Sensory pathways of muscle phenotypic plasticity: Calcium signalling through CaMKII

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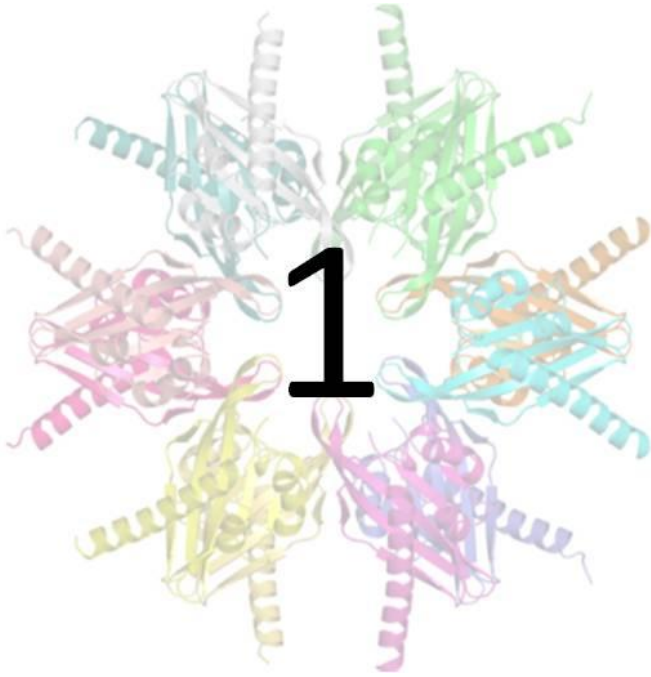
2013

General introduction



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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 might be linked to changes in gene expression. 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 ɣ 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*.

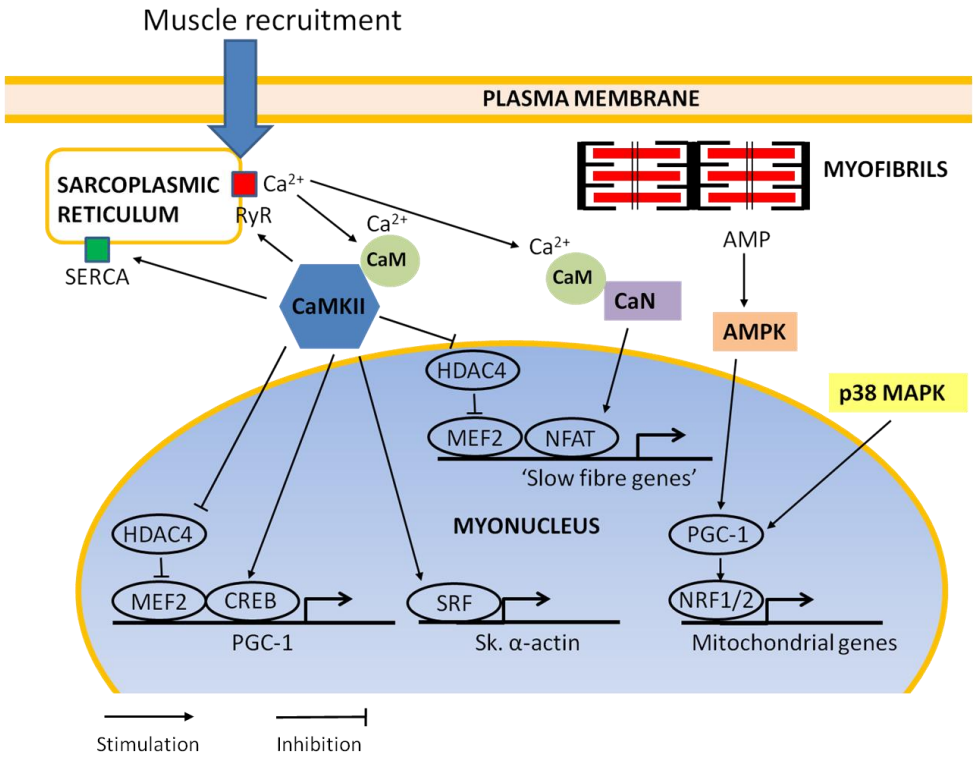

Figure 1: Potential intracellular signalling pathways involved in skeletal muscle adaptation to exercise
Diagram showing how CaMKII may affect muscle phenotype by altering gene expression. In addition, CaMKII is thought to stimulate calcium release from, and uptake into, the sarcoplasmic reticulum. A 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 Ca^{2+}-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 identity 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.