIV and PGC-1 overexpression (Wu et al., 2002, Jiang et al., 2010), and therefore we hypothesised that COXIV expression would be increased in rat skeletal muscle by CaMKII overexpression. An increase in COXIV expression would not have demonstrated an increase in functional mitochondria, as these consist of many different proteins. However, if mitochondrial volume density had increased, COXIV protein levels would have been increased. As we observed no difference in COXIV protein level between control- and CaMKII-transfected muscles, we presume that the volume of functional mitochondria was also not increased in CaMKII-transfected muscles.

Electroporation was used to induce uptake of plasmid DNA by the muscle fibres. This method is thought to result in the permeabilization of the cell membrane, allowing DNA molecules to enter the cytoplasm. Thus, the method depends on the induction of some degree of damage to the muscle fibres. To identify the effects of CaMKII overexpression, CaMKII-transfected fibres were compared to adjacent non-transfected fibres. As described in chapter 3, separation of these two fibre populations was based on visual inspection of CaMKII immunostaining intensity. Both fibre populations contained centrally-nucleated fibres, indicating ongoing regeneration. Therefore, it is conceivable that both populations have taken up the plasmid DNA, but that the 'non-transfected' fibres expressed the encoded CaMKII at much lower levels. This might be an explanation for the absence of a difference in COXIV expression. However, we did detect a significant difference in SERCA2 immunostaining intensity between transfected and non-transfected muscle fibres. Therefore, it seems likely that the difference in CaMKII expression levels achieved between 'CaMKII-transfected' and 'non-transfected' fibres is of a sufficient magnitude to expose CaMKII-dependent effects on protein expression.

Regulation of CaMKII activity in skeletal muscle

Although CaMKII is known to be activated during exercise in humans (Rose and Hargreaves, 2003), very little is known about the physiological regulation of this activity. In this section, we discuss the factors that regulate activation and deactivation of CaMKII in skeletal muscle.

CaMKII activation

A large degree of variation in levels of phospho^{Thr287}-CaMKII was observed in resting SOL and GM compared to the magnitude of the effect of *in situ* stimulation on the levels of phospho^{Thr287}-CaMKII in these muscles as reported in chapter 2. The relatively high variation in phospho^{Thr287} could be related to the fact that the muscles were not immediately sampled after the contraction protocols. However, this suggestion was not supported by experiments described in chapter 3, whereby transfected muscles were freeze-clamped after two minutes of stimulation. In these experiments, no consistent increases in phospho^{Thr287} levels of the endogenous CaMKII isoforms were detected (Chapter 3, Fig. 2). Combined with the difference in resting levels of phospho^{Thr287}-

CaMKII, this demonstrates that other factors than neural stimulation regulate CaMKII phosphorylation.

Passive stretch of mouse EDL and SOL *ex vivo* (i.e. without intact neural stretch reflex loops) increased phospho^{Thr287}-CaMKII in these muscles (Jensen et al., 2007), and may therefore be one such non-neural factor. However, as the muscles in the 'slack contraction' experiment (chapter 2) were not stretched and a substantial degree of variance in phospho^{Thr287}-CaMKII levels was still observed, this is unlikely to be one of the main factors causing the variance in phospho^{Thr287}-CaMKII levels in the muscles in our experiments. A possibility is that the calmodulin concentration differs in muscles from different animals. As reported in chapter 5, calmodulin concentration may be a limiting factor for CaMKII activation, and therefore a higher calmodulin concentration may lead to a higher CaMKII activity upon neural stimulation. Intramuscular calmodulin concentration doubled within two days of chronic electrostimulation of rabbit EDL (Antipenko et al., 1999), suggesting that calmodulin expression is in fact regulated by long-term muscle activity pattern. However, this would not explain the variation in the resting level of phospho^{Thr287}, as the animals used during our experiments were not trained and had similar activity patterns as they were kept in cages until the measurements were carried out. Therefore, it is still unclear what caused the variation in the phospho^{Thr287}-CaMKII levels in SOL and GM in our experiments.

The regulation of CaMKII activation may depend on the muscle phenotype. The modelling results of chapter 5 suggest that the higher amplitude of fast-twitch (FT) calcium transients activate CaMKII to a greater extent in FT muscle compared to slow-twitch (ST) muscle. This is supported by the experimental data reported in chapter 2, which demonstrated that CaMKII autophosphorylation was increased in fast-twitch GM, but not in slow-twitch SOL after *in situ* stimulation with 100 electrical pulses. To the best of our knowledge, no other study has investigated electrical stimulation-induced CaMKII phosphorylation in different muscle types. Further work is required to determine whether the difference in stimulation-induced phospho^{Thr287}-CaMKII levels in FT and ST muscles persists during longer stimulation protocols reflecting exercise sessions.

In addition to affecting stimulation-induced CaMKII phosphorylation, muscle type also has an effect on basal levels of phospho^{Thr287}-CaMKII. Increased resting levels of phospho^{Thr287}-CaMKII levels were observed in skeletal muscle after a period of one-legged

endurance training in humans (Rose et al., 2007b) and after chronic overloading of chicken muscle (Fluck et al., 2000b). As these training sessions increased the expression of genes involved in regulating oxidative metabolism and muscle size, respectively, these results suggest that the increased basal phospho^{Thr287}-CaMKII levels may be involved in establishing a new muscle phenotype. However, higher levels of basal phospho^{Thr287}-CaMKII were observed in rat low-oxidative white GM compared to high oxidative red GM (chapter 2) and in mouse fast-twitch, low-oxidative EDL muscle compared to slow-twitch high-oxidative SOL (Jensen et al., 2007). Therefore, basal phospho^{Thr287}-CaMKII levels in skeletal muscle is not simply higher as the oxidative capacity of the muscle increases, and different mechanisms may regulate resting CaMKII phosphorylation in trained and untrained muscle. It is currently unclear what the mechanisms are that lead to these increased basal levels of phospho^{Thr287}-CaMKII levels.

The development of autonomous CaMKII activity is dependent on the frequency of exposure of the enzyme to Ca²⁺/CaM (De Koninck and Schulman, 1998). However, we observed no effect of neural activation frequency on phospho^{Thr287}-CaMKII in experiments where muscles at their active slack length were stimulated *in situ* (chapter 2). Furthermore, our simulations of CaMKII activity development suggested that the RyR opening frequency only has an effect on phospho^{Thr287}-CaMKII levels during the first few RyR openings (chapter 5). Therefore, the biochemical ‘Ca²⁺/CaM-frequency decoding’ property of CaMKII appears to be relevant in whole muscle only during the initial RyR openings. This may be explained by the following three mechanisms: 1) the high frequency of calcium release in rat skeletal muscle compared to those used during the *in vitro* experiments by De Koninck & Schulman (13-250 Hz (Hennig and Lomo, 1985) versus 1-4 Hz (De Koninck and Schulman, 1998)), 2) the relatively slow deactivation of phosphorylated CaMKII after muscle stimulation (chapter 2) and 3) the existence of a maximum level of autophosphorylated CaMKII in skeletal muscle (chapter 5).

One study has claimed that the frequency at which multiple contractions occur determines CaMKII activity. This conclusion was based on mathematical modelling and the observation that KN93, a pharmacological inhibitor of CaMK’s, inhibited calcium release in single fast-twitch mouse muscle fibres when contractions occurred at 100 ms intervals, but not when contractions occurred at 5 second intervals (Aydin et al., 2007). However,

CaMKII phosphorylation and activity were not measured during this study. Therefore, experiments that employ muscle contraction protocols with repeated contractions followed by measurement of CaMKII phosphorylation and/or activity are still required to confirm whether the frequency of muscle contraction has an effect on CaMKII activation.

It cannot be excluded that calcium influx from other sources than the SR is important for CaMKII activation. Extracellular calcium can enter the cytoplasm through store-operated calcium entry (via SR protein stromal interaction molecule 1 and the Orai1 calcium channel), excitation-coupled calcium entry and transient receptor potential channels (TRPC's) (Dirksen, 2009)). Although calcium influx through TRPC3 has been implicated in the activation of the calcineurin - nuclear factor of activated t-cells (NFAT) pathway (Rosenberg et al., 2004), very little is known about the magnitude of calcium influx through these channels and as of yet no implications for CaMKII activation can be derived.

The calcium concentration in myonuclei also increases during electrical stimulation of muscle fibres, but with slower dynamics compared to those in the cytoplasm (Liu et al., 2005). CaMKII-like activity has been demonstrated in nuclei isolated from chicken skeletal muscle (Fluck et al., 2000a). Some studies have claimed that CaMKII in the nucleus is phosphorylated after exercise based on immunohistochemical measures (Smith et al., 2008, Liu et al., 2005) but these data are not convincing as comparisons of staining intensities between different stained sections were made. Therefore, the results are likely to be affected by differences in staining quality and imaging settings between the different sections. It is currently unknown which CaMKII isoforms are within the myonucleus, and therefore they cannot be distinguished on the western blots performed in chapters 2 and 3.

The roles of the different CaMKII isoforms and whether they are differentially localized in muscle fibres (as for example in cardiac myocytes (Ramirez et al., 1997)) requires further investigation. This knowledge will aid in determining which calcium micro-domains are important for their activation, and therefore help relate the physiological contraction-related stimuli to CaMKII-dependent downstream effects. Possibly, this could be done by overexpression of specific CaMKII isoforms, or targeting these isoforms for degradation using RNA interference (RNAi). These RNAi constructs would likely have to be targeted to the variable region within the association domain in order to knock-down individual isoforms (Hudmon and Schulman, 2002).

Oxidation of methionine residues 281/282 gives CaMKII autonomous activity in a mechanism parallel to autophosphorylation (Erickson et al., 2008). Like autophosphorylation of Thr287, oxidation of Met281/282 requires initial binding of $\text{Ca}^{2+}/\text{CaM}$ and prevents blocking of the catalytic domain by the autoinhibitory domain (Erickson et al., 2008). Prevention of CaMKII oxidation by overexpression of methionine sulfoxide reductase A mitigated aldosterone-induced cardiac rupture in mice (He et al., 2011). This suggests that oxidation-dependent CaMKII activity has effects separate from those of autophosphorylation-dependent CaMKII activity in the heart. The role of oxidized CaMKII has not been the subject of any investigation in skeletal muscle. The formation of oxygen radicals is also induced in contracting skeletal muscle (Powers and Jackson, 2008), and it would therefore be of interest to determine whether oxidation of CaMKII occurs in skeletal muscle.

CaMKII de-activation

Much like the factors that regulate the activation of CaMKII, the factors that regulate the de-activation of CaMKII have received very little attention. Although $\text{Ca}^{2+}/\text{CaM}$ is released from CaMKII when the intracellular $[\text{Ca}^{2+}/\text{CaM}]$ is low, phosphorylation of CaMKII can only be reversed by the action of phosphatases. In both the ‘two-minute isometric contractions’ and ‘slack contraction’ experiments described in chapter 2, increased levels of phospho^{Thr287}-CaMKII in rat skeletal muscle were increased after very brief stimulation (approximately 100 pulses) and remained up to 15 minutes after stimulation of rat skeletal muscle. This suggests that CaMKII autophosphorylation enables CaMKII to outlast the $\text{Ca}^{2+}/\text{CaM}$ stimulus in skeletal muscle required for its initial activation.

One study observed increasing CaMKII activity and phospho^{Thr287}-CaMKII levels in rat fast-twitch EDL muscle *in situ* during stimulation up to 3 minutes after which both activity and phosphorylation decreased during continued stimulation (Rose et al., 2007a). In chapter 2, we reported that although phospho^{Thr287}-CaMKII levels for the δ_A -CaMKII isoform were increased after the activity required to determine optimum muscle length, phospho^{Thr287}-CaMKII was not increased for any of the CaMKII isoforms after a further two minute isometric contraction protocol (chapter 2). This suggests that muscle stimulation increases CaMKII phosphorylation, but also activates a pathway that dephosphorylates CaMKII, so that an elevated level of phospho^{Thr287}-CaMKII is only

maintained after brief stimulation, and CaMKII is dephosphorylated during prolonged stimulation. The identity of this pathway for CaMKII dephosphorylation in skeletal muscle is currently unknown and requires further investigation.

CaMKII function in skeletal muscle

The current view in the literature is that CaMKII is involved in the regulation of mitochondrial biogenesis in skeletal muscle. However, our data indicate that basal levels of phospho^{Thr287}-CaMKII are higher in low-oxidative white compared to high-oxidative red rat *m. gastrocnemius medialis* (chapter 2). This result and the observation that CaMKII overexpression did not increase COXIV expression (chapter 3) indicate that increased CaMKII autophosphorylation and protein level are not sufficient to increase COXIV expression, which is essential to increase mitochondrial capacity. A possibility which has not yet been discussed is that CaMKII induced paracrine signalling that stimulated COXIV expression in non-transfected fibres as well as CaMKII-overexpressing fibres in the same muscle. In this case, there would not be a difference in COXIV staining intensity on the level of single fibres, but there would be a difference in COXIV expression between control- and CaMKII-transfected muscles detected by western blot. However, we observed no difference on COXIV levels between control- and CaMKII-transfected muscles on a western blot (Fig. 1).

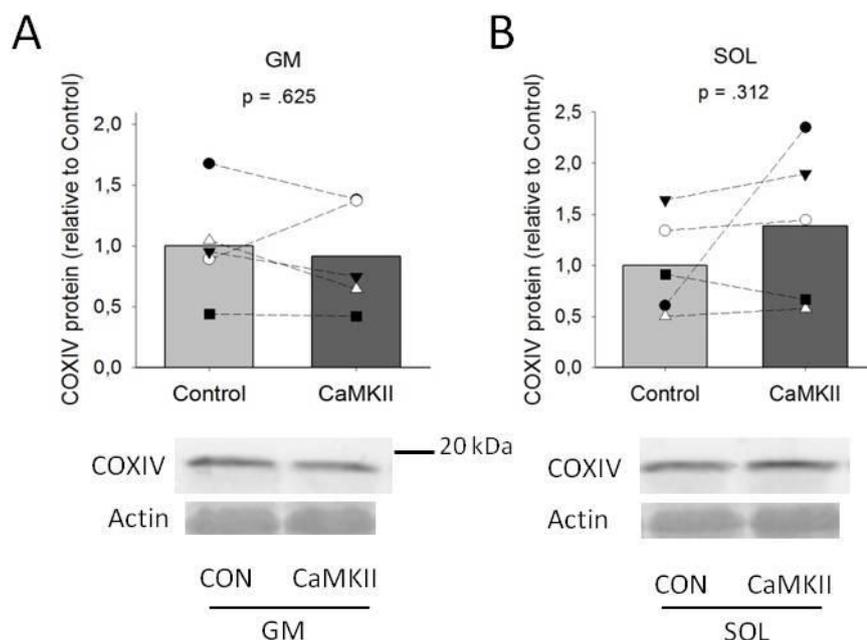


Figure 1: The effect of CaMKII overexpression on COXIV protein levels in whole muscle

Graphs display COXIV protein levels in control- (CON) and CaMKII-transfected GM (A) and SOL (B) as determined by western blotting followed by immunodetection. Bars represent mean COXIV and points represent the data obtained from individual samples. Points connected by a dashed line represent intra-animal muscle pairs. Significance of the difference between control- and CaMKII-transfected muscle s is indicated. Example blots are shown below the graphs. Actin was detected by ponceau s staining of the blot and serves as a loading control.

CaMKII per se may not be sufficient to induce mitochondrial biogenesis but it might be required in combination with other stimuli. This was indirectly suggested by the observation that the pharmacological CaMK inhibitor KN93 inhibited calcium-dependent activation of p38 mitogen-activated protein kinase (MAPK) in mouse epitrochlearis muscle *ex vivo* (Wright et al., 2007). MAPK inhibition had been demonstrated in the same study to inhibit calcium-dependent mitochondrial gene expression. However, this study did not measure the effect of CaMKII inhibition on mitochondrial expression directly. Inhibition of CaMKII in mouse skeletal muscle *in vivo* has been carried out, but no data on mitochondrial gene expression have been reported (Murgia et al., 2009). It is therefore still unclear whether CaMKII is required for mitochondrial gene expression *in vivo*.

In contrast, the data presented in chapters 3 and 4 suggest that CaMKII overexpression is sufficient to increase SERCA2 protein expression and decrease skeletal alpha-actin promoter activity. This suggests that CaMKII does influence gene expression in skeletal muscle. Can putative CaMKII-dependent pathways of gene regulation explain increased SERCA2 expression? The SERCA2a promoter contains a serum response element (SRE) (Baker et al., 1998), and SRF overexpression resulted in decreased SERCA2 mRNA levels in cardiomyocytes (Zhang et al., 2001). This suggests that SRF might have an inhibitory effect on SERCA2 transcription. As CaMKII phosphorylates SRF at sites which either inhibit or stimulate its DNA binding (Fluck et al., 2000a, Rivera et al., 1993, Wheaton and Riabowol, 2004), our results suggest that CaMKII overexpression inhibited SRF DNA binding and decreased the activity of the SRF promoter construct (which contains three SREs (chapter 4)). Possibly by the same mechanism, CaMKII overexpression increased SERCA2 expression (chapter 3). In addition to SRF, MEF2 might be a CaMKII-regulated factor which mediates increases in SERCA expression (Lu et al., 2000a). A MEF2 site is present on the SERCA promoter, but SERCA promoter activity in cardiomyocytes is only

increased when MEF2 is co-overexpressed with NFAT (Vlasblom et al., 2004), which is not known to be regulated by CaMKII. It should be noted that no increase in SERCA mRNA was observed in the CaMKII overexpression experiments in chapter 3. In conclusion, our data point to SRF as a possible mediator of CaMKII-dependent signalling in skeletal muscle *in vivo*, but its actual involvement still requires experimental verification.

Interestingly, δ -CaMKII expression increases in regenerating muscle (Abraham and Shaw, 2006). Increased calcium influx through damaged muscle membranes is thought to be an important factor in the pathophysiology of dystrophic muscle, as it may activate calcium-dependent proteases such as calpains (Whitehead et al., 2006). Stimulation of calcium removal from the cytoplasm by SERCA overexpression mitigates the dystrophic phenotype in skeletal muscle of mdx mice (Goonasekera et al., 2011). In the electroporated muscles in chapter 3, SERCA2 expression was decreased in the damaged electroporated region compared to the non-electroporated region (Fig. 2). SERCA2 expression in *m. soleus* is also decreased during myotoxin-induced regeneration (Zador et al., 1996). In contrast, CaMKII overexpression increased SERCA2 protein expression (chapter 3, Fig. 6). Furthermore we observed a normalization of twitch contraction and relaxation time (chapter 3, Fig. 5). However, no improvements in maximal force of the CaMKII-overexpressing muscles were observed seven days after transfection. Whether CaMKII overexpression decreases resting calcium level or calcium transients during muscle stimulation will require experimental verification. CaMKII could be co-overexpressed with a calcium-sensitive probe which allows for *in vivo* imaging (Rudolf et al., 2004). A comparison can then be made with the contra-lateral muscle overexpressing the probe only. To conclude, our results suggest that CaMKII may stimulate muscle regeneration by increasing SERCA2 expression.

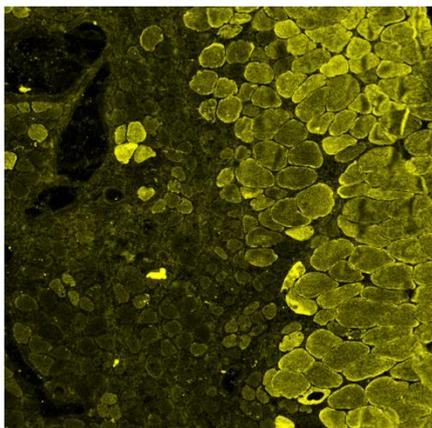


Figure 2: Decreased SERCA2 protein in damaged SOL fibres
Immunofluorescence image of SOL subjected to electroporation procedure and stained for SERCA2. The left half of the image contains small regenerating fibres, whereas the right half of the fibres is undamaged. The lower staining intensity in the regenerating fibres indicates lower SERCA2 levels in these fibres.

The combined results of the stimulation and transfection studies in this thesis suggest that CaMKII regulates gene expression in skeletal muscle, but that its muscle recruitment-dependent activity is limited to brief periods during and after muscle recruitment. The CaMKII activity pattern suggests that it is mostly involved in regulating acute processes during muscle contraction, rather than regulating processes such as the changes in gene expression during the recovery period after exercise. How can this apparent contradiction be explained? Firstly, it is not quite clear what the role of basal CaMKII activity in muscle is. As shown in chapter 2, there are differences in basal CaMKII phosphorylation between different muscle fibre types, and some studies have demonstrated an increase in basal CaMKII phosphorylation after a period of training (Rose et al., 2007b, Fluck et al., 2000b). It may be that changes in basal activity and expression are related to adaptation (though not of oxidative capacity), and that contraction-induced activity is related to acute functions such as glycogen metabolism and calcium release. The latter hypothesis is supported by the modelling results described in chapter 5, which show that the increase in CaMKII activity is largest near substrates involved in regulating glycogen metabolism and calcium release (Singh et al., 2004, Leddy et al., 1993). Assessment of the impact of changes in basal CaMKII activity on muscle phenotype requires experiments in which skeletal muscles *in vivo* are overexpressing a constitutively active CaMKII-mutant. As of yet such experiments have not been carried out, but using the methods described in this thesis will provide relevant information regarding the role of CaMKII in skeletal muscle adaptation.

The role of calcium in mitochondrial gene expression

Calcium influx into skeletal muscle is generally considered to be a factor which stimulates mitochondrial biogenesis (Ojuka et al., 2003, Wright et al., 2007, Bruton et al., 2010). This raises the question as to what the mechanism is through which the calcium-dependent increases in mitochondrial biogenesis are achieved. Constitutively active calcineurin increases mitochondrial biogenesis in mouse skeletal muscle (Long et al., 2007, Jiang et al., 2010), and thus the effects of increasing calcium concentration may be mediated by calcineurin. However, an alternative explanation is that the effects of chronically elevated calcium concentration may be caused by indirect effects (i.e. not requiring calcium-dependent molecules). Increased cellular energy expenditure is likely required because calcium needs to be actively removed from the cytoplasm as continuously elevated calcium concentrations are associated with skeletal muscle pathology (Oberc and Engel, 1977). The

resulting increase in intracellular AMP, and decrease in ATP concentration could activate AMPK, which appears to be sufficient to increase mitochondrial gene expression (Garcia-Roves et al., 2008). Alternatively, the increased calcium levels may have activated CaMK kinase (CaMKK), which activate AMPK in skeletal muscle (Jensen et al., 2007). CaMKK can be inhibited by KN93 (Jensen et al., 2007), and this might therefore explain the observed KN93-dependent attenuation of mitochondrial gene expression in response to calcium (Wright et al., 2007).

A promising approach to test the physiological role of calcium in muscle adaptation without chronically elevating the intracellular calcium concentration is to use actomyosin-ATPase inhibitors to separate the calcium influx from muscle contraction (Dentel et al., 2005). This experiment cannot be carried out *in situ* or *in vivo*, as the actomyosin inhibitor would paralyse the diaphragm. Isolated muscles should be incubated with or without the inhibitor, and stimulated with a protocol that stimulates mitochondrial gene expression. A comparison with unstimulated muscles should identify whether physiologically relevant calcium transients can stimulate mitochondrial gene expression to the same extent as observed in contracting skeletal muscle.

Conclusion

The aim of this thesis was to elucidate the role of CaMKII in skeletal muscle adaptation *in vivo*. We conclude that CaMKII is activated by very brief stimulation in a recruitment frequency-independent manner, and that increased CaMKII protein levels increase SERCA2 expression, but not mitochondrial gene expression. We speculate that contraction-induced CaMKII activity may be mainly involved in regulating acute processes in skeletal muscle, and that chronic increases in CaMKII activity regulate long-term adaptation of skeletal muscle. The adaptations regulated by CaMKII are still unclear and deserve further investigation.

Conference presentations

W. Eilers, R.T. Jaspers, A de Haan & Martin Flueck. CaMKII regulates excitation-contraction coupling, not mitochondrial biogenesis in rat skeletal muscle (2011). Oral presentation: European Muscle Conference, Max Delbrück Center for Molecular Medicine, Berlin-Buch.

W. Eilers, R.T. Jaspers, A. de Haan & Martin Flueck. CaMKII overexpression reverses the effects of electroporation-induced damage on skeletal muscle twitch parameters (2011). Oral presentation; Physiological Society Main Meeting, University of Oxford.

W. Eilers, D. van Overbeek, A. de Haan & M. Flueck. CaMKII is not sufficient to induce mitochondrial biogenesis in rat skeletal muscle (2011). Oral presentation; Atmungsphysiologische Arbeitstagung 2011, Hannover Medical School.

W. Eilers, D. van Overbeek, A. de Haan & M. Flueck. Increased activity of Calcium/Calmodulin-dependent Protein Kinase II is not sufficient to induce mitochondrial biogenesis in rat skeletal muscle (2010). Poster presentation; Physiological Society Main Meeting, University of Manchester.

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