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## The role of Calcium/calmodulin-dependent kinase and Calcineurin pathways in activity-dependent gene regulation in skeletal muscle

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DATA CONSEGNA TESI

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## MOST USED ABBREVIATIONS

AMPK: AMP-activated protein kinase

C.a.: constitutively active

$a^{2+}$: calcium

CaM: calmodulin

CaMK: calcium/calmodulin-dependent kinase

CAT: chloramphenicol acetyltransferase

Cn: calcineurin

CS: citrate synthase

EDL: *extensor digitorum longus*

Enh: enhancer

Fig.: figure

GLUT4: glucose transporter 4

$IP_3$ receptor: $Ins(1,4,5)P_3R$

KIIN: CaMKII inhibitor

LUC: luciferase

MEF2: myocyte enhancer factor

MyHC: myosin heavy chain

NFAT: nuclear factor of activated T cell

PGC-1α: peroxisome proliferator-activated-receptor-γ coactivator-1

RyR: ryanodine receptor

SR: sarcoplasmic reticulum

TA: *tibialis anterior*

TFAM or mtTFA: mitochondrial transcriptional factor A

TCR: T cell receptor

T2D: Type 2 diabetes

## ABSTRACT

Motor neuron activity is a fundamental controller of skeletal muscle growth and differentiation. Excitation-transcription coupling is the process whereby activity causes specific changes in gene transcription through an increase in the concentration of sarcolemmal calcium ($Ca^{2+}$). The molecular mechanisms that relay plasma membrane depolarization to activity-dependent gene transcription is not yet fully understood. Two main $Ca^{2+}$-dependent pathways are known to transduce $Ca^{2+}$ signals into changes in gene expression, the Cn-NFAT and CaMK pathways. We have focused on Cn-NFAT and CaMKII, which is the major CaMK isoform in skeletal muscle. We have studied the effects of the inhibition of these pathways on the expression of $Ca^{2+}$-dependent genes involved in muscle metabolism, such as glucose transporter 4 (GLUT4), mitochondrial transcription factor A (TFAM) and citrate synthase (CS), or in muscle contraction and specific of slow or fast IIB fibers, such as myosin heavy chain-slow and -2B (MyHC-slow and -2B). To this purpose we have used a loss-of-function approach by co-transfecting vectors, coding for natural peptide inhibitors of Cn and CaMKII pathways, together with Luciferase (LUC) reporters in rat skeletal muscle, through *in vivo* electroporation. We have analyzed the effects of these inhibitors on activity-dependent gene expression. Our results indicate that CaMKII controls not only muscle metabolism, by regulating the expression of GLUT4, TFAM and CS, but also the expression of contractile proteins, such as MyHC-slow and -2B. The Cn-NFAT signalling pathway has been widely demonstrated to be a fundamental regulator of muscle fiber slow program. Interestingly our data indicate that Cn also controls the expression of genes involved in muscle metabolism such as GLUT4 and CS. A question remains open as to whether CaMKII and Cn pathways can synergistically control the expression of shared activity-dependent genes. If synergy exists it will be interesting to investigate which is the molecular target underlying CaMKII and Cn synergistic cooperation.

# RIASSUNTO

L'attivita' dei motoneuroni ha un ruolo fondamentale nella crescita e nel differenziamento del muscolo scheletrico. Il cosiddetto processo di accoppiamento eccitazione-trascrizione, attraverso l'aumento del livello di calcio ($Ca^{2+}$) nel sarcolemma, causa specifici mbiamenti attivita'-dipendenti nella trascrizione genica. I meccanismi molecolari che convertono la depolarizzazione della membrana plasmatica in specifiche variazioni nella trascrizione genica-attività dipendente, non sono ancora del tutto chiari. Nel muscolo scheletrico, sono note due principali vie $Ca^{2+}$/calmodulina-dipendenti che accoppiano i segnali del $Ca^{2+}$ a cambiamenti dell'espressione genica: esse sono le vie calcineurina (Cn)-NFAT e chinasi $Ca^{2+}$/calmodulina-dipendenti (CaMK). In questo lavoro abbiamo incentrato la nostra attenzione sulla via Cn-NFAT e su CaMKII, la principale isoforma di CaMK nel muscolo scheletrico. Abbiamo studiato gli effetti dell'inibizione di queste vie sull'espressione di alcuni geni $Ca^{2+}$-dipendenti implicati nel metabolismo muscolare, come il trasportatore del glucosio 4 (GLUT4), il fattore di trascrizione mitocondriale A (TFAM) e la citrato sintasi (CS), e nella contrazione muscolare e specifici per le fibre slow o rapide 2B, come il gene della catena pesante della miosina-lenta e -2B (MyHC-*slow* e -2B). A tale scopo, abbiamo utilizzato un approccio *loss-of-function*, co-trasfettando vettori, codificanti inibitori endogeni delle vie Cn e CaMKII, insieme a sensori luciferasi (LUC), mediante elettroporazione *in vivo*. Abbiamo analizzato gli effetti di tali inibitori sull'espressione di geni attività-dipendenti. I nostri risultati indicano che l'attività di CaMKII controlla non solo il metabolismo muscolare, regolando l'espressione di GLUT4, TFAM e CS, ma inaspettatamente abbiamo dimostrato che ha un effetto anche sull'espressione di proteine contrattili quali MyHC-*slow* e -2B. E' stato largamente dimostrato che la via di segnale Cn-NFAT ha un ruolo fondamentale nella regolazione del programma lento della fibra muscolare. I nostri dati suggeriscono che Cn controlla anche l'espressione di geni metabolici, come per esempio GLUT4 e CS. Stiamo cercando di chiarire se le vie CaMKII e Cn possono controllare sinergicamente l'espressione di geni bersaglio condivisi. Nel caso in cui si verifichi tale sinergia, cercheremo di caratterizzare il meccanismo molecolare alla base di tale sinergia.

# 1. INTRODUCTION

## *1.1 Skeletal muscle fiber properties*

In vertebrates three different muscular tissues can be distinguished: smooth, cardiac and skeletal. Smooth muscle surrounds internal organs and major blood vessels, is innervated by the autonomous nervous system and it is made of mono-nucleated cells (called myocytes). The cells of cardiac muscle are mono-nucleated, and together with skeletal muscles form the complex of striated muscles.

The functional units of skeletal muscles are the muscle fibers, long cylindrical multinucleated, with about 100 nuclei cells, 10-50 μm of diameter, 1-10 mm of length in rat muscles. The great majority of the cytoplasm of these cells is occupied by fascicles of longitudinal filaments called myofibrils, subdivided in light and dark bands, called I band and A band respectively. The basic structural and functional unit of skeletal muscle is called sarcomer: this repeated element has a length of 2 μm. Adjacent sarcomeric units are perfectly aligned, giving rise to the characteristic striated morphology that can be seen with the microscope. Each sarcomer contains two kinds of filaments: thin filaments, made of actin (that constitutes the light I band), thick filaments, made of myosin (dark A band). Contraction speed is related to the ATPase activity of myosin, and to the enzymes of the sarcoplasmic reticulum (SR) that sequester calcium ($Ca^{2+}$). Myosins are composed of three couple of subunits: two heavy chains (MyHC), two light chains (MLC) and two essential or alkaline chains. The two C-terminal domains of the MyHC subunits are coiled to form an α-helix tails, responsible for the association of many other MyHC dimers in a single filament. ATPasic activity and actin binding site are localized within the globular region of the protein (head).

### *1.1.1 Myofiber diversity*

Muscle fibers vary considerably with respect to their morphological, biochemical and physiological properties, enabling different muscles to fulfill a variety of functions, from maintaining the body posture to performing a wide range of movements and motions.

Mammalian skeletal muscles are composed of two major fiber-types (I and II), basically distinguished on the basis of the myosin heavy chain isoform (MyHC) that they express, and differ in terms of size, metabolism, and contractile properties. MyHCs

determine the contractile properties of the fibers, while fiber metabolism determines their fatiguability. Slow (type I) fibers are characterised by slow velocity of shortening and an high content in mitochondria, exhibiting a predominantly oxidative metabolism and resistance to fatigue. They are recruited for sustained, tonic contraction events. Fast (type II) fibers fatigue and contract rapidly and thus have low resistance to fatigue. Type II fibers are further grouped into three subtypes, IIA (or 2A), IIX/D (or 2X/D), IIB (or 2B) differing with respect to their mitochondrial content. IIB fibers are the most glycolityc and most fatigable ones while IIA, being rich in mitochodria, are more oxidative and relatively slower. IIX/D fibers have intermediate properties between IIA and IIB fibers. Type II-fast fibers are required for sudden rapid movements.

Although fibers are roughly divided into these general categories, a wide spectrum of fiber-types exists, from the specialized extremes to the intermediate fiber-types, expressing more than one MyHC isoform (e.g. I and IIA, or IIA and IIX, or IIX and IIB). In addition to the variety of muscle fiber intrinsic composition, the pattern of expression of the MyHC varies between species.

### *1.1.2 Myofiber plasticity*

Myofiber composition is determined by developmental cues and neuronal activity. During embryogenesis, primary myofibers can mature into slow or fast fiber independent of innervation, with a mechanism not yet identified. When primary myofibers mature into secondary myofibers, embryonic MyHC isoforms are progressively replaced by adult IIA, IIX, and IIB isoforms (De Nardi et al., 1993). At this point innervation is required for skeletal muscle growth and survival, and, in addition, controls fiber-type properties.

Skeletal muscle owns a remarkable capacity to remodel in response to specific environmental and physiological stimuli. This powerful adaptability results from specific patterns of nerve activity and the diversity of muscle fiber-types. Specific impulse patterns delivered by motor neurons have been known for years to exert phenotypic changes on muscle fibers which they innervate, thus remodelling myofibers and maintaining their size and function. The determinant role of nerve activity in switching muscle fiber properties has been demonstrated by a surgical switch of the innervation between the slow soleus and the fast EDL muscles (cross-innervation). After 11-15 months soleus acquires most properties of a fast muscle and EDL of a slow muscle (Barany and Close, 1971; Vrbova,

1963). These data indicate that, even if innervation has a great influence, probably there are some intrinsic differences, related to different embryonic lineages (Pette and Staron, 1997). Subsequently, electrical stimulation experiments corroborated the cross-innervation data, demonstrating that impulse patterns, applied directly on the nerve or on denervated muscles, and mimicking the firing pattern of slow and fast motor neurons, could induce changes in muscle fibe-type composition (Pette and Vrbova, 1992; Pette, 2001). A firing pattern mimicking fast motor neuron activity can cause a slow-to-fast switch in the direction I → IIA → IIX → IIB whereas an opposite switch, fast-to-slow, takes place using a firing patter resembling that one of slow motor neurons and it follows the obligatory sequence IIB → IIX → IIA → I. However, electrostimulation usually produces an incomplete transformation of the muscle fiber-type because the range of adaptability is limited by intrinsic differences between muscles, thus a fast muscle can adapt in the range IIB ↔ IIX ↔ IIA and slow muscle in the range I ↔ IIA ↔ IIB (Ausoni et al., 1990). The limit of these experimental approaches is that changes in protein (myofibrillar and SR proteins as well as metabolic enzymes) expression in response to chronic electrical stimulation require weeks or months or even years (Pette and Staron, 1997), while changes at the transcript levels occur more rapidly (Huber and Pette, 1996). Also during pathological conditions complete switches in muscle fiber-type appear, e.g. long term spinal cord injury involves disappearance of type I fibers (Grimby et al., 1976). In fact during denervation a "default" program takes place in both fast and slow muscles, giving rise to a slow-to-fast switch (Butler-Browne et al., 1982), in addition to muscle atrophy, as a consequence of the lack of electrical stimulation (Spector et al., 1985 *a* and *b*).

### *1.2 $Ca^{2+}$-dependent events in skeletal muscle*

#### *1.2.1 Excitation-contraction coupling*

Excitation-contraction coupling is the process whereby membrane depolarization triggers force production (Fig. 1). Membrane depolarization, triggered by a neuronal stimulus, activates $Ca_v1.1$ in the plasma membrane of skeletal muscle, which are voltage-gated L-type $Ca^{2+}$ channels, also known as dihydropyridine receptors (DHPRs), because dihydropyridine is a blocker of this channel (Glossmann et al., 1985). Many L-type channels are situated on t-tubules, deep invaginations of the plasma membrane (Fig. 1),

perpendicular to the long axis which allow depolarization to quickly reach the interior of the cell (Curtis and Catterall, 1984). L-type channels are responsible for the excitation-contraction coupling, due to the interaction of their $\alpha_1$ subunit with the ryanodine receptor RyR (in skeletal muscle the RyR1 isoform), thus connecting the tubular to the SR membranes and forming the junctions between the t-tubule and SR (t-SR junctions). In particular, every RyR1 is associated with a tetrade of four $Ca_v1.1$ channel (Zalk et al., 2007).

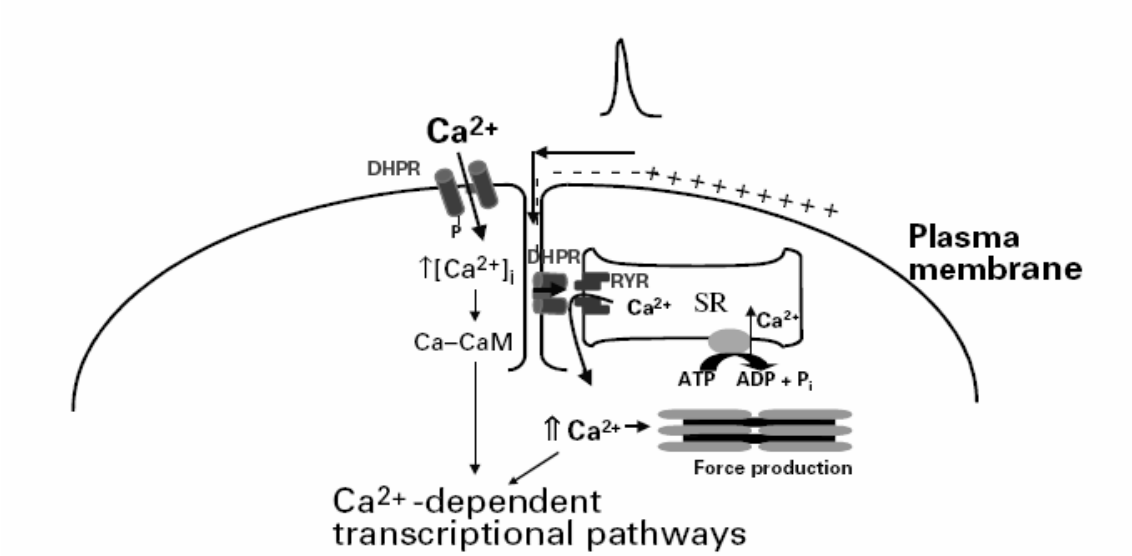


**Fig. 1. Skeletal muscle excitation-contraction and excitation-transcription coupling.** Activation of muscle contraction results in depolarization of the plasma membrane and the transverse tubule (t-tubule) system. The t-tubule system carries the signal to the interior of the fibre where the voltage-sensing dihydropyridine receptor (DHPR) detects the change in membrane potential and transmits the signal to the calcium-release channel, also known as the ryanodine receptor (RYR). Release of calcium from the internal stores (sarcoplasmic reticulum; SR) into the myoplasm results in actin–myosin interaction, fibre shortening and force production. Skeletal muscle calcium levels are also elevated by ligand-mediated activation of L-type calcium channels by signalling molecules such as insulin-like growth factor-I, which result in influx of calcium via the plasma membrane. Both sources of intracellular calcium are thought to play a role in excitation–transcription coupling. $[Ca^{2+}]i$, intracellular free calcium ion concentrations; CaM, calmodulin (from Chin, 2004).

RyR1 is the major channel for $Ca^{2+}$ release from the SR, once membrane depolarization reaches t-tubules. It is believed that an electromechanical coupling exists, which converts an electrical signal into structural changes first in the $Ca_v1.1$ channels. These changes in turn induce a structural alteration in the RyR1s, which finally triggers the opening of the RyR1s (Schneider and Chandler, 1987; Rios and Pizarro, 1991). $Ca^{2+}$ released from RyR1s binds to diverse targets, including troponin C, which triggers muscle contraction through the actin-myosin system. Following contraction, $Ca^{2+}$ uptake from the

cytoplasm into the SR is carried out by the SR $Ca^{2+}$-ATP-ase (SERCA) pump (MacLennan et al., 1985).

### *1.2.2. Excitation-transcription coupling*

The process whereby a given electrical impulse, causing plasma membrane depolarization, leads to specific gene activation or inactivation programs is called excitation-transcription coupling, in analogy with excitation-contraction coupling. The molecular mechanisms responsible for coupling nerve activity to transcriptional changes have not been fully defined. Motor neuron activity results in a marked elevation of intracellular $Ca^{2+}$ and this increase seems to be the primary regulator of altered gene expression in skeletal muscle (Chin et al., 2004). Type I fibers are stimulated by frequencies of 10-30 Hz, whereas type II fibers by 80-150 Hz (Hennig and Lomo, 1985). The frequency and the duration of the stimulus determine the amplitude and duration of the $Ca^{2+}$ transients, which ultimately regulates the force produced by the muscle. It is thought that the amplitude and duration of the $Ca^{2+}$ transient are decoded by muscle cells through $Ca^{2+}$-dependent molecular transducers (McCullagh et al., 2004; Serrano et al., 2001; see paragraph 1.3.2), triggering different gene expression programs depending on the specific $Ca^{2+}$ transient sensed by the fiber.

$Ca^{2+}$ acts as a second messenger in skeletal muscle and the downstream pathways, which convert this signal into changes in protein expression, include the $Ca^{2+}$-dependent phosphatase calcineurin (Cn) (Chin et al., 1998; McCullagh et al., 2004; Serrano et al., 2001), $Ca^{2+}$/calmodulin-dependent kinases (CaMK) (Chin, 2004; Ojuka, 2003) and $Ca^{2+}$-dependent protein kinase C (PKC) (Rose et al., 2004).

Our attention has focused on Cn, which has emerged as a prominent pathway in the excitation-transcription coupling process in the last few years (McCullagh et al., 2004; Serrano et al., 2001; see paragraph 1.3.2), and on CaMKII pathways, another important $Ca^{2+}$-dependent pathway in skeletal muscle (Rose et al., 2006). We have also studied their potential cooperation in the regulation of activity-dependent gene expression. Both pathways are $Ca^{2+}$- and calmodulin-dependent. Calmodulin (CaM) means CALcium MODULated proteIN, in fact this protein senses $Ca^{2+}$ intracellular level, and once activated by $Ca^{2+}$, it activates in turn a number of target enzymes, including calcineurin and CaMK.

### *1.3 Calcineurin (Cn)*

Cn is the only serine/threonine protein phosphatase that is $Ca^{2+}$ and CaM-dependent but this property makes it one of the most common intracellular transducers of $Ca^{2+}$ signalling pathways. Its name derives from its ability to bind $Ca^{2+}$ and the predominant localization in nervous tissue.

Cn is an heterodimer consisting of a 58-64 kDa catalytic subunit, called calcineurin A (CnA; Fig. 2), and a 19 kDa regulatory subunit, named calcineurin B (CnB). Mammalian CnA exists in three isoforms, CnAα, CnAβ and CnAγ, while there are two isoforms of the regulatory subunits, CnB1 and CnB2. CnAγ is expressed only in testis and brain, whereas CnAα and CnAβ are ubiquitous. CnB1 is expressed in the same tissues that express also CnAα and CnAβ, while CnB2 is expressed mainly in testis and also in brain (Kincaid, 1993).

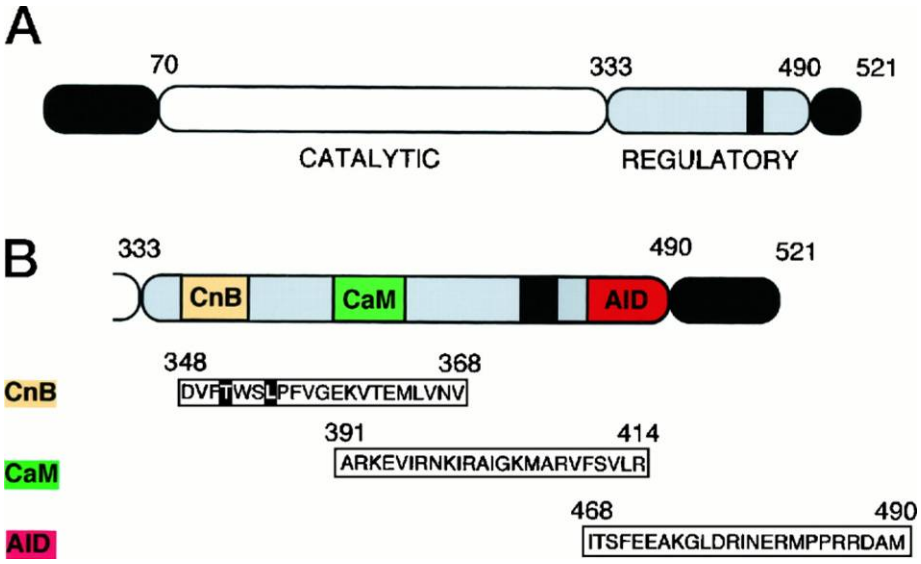


**Fig. 2. Functional domain organization of calcineurin A.** *A.* Schematic representation of the three mammalian isoforms of calcineurin A. The variable regions and 10-amino acid insert, resulting from alternative splicing, in the α and β isoforms of mammalian calcineurin A are shown in *black*. *B.* Extended representation of the regulatory domain; the amino acid sequences of the calcineurin B-binding helix, the calmodulin-binding domain, and the auto inhibitory peptide are *boxed*. The numbering of the amino acids is that of calcineurin Aα. Residues critical for interaction with cyclophilin and FKBP are represented by *white* on *black letters* (from Klee et al, 1998).

Cn was first purified in brain, being highly concentrated in neurons (Klee et al., 1979), but it is also broadly distributed in other tissues. Regardless of its source, CnA is always tightly bound to CnB (even in the presence of only nanomolar concentrations of

$Ca^{2+}$; Klee et al., 1988). This two-subunit structure is unique among phosphatases and it is conserved from yeast to man (Kincaid, 1993), being fundamental for the activity of Cn.

As mentioned before, Cn activity is controlled by $Ca^{2+}$ and CaM. CnB has a highly conserved structure which is similar to that of CaM, with two lobes, each composed of two adjacent $Ca^{2+}$-binding loops connected by a flexible helix linker (Anglister et al., 1994). With regard to sequence homology, CnB is similar to "EF-hand" $Ca^{2+}$-binding protein. CnB has its one high-affinity site occupied by $Ca^{2+}$, even if $Ca^{2+}$ is present at nanomolar concentrations ($10^{-7}$ M), but the enzyme is inactive. When $Ca^{2+}$ concentration rises between 0.5 to 1 μM, the three low-affinity sites of CnB are also bound to $Ca^{2+}$, and Cn is weakly activated. This activity can be enhanced of about 20-folds by putting a equimolar concentration of CaM (Klee et al., 1998); in fact, the higher the amount of CaM, the lower the $Ca^{2+}$ concentration needed to fully activate Cn. Conversely the higher the $Ca^{2+}$ concentration, the lower the CaM necessary for the activation of Cn. In other words, the same stimulus in terms of $Ca^{2+}$ concentration can produce different responses in dependence of the CaM concentration.

Besides containing a CnB- and a CaM-binding site, CnA regulatory domain has an autoinhibitory domain (AID; Fig. 2B), which inhibits the phosphatase activity (Hashimoto et al., 1990) if $Ca^{2+}$ is not bound to CnB and CaM.

One of the hypothesis about the activation mechanism of Cn, suggests that the binding between CaM and CnA causes conformational changes in CnA, so that a displacement of the AID of CnA from the catalytic site is produced (Klee et al., 1988). The mechanism through which CnB, binding to $Ca^{2+}$, activates Cn is still not clear. It is well established that CnB must be present to have the activation of the enzyme. Moreover, $Ca^{2+}$ must be bound to high-affinity sites of CnB, although the two subunits dissociate. $Ca^{2+}$ binding to low-affinity sites is apparently responsible not only for the CaM-independent Cn activity (low), but it is also involved in the CaM-dependent Cn activity (high).

The activity of Cn is controlled not only by $Ca^{2+}$ and CaM but also by several Cn inhibitors, which have been identified during the past few years. The function of Cn has remained unclear until it was identified as the target of the immunosuppressants cyclosporine A and FK506, inhibiting its catalytic activity (Kiani et al., 2000). Physiological Cn inhibitors have been identified, which control the duration and intensity of Cn activity. Among such endogenous inhibitory proteins are calcineurin-binding protein

1 (CABIN1, also known as CAIN), the A-kinase anchor protein AKAP79 (also known as AKAP5) and members of the Down's syndrome critical region (DSCR)/modulatory calcineurin-interacting protein (MCIP) family of Cn inhibitors, which are also known as calcipressins (Crabtree, 2001).

Cn is known to play an important role in the regulation of gene expression in mammalian cells (Klee et al., 1998, Wang et al., 1999; Sussman et al., 1998; De La Pompa et al., 1998; Graef et al., 2001). A well characterized example of Cn transcriptional control is its role in the maintenance and induction of the slow fiber program in skeletal muscles (Chin et al., 1998; Serrano et al., 2001; Meissner et al., 2006; Wu et al., 2000). McCullagh et al. have shown that the latter effect of Cn is mediated by the nuclear factor of activated T cells (NFAT; see paragraph 1.3.2), the main downstream substrate of Cn (see next paragraph). Cn-NFAT pathway and the mechanism by which Cn is activated have been extensively investigated in the immune system, in particular in lymphocytes. Indeed, NFAT has been initially identified as an inducible nuclear factor that could bind to the interleukin-2 promoter (IL-2) in activated T cells (Shaw et al., 1988). Briefly, upon binding of T cell receptor (TCR) ligand, the intracellular concentration of $Ca^{2+}$ increases, thus activating Cn, which in turn desphosphorylates NFAT and induces its translocation to the nucleus where it activates the transcription of genes coding for cytokines, chemokines and cell surface receptors that promote a productive immune response (Rao et al., 1997). The ability of NFAT to interact with different transcriptional partners determines the activation or deactivation of specific gene expression programs (Macian, 2005).

### *1.3.1 NFAT family*

NFAT is a multigene family gene composed of five distinct elements, NFATc1, NFATc2, NFATc3, NFATc4 and NFAT5. Even if the latter has extensive homology with the other NFATs, it is not activated by Cn (Lopez-Rodriguez et al., 1999) and will not be further considered in this thesis work. The length of all NFAT proteins is comprised between 700 and about 1000 amino acids, with different splice variants. Each of the Cn-activated NFAT proteins share a similar structure, roughly divided in three regions: a moderately conserved N-terminal region (also known as NFAT homology region, or NHR), which is the regulatory domain that controls NFAT cellular distribution and

transcriptional activation, a central region that mediates for DNA-binding (known as Rel homology region, or RHR), and a poorly conserved C-terminal domain (Fig. 3).

The NHR region contains many serine residues (distributed in the two serine rich domain and serine-proline-repeats motives) that are phosphorylated under basal conditions, and that become de-phosphorylated upon Cn activation. Cn binds to NFAT through a very conserved site, named PxIxIT (where x means any amino acids), within this region, the Cn binding peptide A, or CnBP-A (Fig. 3; Garcia-Cozar et al., 1998), and also to another conserved site, LxVP, known as CnBP-B (Fig. 3; Park et al., 2000).

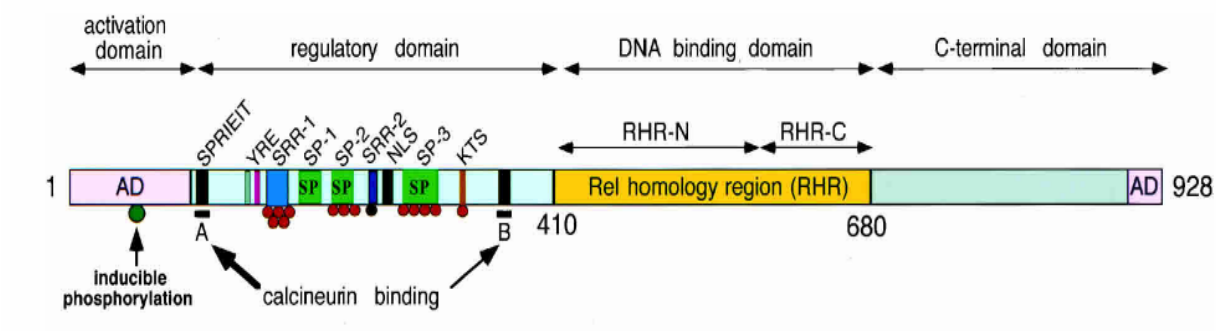


**Fig. 3. Schematic representation of NFAT (based on murine NFATc2).** Regulatory-domain phosphorylations in conserved sequence motifs are shown as circles *below* the motif. Red circles indicate phosphate groups that are removed by calcineurin, whereas the black circle in the SRR-2 region is not susceptible to dephosphorylation by calcineurin. The two regions involved in contacting calcineurin are indicated. (AD) Activation domains; (NLS) nuclear localization signal in the regulatory domain. A subsidiary NLS is located in the RHR-C domain (from Hogan et al. 2003).

It has been shown that the stronger the binding of Cn in CnBP-A is, the weaker the binding in CnBP-B is, and *vice versa* (Park et al., 2000). Interestingly, some endogenous Cn inhibitors bind to Cn using a signal similar to that of NFAT. In this work, we have used another inhibitor of the Cn-NFAT pathway, the peptide VIVIT (Aramburu et al., 1999). VIVIT represents the central part of an artificial 16-mer peptide (the complete sequence is MAGPHPVIVITGPHEE), which mimics the sequence that mediates the interaction between NFAT and Cn (PxIxIT, CnBP-A), and is common to all NFATs (Garcia-Cozar et al., 1998). This peptide was selected for its high affinity and strength of binding to Cn, and can prevent NFAT activation even stronger than CsA. In respect to the NFAT pathway, VIVIT is a competitive inhibitor of Cn. It has been demonstrated that the transcription of Cn-dependent but NFAT-independent genes is blocked by CsA but not by VIVIT, which affects only the expression of NFAT-dependent genes (Aramburu et al., 1999). VIVIT can then be used as a specific inhibitor of the Cn-NFAT pathway, allowing to distinguish

between Cn-dependent and NFAT-dependent or -independent pathways, also bypassing the problems related to the usage of CsA of FK506, which blocks the catalytic activity of Cn, resulting in many side-effects (Kiani et al., 2000).

All NFATs bind to the same sequence in the DNA, which is TGGAAA (Serfling et al., 2004). The RHR domain shares structural homology with REL proteins (such as NF-κB and cRel) and confers DNA-binding specificity that characterizes NFAT-family members (Aramburu et al., 1998).

NFAT family members share high similarity in their RHRs, including their DNA binding domains, which can implicate the existence of functional redundancy among NFAT proteins. In fact knockout mice for individual NFAT member have only a mild alteration in immune functions but, when more than NFAT isoforms has been eliminated many immune cells and functions are severely compromised (Hodge et al, 1996). Although the functional redundancy is undeniable, the knockout-mice data and relative expression level of diverse NFAT members, which, vary in different cell types, indicate that some NFAT functions rely on the balance of specific combinations of NFAT proteins and that their dependent gene expression is highly-tissue specific (e.g. NFATc3 is the most expressed NFAT isoform in thymocytes, while its level is low in peripheral T cells compared with that of NFATc1 or NFATc2). The issue is still open and our group's emerging data suggest that NFAT family members are likely differentially involved in the control of MyHC expression in skeletal muscles (see paragraph 1.3.2).

Moreover NFATs have the ability to interact with different co-activators through their less conserved N- and C-terminal domains, thus supporting the notion that certain T-cell functions are specifically regulated by different NFAT members, as indicated also by the data obtained with NFAT isoform-specific knockout-mice (Hodge et al., 1996; Horsley et al., 2001). The transcriptional activity of NFATs may be either activatory or inhibitory depending on their binding partners (Macian, 2005).

*1.3.2 Cn-NFAT in skeletal muscle*

It has been shown that Cn Aα is the predominant isoform of Cn expressed in skeletal muscle, even if CnAβ is also expressed (Parsons et al., 2003). CnAγ is not present in skeletal muscle and regarding CnB, only CnB1, but not CnB2, is expressed in skeletal muscle.

All NFAT isoforms are expressed in adult skeletal muscle (Hoey et al., 1995), both in slow (soleus) and fast (*extensor digitorum longus*) (Calabria et al., submitted). In addition our data indicate that there are no significant differences in the level of expression between the two muscles of each NFAT, with the exception of NFATc3, which is significantly more expressed in EDL muscles.

Growing evidence indicates that NFAT family members are implicated in several stages of the life cycle of a skeletal muscle fiber and it is likely that different NFAT isoforms are not redundant in regard to their function (Rao et al., 1997). The main evidence that the NFAT family members play important roles during muscle development *in vivo* has been obtained with phenotypic data from knockout mice (Horsley et al., 2001; Kegley et al., 2001) but further investigations are needed to attribute a specific role to each NFAT isoform.

Cn-NFAT pathway has been largely demonstrated to control fiber-type gene expression in skeletal muscle. The first evidence that Cn-NFAT is involved in fiber-type regulation has come from a study showing that a constitutively active isoform of Cn (c.a. CnA) was able to drive the expression of myoglobin (MB) and troponin I slow (TnIs) when transfected in cultured C2C12 myotubes. Moreover, transactivation of these genes required the integrity of NFAT-binding sites in their promoter regions *in vitro*, and the administration of CsA to adult rats induced a slow-to-fast fiber switching (Chin et al., 1998). C.a. CnA is able to induce also the expression of slow myosin heavy chain (MyHC-slow) in cultured C2C12 myotubes, and this activation is attenuated by either CsA administration or transfection of VIVIT (Torgan and Daniels, 2001), which is a NFAT competitor for the binding with Cn (see paragraph 1.3.1). The induction of fast genes is not mediated by NFATc1, which is able to activate only typical slow genes (Swoap et al., 2000, McCullagh et al., 2004).

It has also been shown that fibers isolated from *flexor digitorum brevis* (FDB), transfected with c.a. Cn and NFATc1 conjugated to the green fluorescent protein,

(NFATc1-GFP), undergo a fast-to-slow switch of gene expression, which is greater compared to fibers expressing NFATc1-GFP alone (Mu et al., 2007). Constitutively active NFAT or Cn are able to stimulate the activity of the MyHC-2A promoter stronger than that of MyHC-2X and -2B in C2C12 myotubes (Allen and Leinwand, 2002). The effect of c.a. NFAT is not as strong as the effect of c.a. CnA, indicating that Cn may mediate its effects also through effectors different from NFAT (Allen et al., 2001). MEF2 can be considered a good candidate: Cn can directly regulate MEF2 activity, and NFAT and MEF2 can physically interact thus promoting the transcription target genes (Chin et al., 1998; Youn et al., 2000; Wu et al., 2000).

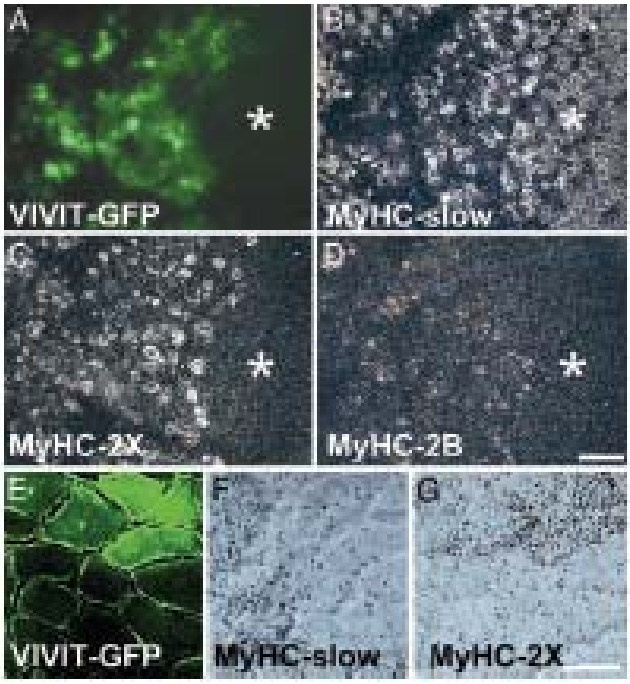


**Fig. 4. VIVIT down-regulates MyHC-slow and up-regulates MyHC-2X gene expression in adult soleus muscle.** (*A–D*) Serial transverse sections of adult soleus muscle transfected with VIVIT-GFP were examined for GFP fluorescence (*A*) or processed for *in situ* hybridization with probes specific for MyHC-slow (*B*), MyHC-2X (*C*), or MyHC-2B (*D*) transcripts. Note that MyHC-2X and -2B transcripts are absent in untransfected areas (asterisk) but are expressed in the VIVIT-GFP transfected area. In contrast, MyHC-slow transcripts are present in most fibers in untransfected areas but are less abundant in the transfected area. (Bar: 200μm.) (*E–G*) Same as *A–C*, shown at higher magnification. Note that fibers expressing VIVIT-GFP (*E*, upper right) contain MyHC-2X (*G*) but not MyHC-slow (*F*) transcripts (from McCullagh et al., 2004).

*In vivo* studies confirmed that the Cn-NFAT pathway plays an important role in skeletal muscle, and in particular in fiber-type specification. In our laboratory, we have used both a genetic (Cain, VIVIT) and a pharmacologic (CsA, FK506) approach to inhibit Cn activity *in vivo*. We showed that MyHC-slow and -2A transcripts are down-regulated,

while fast MyHC-2B and 2X are up-regulated, when Cn is inhibited with Cain. This indicates that Cn controls not only the induction, but also the maintenance of the slow gene program induced by motor neuron activity (Serrano et al., 2001). Moreover, c.a. NFATc1 over-expression in regenerating denervated *extensor digitorum longus* (EDL) muscles is able to induce the expression of MyHC-slow (which normally does not occur), while the transfection of VIVIT in a regenerating innervated soleus prevents the induction of MyHC-slow. Moreover, transfection of VIVIT in a normal soleus down-regulates MyHC-slow transcripts (Fig. 4), while it up-regulates the transcription of MyHC-2X and -2B (McCullagh et al., 2004).

Another recent paper has showed that NFATc1 is predominantly nuclear in slow twitch soleus muscle, while it is predominantly cytoplasmic in the fast *tibialis anterior* (TA) muscle. Denervation or anaesthesia promote nuclear export of NFATc1 also in the soleus, and *in vivo* electrical stimulation with a 20 Hz pattern (typical of slow motor neurons), but not with a 100 Hz fast pattern, induces NFATc1 nuclear translocation. NFATc1 goes into the nucleus in a Cn-dependent manner (Fig. 5), since the co-transfection of cain abolishes NFATc1 translocation (Tothova et al., 2006). This work corroborates previous data obtained *in vitro*, which have demonstrated that the treatment with the $Ca^{2+}$ ionophore A23187 causes a translocation of NFATc1 into the nuclei of cultured myotubes, detected by immunofluorescence. When CsA was added to A23187, the fluorescence signal in the nuclei disappears, while the cytoplasm is positively stained (Meissner et al., 2001). Furthermore our unpublished *in vivo* data show that NFATc4 remains nuclear within fibers of denervated EDL electrostimulated with a tonic 20 Hz pattern. NFATc4 has a completely different distribution: it is nuclear both in soleus and in EDL muscles. How NFATc4 maintains its nuclear localization even in conditions of Cn inactivation (i.e. denervation) remains to be elucidated (Calabria et al., submitted).

Although the involvement of NFAT in mediating the induction and the maintenance of slow muscle phenotype, and also the repression of fast gene expression in slow muscles (McCullagh et al., 2004) have been demonstrated, the role of each of the four Cn-regulated NFATs (NFATc1-4), has not been studied in detail. Our group has analysed the role of NFAT family members in skeletal muscle *in vivo* using a loss-of-function approach by RNA interference. Results obtained by our group show that, depending on the applied activity pattern (slow-like, 20 Hz or fast-like, 100 Hz), different combinations of

NFAT family members translocate to the nucleus contributing to the transcription of fiber type-specific genes. The transcription of slow and fast myosin heavy chain (MyHC) genes uses different combinations of NFAT family members, ranging from MyHC-slow which uses all four NFAT isoforms to MyHC-2B which only uses NFATc4 (Calabria et al., submitted). Collectively, these results indicate that NFAT factors are not redundant in regard to their functions in skeletal muscle, and that they are likely differentially involved in the regulation of type II myosins. How different NFATs can be responsible for the activation of different MyHC genes it is still not clear. One possibility is that different NFATs have different partners that participate to DNA binding (Macian et al., 2005).

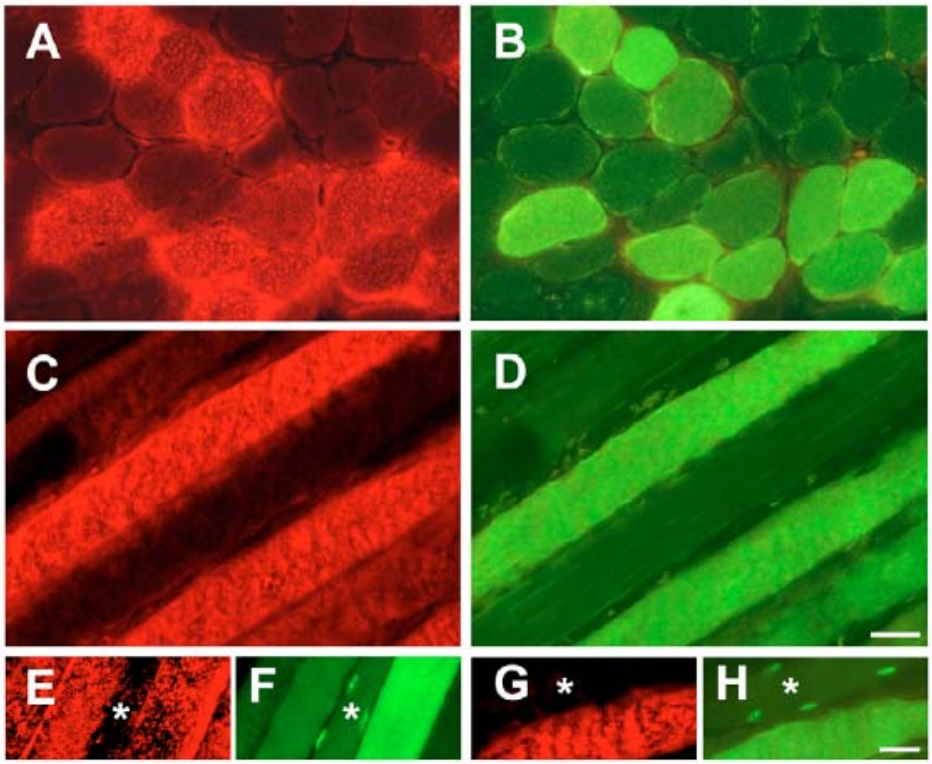


**Fig. 5. The calcineurin inhibitor cain blocks the nuclear translocation of NFATc1-GFP.** (A,B) Soleus muscles co-transfected with plasmids coding for NFATc1-GFP and myc-tagged cain. (C,D) TA muscles co-transfected with plasmids coding for NFATc1-GFP and myc-tagged cain and electrostimulated for 2 hours with a 20 Hz impulse pattern. Serial sections were either stained with anti-myc (A,C,E,G) or examined for GFP fluorescence (B,D,F,H). (E-H) Occasional fibers in electrostimulated TA muscles that do not express cain (asterisks) maintain a nuclear localization of NFATc1, whereas neighboring fibers that do express cain show a cytoplasmic localization of NFATc1-GFP. Bar, 30 μm (from Tothova et al., 2006).

### *1.4 CaMK pathway*

CaMKs are a family of serine/threonine kinases which can be divided into two general categories on the basis of their substrate specificity: dedicated kinases have very restricted substrate specificity and multifunctional kinases phosphorylate diverse targets (Soderling and Stull, 2001). CaMK kinase (CaMKK), CaMKI, CaMKIV and CaMKII belong to the class of multifunctional kinases, while CaMKIII (or eEF-2K) and Myosin light chain kinase (MLCK) are dedicated kinase, having only one target substrate. All CaMKs share some common features: they have a similar domain organization, except CaMKIII which belongs to a unique family of Ser/Thr protein kinases; they are autoinhibited in the absence of $Ca^{2+}$/CaM; finally CaMKs are further regulated by phosphorylation (Soderling and Stull, 2001).

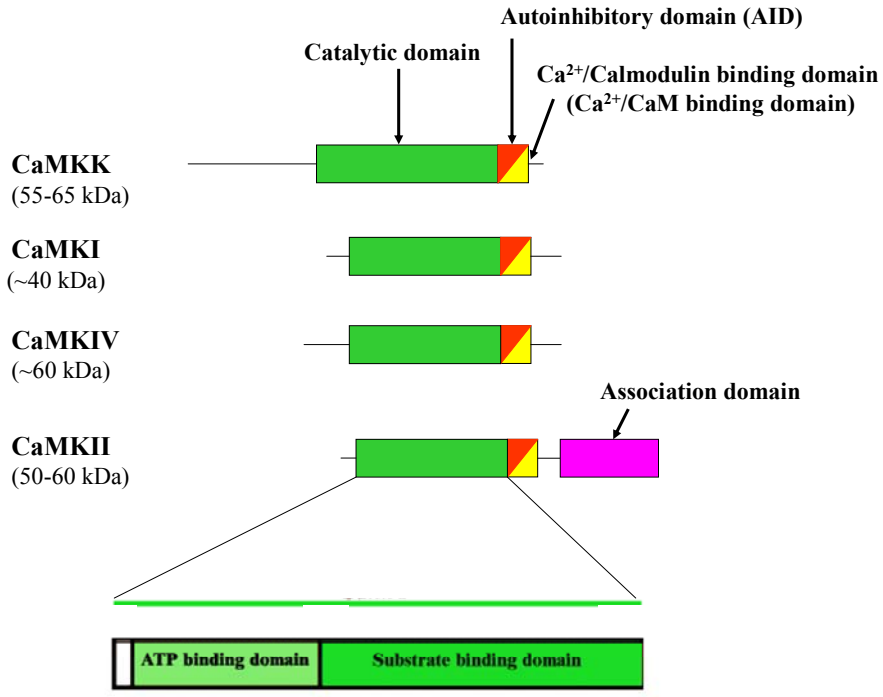


**Fig. 6. Domain organization for multifunctional members of the $Ca^{2+}$/calmodulin (CaM)-dependent protein kinases.** The domains are shown as as follows: catalytic domain, dark green; autoinhibitory domain (AID), orange; $Ca^{2+}$/Calmodulin binding domain ($Ca^{2+}$/CaM binding domain), yellow; the catalytic binding domain comprises the ATP and the substrate binding domain and this domain organization is common for all the multifunctional kinases, not only for CaMKII. The latter kinase contains a supplementary domain, the association domain which is indicated in magenta. The bars represent the non conserved regions. Splicing variants for each kinase isoform are not shown. The molecular weights of the mammalian multifunctional kinases are reported in brackets.

CaMKK is the upstream kinase which activates CaMKI and CaMKIV, which has been first identified in brain extracts as an "activating factor", upon incubation with $Ca^{2+}$/CaM plus $Mg^{2+}$/ATP, because it has dramatically increased their activity (Lee and Edelman, 1994). CaMKKs are monomeric, exist in two isoforms, named α and β, which are more abundant in testis, spleen and brain and display a cytoplasmic and nuclear cellular localization respectively (Anderson et al., 1998; Tokumitsu and Soderling, 1995). They have a general domain organization similar to other multifunctional CaMKs (Fig. 6). In basal condition (unbound $Ca^{2+}$/CaM) the autoinhibitory (AID) interacts with and inhibits the catalytic domain (Kobe et al., 1997). Once $Ca^{2+}$/CaM complex binds to the Calcium/calmodulin binding domain ($Ca^{2+}$/CaM binding domain), which partially overlaps the AID (Tokumitsu et al., 1997; Fig. 6), probably it causes a conformational change (Soderling and Stull, 2001). This conformational change disrupts the interaction of the AID with the catalytic domain, thus activating the kinase. Being a feature of CaMKKs but also of CaMKs, it is necessary that $Ca^{2+}$/CaM complex binds to both CaMKK and its downstream substrate CaMK (Tokumitsu and Soderling, 1995).

### *1.4.1 CaMKII*

#### 1.4.1.1 CaMKII structure

CaMKII is the main multifunctional CaMK, encoded by four genes (α, β, γ, and δ) whose mRNAs are processed into a variety of alternative splice variants (Braun and Schulman, 1995). The major differences between CaMKII splice variants derive from variable inserts, C-terminal to $Ca^{2+}$/CaM binding domain and within the association domain which resides in the so called variable region (V in Fig. 7A; see also Fig. 10). The translated protein products are generally 50-60 kDa in size and have a domain organization similar to other CaMKs, that is a N-terminal catalytic domain, a regulatory domain which comprises the AID and the $Ca^{2+}$/CaM binding domain (Fig. 7A), but it differs for its C-terminal extension that is involved in CaMKII subunit association (Fig. 7A; Soderling, 1996). CaMKII isoforms have conserved phosphorylation sites in the regulatory region, which are critical for the regulation of its activity (Fig. 10). CaMKII mechanism of activation will be discussed later.

CaMKII is a holoenzyme of 12 subunits arranged as two stacked hexameric rings, whose the central core of each ring is formed by the C-terminal association domain with

N-terminal catalytic domain projecting outward (Kolodziej et al., 2000; Fig. 7B). All CaMKII isoforms studied to date can form homo- or hetero-multimers *via* the C-terminal association domain, with a subunit composition dictated in a stochastic manner by relative isoform expression levels (Colbran, 2004).

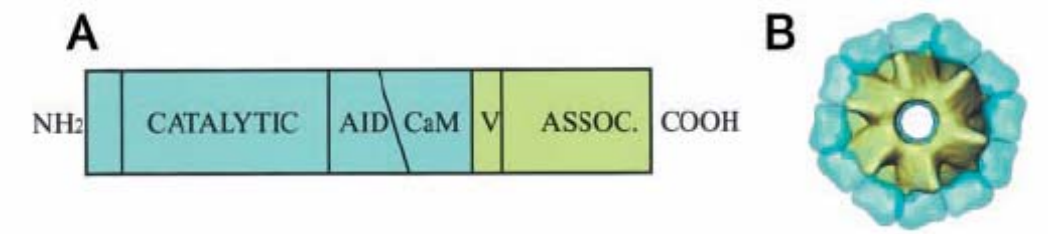


**Fig. 7. Structure of CaM-KII.** A. CaMKII subunit domain organization. All of the isoforms and alternative splice variants of CaMKII have a similar overall subunit organization with an N-terminal catalytic domain, a central regulatory region with overlapping autoinhibitory (*AID*) and CaM-binding (*CaM*) domains, a variable region (*V*) with inserts or deletions, and a C-terminal subunit association region. The color coding corresponds with the domain organization of the holoenzyme in *B*. B holoenzyme structure. CaMKII a holoenzyme consists of a gear-shaped body; 140 Å in diameter with a height of 100 Å and contains 12 subunits arranged in two sets of six subunits that form stacked hexagonally shaped rings. The central structure is comprised of the association domains (*green*), and the foot-like protrusions (*aqua blue*) contain the catalytic and regulatory domains. This top view of reconstructed CaMKII, based on three-dimensional electron microscopy, is reproduced with permission from Kolodziej *et al.* (from Soderling et al., 2001).

1.4.1.2 CaMKII in brain

CaMKII is ubiquitously expressed but it is more abundant in excitable tissues, such as skeletal muscle, brain and heart (Bayer et al., 1999; Rose and Hargreaves, 2003; Tobimatsu and Fujisawa, 1989). The γ and δ isoforms are expressed in most tissues, whereas α and β isoforms are most prominent in neural tissues and they comprise up to 2% of total protein content in hippocampus. Being a very abundant enzyme, its cellular localization is critical in order to achieve specificity in action and increases CaMKII efficiency by elevating its local concentration. The subcellular localization of CaMKII permits to match its localization with that of the $Ca^{2+}$ signal to which CaMKII is intended to respond and to co-localize with the correct substrate in order to provide the appropriate cellular response, as it has been found in brain (Soderling et al., 2001). Brain is particularly abundant in CaMKII, where it has an important role in synaptic plasticity, which is the capability of neurons to modulate the efficiency of synaptic transmission. It has been demonstrated that CaMKII seems to be involved in the induction of hippocampal long term potentiation (LTP), a form of synaptic plasticity, but this issue will not be treated (for review Fox, 2003).

1.4.1.3 CaMKII as a frequency decoder

Among CaMKs, CaMKII has a unique structure which endows it with the capacity to decode the frequency of synaptic stimulation and to yield a prolonged activation beyond the initial stimulus (Soderling et al., 2001), two features fundamental for the generation of synaptic plasticity (Yang et al., 1999). CaMKII is organized into two stacked hexameric rings and such a structural organization facilitates decoding of $Ca^{2+}$ spikes into distinct amounts of CaMKII activity. Similarly to CaMKI and CaMKIV, has an AID in its regulatory region (Fig. 7A), which inhibits kinase activity in the absence of $Ca^{2+}$/CaM binding, being a pseudosubstrate interacting with ATP- and substrate-binding pocket. In particular, AID residues 297-300 occupy the substrate binding site (S-site), whereas a more N-terminal portion, residues 282-294, make additional interactions with a hydrophobic pocket on the C-terminal lobe of the catalytic domain (T-site). Interactions with the T-site are believed to induce secondary conformational changes that interfere with ATP-binding to the kinase (for review see Colbran, 2004). $Ca^{2+}$/CaM binding produces maximal CaMKII activity, unlike CaMKI and CaMKIV which need to be phosphorylated in their activation loop for maximal activity. Upon activation by $Ca^{2+}$/CaM binding, the kinase undergoes an immediate autosphorylation on Thr286 (numbering is and will referred to CaMKIIα isoform). In fact $Ca^{2+}$/CaM binding produces a conformational change which exposes Thr286 (Colbran et al, 1988), a conserved phosphorylation site which is present in all CaMKII isoforms but αKAP. The phosphorylation of this residue decreases the inhibitory potency of AID (Colbran et al., 1989). This autophosphorylation event occurs in an intramolecular contex but also intermolecular, because it takes place between adjacent subunits that have bound $Ca^{2+}$/CaM complex (Hanson et al., 1999). The consequences of CaMKII autophosphorylation are the following: first, the subsequent dissociation rate for $Ca^{2+}$/CaM, upon removal of $Ca^{2+}$, is decreased by several orders of magnitude with a correspondent 1000 fold increase in $Ca^{2+}$/CaM affinity, a process called “CaM trapping” (Meyer et al., 1992). It seems that autophosphorylation on Thr286 induces a conformational change in CaMKII structure, thus exposing residues in the $Ca^{2+}$/CaM binding domain which stabilize the binding $Ca^{2+}$/CaM complex, ultimately producing a high-affinity binding (Putkey and Waxham, 1996). Second, even when $Ca^{2+}$/CaM complex is fully dissociated, the kinase retains partial activity ($Ca^{2+}$/CaM-indipendent binding activity or constitutive activity or autonomous activity). Presumably the complex structure

of this holoenzyme endows it these regulatory properties (Soderling and Stull, 2001) and the ability of CaMKII to be a frequency decoder (Fig. 8).

It has been demonstrated that autophosphorylation follows a frequency-response function: different amounts of autonomous activity result from various frequency of $Ca^{2+}$ pulses (De Koninck and Schulman, 1998; Fig. 9).

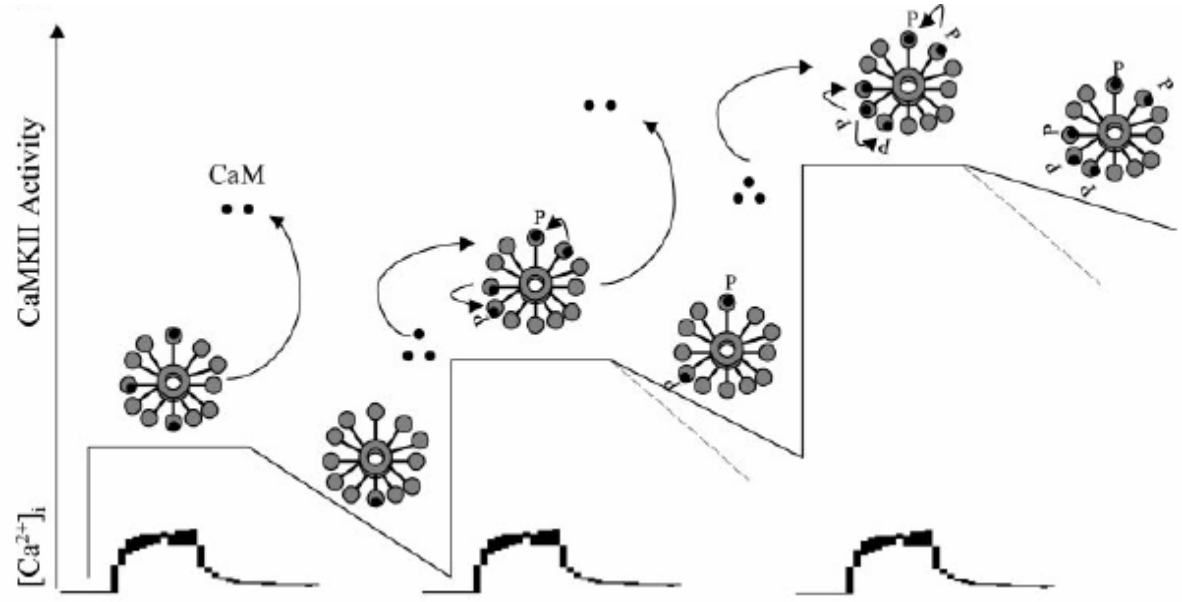


**Fig. 8. $Ca^{2+}$ frequency decoding mechanism by CaMKII autophosphorylation.** This figure illustrates under conditions where CaM is limiting relative to CaMKII (depicted for simplicity as a decamer) three $Ca^{2+}$ spikes at a frequency and amplitude such that not all $Ca^{2+}$/CaM has dissociated from CaMKII after the first spike. The magnitude and duration of each spike allows three subunits of CaMKII to bind $Ca^{2+}$/CaM. During the first spike, by chance, no adjacent subunits bind CaM; therefore, no Thr286 autophosphorylation occurs, and at the end of the spike the $Ca^{2+}$/CaM rapidly dissociates. The second spike occurs at a frequency when one nonphosphorylated subunit still has bound $Ca^{2+}$/CaM, increasing the probability that a proximal subunit would bind one of the three additional $Ca^{2+}$/CaM and undergo intersubunit autophosphorylation (P). Between the second and third $Ca^{2+}$ spikes, $Ca^{2+}$/CaM dissociates more slowly from autophosphorylated subunits than from nonphosphorylated subunit. During the third $Ca^{2+}$ spike additional adjacent subunits autophosphorylate. The constitutive activity of the CaMKII holoenzyme is the summation of the autophosphorylated subunits, which in turn depends on the magnitude, frequency, and duration of the $Ca^{2+}$ spikes (from Chin, 2005; Soderling et al., 2001).

The frequency-response function is also modulated by the amplitude and duration of $Ca^{2+}$ spikes: with higher amplitudes and prolonged duration of $Ca^{2+}$ pulses, a larger number of subunits become phosphorylated through an intramolecular and intermolecular phosphorylation reaction within the holoenzyme. In addition diverse isoforms of CaMKII seem to differently sense $Ca^{2+}$ pulse frequencies (Bayer et al., 2002). As result CaMKII can decode the frequency, duration and amplitude of $Ca^{2+}$ oscillations into diverse levels of kinase activity and provide a sort of cellular memory of the previous level of activation,

thus giving a prolonged readout beyond the initial stimulus. In fact when the activating stimulus is terminated and $Ca^{2+}$ intracellular concentration decreases to basal levels, an increase in kinase activity persists thanks to autophosphorylation (Fig. 8). These CaMKII activity properties are essential for a molecule which is involved in synaptic plasticity, such as LTP (see paragraph 1.4.1.2).

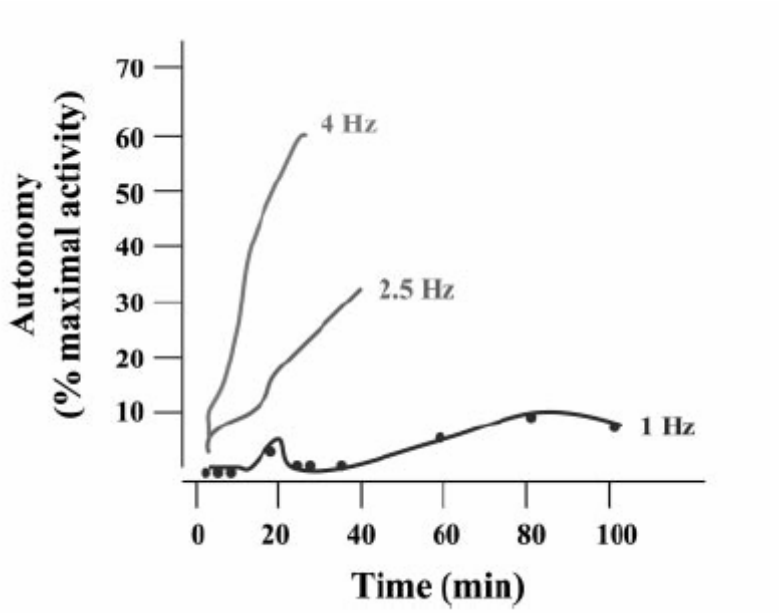


**Fig. 9. Frequency dependence of CaMKII autophosphorylation induced by pulse stimulation with $Ca^{2+}$ and calmodulin.** Four to 100 stimulus pulses (200ms) were delivered at 1, 2.5, or 4 Hz by flowing perfusion solution containing either $Ca^{2+}$ (500 mM), calmodulin (100 nM), and ATP (250 mM) or EGTA (500 mM). CaMKII can decode frequency oscillations of $Ca^{2+}$ into discrete levels of kinase activity: increases in frequency $Ca^{2+}$ pulses result in enhancements of autonomous kinase activity (from Chin, 2005).

Two requisites are needed for intersubunit phosphorylation on Thr 286: $Ca^{2+}$/CaM binding on the "kinase" subunit and as well as the subunit acting as substrate (Rich and Schulman, 1998). Following autophosphorylation on Thr286, CaMKII undergoes further autophosphorylation which inhibits subsequent reactivation of the enzyme by $Ca^{2+}$/CaM binding, a process termed "CaM capping" (Hanson and Schulman, 1992). The sites involved in this process are Thr305 and Thr306 in the $Ca^{2+}$/CaM binding domain (Fig. 10). Autophosphorylation gives CaMKII the unique capacity of modifying its own affinity for CaM during periods in which $Ca^{2+}$ levels are low (Hanson and Schulman, 1992).

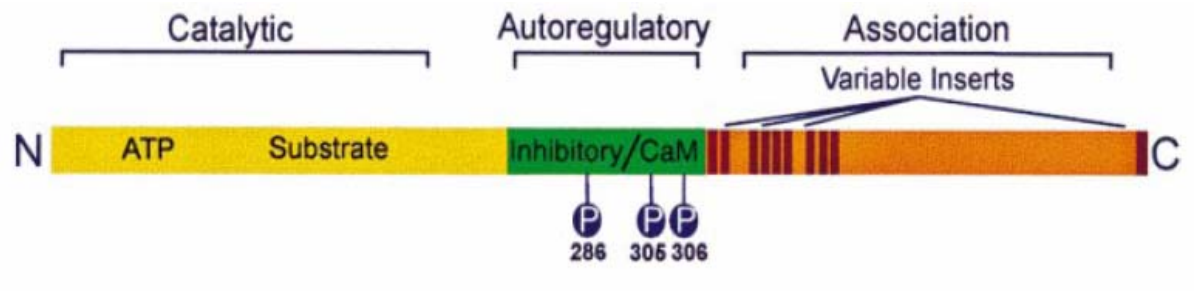


**Fig. 10. Linear diagram of CaMKII organization domains, showing its conserved phosphorylation sites.** The catalytic domain (yellow) binds ATP and CaMKII substrates. Isoform differences of CaMKII are contributed primarily a region of multiple alternatively spliced sequences, the variable inserts (red), which reside in the association domain represented in orange. Conserved sites of autophosphorylation are indicated in the autoregulatory region (green): Thr286 resides in the inhibitory domain, while Thr 305 and Thr 306 are located in the $Ca^{2+}$/CaM binding domain (from Hudmon and Schulman, 2002).

It is likely that the physiological role of autophosphorylation on Thr in the $Ca^{2+}$/CaM binding domain is to mark active/autophosphorylated subunits from those ones which are inactive, in order to avoid competition for CaM binding. CaMKII might be unique in enhancing CaM binding on the autophosphorylated subunits. Thus the "population" of active subunits is excluded from competing with, not only CaMKII inactive subunits, but also the other specific subcellular CaM targets.

It has also emerged that phosphorylation on Thr286 and Thr305/6 has an important role in determining CaMKII association with the postsynaptic density (PSD) (Soderling et al., 2001), and this association itself is critical for the level of CaMKII autophosphorylation (Davies et al., 2007). CaMKII association is reversed by the opposing effect of Ser/Thr protein phosphatases (PPs) on CaMKII autophosphorylation (Griffith et al., 2003). Targeting of CaMKII to specific subcellular compartments may also modulate its avaibility to different PPs (for review see Colbran, 2004).

1.4.1.4 CaMKII in skeletal muscle

CaMKII activity is detectable in skeletal muscle and immunoblotting has revealed the presence of the γ and δ isozymes and a splice variant of the β isozyme designated $\beta_M$ (Rose and Hargreaves, 2003). A variant of the α isozyme, named αKAP, has also been identified in skeletal muscle, which lacks catalytic and regulatory domains, containing an hydrophobic N-terminus fused to the C-terminal association domain. The latter domain can form heteromer with all CaMKII isoforms. αKAP is highly expressed in skeletal muscle where the αKAP/CaMKII complex is anchored to the SR membrane through αKAP N-

terminal domain. Binding to αKAP, CaMKII is tethered to in close proximity to its skeletal muscle substrate, including phospholamban (PLB), RyR and SR $Ca^{2+}$-ATPase (SERCA) pump. Phosphorylation of PLB is believed to relieve its physical inhibitory interaction with SERCA (for review see Simmerman and Jones, 1988). PLB has been found to be phosphorylated in human skeletal muscle during contraction. Thus it may be a mechanism to accelerate $Ca^{2+}$-uptake rates during exercise to maintain normal homeostasis (Rose et al., 2006).

It has been reported that another downstream substrate of CaMKII is RyR but the physiological effects of CaMKII phosphorylation remain controversial (Hain et al., 1995; Wang and Best, 1992; Witcher et al., 1991).

In rat muscle, a further CaMKII substrate has been recently found to be phosphorylated by this enzyme, namely trisk95 (Rose et al., 2007*a*), an integral membrane protein within the junctional SR in rat skeletal muscle which interacts with RyR. It is likely that it inhibits excitation-induced $Ca^{2+}$ release probably by decreasing RyR open probability (Rose et al., 2007*a*).

#### 1.4.1.5 CaMKII regulation of gene transcription

CaMKII has also been described to regulate nuclear gene transcription. The molecular weight of CaMKII prevents its passive diffusion into the nucleus, nonetheless, in early studies, CaMKII activity was observed in neuronal nuclei (Sahyoun et al., 1984). More recent molecular cloning studies have detected nuclear localization signal in the variable region of some α, γ and δ alternatively spliced variants ($\alpha_B$, $\delta_B$ and $\gamma_A$; Brocke et al., 1995; Srinivasan et al., 1994). Nuclear entry of these CaMKII is regulated by several mechanisms (Fig. 11).

CaMKII can form homo- and hetero-multimers and in this last case the ability of CaMKII to enter the nucleus is dictated by the ratio of nuclear (containing the NLS)/cytoplasmic (lacking of NLS) subunits within the holoenzyme (Srinivasan et al., 1994). In mammals, nuclear CaMKII isoforms have their NLS followed by a string of 4 Ser residues. Phosphorylation of the first Ser by CaMKI or CaMKIV blocks nuclear translocation of $\alpha_B$ (Heist et al., 1998), thus constituting another way for regulating CaMKII nuclear entry.

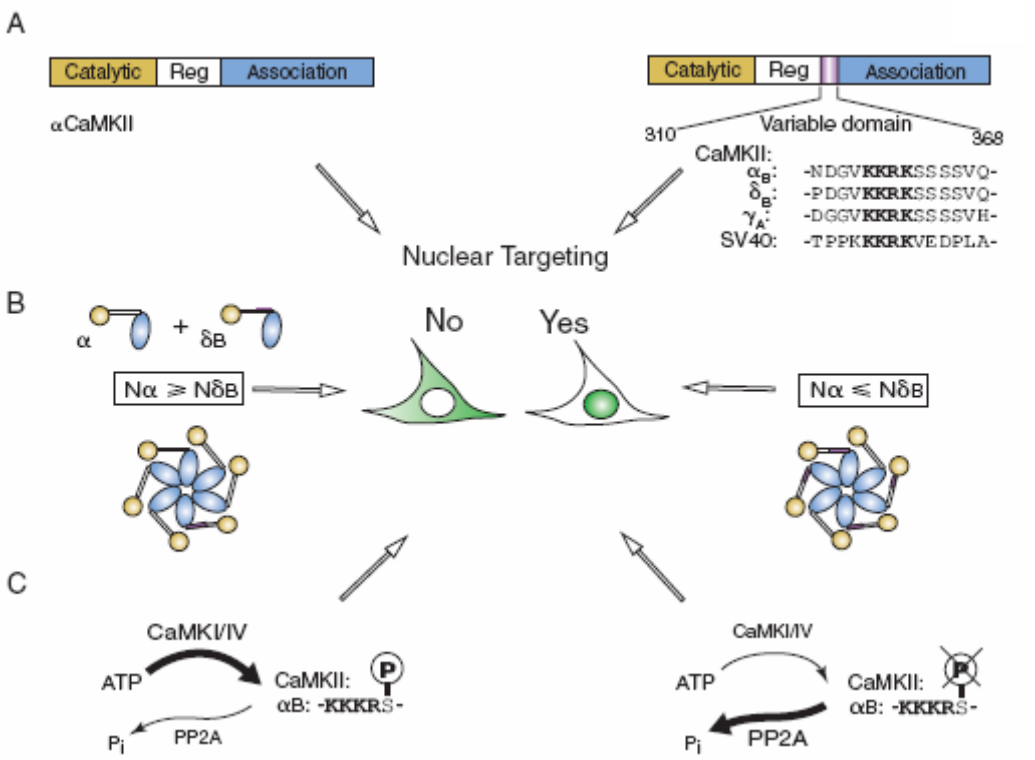


**Fig. 11. Mechanism for nuclear targeting of CaMKII.** A. CaMKII isoforms that contain a nuclear localization sequence (NLS) target nuclear entry. The prototype NLS of SV40 T antigen is included for sequence comparison. B. The ratio of cytosolic isoform vs. nuclear isoform determines the subcellular localization of $\delta_B$CaMKII. C. Phosphorylation close to the NLS by CaMKI or CaMKIV prevents nuclear entry of $\alpha_B$CaMKII, whereas an increased level of PP2A was associated with nuclear targeting of $\delta_B$CaMKII (from Griffith et al., 2003).

Even in the absence of the NLS, CaMKII has been shown to enter into the nucleus. In this case its translocation could be regulated posttranslationally by sumoylation (Long and Griffith, 2000). In addition autophosphorylation on Thr286 in the regulatory domain of CaMKII may also influence its nuclear localization (Bayer and Schulman, 2001). Recent evidence supports the idea that nuclear localization of CaMKII is affected by its activation state: it has been reported that an activated mutated $\delta_B$CaMKII (containing NLS), with a Thr287Asp (Thr287 corresponds to Thr286 in the $\delta_B$ isoform) mutation which mimics autosphorylation of the kinase, has been predominantly localized in the cytosol, while wt (inactive) $\delta_B$CaMKII has been mainly detected in the nucleus (Backs et al., 2006). The mutated form of the kinase co-localizes with a hystone deacetylase of class II, HDAC4, in COS cells. The latter has a unique docking site for CaMKII which is accessible to the kinase only when is $Ca^{2+}$/CaM activated, suggesting that autosphorylation may induce a conformational change in CaMKII which enables it to bind to HDAC4 (Backs et al., 2006).

Previous *ex vivo* experiments in single fibers isolated from FDB indicate that a stimulation pattern, which resembles the firing pattern of slow motor neurons, but not that of fast motor neurons, causes a marked translocation of HDAC4 to the cytoplasm (Liu et al., 2005). Thus, collectively these data indicate that only slow fiber stimulation activates CaMKII in skeletal muscles by inducing CaMKII interaction with HDAC4 and the subsequent exit of the complex from the nucleus. As HDAC4 is implicated in gene expression regulation, these data could also suggest that CaMKII has a fundamental role in controlling gene expression in slow muscle fiber through HDAC4.

The first evidence that CaMKII can regulate gene transcription in skeletal muscle was found analysing serum response factor (SRF; Fluck et al., 2000*b*), which is a transcription factor involved in skeletal muscle hypertrophy (Croissant et al., 1996). Another work has shown that CaMKII is enriched in skeletal muscle nuclei and that nuclear CaMKII is activated with contraction (Fluck et al., 2000*a*). In the light of these data, CaMKII phosphorylation activity toward SRF was investigated during muscle contraction. The results indicate that the SRF phosphorylation, but not its expression, was higher in trained muscles respect to resting muscles (Rose et al., 2007*b*).

Another CaMKII substrate is the cAMP response element binding protein (CREB), which is a transcription factor that can be activated by the phosphorylation on its Ser133 by protein kinase A (PKA; Gonzalez and Montminy, 1989), CaMKI and CaMKIV (Sheng et al., 1991). This phosphorylation allows CREB to recruit its coactivator, CREB binding protein (CBP), which is critical to induce CREB-mediated gene transcription. CaMKII can also phosphorylate CREB on Ser133, although without inducing CREB-mediated transcription, but inhibiting it (Sun et al., 1994). Phosphopeptide mapping indicates that CaMKII phosphorylates another site in CREB, Ser142, besides Ser133. Nuclear magnetic resonance studies have demonstrated that, when Ser142 is phosphorylated by CaMKII, the affinity of CREB to CBP markedly decreases (Parker et al., 1998). CREB dimerization, which acts as a trascriptional switch, is lost when Ser142 is phosphorylated. This loss in CREB dimerization prevents CBP association to CREB, thus giving a possible mechanism to explain the inhibition of CREB transcriptional activity by CaMKII (Wu and McMurray). Moreover the inhibiting effect of Ser142 phosphorylation is dominant on Ser133 activating phosphorylation on CREB (Sun et al., 1994).

### *1.5 MEF2 transcriptional regulation*

Another important CaMKII target, involved in transcriptional gene regulation, is the myocyte enhancer factor 2 (MEF2). MEF2 family of transcription factors plays critical roles in skeletal muscle differentiation and fiber-type programming, myogenesis, cardiac development and hypertrophy, vascular development, smooth muscle proliferation (Potthoff and Olson, 2002). In mammals MEF2 exists in four isoforms: A, B, C and D but only A, C and D isoforms are highly expressed in mature skeletal muscle (McKinsey et al., 2002). The four MEF2 proteins share homology in an N-terminal MCM1 agamous deficiens serum response factor (MADS) domain which mediates DNA binding, dimerization and cofactor interaction, while a transcriptional activated domain (TAD) resides at the C-terminus (Black and Olson, 1998).

The transcriptional activity of MEF is regulated by a complex balance between co-repression, co-activation and phosphorylation. One mechanism which controls gene expression is histone acetylation/deacetylation. Histone acetyl transferases (HATs) catalyze the acetylation on Lys residues of nucleosome core histones, which relaxes chromatin structure, favouring gene transcription (Roth et al., 2001). Conversely, in the deacetylated state, positively charged histone tails interact with negatively charged DNA backbone (Grozinger and Schreiber, 2002). These interactions cause chromatin condensation, resulting in inhibition of transcription. Mammalian HDACs can be categorized into three different classes according to their sequence homology, size and formation of different complexes. Class IIa HDACs (HDACIIa), comprising HDAC-4, -5, -7, -9, are most abundantly expressed in heart, brain and skeletal muscle (Verdin et al., 2003), the same tissues as MEF2 is expressed at highest levels (Black and Olson, 1998). HDACIIa shares a common structure, with a C-terminal catalytic domain and an N-terminal regulatory domain. The latter mediates interactions with transcription factors, co-activators and co-repressors (Verdin et al., 2003). The N-terminal regions of HDACIIa also control their subcellular localization and confer signal responsiveness to downstream target genes (Gronziger and Schreiber, 2002; McKinsey et al., 2000*a*). This is due to a set of conserved Ser residues which, if phosphorylated, create binding sites for the chaperone protein 14-3-3. This interaction exposes a nuclear export signal on HDACIIa, determining the exit of HDACIIa from the nucleus to the cytoplasm through the CRM1-dependent nuclear export system. HDACIIa contains a unique amino-terminal extension that mediates

association with MEF2 factors in the basal state (Lu et al., 2000; Sparrow et al., 1999), but, once phosphorylated, HDACIIa moves out from the nucleus releasing its inhibition on MEF2 and consequently on MEF2-dependent transcription. Phosphorylation of HDACIIa serves to convert extracellular stimuli into changes in gene expression of HDACIIa target genes. In fact a characteristic feature of these HDACs is their signal-dependent regulation of their nucleocytoplasmic trafficking, by phosphorylation of the conserved Ser residues in the N-terminal extensions to promote association with 14-3-3 protein and finally nuclear export. Protein kinase D (PKD; Kim et al., 2008), protein kinase C (PKC, Vega et al., 2004), AMP-activated protein kinase (AMPK; McGee et al., 2008), salt-induced kinase 1(SIK1; Berdeaux et al., 2007) and various CaMKs (Backs et al., 2006; Davis et al., 2003; Liu et al., 2005; McKinsey et al., 2000*a*,*b*) have been reported to transduce signals from extracellular stimuli, into post-translational modification, by phosphorylation of HDACIIa. All these HDACIIa kinases are expressed and activated by contraction in skeletal muscle and supposed to control HDAC shuttling according to the scheme represented in Fig. 12.

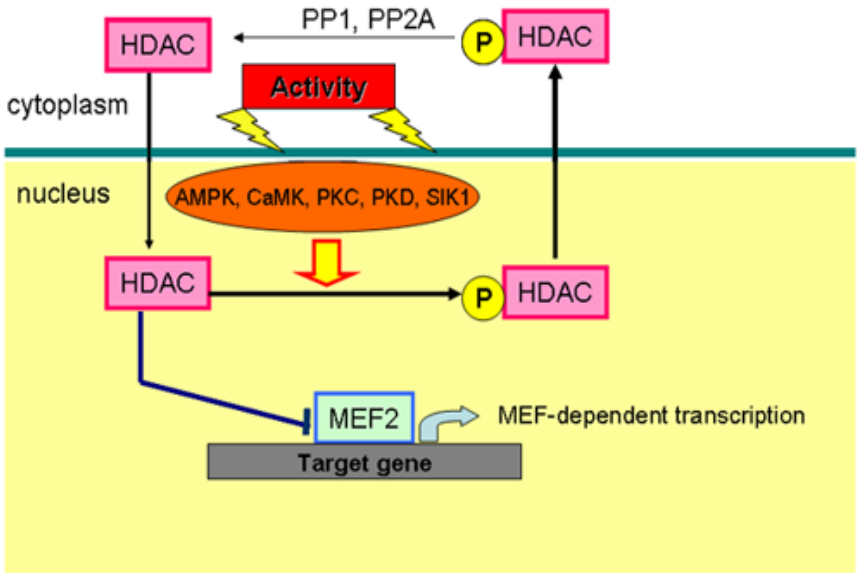


**Fig. 12. Scheme of cellular nucleocytoplasmic shuttling of HDAC in active skeletal muscle.** When AMPK, CaMK, PKC, PKD and SIK1 are activated by muscle contraction, nuclear HDAC is phosphorylated and it moves out from the nucleus, thus removing MEF2-dependent transcription. Phosphatase activity dephosphorylates HDAC, resulting in HDAC entry into the nucleus and inhibitory association with MEF2 transcription factor.

*1.5.1 CaMKII-HDAC signalling on MEF2 transcriptional activity*

CaMK isoforms, which have initially been found to phosphorylate HDACIIa, are CaMKI and CaMKIV (McKinsey et al., 2000*a*) and subsequently CaMKII (Backs et al., 2006; Davis et al., 2003; Liu et al., 2005). However, it has been reported that CaMKI and CaMKIV are not expressed in adult skeletal muscle (Rose et al., 2006), so it seems that CaMKII may be the only CaMK that controls MEF-dependent transcription in skeletal muscle. However, we do detect the mRNA of CaMKI in adult skeletal muscle (our unpublished observation) and are planning to further investigate this issue.

It has been recently found that electrostimulation of *ex vivo* single fibers isolated from FDB induced HDAC4 nuclear efflux (Liu et al., 2005). In the presence of CaMK inhibitor, KN62, there were no changes in either nuclear or cytoplasmic fluorescence of transfected HDAC4 fused to the green fluorescent protein (HDAC4-GFP). In the same work, it has been shown that CaMKII and MEF2 transcription is activated by fiber electrostimulation, detected by an anti-autophosphorylated CaMKII antibody and luciferase assay respectively. KN62 completely blocks stimulation-dependent increase in MEF2 transcriptional activity, by completely inhibiting stimulation-dependent efflux of HDAC4-GFP from fiber nuclei. In addition the pattern of stimulation affects HDAC4 efflux from the nuclei: 10 Hz continuos stimulation, typical of slow fibers, causes a marked translocation of HDAC-GFP from the nucleus to the cytoplasm, while 100 Hz every 50 seconds, which resembles the stimulation pattern of fast fibers, did not cause any detectable change in nuclear fluorescence. Thus HDAC4 export from the nucleus to the cytoplasm is determined by the pattern of activity experienced by muscle fibers, and in particular, only slow pattern of stimulation induces nuclear efflux of HDAC4. Treatment of the fibers with Leptomycin B, which blocks CRM-1 mediated nuclear export, has shown that a balanced nuclear influx and efflux of HDAC4 exists in resting fibers. In resting fibers CaMKII activity does not mediate HDAC4 phosphorylation required for its shuttling out of the nuclei, which is likely mediated by another kinase. In fact staurosporine, a broad-spectrum kinase inhibitor, increases fluorescence signal of HDAC-GFP in the nuclei, without affecting cytoplasmic fluorescence in unstimulated fibers (Liu et al., 2005). In conclusion it seems that in unstimulated fibers there is a balanced resting shuttling of HDAC4 nuclear efflux and HDAC nuclear influx, which determines the resting level of nuclear HDAC4. In addition the nuclear efflux of HDAC4 in resting fibers does not

involve CaMKII, but a yet unidentified kinase. It has also been demonstrated that the rate of nuclear export of HDAC4 from the nucleus is equal in both conditions (stimulated and resting). A dual regulation mechanism may exist for the nuclear level of HDAC4, enabling muscle fibers to set nuclear level of HDAC4 independently during rest and contraction, by turning on different kinases to export HDAC4. After fiber stimulation nuclear HDAC-GFP fluorescence begins to increase, and, using calyculin A (a PP1 and PP2A inhibitor), it was demonstrated that almost all of the influx of HDAC4 into nuclei during shuttling in resting fibers is mediated by PP1 or PP2A. CsA did not block HDAC4 nuclear influx, so it can be assumed that Cn does not play any role in HDAC4 shuttling.

Some groups have shown that HDAC5 does not exhibit stimulation-dependent nuclear efflux (Liu et al, 2005), but others have reported opposing results. McGee and Hargreaves (2004) have found that in human contracting skeletal muscle HDAC5 nuclear level decreases without any changes in total HDAC5 protein content, suggesting that HDAC5 has been exported from the nucleus. CaMKII has not been detected in the muscle nuclear fraction during rest or after exercise either. They suggest that a potential HDAC5 kinase could be AMPK, which shares very similar substrate specificity to CaMK. In addition AMPK $\alpha_2$ has been found to translocate into the nucleus (McGee et al., 2003), in response to the same exercise protocol used in their aforementioned work (McGee and Hargreaves, 2004). Subsequently the same group has evidenced that AMPK is able to phosphorylate HDAC5 in primary myotubes (McGee et al., 2008), although it has not demonstrated that this event occurs during fiber contraction.

It has been shown that autophosphorylated activated CaMKII signals specifically to HDAC4 by binding to a unique docking site which resides on only this member belonging to class IIa of HDACs. HDAC5 lacks of this unique domain that mediates strong interactions with CaMKII, accounting for HDAC5 insensitivity to this kinase (Backs et al., 2006).

The apparent discrepancy in the responsiveness of both HDAC4 and HDAC5 to CaMKII may be explained by recent data which have shown that, although HDAC5 is unresponsive to CaMKII, it becomes responsive to CaMKII in the presence of HDAC4 (Backs et al., 2008). Thus CaMKII can also signal to HDAC5 in virtue of the oligomerization with HDAC4. The level of HDAC4 is essential to determine the extent of nuclear export of HDAC5, and thereby the extent of gene transcriptional derepression by

CaMKII. In conclusion CaMKII is a key regulator of HDACIIa gene repression. Recently it has been shown that ubiquitination of HDACIIa is implicated in determining the level of HDACIIa in skeletal muscles, where its deacetylating activity has been found to control muscle fiber-type by regulating MEF2 transcriptional activity (Potthoff et al., 2007).

### *1.6 Exercise and Type 2 diabetes (T2D)*

#### *1.6.1 Glucose transport in skeletal muscle*

Individuals with insulin resistance and Type 2 diabetes are characterized by impaired insulin action on whole body glucose uptake, mainly due to impaired insulin stimulated glucose uptake in skeletal muscle (Zierath et al., 1996). The main glucose transporter in skeletal muscle and adipose tissue is GLUT4. It belongs to a family of 13 sugar transporter proteins encoded in the human genome through which sugars are transported across the membrane by facilitative diffusion. The glucose transporters differ for their kinetics and respective substrate specificities.

Skeletal muscle is the major tissue for glucose metabolism, accounting for ~75% of whole-body insulin-stimulated glucose uptake (DeFronzo et al., 1981). Thus GLUT4 is the major mediator of glucose removal from the circulation and a key regulator of whole-body glucose homeostasis. Insulin and exercise are the most relevant physiological stimulators of glucose transport in skeletal muscle (Goodyear and Khan, 1998; Hayashi et al., 1997). The translocation of GLUT4 from an intracellular vesicular pool to the plasma membrane is the major mechanism through which insulin and exercise increase glucose uptake (Goodyear and Khan, 1998; Hayashi et al., 1997). Several lines of evidence show that muscle contraction stimulates glucose uptake in the complete absence of insulin; that the maximal effects of contraction and insulin are additive; and that contraction and insulin stimulate glucose transport by separate pathways (Hawley and Lessard, 2008) which lead to enhanced glucose transport. In fact in T2D insulin- but not contraction-stimulated glucose transport is impaired (Kennedy et al., 1999), and this is the basis to explain why regular exercise can prevent the onset of T2D and ameliorate insulin resistance.

There are evidences that implicate CaMK activity as a regulator of glucose uptake, independently from insulin pathway. The increase in intracellular $Ca^{2+}$ activates multifunctional CaMKs and an inhibition of CaMKs by KN93 leads to partial reduction in contraction-stimulated glucose transport in epitrochlearis (Wright et al., 2004) and

complete abolition in soleus muscle (Wright et al., 2005). Conversely KN62, another CaMK inhibitor, does not prevent insulin-stimulated glucose uptake, demonstrating that CaMK activity does not influence insulin-dependent glucose transport (Wright et al., 2004).

Importantly CaMKII activity is increased in contracting muscle of humans (Rose and Hargreaves, 2003) and rodents (Rose et al., 2007*a*). KN62/KN93 impair the activation of CaMKII by contraction (Wright et al., 2005). It must be considered that these inhibitors can inhibit other multifunctional CaMKs, such as CaMKI and CaMKIV, so the involvement of other CaMK isoforms can not be ruled out. Adult skeletal muscle has not been shown to express CaMKI and CaMKIV (Rose et al., 2006) but, as mentioned previously, we could detect the mRNAs and the physiological significance of this observation needs to be further investigated.

### *1.6.2 Exercise-induced GLUT4 expression in skeletal muscle*

Besides stimulating GLUT4 translocation, exercise training results in a rapid increase in the expression of both GLUT4 mRNA and protein in skeletal muscle and these changes have been associated with improved glucose uptake (Hawley and Lessard, 2008). In diabetic transgenic mice the effect of T2D can be alleviated by increasing GLUT4 expression (Gibbs et al., 1995; Tozzo et al., 1997). This suggests that the knowledge of GLUT4 gene regulation may represent a mean to detect potential intervention points for treatment of T2D.

The expression of GLUT4 is highly regulated depending on the developmental stage (Daugaard et al., 2000; Santalucia et al., 1992). Skeletal muscle expression of GLUT4 is low in the foetal rat and a continuos induction of GLUT4 mRNA and protein takes place in the perinatal phase (Zorzano et al., 2005). This induction is regulated by thyroid hormones, contractile activity and neural-derived trophic factors. Thyroid hormone seems to stimulate GLUT4 expression in regenerating muscle but it may play an inhibitory role in adult muscle for yet unexplained reasons (see also below; Zorzano et al., 2005). Low-frequency stimulation of skeletal muscle specifically causes the induction of GLUT4 (Daugaard et al., 2000; Zorzano et al., 2005). Motor neurons contribute in various ways to the control glucose transporter expression: chronic incubations of L6 cells with neuregulins

markedly alters the expression of glucose transporter expression, which are released from neuronal or skeletal muscle cells in physiological condition (Zorzano et al., 2005).

Studies using transgenic mice have evidenced that there are specific skeletal muscle elements located within 730 bp of GLUT4 5'-flanking DNA. In particular GLUT4 muscle-specific expression is conferred by a DNA sequence located between -502 and -420 bp of rat GLUT4, which is a transcriptional enhancer (this region has 80% similarity with the human and mouse sequence). In this regard, a MEF2 binding site has been detected in the GLUT4 enhancer and it has been found to be essential for the specific expression of GLUT4 in skeletal muscle. The same region of GLUT4 promoter contains also a low-affinity thyroid hormone receptor and MyoD binding site (TRE and E-box respectively) next to the MEF2 site. The existence of a co-operation between MEF2, MyoD and thyroid hormone receptor has been reported, defined as a triple synergistic effect producing maximal activation of GLUT4 enhancer when these three factors act together on their respective binding sites in L6 muscle cells (Santalucia et al., 2001). However in adult skeletal muscle the mutation of TRE induces an increase of the enhancer activity, indicating that in normal condition the enhancer is subjected to repression via the TRE in adult skeletal muscle (Moreno et al., 2003). The E-box remains inactive in adult skeletal muscle, whereas it is functional in regenerating muscle, a condition which recapitules skeletal muscle development. This is consistent which the low level of expression of muscle regulatory factors (MRFs), among which MyoD, in adult skeletal muscle and with their induction during regeneration. MEF2 binding site is requested, together with the E-box and TRE, for normal activity of GLUT4 enhancer in regenerating muscle.

The integrity of the MEF2 site is essential especially in adult skeletal muscle, where its mutation causes a marked reduction of the enhancer activity (Moreno et al., 2003). It has been suggested that a MEF2A-MEF2D heterodimer is responsible for the regulation of GLUT4 gene expression skeletal muscle in basal conditions (Mora and Pessin, 2000). Exercise has been found to increase DNA-binding activities of MEF2A-MEF2D heterodimer (McGee et al., 2005). Increases in the content of MEF2A and MEF2D have been found in L6 cells after their stimulation with caffeine (Ojuka et al., 2002), which induces $Ca^{2+}$ release from the RyR in skeletal muscle SR. Thus, these data suggest that an increment in MEF2 proteins may regulate GLUT4 expression, although contrasting results have been reported (Smith et al., 2006).

Interestingly, KN93 completely inhibits the increase in MEF2A and MEF2D and the $Ca^{2+}$-mediated increase in GLUT4 protein levels in L6 cells (Ojuka et al., 2002). The activation of AMPK produces opposite effects with respect to KN93 (Ojuka et al, 2002). Thus AMPK activation and the increase in intracellular $Ca^{2+}$, both of which occur during exercise, seem to induce an increase in GLUT4, MEF2A and MEF2D content in L6 myotubes. Other studies indicate a role for AMPK in mediating exercise-induced increase in GLUT4 expression in skeletal muscles (McGee et al., 2006,2008), whereas others have shown that AMPK is not involved in GLUT4 increase during exercise but only in basal conditions (Jorgensen et al., 2007). It thus seems that the role of AMPK in the exercise-induced increase of GLUT4, although well established, is still controversial at the mechanistic level.

Caffeine induces an increase in GLUT4 and MEF2 proteins *in vivo* (rat epitrochlearis muscles) but a role for CaMK has not been investigated in this work (Ojuka et al., 2002). A role for CaMK in regulating GLUT4 expression has been demonstrated in another work: MEF2A binding to the GLUT4 promoter was higher in exercised rat triceps, followed by an increase in GLUT4 mRNA. CaMKII activity was increased after the same protocol of exercise and CaMK signalling has been shown to mediate MEF2A binding on GLUT4 promoter *in vitro* (Smith et al., 2006).

The molecular mechanism whereby caffeine-induced CaMKII activity mediates the binding of MEF2A on its GLUT4 promoter binding site has recently been elucidated. Exercise induces acetylation of histone H3 at the MEF2 binding site on the GLUT4 promoter and this effect is blocked by CaMKII inhibition in rat triceps muscle. This indicates that the increase in MEF2 accessibility on its *cis* element on GLUT4 gene is mediated by exercise-induced activation through CaMKII signalling (Smith et al., 2008). However, it cannot be excluded that the increase in MEF2 binding on GLUT4 promoter can also occur by an increase in MEF2 translocation and that other mechanism co-operate in the regulation of MEF2 transcriptional activity, such as ubiquitination (Potthoff et al., 2007; see paragraph 1.5.1).

It must be considered that MEF2 regulation of transcriptional activity may be very complex. MEF2 represents a point of convergence of different signalling pathways, such as those activated by exercise, i.e. CaMK, AMPK and Cn (Ojuka et al., 2002; Rockl et al., 2008), which ultimately determine its transcriptional activation, binding and accessibility

to the DNA and to co-activators (McGee and Hargreaves, 2006). In addition other HDAC kinases have been reported to influence MEF2-dependent transcription, like SIK1 (Berdeaux et al., 2007) and PKD (Kim et al., 2008).

### *1.6.3 Benefits of exercise in skeletal muscle*

Other important muscle adaptations to exercise are fiber-type transition and increase in mitochondrial activity and content. There is an association between fiber-type and mitochondrial content, with type IIB fibers having the lowest and type I, IIA fibers the highest abundance of mitochondria. Endurance exercise has been shown to induce an increase in mitochondrial content and activity within the same fiber-type, but also a shift in MyHC expression (Rockl et al., 2008), provoking a fiber transformation from IIB towards more oxidative type IIX and IIA fibers (Pette and Staron, 2001).

Interestingly individuals with insulin resistance or T2D have decreased content of slow oxidative muscle fibers (Marin et al., 1994; Nyholm et al., 1997). The master regulator of mitochondrial biogenesis is PGC-1α transcriptional co-activator which interacts with a variety of transcription factors, such as NRF-1, NRF-2, PPARα and PPARγ resulting in an increase of expression of genes involved in fatty acid oxidation and in respiratory chain (Vega et al., 2000; Wu et al., 1999), PGC-1 α is also known to drive muscle fiber-type transformation from type II to type I fibers (Lin et al., 2002), which indicates a coordinate fiber-type and metabolism switch. However, it must be emphasized that mitochondrial biogenesis and fiber-type transformation can occur independent from each other, suggesting the existence of different signalling mechanisms for these adaptive responses (Rockl et al., 2008).

Many pathways seem to impinge on PGC-1α activation (Akimoto et al., 2008; Rockl et al., 2008), including increasing in intracellular $Ca^{2+}$, e.g. induced by caffeine (Ojuka et al., 2002) or by exercise (Pilegaard et al., 2003). To date, the specific pathways through which increases in intracellular $Ca^{2+}$ induce mitochondrial biogenesis have been not fully understood. A possible candidate seems to be CaMK, as c.a. CaMKIV increases mitochondrial biogenesis in skeletal muscle (Wu et al., 2002) and KN93 prevents the increase in GLUT4, MEF2, PGC-1α and mitochondrial marker proteins (Ojuka et al., 2003) such as mitochondrial transcription factor A (mtTFA or TFAM), which controls mitochondrial DNA replication and transcription (Virbasius and Scarpulla, 1994).

Moreover c.a.Cn results in increses in PGC-1α and GLUT4 expression in skeletal muscle (Long et al., 2007; Ryder et al. 2003), thus implicating Cn as a possible important mediator of skeletal muscle metabolism. Nevertheless, the role of Cn in mitochondrial biogenesis remains controversial, e.g. overexepression of a Cn protein inhibitor, RCAN1, results in a transformation of muscle fiber-type without alterations in oxidative capacity or mitochondrial content in skeletal muscle (Long and Zierath, 2008; Rockl et al., 2008) and it seems that, besides Cn, other upstream signalling molecules (e.g AMPK, CaMK, p38 MAPK) can regulate PCG-1α activity and expression (Rockl et al., 2008).

Although exercise is beneficial and a fundamental component of a healthy lifestyle, by increasing GLUT4 expression and muscle mitochondria level, many individuals are unable or unwilling to exercise, also because these adaptations require a certain degree of intensity and duration. Understanding the signalling pathways that mediate exercise-induced adaptations could thus allow the design of drugs with exercise-mimetic effects, to pharmacologically mimic the beneficial effects of exercise on skeletal muscle.

# 2. MATERIALS AND METHODS

## *2.1 Vectors*

| Reporter constructs/expression vectors | Expressed gene | Reference |
|---|---|---|
| Cain | Cain (full length) | Friday et al., 2000 |
| c.a. CaMKIV | Constitutively active CaMKIV | Passier et al., 2000 |
| GLUT4 enhancer (GLUT4 enh) | Luciferase | Liu et al., 1994 |
| GLUT4-CAT | CAT | Liu et al., 1992 |
| CS-LUC | Luciferase | McClelland et al., 2004 |
| KIIN construct | KIIN | Chang et al., 1998 |
| myc-Cain | myc-tagged Cain | Lai et al., 1998 |
| MyHC slow-LUC | Luciferase | Hasegawa et al., 1997 |
| MyHC 2B-LUC | Luciferase | Swoap, 1998 |
| MEF2-LUC | Luciferase | Naya et al., 1999 |
| TFAM-LUC | Luciferase | Choi et al, 2004 |
| pcDNA3 (vector) | --- | Buelow et al., 1992 |
| PGC-1α | FLAG-tagged PGC-1α | Monsalve et al., 2000 |
| Tk-Rn | *Renilla* Luciferase | Promega |
| VIVIT | VIVIT-GFP | Aramburu et al., 1999 |

### *2.2 Bacterial transformation*

MATERIALS:-*E. coli* cells, XL1-Blue strain;

-Luria-Bertani medium (LB, bacto-tryptone 10 g/l; yeast extract 5 g/l; NaCl 10 g/l in distilled water).

-ampicillin stock 50 mg/ml; kanamycin stock 50 mg/ml.

-100 ng of plasmidic DNA

-Petri's dishes (filled with about 20 ml of LB added with ampicillin 50 μg/ml or kanamycin 50 μg/ml and with 1.5% of bacto-agar).

METHOD: XL1-Blue competent cells have been mixed with 100 ng of plasmidic DNA and kept on ice for 30 minutes. Heat shock: 45 seconds at 42°C. Then cells have been incubated 1 h at 37 °C under constant rotation in 3 ml of LB. Finally cells have been plated on Petri's dishes and incubated overnight at 37°C.

### *2.3 Plasmidic DNA preparation (Maxiprep)*

MATERIALS:-transformed bacteria;

-Luria-Bertani medium (LB, bacto-tryptone 10 g/l; yeast extract 5 g/l; NaCl 10 g/l in distilled water), sterilized in autoclave and added with ampicillin 50 μg/ml or kanamycin 50 μg/ml;

-Invitrogen Equilibration Buffer (EQ1);

-Invitrogen Resuspension buffer (R3);

-Invitrogen RNase A (20 mg/ml);

-Invitrogen Lysis Buffer (L7);

-Invitrogen Precipitation Buffer (N3);

-Invitrogen HiPure Filter Maxi Column;

-Invitrogen Wash Buffer (W8);

-Invitrogen Eluition buffer (E4);

-isopropanol;

-ethanol (etOH) 70%;

-TE (10mM Tris-HCl pH8, 1 mM EDTA pH8).

METHOD: once equilibrated HiPure Filter Maxi Column with 30 ml of EQ1 buffer, starting from a 200 ml culture of bacteria grown overnight at 37°C, cells have been harvested at 4,000 x g for 10 min at 4°C. Then bacteria have been resuspended in 10 ml of R3 buffer with RNase A, and lysated adding 10 ml of L7 buffer. After 5 min, the reaction

has been stopped by adding N3 precipitation buffer, and each sample lysate has been transferred into the equilibrated HiPure Maxi Column. After the HiPure Maxi Column has stopped dripping, the inner Filtration Cartridge has been removed from the column and the Maxi Column has been washed with 50 ml of W8 buffer. Then plasmidic DNA has been eluted with 15 ml of E4 buffer, and collected on a tube. Once eluition has stopped, 10.5 ml of isopropanol have been added to precipitate the DNA. The solution has been then centrifuged at >15,000 x g for 30 min at 4°C. The pellet has been washed with 5 ml of ethanol 70%, centrifuged for 10 minutes at >15,000 x g for 5 min at 4°C. Then the pellet has been air-dried, and finally resuspended in 200-400 µl of $H_2O$.

### *2.4 In vivo transfection by electroporation*

MATERIALS:-adult Wistar rats (150-250 gr) or C57BL/6 mice (20-40 gr);

-anaesthetic: 1:5 mixture of Zoletil 100® (a combination of Zolazapam and Tiletamine, 1:1, 10 mg/kg, Laboratoire Virbac) and Rompun® (Xilazine 2%, 0.06 ml/kg, Bayer).

-alternatively: gas anaesthesia with oxygen and 2% mix of isofluorane, on a Fluovac gas anaesthesia machine (Harvard Scientific instruments)

-50 µg of plasmidic DNA diluted in 50 µl of total volume of a 0.9% NaCl solution;

-electroconductive gel;

-ECM 830 Electroporator (BTX, Harvard Bioscience company), parameters set as follows: voltage: 220 V/cm;

pulses number: 6;

pulse length: 20 ms;

interval between pulses: 176 ms.

METHOD: rats or mice have been anaesthetized with Zoletil plus Xilor solution (5:1 ratio), or with gas anaesthesia. Solues and EDL muscles have been exposed and injected with a solution containing plasmidic DNA (usually 20 µg of reporter, 20 µg of inhibitor, 10 µg of co-transfectant to normalize for transfection efficiency, with a Hamilton syringe

(Fig. A). As co-transfectanct, which constitutes our internal control, in all experiments we have used a *Renilla* Luciferase plasmid whose expression is controlled by a thymidine kinase promoter (Tk-Rn, Promega).

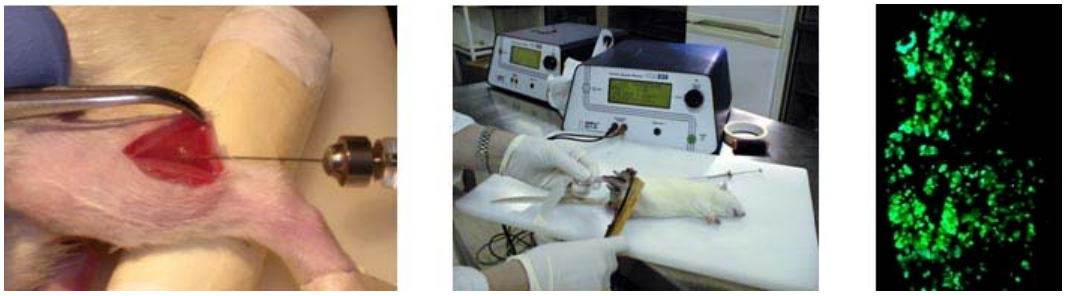

**Figure A. *In vivo* transfection of adult rat muscles.** Injection of DNA in the soleus muscle (*left*) and electroporation (*centre*). Example of a muscle tranfected with GFP (*right*), seven days after transfection.

Skin has been sutured, then muscles have been electroporated, as described by Mir et al. (1999). Muscles has been collected 7 days after transfection, frozen in liquid nitrogen cooled liquid isopentane, and kept at –80°C.

### *2.5 Checking DNA with restriction enzymes*

MATERIALS:-0.5 µg of DNA;

-2 µl of reaction buffer 10X;

-0.5 µl of restriction enzym (about 1 U/µg of DNA/cutting site);

-distilled sterile water to a final volume of 20 µl.

METHOD: to be sure that the DNA obtained with a MaxiPrep is correct, DNA has been digested with specific restriction enzymes, to produce linear double stranded fragments, which have been predicted by using restriction maps. Reaction has been done at 37°C for 1 h.

### *2.6 Agarose gel electrophoresis*

MATERIALS:-agarose;

-TBE running buffer (1 mM Tris, 0.9 mM boric acid, 0.01 EDTA, pH 8.4);

-etidium Bromide (0.5 µg/ml final concentration);

-5X Loading buffer III and Ladder 1 kbp (weight marker).

METHOD: agarose has been melted in TBE (1% final concentration) and added with EtBr. Digested DNA has been then mixed with loading buffer and loaded into the gel. Then electrophoresis has been obtained by applying a 100 V voltage for 30 min. Bands have been revealed under UV light.

### *2.7 Luciferase assay*

MATERIALS: -luminometer (TD 20/20 Turner Design).

-Dual luciferase kit (E1960, Promega Corp.,Madison WI, U.S.A.), composed of:

-Passive lysis buffer 5X;

-A: LarII substrate for Firefly luciferase;

-B: Stop and Glow substrate for *Renilla* luciferase.

METHOD: transfected muscles have been crushed with a pestle and mortar cooled with liquid nitrogen. Powder has been weighted and added with 2.5 µl of lysis buffer each mg of tissue. Lysates have been frozen with liquid nitrogen and thawed at 4°C (twice). Then lysates have been centrifuged for 20 min at 13.000 rpm at 4°C, and supernatants have been collected. 2-10 µl of supernatant have been added to 50 µl of LarII buffer, and Firefly luciferase activity has been measured. Stop and Glow buffer has been added, and *Renilla* luciferase measured.

### *2.8 MyHC/FLAG immunohistochemistries*

MATERIALS:-anti-MyHC I and anti-MyHC IIA, monoclonal primary antibodies; dilution 1:500 (Schiaffino et al., 1989)/anti-FLAG F7425 (Sigma); polyclonal primary antibody; dilution 1:500;

-anti-mouse Cy3 conjugated made in goat secondary antibody; dilution 1:200/anti-rabbit Cy3 conjugated made in goat secondary antibody; dilution 1:250;

-PBS 10X (quantities for 1 l): NaCl 80 g, KCl 2 g, $Na_2HPO_4$ 14.4 g, $KH_2PO_4$ 2.4 g. pH of the working solution (1X) must be 7.3-7.4;

-primary antibodies buffer: PBS 1X added with 0.5% (w/v) bovine serum albumin (BSA);

-secondary antibodies buffer: PBS 1X added with 0.5% (w/v) BSA and goat serum (1:200/1:250 dilution);

-Elvanol, mounting medium.

METHOD: muscle sections (10 μm thick have been incubated with the anti-MyHC antibodies for 1 h at room temperature and with the anti-FLAG antibody for 2 h at room temperature. Sections have been then washed 3 times with PBS 5 min each, incubated with secondary antibodies for 1 h at room temperature, then washed 3 times with PBS 5 min each. Finally, sections have been mounted with elvanol.

### *2.8 Succinate dehydrogenase (SDH) assay*

MATERIALS:-0.2 M sodium succinate solution:

sodium succinate: 5.4 g;

distilled Water (DW): 100 ml;

-0.2 M Phosphate buffer, pH 7.4:

$Na_2HPO_4$ 11.36 g/400 ml DW;

$KH_2PO_4$ 2.72 g/100 ml DW;

-SDH stock solution:

0.2 M sodium succinate:100 ml;

0.2 M phosphate buffer, pH 7.4: 100 ml;

-SDH incubating solution:

Nitroblue tetrazolium (NBT): 10 mg;

SDH stock solution: 10 ml;

Adjust to pH 7.2 to 7.6 with 0.1 N HCl or NaOH;

-acetone 30% and 60%;

-ethanol 50%, 70%, 90%, 100%;

-xylene;

-Entellan, mounting medium.

METHOD: muscle sections have been incubated in SDH incubating solution for 60 minutes at 37 °C and rinsed free times in DW. Sections have been dipped in 30%, 60%, 30% acetone, one rapid dip each. After this they have been washed in DW. Then sections have been dehydrated in ethanol: 50%, 70%, 90%, 100%, 100% and finally cleared in xylene. Sections have been mounted in Entellan.

# 3. RESULTS

## *3.1 KIIN is a functional CaMKII inhibitor in skeletal muscle*

To investigate the role of CaMKII in controlling the expression of activity-dependent genes we have used a loss of function approach based on a natural inhibitor protein of CaMKII which has been originally identified in brain by a yeast two-hybrid screen. The characterization of KIIN inhibitory activity has shown that it is a potent and specific inhibitor of CaMKII, but not of other multifunctional CaMKs or kinases such as PKA, PKC or MAPK (Chang et al., 1998). Thus its specificity for CaMKII permits to avoid off-target effects and to attribute the effects observed only to CaMKII activity.

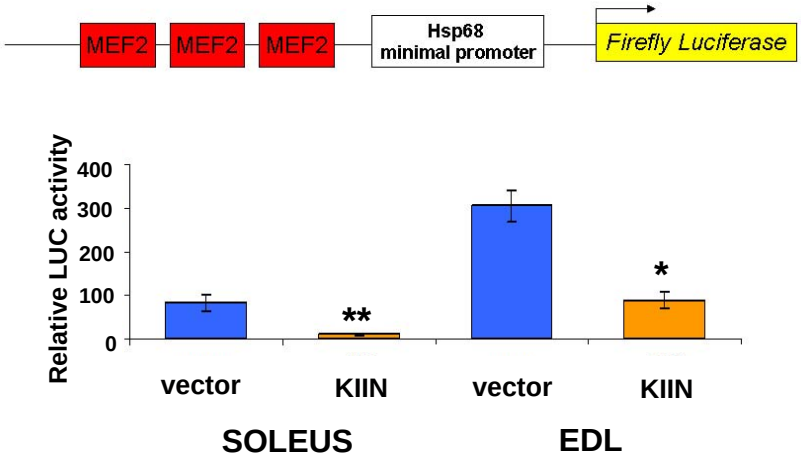


**Fig. 13. KIIN inhibits CaMKII activity in skeletal muscles.** On the top of the figure a simplified scheme of MEF2-LUC sensor: upstream of the Hsp68 minimal promoter there are three MEF2 binding sites, which control the expression of the Luciferase (LUC) gene. Rat soleus and EDL muscles have been transfected with MEF2-LUC sensor and the plasmid containing the gene coding for KIIN (KIIN construct) or with an empty vector (vector). Co-transfection with Tk-Rn vector has been used to normalize the efficiency of transfection thus obtaining relative LUC activity values. Values are means ± SD of one experiment of five muscles for each type of transfection. * and ** mean statistically difference of relative LUC values between muscles transfected with vector and KIIN construct, $P < 0.05$ and $P < 0.01$ respectively.

To test if KIIN is a functional inhibitor in skeletal muscle *in vivo*, we have used as readout a MEF2 sensor, which contains three tandem MEF2 binding sites from the desmin enhancer (Naya et al., 1999) upstream of the luciferase gene (MEF2-LUC sensor). Upon phosphorylation of HDACs by CaMKII, HDAC moves out from the nucleus, thus releasing its inhibition on MEF2. Therefore, if CaMKII activity is blocked, MEF2-dependent transcription will be inhibited. Our results indicate that KIIN works in skeletal muscle as a CaMKII inhibitor, since the relative LUC activity of the sensor is

downregulated in muscles transfected with KIIN (Fig. 13). Interestingly, we have observed that MEF2 transcriptional activity is higher in EDL muscles than in soleus. This is in contrast with previous hypotheses on the role of MEF2 in slow muscle (Wu et al., 2000) but the reasons for this discrepancy have not been investigated.

### *3.2. CaMKII regulates GLUT4 expression in skeletal muscle*

We have investigated the role of CaMKII in controlling the expression of GLUT4. Soleus and EDL muscles have been transfected with the plasmid coding for KIIN together with two different portions of the GLUT4 promoter driving the expression of reporter genes, namely a) the so called GLUT4 enhancer (-502 to -420 bp) fused to luciferase (GLUT4-LUC) and b) 2,8 kbp of the GLUT4 promoter fused to CAT (GLUT4-CAT).

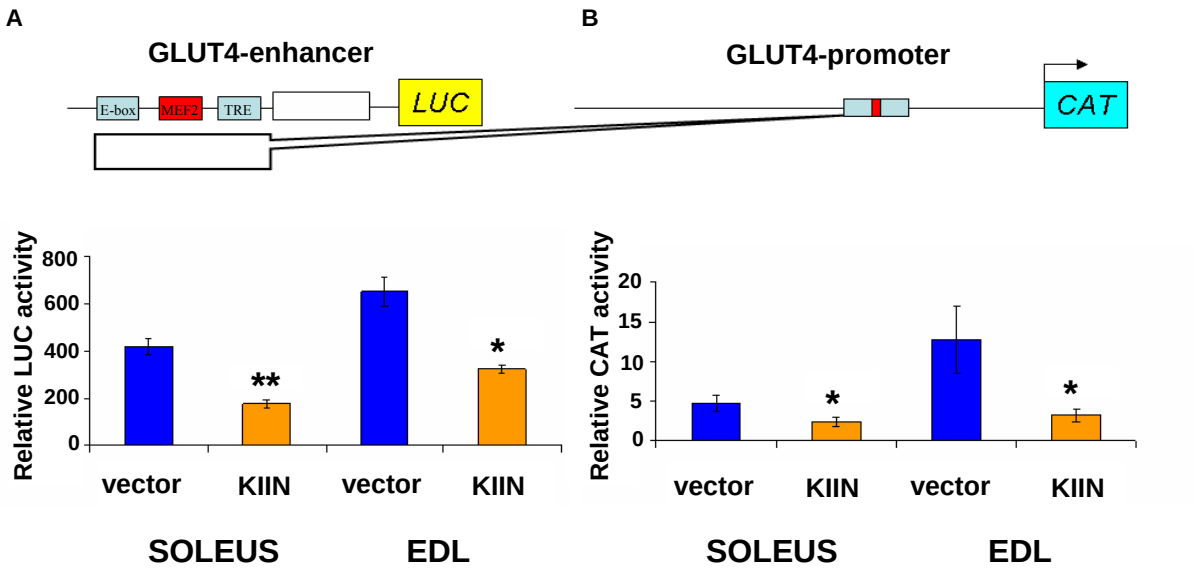


**Figure 14. KIIN negatively affects GLUT4 expression in skeletal muscle.** On top**,** simplified scheme of GLUT4-enhancer LUC reporter (A) and GLUT4-promoter CAT reporter (B). A. GLUT4 enhancer (enh) contains, besides a MEF2 binding site, an E-box and a thyroid response element upstream of a minimal promoter (empty rectangle). B. GLUT4 promoter, which contains the enh, controls CAT gene expression. Rat adult soleus and EDL muscles have been transfected with one of the GLUT4 reporters along with the plasmid containing the gene coding for KIIN (KIIN construct) or with an empty vector (vector). Co-transfection with Tk-Rn vector, has been used to normalize the efficiency of transfection thus obtaining relative LUC activity values. Values are means ± SD of one experiment of five muscles for each type of transfection. * and ** mean statistically difference of relative LUC values between muscles transfected with vector and KIIN construct, $P < 0.05$ and $P < 0.01$ respectively.

The enhancer has been previously shown to retain many of the transcriptional properties of the longer promoter (Moreno et al., 2003) and it is more amenable to mechanistic studies. As shown in Fig. 14, CaMKII inhibition by KIIN causes a strong inhibition of LUC and CAT activities of both reporters, thus indicating that transcription of GLUT4 is controlled by CaMKII in skeletal muscle *in vivo*. It also confirms that the GLUT4 enhancer maintains most properties of the longer promoter. We have therefore used the GLUT4-LUC in most of the experiments shown in this thesis. As observed with the MEF2 sensor (Fig. 14), the activity of GLUT4-LUC is also higher in EDL than in soleus.

### *3.3. Calcineurin (Cn) regulates GLUT4 expression in skeletal muscle*

Cn has a fundamental role in the control of muscle fiber-type (McCullagh et al., 2004; Serrano et al., 2001), but also affects muscle metabolism (Long et al., 2007; Ryder et al., 2003). We asked whether Cn can influence GLUT4 expression by co-transfecting Cain, a known Cn inhibitor (Lai et al., 1998), together with GLUT4-LUC in skeletal muscle.

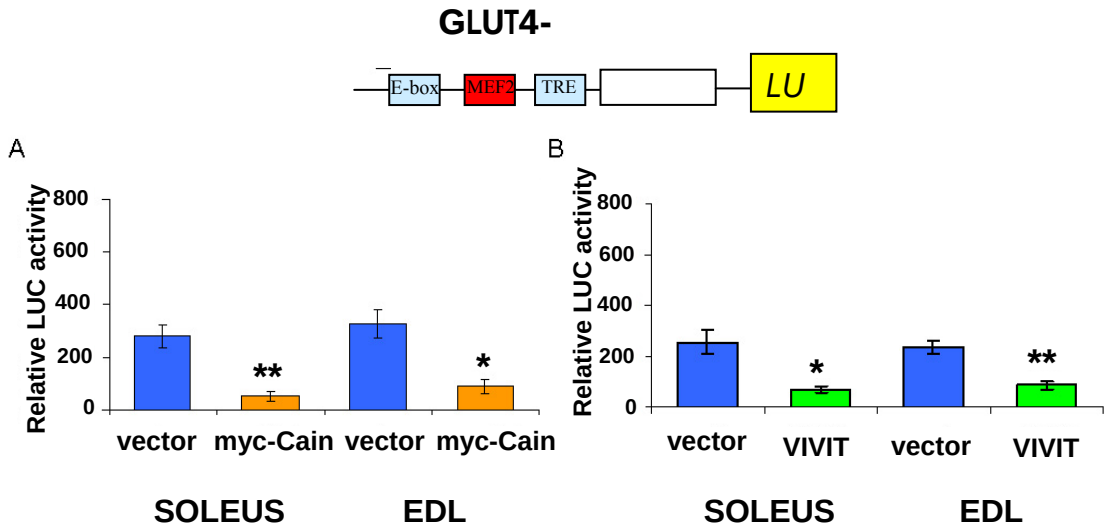


**Fig. 15. GLUT4 expression is affected by Cn activity in skeletal muscle.** Rat adult soleus and EDL muscles have been transfected with the GLUT4 LUC-reporter along with the plasmid containing the gene coding for Cain (a Cn inhibitor) with a myc-tag, named myc-Cain (A), or for VIVIT, an NFAT inhibitor, (B) or with an empty vector (vector). Co-transfection with Tk-Rn vector, has been used to normalize the efficiency of transfection and thus obtaining relative LUC activity values. Values are means ± SD of one experiment of five muscles for each type of transfection. * and ** mean statistically difference of relative LUC values between muscles transfected with vector and the construct coding for myc-Cain or VIVIT, $P < 0.05$ and $P < 0.01$ respectively.

Our results show that Cn is a positive regulator of GLUT4 expression, because Cain strongly reduces GLUT4-LUC activity (Fig. 15A). Since NFAT is a well known Cn target in skeletal muscle (McCullagh et al., 2004), we have then investigated if the effects of Cain on GLUT4 expression are due to NFAT inhibition. To this purpose, we have used VIVIT, a peptide inhibitor of NFAT (Aramburo et al., 1999), fused to GFP. Co-transfection with VIVIT-GFP causes a strong inhibition of GLUT4-LUC activity (Fig. 15B), suggesting that the transcriptional effects of Cn on GLUT4 expression are mediated by NFAT. Our results thus indicate that Cn-NFAT pathway is an important controller of GLUT4 expression in skeletal muscle. Altogether, these data support the idea that two major $Ca^{2+}$-dependent signalling pathways, CaMKII and Cn, cooperate in the transcriptional control of GLUT4 in skeletal muscle.

### *3.4 CaMKII and Cn have an additive effect on GLUT4 expression*

We have next assessed if CaMKII and Cn synergistically influence GLUT4 expression by transfecting the two inhibitors, KIIN and Cain, alone or together in the same muscle. When the two inhibitors are co-expressed in the same muscle, the inhibitory effect on GLUT4-LUC activity is stronger than when the inhibitors are separately transfected. KIIN and Cain are equally potent in inhibiting CaMKII and Cn respectively, thus showing that both pathways are active on GLUT4 expression under these conditions. The fact that GLUT4-LUC activity is not completely abolished when both CaMKII and Cn are blocked suggests that other pathways can regulate GLUT4 expression both in soleus and EDL.

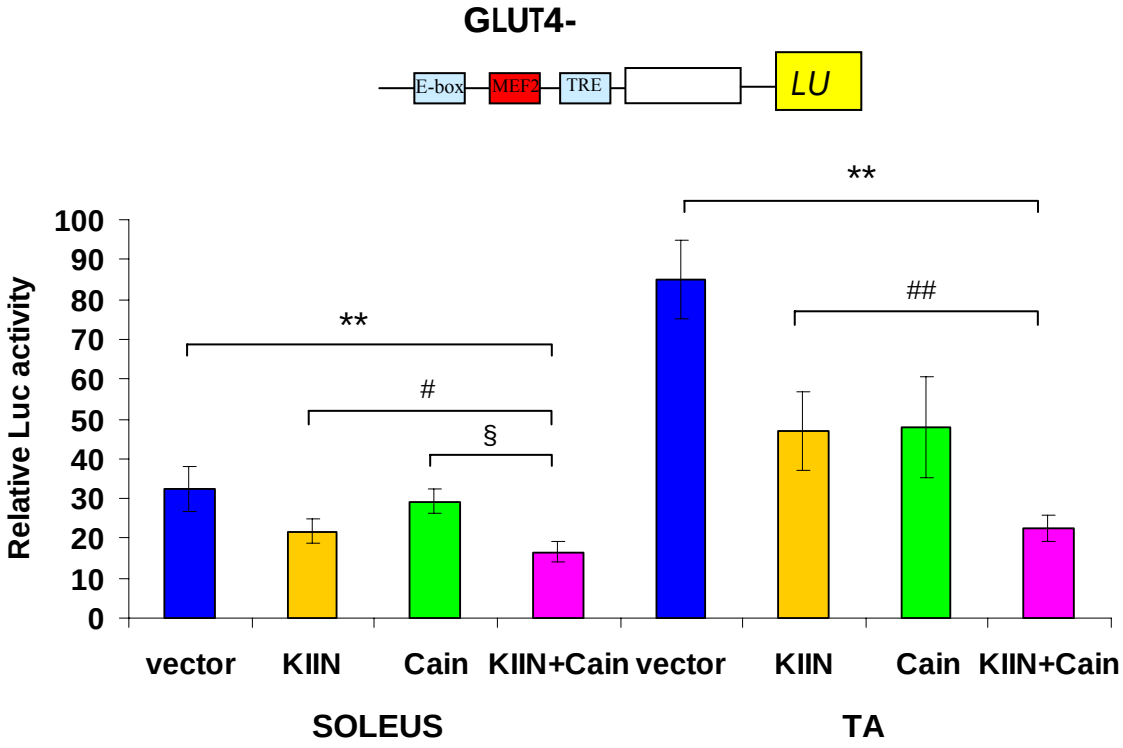


**Fig. 16. CaMKII and Cn synergistically regulate GLUT4 enhancer activity in skeletal muscle.** GLUT4-enhancer LUC reporter has been transfected in mouse adult soleus and *tibialis anterior* (TA) skeletal muscles together with the plasmid coding for KIIN (KIIN) or Cain full length (Cain) or both of the two constructs or with an empty vector (vector). All combinations of these constructs have been co-transfected with Tk-Rn vector, in order to normalize the efficiency of transfection, thus obtaining relative LUC activity values. Values are means ± SD of two experiments of five mouse adult soleus and TA muscles for each type of transfection. ** means statistically difference of relative LUC values between muscles transfected with vector and KIIN+Cain, $P < 0.01$; # and ## mean statistically difference of relative LUC values between muscles transfected with KIIN and KIIN+Cain, $P < 0.05$ and $P < 0.01$ respectively; § means statistically difference of relative LUCbetween muscles transfected with Cain and KIIN+Cain, $P < 0.05$.

### *3.5 CaMKII and Cn signalling to mitochondrial metabolism in skeletal muscle*

We have demonstrated that CaMKII regulates the expression of GLUT4, which is the major glucose transporter in skeletal muscles. We have further examined the role of CaMKII in skeletal muscle metabolism, in particular in mitochondrial metabolism, by assessing the role of CaMKII inhibition on the expression of mitochondrial transcription factor A (mTFA or TFAM). TFAM is a nuclear encoded transcription factor essential for mitochondrial DNA transcription and replication and, consequently, for mitochondrial biogenesis (Shadel and Clayton, 1997).

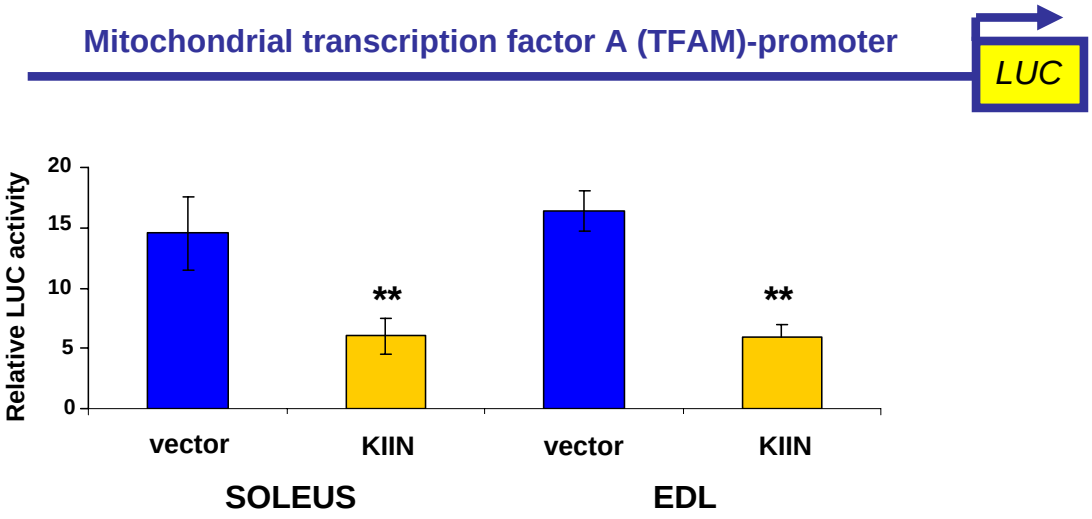


**Fig. 17. TFAM expression is controlled by CaMKII in skeletal muscle.** On the top of the figure a simplified scheme of TFAM-LUC reporter is represented: TFAM promoter controls the expression of the downstream LUC gene. Rat adult soleus and EDL muscles have been transfected with TFAM-LUC sensor along with plasmid containing the gene coding for KIIN (KIIN) or with an empty vector (vector). Co-transfection with Tk-Rn vector has been used to normalize the efficiency of transfection thus obtaining relative LUC activity values. Values are means ± SD of one experiment of five muscles for each type of transfection. ** means statistically difference of relative LUC values between muscles transfected with vector and KIIN, $P < 0.01$.

We have co-transfected the construct coding for KIIN together with a LUC reporter containing the human TFAM promoter (TFAM-LUC) in adult soleus and EDL muscles (Choi et al., 2004). TFAM-LUC activity is strongly and equally inhibited by KIIN in both muscles (Fig. 17). We were surprised by the observation that TFAM promoter activity is similar in soleus and EDL, because one would expect slow muscles, such as soleus, to have a higher mitochondrial content than fast muscles like EDL, and thus a higher TFAM promoter activity. However, it should be considered that IIA and IIX fibers, which constitute a large percentage of the total number of EDL fibers are also very rich in mitochondria (Fig. 20B, SDH staining). Altogether, our results indicate that CaMKII can regulate muscle mitochondrial metabolism, possibly by affecting mitochondrial biogenesis through its control on TFAM expression.

In order to confirm that CaMK activity can stimulate TFAM-LUC expression in skeletal muscle, we have used a constitutively active (c.a.) form of CaMKIV, which lacks the C-terminal autoinhibitory domain (Passier et al., 2000). We have chosen to use CaMKIV as a surrogate of CaMKII, for different reasons: i) CaMKIV is a monomeric multifunctional kinase; ii) CaMKII is a holoenzyme which exists as an homo- or hetero-multimer (Hudmon and Schulman, 2002) and overexpressing one single subunit in a

constitutively active form could significantly alter the stoichiometry of endogenous isoforms, with hardly predictable effects; iii) CaMKII and CaMKIV phosphorylate many common substrates, such as CREB and HDAC (Bito et al., 1996; Karanboulas et al., 2006; Liu et al., 2005; Sun et al., 2004); iv) CaMKIV is not present in skeletal muscle (Rose and Hargreaves, 2003), but it has been demonstrated to be functional when overexpressed in transgenic mice (Wu et al., 2002).

As shown in Fig. 18, c.a. CaMKIV induces an increase in TFAM-LUC activity in soleus but not in EDL muscles. It is puzzling that the effects of the c.a. CaMKIV do not exactly mirror those of the KIIN inhibitor. One could hypothesize that a CaMK substrate which is important for TFAM expression is rate-limiting in EDL but not in soleus. i.e. more concentrated in slow then in fast fibers. Fast fibers, however, are not intrinsically resistant to the effects of CaMKIV, since this kinase promotes mitochondrial biogenesis in the fast-twitch *plantaris* muscle (Wu et al., 2002). Part of this discrepancy could indeed be due to different substrate-specificities of the two kinases.

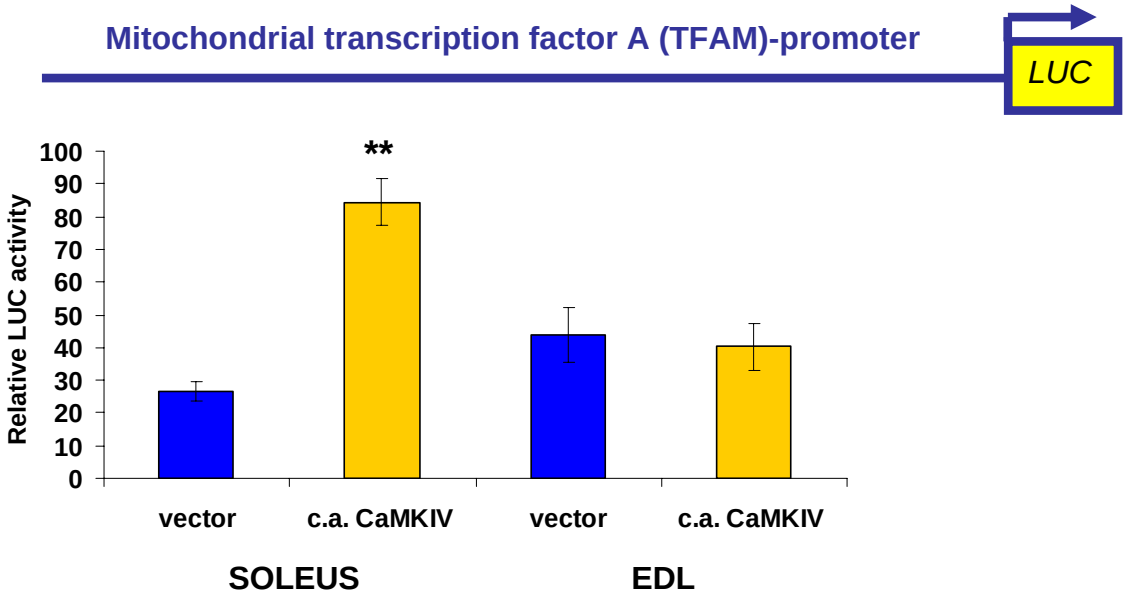


**Fig. 18. CaMK activity upregulates TFAM expression in skeletal muscle.** On the top of the figure a simplified scheme of TFAM-LUC reporter is represented: TFAM promoter controls the expression of the downstream LUC gene. Rat adult soleus and EDL muscles have been transfected with TFAM-LUC sensor along with plasmid containing the gene coding for a constitutively active form (c.a.) of CaMKIV, lacking of its COOH-terminus autoinhibitory domain, or with an empty vector (vector). Co-transfection with Tk-Rn vector has been used to normalize the efficiency of transfection thus obtaining relative LUC activity values. Values are means ± SD of one experiment of five muscles for each type of transfection. ** means statistically difference of relative LUC values between muscles transfected with vector and the construct coding for c.a. CaMKIV, $P < 0.01$.

To confirm the role of CaMKII in regulating muscle mitochondrial metabolism we have also analyzed the effect of KIIN on the expression of the mitochondrial enzyme citrate synthase (CS), using a human CS promoter-luciferase reporter (CS-LUC) (McClelland et al., 2004). CS is a key mitochondrial enzyme which catalyzes the first reaction of the Krebs cycle (Wiegand and Remington, 1986).

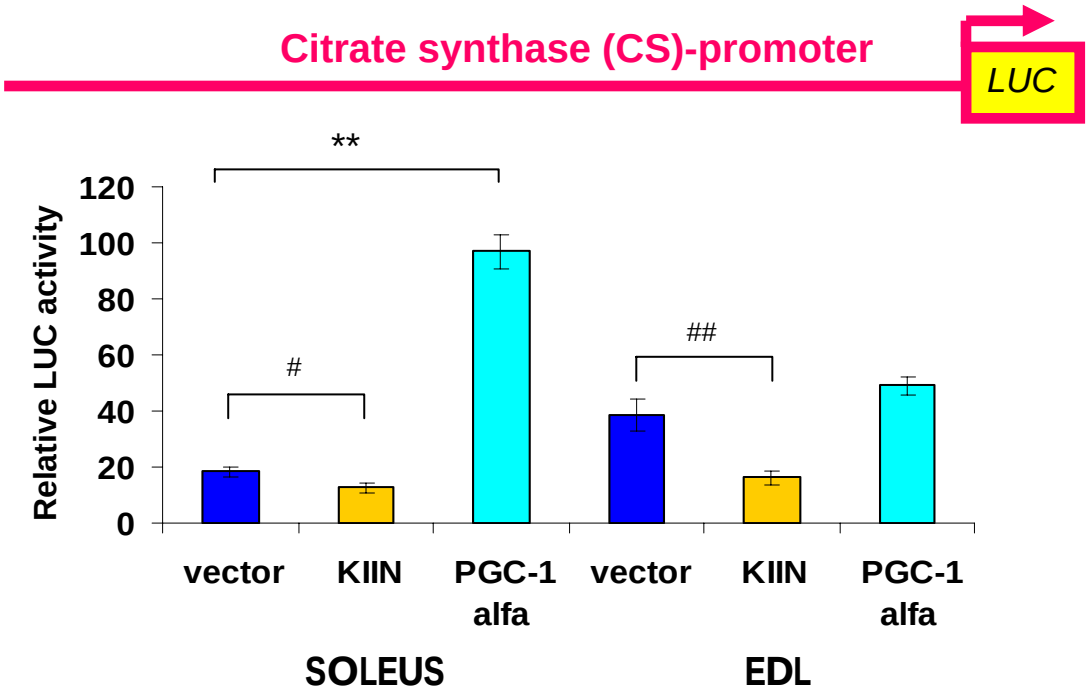


**Fig. 19. CaMKII positively regulates CS expression.** On the top of the figure a simplified scheme of CS-LUC reporter is represented: CS promoter controls the expression of the downstream LUC gene. Rat adult soleus and EDL muscles have been transfected with CS-LUC sensor along with plasmid containing the gene coding for KIIN (KIIN) or PCG-1α (PGC-1 alfa) or with an empty vector (vector). Co-transfection with Tk-Rn vector, has been used to normalize the efficiency of transfection thus obtaining relative LUC activity values. Values are means ± SD of one experiment of five muscles for each type of transfection. ** means statistically difference of relative LUC values between muscles transfected with vector and PGC-1 alfa, $P < 0.05$; # and ## mean statistically difference of relative LUC values between muscles transfected with vector and KIIN, $P < 0.05$ and $P < 0.01$ respectively.

CaMKII inhibition by KIIN potently decreases CS-LUC activity, thus confirming the results obtained with TFAM. Surprisingly again, the relative LUC activity of CS reporter is higher in EDL than soleus muscle (Fig. 19). To confirm that the transcriptional control of the CS-LUC resembles that of the endogenous CS gene under our experimental conditions, we have used as a proof of principle the transcriptional coactivator PPARgamma coactivator-1alpha (PGC-1α), which is a well known stimulator of mitochondrial biogenesis and OXPHOS genes (Wu et al., 1999). Intriguingly, PGC-1α

strongly activates the promoter activity in soleus but not EDL muscles (Fig. 19). It is known that PGC-1α docks to specific transcription factors, such as nuclear respiratory factor (NRF) -1 and -2 (Wu et al., 1999), which in turn activate TFAM. NRFs are known to control the expression of other nuclear genes, i.e. encoding respiratory chain subunits and other mitochondrial proteins. Together with the results obtained with TFAM-LUC and c.a. CaMKIV, these data suggest that a component of the PGC-1α/NRF-dependent transcriptional complex, present in soleus but not in EDL, could be a target of CaMKII signalling.

Mitochondrial content can be easily visualized with a histochemical staining for succinate dehydrogenase (SDH), one of the Krebs cycle enzymes and a member of the electron transport chain (Rustin et al., 2002). In skeletal muscle, SDH staining labels strongly slow and 2A fibers, which are reachest in mitochondria. To assess whether PGC-1α is working correctly on mitochondrial biogenesis in our conditions, we have analyzed PGC 1α -transfected muscle fibers by SDH staining. To recognize transfected PGC-1α we have performed an immunohistochemical staining on serial sections using an antibody against the FLAG molecular tag which is fused to PCG-1α. We have further stained serial sections with antibodies specific for MyHC-slow, which stain slow muscle fibers, and for MyHC-2A which stain 2A muscle fibers. Indeed, the results in Fig. 19 could also be explained by a failure to overexpress PGC-1α in EDL; this, however, is not the case. As shown in Fig. 20, fibers positive for PGC-1α show the expected nuclear localization of this coactivator and invariably show a dark SDH staining, i.e. an elevated mitochondrial content, both in soleus and in EDL. Interestingly, not only slow and 2A fibers transfected with PGC-1α are reach in mitochondria, but also fibers which are negative for both antibodies and could be either 2X or glycolytic 2B. In synthesis, our SDH results suggest that PGC-1α increases mitochondrial biogenesis in slow as well as in fast fibers under our experimental conditions, as previously shown (Wu et al., 2002). We thus cannot explain at present the lack of effect of PGC-1α on CS-LUC in EDL.

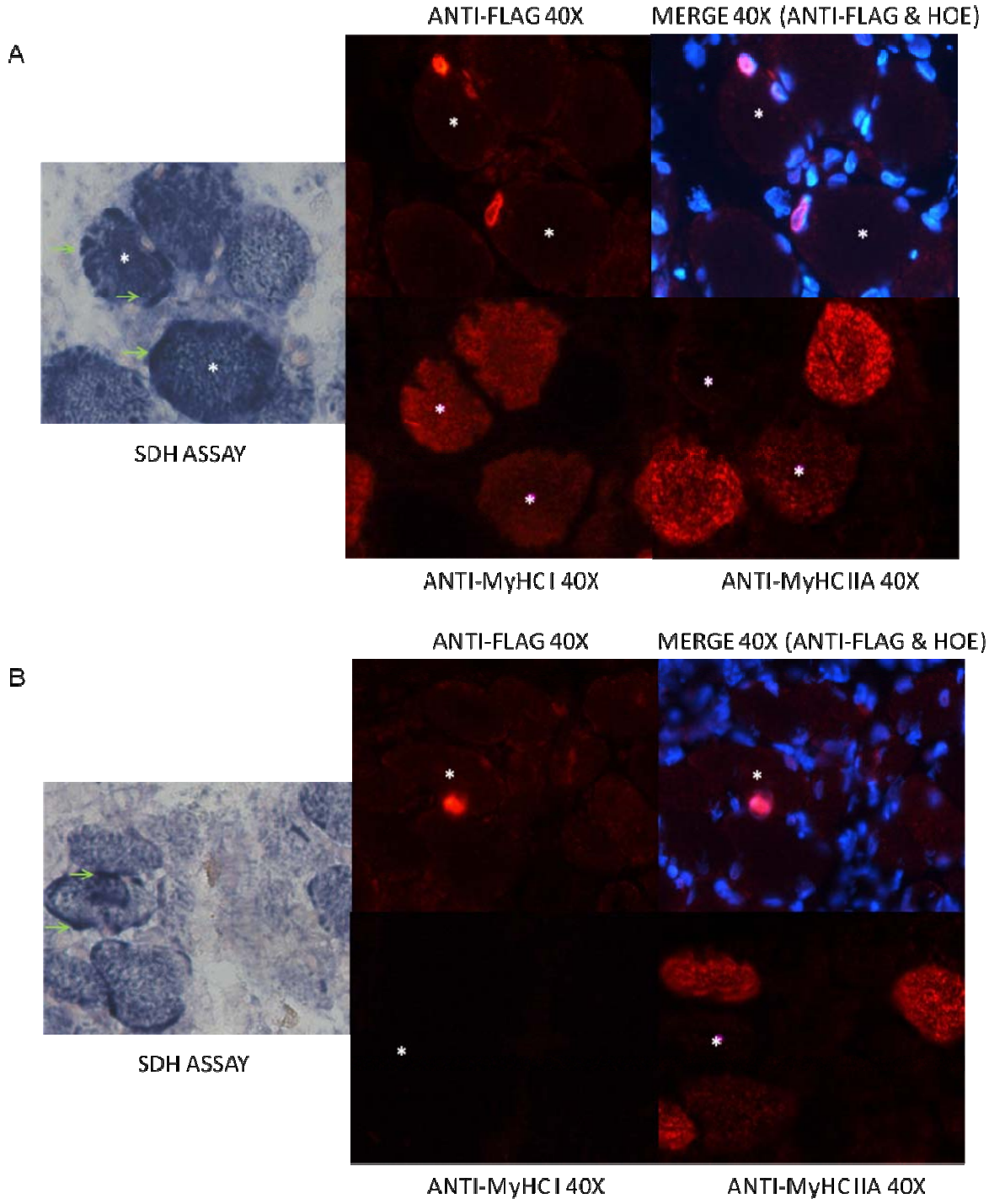


**Fig. 20. Muscle fibers transfected with PGC-1α have an increased SDH activity.** Rat adult soleus (A) and EDL (B) muscles have been transfected with the construct coding for PGC-1α tagged with an N-terminal FLAG. On the left of each group of figures the results of the SDH assay are showed using a 40X magnification. Immunohistochemistries were performed with three different antibodies: anti-FLAG, anti-MyHC I and IIA (40X magnification). On the upper part and on the right of each group of figures merge between the anti-FLAG immunehistochemistry and the fluorescent staining with Hoechst dye (merge anti-FLAG & Hoe) is showed (40X magnification). The green arrows indicate some clusters of subsarcolemma mitochondria which result dark stained by SDH assay in PGC-1α transfected fibers.

Since both CaMKII and Cn contribute to the transcriptional control of GLUT4, we asked whether Cn also plays a role, together with CaMKII, in mitochondrial metabolism. Muscles transfected with myc-Cain show a reduced CS-LUC activity in soleus but not EDL muscle (Fig. 21). It remains to be established whether inhibition of both CaMKII and Cn has a cumulative effect on the downregulation of CS-LUC, as seen with GLUT4-LUC (Fig. 16).

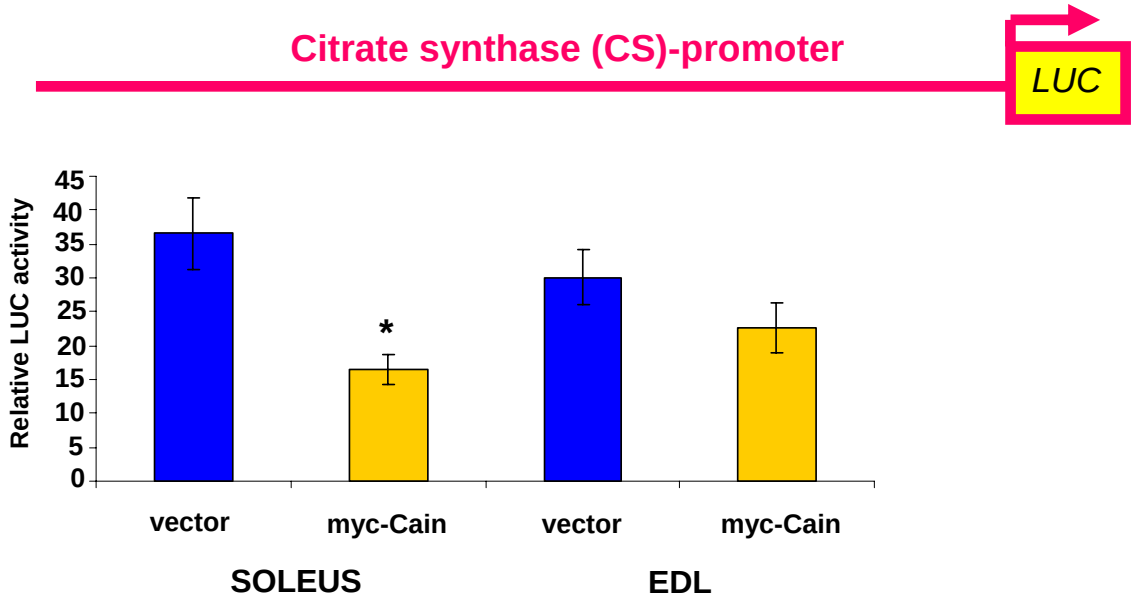


**Fig. 21. Myc-Cain inhibits CS expression in soleus muscle.** Rat adult soleus and EDL muscles have been transfected with CS-LUC sensor along with plasmid containing the gene coding for myc-Cain (Cain) or an empty vector (vector). Co-transfection with Tk-Rn vector has beeen used to normalize the efficiency of transfection thus obtaining relative LUC activity values. Values are means ± SD of one experiment of five muscles for each type of transfection. * means statistically difference of relative LUC values between muscles transfected with vector and Cain. $P < 0.05$.

### *3.6 MyHC expression is regulated by CaMKII in skeletal muscle*

The fact that CaMKII and Cn control together the expression of metabolic genes such as GLUT4 and CS has prompted us to verify whether these signalling pathways also collaborate in the control of other fundamental muscle genes, such as those of contractile proteins. It has been shown that Cn is a mediator of slow nerve activity and directs the transcription of MyHC-slow. (McCullagh et al., 2004; Serrano et al., 2000). We asked whether CaMKII also regulates MyHC gene expression. We have thus co-transfected the MyHC-slow promoter driving the expression of luciferase (MyHC-LUC) (Hasegawa et al., 1997; Ojamaa et al., 1996), with the plasmid coding for KIIN. Fig. 22 shows that

expression of MyHC-slow is also controlled by CaMKII, since KIIN strongly inhibits MyHC promoter activity in soleus. No inhibition can be seen in EDL, however the expression of MyHC-slow is so low in this fast muscle that it can be considered very close to background level.

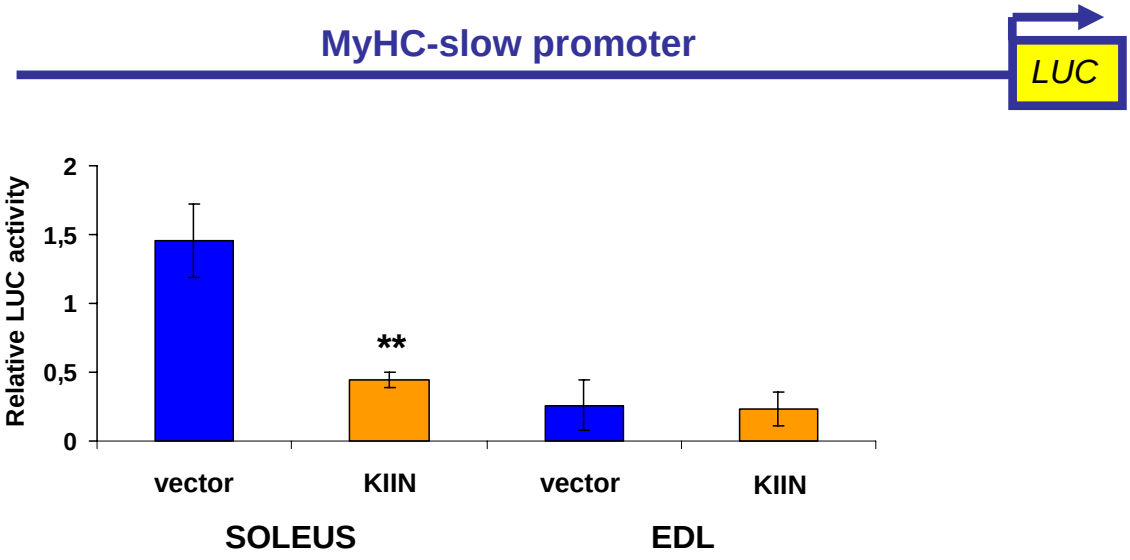


**Fig. 22. CaMKII is implicated in the control of muscle fiber-type in skeletal muscle.** On the top of the figure a simplified scheme of MyHC slow-LUC reporter is represented: the rat MyHC slow- promoter controls the expression of the downstream LUC gene. Rat adult soleus and EDL muscles have been transfected with MyHC slow-LUC sensor along with plasmid containing the gene coding for KIIN (KIIN construct) or with an empty vector (vector). Co-transfection with Tk-Rn vector has been used to normalize the efficiency of transfection thus obtaining relative LUC activity values. Values are means ± SD of one experiment of five muscles for each type of transfection. ** means statistically difference of relative LUC values between muscles transfected with vector and KIIN construct , $P < 0.01$.

Besides promoting the slow fiber program by affecting the activity of MyHC-slow gene, Cn-NFAT signalling is also known to control the expression of MyHC-2A in myotubes (Allen and Leinwand, 2002) and to transcriptionally inhibit MyHC-2B expression in adult skeletal muscle (McCullagh et al., 2004; Serrano et al., 2001). We have investigated a possible role of CaMKII in the control of MyHC-2B. Our results indicate that KIIN strongly downregulates the activity of the MyHC-2B LUC promoter in EDL muscle (Fig. 23). These data suggest that CaMKII is a possible positive regulator of MyHC-2B expression.

It has not yet been elucidated how CaMKII can influence MyHC gene expression. Bioinformatic analysis shows the existence of more than one MEF2 and cAMP response element (CRE) binding sites on the MyHC-slow and -2B promoters, which bind

transcription factors that are known targets of CaMKII. Further studies are needed to elucidate if CaMKII controls the expression of distinct MyHCs by acting on different combinations of transcription factors, such as MEF2 or CRE binding protein (CREB). Considering that CaMKII is a frequency decoder in brain (De Koninck and Schulman, 1998) it cannot be excluded that different MyHC expression programs are determined by diverse levels of CaMKII activity, according to $Ca^{2+}$ transients resulting from motor neuron activity. In fact it is known that neuronal firing pattern plays a prominent role in determining the pattern of genes expressed in skeletal muscles (Bassel-Duby and Olson, 2006; Schiaffino et al., 2007).

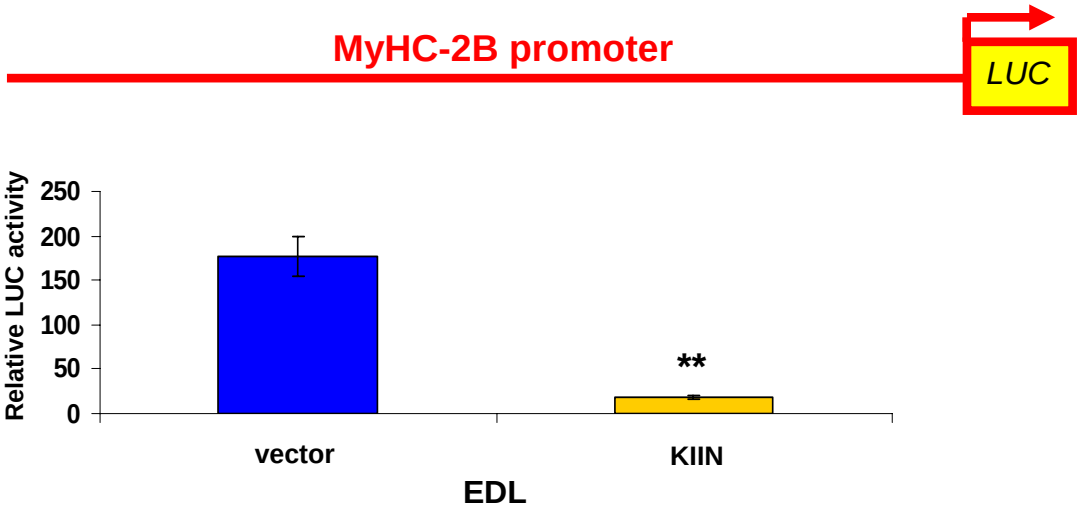


**Fig. 23. CaMKII is implicated in the control of muscle fiber type in skeletal muscle.** On the top of the figure a simplified scheme of MyHC 2B-LUC reporter is represented: the rat MyHC 2B- promoter controls the expression of the downstream LUC gene. Rat adult EDL muscles have been transfected with MyHC 2B-LUC sensor along with plasmid containing the gene coding for KIIN (KIIN construct) or with an empty vector (vector). Co-transfection with Tk-Rn vector has been used to normalize the efficiency of transfection thus obtaining relative LUC activity values. Values are means ± SD of one experiment of five muscles for each type of transfection. ** means statistically difference of relative LUC values between muscles transfected with vector and KIIN construct, $P < 0.01$.

# 4. DISCUSSION

### *4.1 CaMKII and Cn in excitation-transcription coupling: role of MEF2*

The molecular mechanisms underlying excitation-transcription coupling are not yet fully understood. We have investigated whether Cn and CaMKII can affect the expression of activity-dependent genes involved in muscle metabolism or fiber-type specification. To this purpose we have used a loss-of-function approach consisting in the transfection of vectors, coding for CaMKII and Cn peptide inhibitors, together with Luciferase (LUC) reporters in rat skeletal muscle, through *in vivo* electroporation. Using this technique it is possible to measure changes in transcriptional activity induced by alterations, in our case inhibition, of CaMKII and Cn signalling.

The *glut4* gene encodes the major glucose transporter (GLUT4) which mediates exercise- and insulin-stimulated glucose transport in skeletal muscle (Goodyear and Khan, 1998; Hayashi et al., 1997). We have shown, by transfection of a peptide inhibitor (KIIN) specific for CaMKII and functional in skeletal muscle (Fig. 13), that CaMKII inhibition downregulates GLUT4-LUC reporter activity in soleus and EDL muscles (Fig. 14). Previous data have shown that GLUT4 expression is controlled by CaMK activity stimulated by caffeine in L6 rat myotubes and by contraction in epitrochlearis muscle (Ojuka et al., 2002; Wright et al, 2004). However, those studies were based on the pharmacological inhibitors KN62/KN93, which block all multifunction CaMKs. Our data with a specific CaMKII inhibitor indicate that this is due indeed to CaMKII and not to other CaMK family members. Taken together, our and Ojuka and Wright's data, support a role for CaMKII in mediating $Ca^{2+}$-induced increase of GLUT4 expression in skeletal muscle.

CaMKII signalling is known to regulate MEF2 transcriptional activity by phosphorylating HDACs, which then move out from the nucleus and relieve their inhibition on MEF2 (Liu et al., 2005). Thus when CaMKII is inhibited by KIIN, MEF2 transcriptional activity is blocked. GLUT4 enhancer sequence harbours a MEF2 binding site, whose integrity is fundamental for the enhancer activity (Moreno et al., 2003). Therefore, the decrease in GLUT4 LUC activity is likely to be due to MEF2 transcriptional inhibition caused by CaMKII inhibition and, therefore, deficient phosphorylation on HDACs. There are other indications which suggest that MEF2 transcriptional activity plays

an important role in GLUT4 expression: electrophoretic mobility shift assay (EMSA) has revealed that MEF2 binds as an heterodimer on human and rat GLUT4 promoters (Liu et al., 1994; Mora and Pessin, 2000; Thai et al., 1998) and that the amount of bound MEF2 increases with electrical stimulation or exercise (McGee et al., 2005; Smith et al., 2006). GLUT4 mRNA has been found to increase during exercise (Smith et al., 2006). This event is dependent on CaMK activity because myotubes expressing c.a. CaMKIV have an increase in MEF2 binding on GLUT4 promoter, while dominant negative CaMKIV does not cause any increase (Smith et al., 2006). A recent paper has shown that treatment with caffeine of C2C12 myotubes, increases MEF2 expression, binding of MEF2 to the GLUT4 promoter and GLUT4 mRNA content. It also causes hyperacetylation of MEF2 site on GLUT4 promoter, another possible mechanism to increase GLUT4 expression. CaMK inhibition by KN93, a general CaMK inhibitor, abolishes all the caffeine-induced changes (Mukwevho et al., 2008). Thus, also caffeine-stimulated acetylation of MEF2 binding site seems to promote GLUT4 expression *via* a mechanism that involves CaMK.

CaMKII is the major CaMK in skeletal muscle (Rose and Hargreaves, 2003) and probably mediates most $Ca^{2+}$-stimulated effects on GLUT4 expression. Other groups have previously reported that CaMK phosphorylates HDAC and causes their nuclear export (Backs et al., 2008; Liu et al., 2005), which has also been found in response to caffeine (Mukwevho et al., 2008). Thus it is conceivable that, once HDAC is exported from the nucleus to the cytoplasm, nuclear HAT activity increases, promoting chromatin relaxation and consequently increased accessibility of MEF2 factors to their DNA binding sites. Nonetheless, HDAC cellular localization does not constitute the unique mechanism to regulate MEF2 transcriptional activity. HDAC activity has been shown to be regulated through ubiquitination (Potthoff et al., 2007). In addition McGee and Hargreaves (2004) have demonstrated that in human exercised skeletal muscles HDAC association with MEF2 decreases but also that exercise increases PGC-1α and p38 MAPK association with MEF2, thus suggesting that other signalling pathways, besides HDAC, can control MEF2-dependent transcription. In C2C12 myotubes MEF2 phosphorylation status has been shown to be also regulated by Cn, which can stimulate MEF2-dependent transcription by dephosphorylating MEF2 (Wu et al., 2000).

Indeed, CaMKII is not the only $Ca^{2+}$-dependent signal transducer implicated in the control of GLUT4 expression in skeletal muscle. Our data indicate that Cn inhibition

decreases the LUC activity of GLUT4 enhancer reporter (Fig. 15A). NFAT, a known Cn downstream target, is an effector of Cn signalling in the control of GLUT4 expression, since NFAT inhibition diminishes GLUT4 enhancer activity (Fig. 15B). Our results are in agreement with another finding in which transgenic mice overexpressing c.a. Cn show an increased content of GLUT4 protein in skeletal muscle (Ryder et al., 2003). Thus a new role of Cn in muscle metabolism is emerging besides the known role as a master regulator of slow fiber-type program in skeletal muscle (McCullagh et al., 2004; Serrano et al., 2001).

As MEF2-dependent transcription can be regulated by CaMKII and Cn-NFAT pathways, we have asked if these pathways can synergistically control GLUT4 expression. We have shown that GLUT4 enhancer reporter activity is lower when CaMKII and Cn inhibitors are co-transfected in the same muscle than when they are independently transfected (Fig. 16). We hypothesize that MEF2 could be the common target of both pathways for the induction of GLUT4 expression. In this extent, our data indicate that Cain can decrease the activity of MEF2-LUC sensor in skeletal muscle (data not shown). In particular Cn has been demonstrated to affect MEF2-dependent transcription in different ways: 1) Cn can directly act on MEF2 and maintain it in a hyposphorylated state (Wu et al., 2000), which enhances the DNA binding affinity of MEF2 (Mao and Wiedmann, 1999) and also protects it from caspase-mediated cleavage (Li et al., 2001); 2) Cn can also influence MEF2-mediated gene expression indirectly by regulating the subcellular localization of NFAT transcription factors which act as MEF2 co-activators by synergistically recruiting p300/CBP (Garcia-Rodriguez and Rao, 1998; Youn et al., 2000). Our results seem to favour the latter mechanism, since the inhibition of NFAT causes a great reduction of GLUT4 enh sensor activity (Fig. 15B) and this decrease is even greater when CaMKII and Cn-NFAT pathways are both inhibited (Fig. 16).

However a residual activity of GLUT4 enh sensor persists even when both Cn-NFAT and CaMKII pathways are blocked. We postulate that other pathways can stimulate GLUT4 expression independent of CaMKII and Cn pathways. A good candidate could be AMP-activated protein kinase (AMPK), as in human primary myotubes HDAC phosphorylation by AMPK has been found to increase MEF2 transcriptional activity on GLUT4 promoter (McGee et al., 2008). Another work reinforces the hypothesis by showing that AMPK activation induces expression of GLUT4 which occurs in parallel

with an increased expression of MEF2 (Ojuka at al., 2002). Moreover, transgenic mice with muscle-specific expression of kinase-dead AMPK$\alpha_2$ transfected with KIIN or Cain show a further reduction in GLUT4-LUC activity in soleus muscle with respect to control wild-type AMPK mice (data not shown). Therefore AMPK can constitute another pathway that enhances GLUT4 expression probably *via* MEF2 regulation. In addition other HDAC kinases probably impinge on MEF2 transcriptional regulation, like PKD (Kim et al., 2008) and SIK (Berdeaux et al., 2007).

### *4.2 Regulation of fiber metabolism*

Another adaptation of exercised skeletal muscle is an increased expression of enzymes of oxidative metabolism. In order to face physiological demands, as for example during prolonged activity, skeletal muscle remodelling elicits a more oxidative, muscle program, which is far more efficient than aerobic glycolysis, typical of fast fibers. Besides inducing the slow fiber gene program, Cn has been known to exert profound effects on the metabolic properties of skeletal muscle. Constitutively active Cn overexpressed in transgenic mice causes an increase in GLUT4 level, concomitant with elevated insulin-stimulated skeletal muscle glucose uptake (Ryder et al., 2003). Apart from these effects, activation of Cn in skeletal muscle increases protein content of an important transcriptional regulator, PGC-1α, which affects lipid and mitochondrial metabolic gene expression (Long et al., 2007).

Consistent with a role of Cn in the regulation of genes involved in mitochondrial metabolism, we have shown that the inhibition of this $Ca^{2+}$-dependent phosphatase provokes a strong decrease in citrate synthase (CS) expression in skeletal muscle (Fig. 21). These data indicate that Cn could play a role in muscle fiber metabolism by controlling the expression of some important metabolic genes, such as GLUT4 and CS. Cn activity could thus couple $Ca^{2+}$-dependent transcription of contractile and metabolic genes in muscle fibers.

The same conclusions can be derived for CaMKII, since our data indicate that its activity positively controls the expression of metabolic and contractile genes (Fig. 14, 17, 19). Concerning muscle metabolism, a positive role for CaMKs in muscle mitochondrial $Ca^{2+}$-dependent gene expression has first emerged from studies in L6 rat myoblasts using $Ca^{2+}$ ionophores, including A-23187 (Freyssenet et al., 1999), ionomycin (Ojuka et al.,

2003) and caffeine (Ojuka et al., 2002). KN93, a CaMK inhibitor, completely blocks the effects of caffeine. Our *in vivo* data confirm that CaMKII regulates CS expression in skeletal muscle (Fig. 19). We have also shown that PGC-1α increases CS expression (Fig. 19). Furthermore this effect seems to be fiber-specific, since PGC-1α increases CS-LUC activity only in soleus muscles. We hypothesize that CaMKII can control CS expression through PGC-1α, at least in soleus. Considering that the PGC-1α promoter harbours MEF2 sites (Lin et al., 2005), CaMKII could control PGC-1α expression *via* HDAC phosphorylation. The result may be a feed-forward loop for the regulation of GLUT4 expression, since MEF2 binds to GLUT4 and PGC-1α is known to be one of its transcriptional co-activators (Czubryt et al., 2003). It remains to be explained how CaMKII can control CS expression in a fiber-type dependent manner. We hypothesize that PGC-1α may complex with a factor which is present and used in soleus but not in EDL muscle.

PGC-1α is known to be the master regulator of muscle mitochondrial biogenesis (Wu et al., 1999), as shown also in our SDH stainings (Fig. 20A,B). Muscle fibers transfected with a PGC-1α expression vector have a dark staining, indicating the presence of a high mitochondrial content in transfected fibers. Mitochondrial biogenesis requires the concerted expression of genes encoded by mitochondrial and nuclear genome. TFAM (or mTFA) is a nuclear encoded transcription factor which plays a fundamental role in mitochondrial DNA transcription and replication (Shadel and Clayton, 1997). It has been shown that TFAM expression is induced by PGC-1α co-activation of NRF in mitochondrial biogenesis (Wu et al., 1999). Our results indicate that CaMKII may have a role in this process, since KIIN potently inhibits TFAM sensor activity (Fig. 17). Unexpectedly, in our hands, TFAM-LUC activity seems to be equal in soleus and EDL muscles (Fig. 17) although slow muscles are known to have more mitochondria (Lin et al., 2002). To date, we do not have an explanation for this discrepancy.

CaMK involvement in the control of TFAM expression has been confirmed by co-trasfection of TFAM-LUC reporter together with c.a. CaMKIV. This multifunctional CaMK has many substrates in common with CaMKII (Bito et al., 1996; Karanboulas et al., 2006; Liu et al., 2005; Sun et al., 2004) and it is functional in skeletal muscle when ectopically expressed (Wu et al., 1999), even if it is not normally present in skeletal muscle (Rose and Hargreaves, 2003). We have shown that the c.a. form of CaMKIV strongly increases TFAM-LUC sensor activity, only in soleus muscle (Fig. 18). As in the case of CS

reporter, the fiber-type effect can be due to a CaMK target factor which is rate-limiting in EDL but not in soleus muscle. There is also the possibility that CaMKII and CaMKIV do not have overlapping targets in soleus and EDL.

Taken together, our data with TFAM and CS sensors indicate that CaMKII can play an important role in muscle mitochondrial metabolism and biogenesis. The latter needs DNA replication and a coordinated expression of nuclear and mitochondrial genes. Previous work has shown that caffeine-induced CaMK activity provides the initiating signal leading to mitochondrial biogenesis. Indeed, KN93, a general CaMK inhibitor, blocks caffeine-induced increase in TFAM, PGC-1α, NRF-1 and NRF-2 in L6 myotubes (Ojuka et al., 2003). These adaptations are critical during exercise, such as prolonged running or swimming, because they induce an increase in mitochondria which results in an enhanced capacity to generate ATP via oxidative phosphorylation (Wright, 2007).

### *4.3 Regulation of contractile phenotype*

Exercise can induce an increase in GLUT4 expression (McGee and Hargreaves, 2004; Smith et al., 2006) but also changes in muscle fiber-type. A principle of muscle biology, indeed, is that motor neuron activity has a prominent role in the regulation of fiber-specific gene programs (Pette and Staron, 2001; Pette and Vrbova, 1992). A pattern of electrostimulation which resembles that of slow motor units has been proposed to induce slow but sustained levels of intracellular $Ca^{2+}$, which is the neural activity pattern proposed to activate Cn (Bassel-Duby and Olson, 2006; Chin et al., 1998). Several lines of evidence support Cn as an activator of the slow fiber program, by inducing NFAT translocation from the cytoplasm into the nucleus (Crabtree and Olson 2002; Zhu and McKeon, 1999), where it binds to consensus DNA sequences of gene promoter region, as well as to other transcription factors, including MEF2, for the activation of its target genes (Chin et al., 1998; Youn et al., 2000).

A defined sequence of fiber-type switches occurs in response to activity. Fiber transformations from type IIB to IIX and IIA have been detected as an exercise-induced adaptation, whereas transitions to type I are rare (Pette and Staron, 1997,2001). On the other hand, inactivity, such as after experimental denervation or in spinal cord injury, causes loss of slow fibers and transitions toward a fast IIX-IIB phenotype (Butler-Browne et al., 1982).

The molecular mechanisms underlying these adaptations have not been precisely defined. Cn-NFAT has emerged as key regulator for the establishment and maintenance of slow fiber program (McCullagh et al., 2004), Interestingly, transgenic mice, expressing a c.a form of CaMKIV in skeletal muscle also exhibit an increased mitochondrial biogenesis as well as an augmented expression of type I fiber gene, determining a switch toward a slower fiber phenotype (Wu et al., 2002). Since CaMKII has been identified as the major CaMKII in skeletal muscle (Rose and Hargreaves, 2003) and CaMKIV, as previously mentioned, is not expressed in adult muscle fibers, we have asked whether CaMKII plays a role in muscle fiber-type specification. Our results show indeed that CaMKII inhibition downregulates MyHC-slow expression in skeletal muscle (Fig. 22). The fact that a CaMK family kinase controls MyHC expression explaines the results obtained with the CaMKIV transgenics and is in agreement with results obtained in cultured muscle fibers treated with a pharmacological CaMK general inhibitor (Mu et al., 2007).

Taken together, these and our group's previous results (McCullagh et al., 2004; Serrano et al., 2001) show that CaMKII and Cn pathways contribute to fiber transformation. At present a question remains open as to whether these $Ca^{2+}$-dependent pathways act independently or synergistically. Mu et al. (2007) hypothesize that the pathways are independent since inhibition of one pathway has different effects in terms of patterns of gene expression. In addition they show an incomplete fast-to-slow fiber-type transformation when CaMKII or Cn are inhibited, thus considering the possible involvement of some other signalling pathways. In contrast MEF2 transcriptional activity has been found to be enhanced when CaMKIV and c.a. Cn are cotransfected in C2C12 muscle cells, whereas they are less effective alone (Wu et al., 2000). It has been shown that MEF2 transcriptional activity can be stimulated by Cn either directly, by dephosphorylation of MEF2 (Wu et al., 2000), or indirectly by the association of active NFAT with MEF2 bound to DNA (McKinsey et al., 2002). In this extent, NFAT synergizes with MEF2 to recruit co-activators with intrinsic HAT activity, such as p300, in order to promote transcription of genes involved T-lymphocyte apoptosis (Youn et al., 2000; McKinsey et al., 2002). Upon Cn dephosphorylation, the only NFAT family member, which seems to associate with MEF2 (-A and -D), after nuclear translocation, is NFATc2 (Blaeser et al., 2000). Functional cooperation between NFAT and MEF2 has also been demonstrated to play a role in the control of muscle fiber-type (Chin et al., 1998; Wu

et al, 2000). Concerning MEF2, it seems to be more active in the regulation of slow genes, like TnI and MyHC-slow (Chin et al., 1998; Meissner et al., 2006). In this contest, NFATc1 is predominantly nuclear in slow twitch soleus muscle, while it is predominantly cytoplasmic in fast TA muscle. *In vivo* electrical stimulation with a 20 Hz pattern (typical of slow motor neurons), but not with a 100 Hz fast pattern, induces NFATc1 nuclear translocation. NFATc1 goes into the nucleus in a Cn-dependent manner, since the co-transfection of cain abolishes NFATc1 translocation (Tothova et al., 2006)

In the light of these results and of our data obtained by inhibiting both pathways in the same muscles transfected with GLUT4-LUC sensor (Fig. 16), cooperation between CaMK and Cn-NFAT could also be involved in the control of MyHC-slow expression. Interestingly however, CaMKII and Cn pathways seem to positively regulate MyHC-slow (Fig. 22), but to have opposite effects on fast MyHCs. Cn-NFAT promotes the slow fiber program by inducing MyHC-slow expression, but it also represses the fast MyHC-2X and to a minor degree MyHC-2B genes in soleus muscle (McCullagh et al., 2004; Serrano et al., 2001). Likewise, CaMKII positively controls the expression of MyHC-2B in EDL (Fig. 23). We hypothesize that CaMKII is a general regulator of MyHC expression which, unlike calcineurin, is fiber type-independent.

In conclusion these results suggest that cooperation between Cn and CaMKII is mediated by MEF2 and NFAT binding sites, which are both present in the promoter sequence of MyHC-slow (Meissner et al., 2006) and of the other promoter-reporter constructs we have used. MEF2 and NFAT have been shown to synergistically interact to recruit HAT co-factors, such as p300 and CBP (Garcia-Rodriguez and Rao, 1998; Youn et al., 2000). In the case of the short GLUT4 enhancer, which contains a MEF2 site but no NFAT site and is inhibited by cain, we hypothesize that the cooperation between MEF2 and NFAT occurs by protein-protein interaction at the MEF2 site. Furthermore, we cannot rule out that cooperation between CaMKII and Cn could occur through another CaMKII downstream target. CREB is phosphrorylated by CaMKIV on Ser 133 (Sheng et al., 1991) and by CaMKII on Ser 133 and 142. CaMKII phosphorylation on the latter Ser has a predominant inhibitory effect on CREB transcriptional activity, even if CaMK phosphorylation on Ser 133 has an activating effect (Sun et al., 1994). Dimerization of CREB is blocked by CaMKII phosphorylation, which prevents the recruitment of the critical CREB co-activator (CBP; Wu and McMurray, 2001). Recently we have found that

a NFAT sensor, harbouring 9 NFAT binding sites, shows a reduced LUC activity when CaMKII is inhibited (data not shown). In osteoclast precursors, CaMK-CREB pathway enhances NFATc1 induction. Moreover after Cn-mediated dephosphorylation of NFATc1, the latter translocates into the nucleus and interacts with phosphorylated CREB to induce osteoclast gene transcription during the late phase of differentiation (Sato et al., 2006). Therefore, osteoclast differentiation represents an example of functional collaboration between CaMK and Cn pathways. The mechanisms of interaction between CREB and NFAT remain to be elucidated. CBP may represent a possible explanation given that it is able to interact with both transcription factors (Garcia-Rodriguez et al., 1998; Wu and McMurray, 2001).

# 5. CONCLUDING REMARKS

Understanding the signalling pathways that control myofiber remodelling could be important to identify therapeutic targets for the treatment of various human diseases. The incidence of Type 2 (T2D) diabetes is growing at epidemic proportions. Subjects with insulin resistance or T2D diabetes display a reduced percentage of slow oxidative muscle fibers (Marin et al., 1994; Nyholm et al., 1997). Oxidative type I fibers express the highest level of GLUT4 (Daugaard et al., 2000; Kong et al., 1994) and they are rich in mitochondria together with type II A fibers (Schiaffino et al., 2007; Fig. 20A,B). Thus, a positive correlation exists between fiber-type, mitochondrial content and GLUT4 expression. It should be kept in mind, however, that fiber-type and muscle metabolism can be regulated independently (Rockl et al., 2008).

Large body of evidence indicates that exercise training is an effective therapeutic intervention to increase insulin sensivity in obese and insulin-resistant individuals (Hawley and Lessard 2008) and that exercise, therefore, has beneficial effects for the prevention or delay of T2D diabetes. Indeed, exercise can improve glucose tolerance in insulin-resistant patients because contractile activity can stimulate glucose transport through an insulin-independent mechanism.

Contractile activity causes a prolonged effect on glucose tolerance by inducing changes in protein involved in glucose uptake. Sarcolemmal $Ca^{2+}$ increase, caused by motor neuron activity or mimicked by $Ca^{2+}$-releasing agents, has been found to increase GLUT4 mRNA and protein levels in skeletal muscle (Hawley and Lessard, 2008; Ojuka et al., 2002; Smith et al., 2006). $Ca^{2+}$-induced effects on GLUT4 expression seem to be mediated by two separate pathways, AMPK and CaMK (Ojuka et al., 2002; Wright et al., 2004). Our data show that CaMKII is the CaMK isoform implicated in the regulation of GLUT4 expression (Fig. 14). Our results also suggest that another $Ca^{2+}$-dependent pathway may control GLUT4 expression, namely the Cn-NFAT pathway (Fig. 15), probably synergizing with CaMKII to control GLUT4 gene induction (Fig. 16).

MEF2 seems to be the downstream effector of CaMK and AMPK signalling, by binding to the GLUT4 promoter and inducing an increase of GLUT4 content in skeletal muscle cells (Ojuka et al., 2002). Indeed, MEF2 transcriptional activity has a prominent role in GLUT4 expression (McGee et al., 2005; Smith et al., 2006). As we have shown (Fig. 15B), the downstream target of Cn is likely to be NFAT. It cannot be excluded that,

once dephosphorylated by Cn, NFAT can co-activate MEF2 by the recruitment of HAT co-activators (Garcia-Rodriguez and Rao, 1998; Youn et al., 2000).

MEF2 is controlled in a very complex manner and is a nodal point of multiple signalling pathways. Being a transcription factor implicated in a variety of critical processes in skeletal muscle (McKinsey et al., 2002), the knowledge of the molecular pathways which control MEF2 transcriptional activity is fundamental for muscle physiopathology. In this regard, alterations of MEF2 transcriptional activity, through interference with HDAC phosphorylation, results in a dystrophic phenotype (Berdeaux et al., 2007). Insights into the control of MEF2 in muscle may also have implications for T2D, since MEF2 is fundamental for a proper expression of GLUT4 in skeletal muscle. Loss- or gain-of function techniques can give new insights on the molecular mechanisms regulating MEF2 and the interplay between diverse signalling pathways, as shown by our data in the case of GLUT4 regulation by CaMKII and Cn.

Besides the control of GLUT4 expression, CaMKII seems to have also an important role in the regulation of other genes implicated in muscle mitochondrial metabolism, such as CS and TFAM. Strikingly Cn, which has been widely studied in the control of muscle fiber-type (McCullagh ety al., 2004; Serrano et al., 2001), plays a role in the control of CS expression as well. Since the CS promoter harbours MEF2 and NFAT binding sites, a synergy between CaMKII and Cn-NFAT pathways can be envisaged. In addition, NFAT could alternatively interact with MEF2 by direct protein-protein interaction without binding to DNA, as shown (Youn et al., 2000) and also suggested by our data with the GLUT4 enhancer.

Interestingly, our data show that CaMKII also affects the contractile properties of muscle fibers, by controlling the expression of MyHC-slow and MyHC-2B. Thus CaMKII emerges as a general regulator of muscle MyHC expression, in partial contrast with Cn-NFAT which is specifically associated to a slow muscle gene program (McCullagh et al., 2004; Serrano et al., 2001). It remains to be established whether CaMKII, which is an important frequency decoder in brain (De Koninck and Schulman, 1998), regulates MyHC expression in an activity-dependent manner.

All genes we have studied are known to be $Ca^{2+}$-dependent genes (Ojuka et al., 2003; Pette and Staron, 2001; Pilegaard et al., 2003). The knowledge of the molecular mechanisms, which act during physiological activity conditions in skeletal muscle are

fundamental to detect the molecular effectors that determine phenotypic muscle adaptations. One of the most dramatic phenotypic alterations occurs in mitochondria in response to exercise or chronic contractile activity (Hood et al., 2006). These adaptations are highly specific and dependent upon type of exercise (i.e. endurance vs strength training) as well as its frequency, intensity and duration. In this context, CaMKII and Cn may represent two main pathways which transduce $Ca^{2+}$ signals into specific changes in the expression of metabolic and contractile genes in skeletal muscle. Further investigations are needed to understand the precise molecular mechanisms underlying activity-dependent gene regulation and the complex cross-talk between CaMKII, Cn and other signalling pathways in the definition of muscle phenotype and performances.

# 6. REFERENCES


Akimoto T, Li P, Yan Z. (2008) Functional interaction of regulatory factors with the Pgc-1alpha promoter in response to exercise by in vivo imaging. Am J Physiol Cell Physiol 295, 288-292.

Allen DL and Leinwand LA. (2002) Intracellular calcium and myosin isoform transitions. Calcineurin and calcium-calmodulin kinase pathways regulate preferential activation of the IIa myosin heavy chain promoter. J Biol Chem 277, 45323-45330.

Allen DL, Sartorius CA, Sycuro LK, Leinwand LA. (2001) Different pathways regulate expression of the skeletal myosin heavy chain genes. J Biol Chem 276, 43524-43533.

Anderson KA, Means RL, Huang QH, Kemp BE, Goldstein EG, Selbert MA, Edelman AM, Fremeau RT, Means AR. (1998) Components of a calmodulin-dependent protein kinase cascade. Molecular cloning, functional characterization and cellular localization of Ca2+/calmodulin-dependent protein kinase kinase beta. J Biol Chem 273, 31880-3189.

Anglister J, Grzesiek S, Wang AC, Ren H, Klee CB, Bax A. (1994) 1H, 13C, 15N nuclear magnetic resonance backbone assignments and secondary structure of human calcineurin B. Biochemistry 33, 3540-3547.

Aramburu J, Garcia-Cozar F, Raghavan A, Okamura H, Rao A, Hogan PG. (1998) Selective inhibition of NFAT activation by a peptide spanning the calcineurin targeting site of NFAT. Mol Cell 1, 627-637.

Aramburu J, Yaffe MB, Lopez-Rodriguez C, Cantley LC, Hogan PG, Rao A. (1999) Affinity-driven peptide selection of an NFAT inhibitor more selective than cyclosporin A. Science 285, 2129-2133.

Ausoni S, Gorza L, Schiaffino S, Gundersen K, Lomo T. (1990) Expression of myosin heavy chain isoforms in stimulated fast and slow rat muscles. J Neurosci 10, 153-160.

Backs J, Backs T, Bezprozvannaya S, McKinsey TA, Olson EN. (2008) Histone deacetylase 5 acquires calcium/calmodulin-dependent kinase II responsiveness by oligomerization with histone deacetylase 4. Mol Cell Biol 28, 3437-3445.

Backs J, Song K, Bezprozvannaya S, Chang S, Olson EN. (2006) CaM kinase II selectively signals to histone deacetylase 4 during cardiomyocyte hypertrophy. J Clin Invest 116, 1853-1864.

Barany M and Close RI. (1971) The transformation of myosin in cross-innervated rat muscles. J Physiol 213, 455-474.

Bassel-Duby R and Olson EN. (2006) Signaling pathways in skeletal muscle remodeling. Annu Rev Biochem 75, 19-37.

Bayer KU and Schulman H. (2001) Regulation of signal transduction by protein targeting: the case for CaMKII. Biochem Byophysic Res Commun 289, 917-23.

Bayer KU, De Koninck P, Schulman H. (2002) Alternative splicing modulates the frequency-dependent response of CaMKII to Ca(2+) oscillations. EMBO J 21, 3590-3597.

Bayer KU, Löhler J, Schulman H, Harbers K. (1999) Developmental expression of the CaM kinase II isoforms: ubiquitous gamma- and delta-CaM kinase II are the early isoforms and most abundant in the developing nervous system. Brain Res Mol Brain Res 70, 147-154.

Berdeaux R, Goebel N, Banaszynski L, Takemori H, Wandless T, Shelton GD, Montminy M. (2007) SIK1 is a class II HDAC kinase that promotes survival of skeletal myocytes. Nat Med 13, 597-603.

Bito H, Deisseroth K, Tsien RW. (1996) CREB phosphorylation and dephosphorylation: a Ca(2+)- and stimulus duration-dependent switch for hippocampal gene expression. Cell 87, 1203-1214.

Black BL and Olson EN. (1998) Transcriptional control of muscle development by myocyte enhancer factor-2 (MEF2) proteins. Annu Rev Cell Dev Biol 14, 167-196.

Blaeser F, Ho N, Prywes R, Chatila TA. (2000) Ca(2+)-dependent gene expression mediated by MEF2 transcription factors. J Biol Chem 275, 197-209.

Braun AP and Schulman H. (1995) The multifunctional calcium/calmodulin-dependent protein kinase: from form to function. Annu Rev Physiol 57, 417-445.

Brocke L, Srinivasan M, Schulman H. (1995) Developmental and regional expression of multifunctional Ca2+/calmodulin-dependent protein kinase isoforms in rat brain. J Neurosci 15, 6797-67808.

Buelow R, O'Hehir RE, Schreifels R, Kummerehl TJ, Riley G, Lamb JR. (1992) Localization of the immunologic activity in the superantigen Staphylococcal enterotoxin B using truncated recombinant fusion proteins. J Immunol 148, 1-6.

Butler-Browne GS, Bugaiski LB, Cuenoud S, Schwartz K, Whalen RG. (1982) Denervation of new born rat muscles does not block the appearance of adult fast myosin heavy chain. Nature 299, 830-833.

Chang BH, Mukherji S, Soderling TR. (1998) Characterization of a calmodulin kinase II inhibitor protein in brain. Proc Natl Acad Sci U S A 95, 10890-10895.

Chin ER, Olson EN, Yang Q, Shelton J, Bassel-Duby R, Williams RS. (1998) A calcineurin-dependent pathway control skeletal muscle fiber type. Genes and Development 12, 2499-2509.

Chin ER. (2004) The role of calcium and calcium/calmodulin-dependent kinases in skeletal muscle plasticity and mitochondrial biogenesis. Proc Nutr Society 63, 279-286.

Chin ER. (2005) Role of Ca2+/calmodulin-dependent kinases in skeletal muscle plasticity. J Appl Physiol 99, 414-423.

Choi YS, Kim S, Kyu Lee H, Lee KU, Pak YK. (2004) In vitro methylation of nuclear respiratory factor-1 binding site suppresses the promoter activity of mitochondrial transcription factor A. Biochem Biophys Res Commun 314, 118-122.

Colbran RJ, Fong YL, Schworer CM, Soderling TR. (1988) Regulatory interactions of the calmodulin-binding, inhibitory, and autophosphorylation domains of Ca2+/calmodulin-dependent protein kinase II. J Biol Chem 263, 18145-18151.

Colbran RJ, Smith MK, Schworer CM, Fong YL, Soderling TR. (1989) Regulatory domain of calcium/calmodulin-dependent protein kinase II. Mechanism of inhibition and regulation by phosphorylation. J Biol Chem 264, 4800-4804.

Colbran RJ. (2004) Targeting of calcium/calmodulin-dependent protein kinase II. Biochem J 378, 1-16.

Crabtree GR and Olson EN. (2002) NFAT signaling: choreographing the social lives of cells. Cell 109 Suppl, 67-79.

Crabtree GR. (2001) Calcium, Calcineurin, and the Control of Transcription. J Biol Chem 276, 2313–2316.

Croissant JD, Kim JH, Eichele G, Goering L, Lough J, Prywes R, Schwartz RJ. (1996) Avian serum response factor expression restricted primarily to muscle cell lineages is required for alpha-actin gene transcription. Dev Biol 177, 250-264.

Curtis BM and Catterall WA. (1984) Purification of the calcium antagonist receptor of the voltage-sensitive calcium channel from skeletal muscle transverse tubules. Biochemistry 23, 2113-2118.

Czubryt MP, McAnally J, Fishman GI, Olson EN. (2003) Regulation of peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1 alpha) and mitochondrial function by MEF2 and HDAC5. Proc Natl Acad Sci U S A 100, 1711-1716.

Daugaard JR, Nielsen JN, Kristiansen S, Andersen JL, Hargreaves M, Richter EA. (2000) Fiber type-specific expression of GLUT4 in human skeletal muscle: influence of exercise training. Diabetes 49, 1092-1095.

Davies KD, Alvestad RM, Coultrap SJ, Browning MD. (2007) alphaCaMKII autophosphorylation levels differ depending on subcellular localization. Brain Res 1158, 39-49.

Davis FJ, Gupta M, Camoretti-Mercado B, Schwartz RJ, Gupta MP. (2003) Calcium/calmodulin-dependent protein kinase activates serum response factor transcription activity by its dissociation from histone deacetylase, HDAC4. Implications in cardiac muscle gene regulation during hypertrophy. J Biol Chem 278, 20047-20058.

De Koninck P and Schulman H. (1998) Sensitivity of CaM kinase II to the frequency of Ca2+ oscillations. Science 279, 227-230.

De la Pompa JL, Timmerman LA, Takimoto H, Yoshida H, Elia AJ, Samper E, Potter J, Wakeham A, Marengere L, Langille BL, Crabtree GR, Mak TW. (1998) Role of the NF-ATc transcription factor in morphogenesis of cardiac valves and septum. Nature 392, 182-186.

De Nardi C, Ausoni S, Moretti P, Gorza L, Velleca M, Buckingham M, Schiaffino S. (1993) Type 2X-myosin heavy chain is coded by a muscle fiber type-specific and developmentally regulated gene. J Cell Biol 123, 823-835.

DeFronzo RA, Jacot E, Jequier E, Maeder E, Wahren J, Felber JP. (1981) The effect of insulin on the disposal of intravenous glucose. Results from indirect calorimetry and hepatic and femoral venous catheterization. Diabetes 30, 1000-1007.

Fluck M, Booth FW, Waxham MN. (2000*a*) Skeletal muscle CaMKII enriches in nuclei and phosphorylates myogenic factor SRF at multiple sites. Biochem Biophys Res Commun 270, 488-494.

Fluck M, Waxham MN, Hamilton MT, Booth FW. (2000*b*) Skeletal muscle $Ca^{(2+)}$-indipendent kinase activity increasing during hypertrophy or running. J Appl Physiol 88, 352-358.

Fox K. (2003) Synaptic Plasticity: The Subcellular Location of CaMKII Controls Plasticity. Curr Biol 13, 143-145.

Freyssenet D, Di Carlo M, Hood DA. (1999) Calcium-dependent regulation of cytochrome c gene expression in skeletal muscle cells. J Biol Chem 274, 9305-9311.

Friday BB, Horsley V, Pavlath GK. (2000) Calcineurin activity is required for the initiation of skeletal muscle differentiation. J Cell Biol 149, 657-666.

Garcia-Cozar FJ, Okamura H, Aramburu JF, Shaw KT, Pelletier L, Showalter R, Villafranca E, Rao A. (1998) Two-site interaction of nuclear factor of activated T cells with activated calcineurin. J Biol Chem 273, 23877-23883.

García-Rodríguez C and Rao A. (1998) Nuclear factor of activated T cells (NFAT)-dependent transactivation regulated by the coactivators p300/CREB-binding protein (CBP). J Exp Med 187, 2031-2036.

Gibbs EM, Stock JL, McCoid SC, Stukenbrok HA, Pessin JE, Stevenson RW, Milici AJ, McNeish JD. (1995) Glycemic improvement in diabetic db/db mice by overexpression of the human insulin-regulatable glucose transporter (GLUT4). J Clin Invest 95, 1512-1518.

Glossmann H, Ferry DR, Goll A, Striessnig J, Zernig G. (1985) Calcium channels and calcium channel drugs: recent biochemical and biophysical findings. Arzneimittelforschung 35, 1917-1935.

Gonzalez GA and Montminy MR. (1989) Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. Cell 59, 675-680.

Goodyear LJ and Kahn BB. (1998) Exercise, glucose transport, and insulin sensitivity. Annu Rev Med 49, 235-261.

Graef IA, Chen F, Chen L, Kuo A, Crabtree GR. (2001) Signals transduced by Ca(2+)/calcineurin and NFATc3/c4 pattern the developing vasculature. Cell 105, 863-875.

Griffith LC, Lu CS, Sun XX. (2003) CaMKII, an enzyme on the move: regulation of temporospatial localization. Mol Interv 3, 386-403.

Grimby G, Broberg C, Krotkiewska I, Krotkwieski M. (1976) Muscle fiber composition in patients with traumatic cord lesion. Scand J Rehabil Med 8, 37-42.

Grozinger CM and Schreiber SL. (2002) Deacetylase enzymes: biological functions and the use of small-molecule inhibitors. Chem Biol 9, 3-16.

Hain J, Onoue H, Mayrleitner M, Fleischer S, Schindler H. (2001) Phosphorylation modulates the function of the calcium release channel of sarcoplasmic reticulum from cardiac muscle. J Biol Chem 270, 2074-2081.

Hanson PI and Schulman H. (1992) Inhibitory autophosphorylation of multifunctional Ca2+/calmodulin-dependent protein kinase analyzed by site-directed mutagenesis. J Biol Chem 267,17216-24.

Hanson PI, Meyer T, Stryer L, Schulman H. (1999) Dual role of calmodulin in autophosphorylation of multifunctional CaM kinase may underlie decoding of calcium signals. Neuron 12, 943-956.

Hasegawa K, Lee SJ, Jobe SM, Markham BE, Kitsis RN. (1997) cis-Acting sequences that mediate induction of beta-myosin heavy chain gene expression during left ventricular hypertrophy due to aortic constriction. Circulation 96, 3943-3953.

Hashimoto Y, Perrino BA, Soderling TR. (1990) Identification of an autoinhibitory domain in calcineurin. J Biol Chem 265, 1924-1927.

Hawley JA and Lessard SJ. (2008) Exercise training-induced improvements in insulin action. Acta Physiol 192, 127–135.

Hayashi T, Wojtaszewski JF, Goodyear LJ. (1997) Exercise regulation of glucose transport in skeletal muscle. Am J Physiol 273, 1039-1051.

Heist EK, Srinivasan M, Schulman H. (1998) Phosphorylation at the nuclear localization signal of Ca2+/calmodulin-dependent protein kinase II blocks its nuclear targeting. J Biol Chem 273, 19763-19771.

Hennig R and Lomo T. (1985) Firing pattern of motor units in normal rats. Nature 314, 164-166.

Hodge MR, Ranger AM, Charles de la Brousse F, Hoey T, Grusby MJ, Glimcher LH. (1996) Hyperproliferation and dysregulation of IL-4 expression in NF-ATp-deficient mice. Immunity 4, 397-405.

Hoey T, Sun YL, Williamson K, Xu X. (1995) Isolation of two new members of the NF-AT gene family and functional characterization of the NF-AT proteins. Immunity 2, 461-472.

Hogan PG, Chen L, Nardone J, Rao A. (2003) Transcriptional regulation by calcium, calcineurin, and NFAT. Genes Dev 17, 2205-2232.

Hood DA, Irrcher I, Ljubicic V, Joseph AM. (2006) Coordination of metabolic plasticity in skeletal muscle. J Exp Biol 209, 2265-2275.

Horsley V, Friday BB, Matteson S, Kegley KM, Gephart J, Pavlath GK. (2001) Regulation of the growth of multinucleated muscle cells by an NFATC2-dependent pathway. J Cell Biol 153, 329-338.

Huber B and Pette D. (1996) Dynamics of parvabulmin expression in low-frequency-stimulated fast-twitch rat muscle. Eur J Biochem 236, 814-819.

Hudmon A and Schulman H. (2002) Structure-function of the multifunctional Ca2+/calmodulin-dependent protein kinase II. Biochem J 364, 593-611.

Jørgensen SB, Wojtaszewski JF, Viollet B, Andreelli F, Birk JB, Hellsten Y, Schjerling P, Vaulont S, Neufer PD, Richter EA, Pilegaard H. (2007) Effects of alpha-AMPK knockout on exercise-induced gene activation in mouse skeletal muscle. FASEB J 19, 1146-1148.

Karamboulas C, Swedani A, Ward C, Al-Madhoun AS, Wilton S, Boisvenue S, Ridgeway AG, Skerjanc IS. (2006) HDAC activity regulates entry of mesoderm cells into the cardiac muscle lineage. J Cell Sci 119, 4305-4314.

Kegley KM, Gephart J, Warren GL, Pavlath GK. (2001) Altered primary myogenesis in NFATC3(-/-) mice leads to decreased muscle size in the adult. Dev Biol 232, 115-126.

Kennedy MB. (1997) Signal transduction molecules at the glutamatergic postsynaptic membrane. Brain Res Brain Res Rev 26, 243-257.

Kiani A, Rao A, Aramburu J. (2000) Manipulating immune responses with immunosuppressive agents that target NFAT. Immunity 12, 359-372.

Kim MS, Wang F, Puthanveetil P, Kewalramani G, Hosseini-Beheshti E, Ng N, Wang Y, Kumar U, Innis S, Proud CG, Abrahani A, Rodrigues B. (2008) Protein kinase D is a key regulator of cardiomyocyte lipoprotein lipase secretion after diabetes. Circ Res 103, 252-260.

Kincaid R (1993). Calmodulin-dependent protein phosphatases from microorganisms to man. A study in structural conservatism and biological diversity. Advances in second messenger and phosphoprotein research 27, 1-23.

Klee CB, Crouch TH, Krinks MH. (1979) Calcineurin: a calcium- and calmodulin-binding protein of the nervous system. Proc Natl Acad Sci U S A 76, 6270-6273.

Klee CB, Draetta GF, Hubbard MJ. (1988) Calcineurin. Advances in enzymology and related areas of molecular biology 61, 149-200.

Klee CB, Ren H, Wang X. (1998). Regulation of the calmodulin-stimulated protein phosphatase, calcineurin. J Biol Chem 273, 13367-13370.

Kobe B, Heierhorst J, Kemp BE. (1997) Intrasteric regulation of protein kinases. Adv Second Messenger Phosphoprotein Res 31, 29-40.

Kolodziej SJ, Hudmon A, Waxham MN, Stoops JK. (2000) Three-dimensional reconstructions of calcium/calmodulin-dependent (CaM) kinase IIalpha and truncated CaM kinase IIalpha reveal a unique organization for its structural core and functional domains. J Biol Chem 275, 14354-14359.

Kong X, Manchester J, Salmons S, Lawrence JC Jr. (1994) Glucose transporters in single skeletal muscle fibers. Relationship to hexokinase and regulation by contractile activity. J Biol Chem 269, 12963-12967.

Lai MM, Burnett PE, Wolosker H, Blackshaw S, Snyder SH. (1998) Cain, a novel physiologic protein inhibitor of calcineurin. J Biol Chem 273, 18325-18331.

Lee JC and Edelman AM. (1994) A protein activator of Ca(2+)-calmodulin-dependent protein kinase Ia. J Biol Chem 269, 2158-2164.

Li M, Linseman DA, Allen MP, Meintzer MK, Wang X, Laessig T, Wierman ME, Heidenreich KA. (2001) Myocyte enhancer factor 2A and 2D undergo phosphorylation and caspase-mediated degradation during apoptosis of rat cerebellar granule neurons. J Neurosci 21, 6544-6552.

Lin J, Handschin C, Spiegelman BM. (2005) Metabolic control through the PGC-1 family of transcription coactivators. Cell Metab 1, 361-370.

Lin J, Wu H, Tarr PT, Zhang CY, Wu Z, Boss O, Michael LF, Puigserver P, Isotani E, Olson EN, Lowell BB, Bassel-Duby R, Spiegelman BM. (2002) Transcriptional co-activator PGC-1 alpha drives the formation of slow-twitch muscle fibres. Nature 418, 797-801.

Liu ML, Olson AL, Edgington NP, Moye-Rowley WS, Pessin JE. (1994) Myocyte enhancer factor 2 (MEF2) binding site is essential for C2C12 myotube-specific expression of the rat GLUT4/muscle-adipose facilitative glucose transporter gene. J Biol Chem 269, 28514-28521.

Liu ML, Olson AL, Moye-Rowley WS, Buse JB, Bell GI, Pessin JE. (1992) Expression and regulation of the human GLUT4/muscle-fat facilitative glucose transporter gene in transgenic mice. J Biol Chem 267, 11673-11676.

Liu Y, Shen T, Randall WR, Schneider MF. (2005) Signaling pathways in activity-dependent fiber type plasticity in adult skeletal muscle. J Muscle Res Cell Motil 26, 13-21.

Long X and Griffith LC. (2000) Identification and characterization of a SUMO-1 conjugation system that modifies neuronal calcium/calmodulin-dependent protein kinase II in Drosophila melanogaster. J Biol Chem 275, 40765-40776.

Long YC and Zierath JR. (2008) Influence of AMP-activated protein kinase and calcineurin on metabolic networks in skeletal muscle. Am J Physiol Endocrinol Metab 295, 545-552.

Long YC, Glund S, Garcia-Roves PM, Zierath JR. (2007) Calcineurin regulates skeletal muscle metabolism via coordinated changes in gene expression. J Biol Chem 282, 1607-1614.

Lopez-Rodriguez C, Aramburu J, Rakeman AS, Rao A. (1999) NFAT5, a constitutively nuclear NFAT protein that does not cooperate with Fos and Jun. Proc Natl Acad Sci U S A 96, 7214-7219.

Lu J, McKinsey TA, Nicol RL, Olson EN. (2000) Signal-dependent activation of the MEF2 transcription factor by dissociation from histone deacetylases. Proc Natl Acad Sci U S A 97, 4070-4075.

Macian F. (2005) NFAT proteins: key regulators of T-cell development and function. Nat Rev Immunol 5, 472-484.

MacLennan DH, Brandl CJ, Korczak B, Green NM. (1985) Amino-acid sequence of a Ca2+/Mg2+-dependent ATPase from rabbit muscle sarcoplasmic reticulum, deduced from its complementary DNA sequence. Nature 316:696-700.

Mao Z and Wiedmann M. (1999) Calcineurin enhances MEF2 DNA binding activity in calcium-dependent survival of cerebellar granule neurons. J Biol Chem 274, 31102-31107.

Marin P, Andersson B, Krotkiewski M, Björntorp P. (1994) Muscle fiber composition and capillary density in women and men with NIDDM. Diabetes Care 17, 382-386.

McClelland GB, Kraft CS, Michaud D, Russell JC, Mueller CR, Moyes CD. (2004) Leptin and the control of respiratory gene expression in muscle. Biochim Biophys Acta 1688, 86-93.

McCullagh KJ, Calabria E, Pallafacchina G, Ciciliot S, Serrano AL, Argentini C, Kalhovde JM, Lomo T, Schiaffino S. (2004) NFAT is a nerve activity sensor in skeletal muscle and controls activity-dependent myosin switching. Proc Natl Acad Sci U S A 101, 10590-10595.

McGee SL and Hargreaves M. (2004) Exercise and myocyte enhancer factor 2 regulation in human skeletal muscle. Diabetes 53, 1208-1214.

McGee SL and Hargreaves M. (2006) Exercise and skeletal muscle glucose transporter 4 expression: molecular mechanisms. Clin Exp Pharmacol Physiol. 33, 395-399.

McGee SL, Howlett KF, Starkie RL, Cameron-Smith D, Kemp BE, Hargreaves M. (2003) Exercise increases nuclear AMPK alpha2 in human skeletal muscle. Diabetes 52, 926-928.

McGee SL, Sparling D, Olson AL, Hargreaves M. (2005) Exercise increases MEF2- and GEF DNA-binding activity in human skeletal muscle. FASEB J 20, 348-349.

McGee SL, van Denderen BJ, Howlett KF, Mollica J, Schertzer JD, Kemp BE, Hargreaves M. (2008) AMP-activated protein kinase regulates GLUT4 transcription by phosphorylating histone deacetylase 5. Diabetes 57, 860-867.

McKinsey TA, Zhang CL, Lu J, Olson EN. (2000*a*) Signal-dependent nuclear export of a histone deacetylase regulates muscle differentiation. Nature 408, 106-111.

McKinsey TA, Zhang CL, Olson EN. (2000*b*) Activation of the myocyte enhancer factor-2 transcription factor by calcium/calmodulin-dependent protein kinase-stimulated binding of 14-3-3 to histone deacetylase 5. Proc Natl Acad Sci U S A 97, 14400-14405.

McKinsey TA, Zhang CL, Olson EN. (2002) MEF2: a calcium-dependent regulator of cell division, differentiation and death. Trends Biochem Sci 27, 40-47.

Meissner JD, Gros G, Scheibe RJ, Scholz M, Kubis HP. (2001) Calcineurin regulates slow myosin, but not fast myosin or metabolic enzymes, during fast-to-slow transformation in rabbit skeletal muscle cell culture. J Physiol 533, 215-226.

Meissner JD, Umeda PK, Chang KC, Gros G, Scheibe RJ. (2006) Activation of the beta myosin heavy chain promoter by MEF-2D, MyoD, p300, and the calcineurin/NFATc1 pathway. J Cell Physiol 211, 138-148.

Meyer T, Hanson PI, Stryer L, Schulman H. (1992) Calmodulin trapping by calcium-calmodulin-dependent protein kinase. Science 256, 1199-1202.

Mir LM, Bureau MF, Gehl J, Rangara R, Rouy D, Caillaud JM, Delaere P, Branellec D, Schwartz B, Scherman D. (1999) High-efficiency gene transfer into skeletal muscle mediated by electric pulses. Proc Natl Acad Sci U S A 96, 4262-4267.

Monsalve M, Wu Z, Adelmant G, Puigserver P, Fan M, Spiegelman BM. (2000) Direct coupling of transcription and mRNA processing through the thermogenic coactivator PGC-1. Mol Cell 6, 307-316.

Mora S and Pessin JE. (2000) The MEF2A isoform is required for striated muscle-specific expression of the insulin-responsive GLUT4 glucose transporter. J Biol Chem 275, 16323-16328.

Moreno H, Serrano AL, Santalucía T, Gumá A, Cantó C, Brand NJ, Palacin M, Schiaffino S, Zorzano A. (2003) Differential regulation of the muscle-specific GLUT4 enhancer in regenerating and adult skeletal muscle. J Biol Chem 278, 40557-40564.

Mu X, Brown LD, Liu Y, Schneider MF. (2007) Roles of the calcineurin and CaMK signaling pathways in fast-to-slow fiber type transformation of cultured adult mouse skeletal muscle fibers. Physiol Genomics 30, 300-312.

Mukwevho E, Kohn TA, Lang D, Nyatia E, Smith J, Ojuka EO. (2008) Caffeine induces hyperacetylation of histones at the MEF2 site on the Glut4 promoter and increases MEF2A binding to the site via a CaMK-dependent mechanism. Am J Physiol Endocrinol Metab 294, 582-588.

Naya FJ, Wu C, Richardson JA, Overbeek P, Olson EN. (1999) Transcriptional activity of MEF2 during mouse embryogenesis monitored with a MEF2-dependent transgene. Development 126, 2045-2052.

Nyholm B, Qu Z, Kaal A, Pedersen SB, Gravholt CH, Andersen JL, Saltin B, Schmitz O. (1997) Evidence of an increased number of type IIb muscle fibers in insulin-resistant first-degree relatives of patients with NIDDM. Diabetes 46, 1822-1828.

Ojamaa K, Klemperer JD, MacGilvray SS, Klein I, Samarel A. (1996) Thyroid hormone and hemodynamic regulation of beta-myosin heavy chain promoter in the heart. Endocrinology 137, 802-808.

Ojuka EO, Jones TE, Han DH, Chen M, Holloszy JO. (2003) Raising Ca2+ in L6 myotubes mimics effects of exercise on mitochondrial biogenesis in muscle. FASEB J 17, 675-681.

Ojuka EO, Jones TE, Nolte LA, Chen M, Wamhoff BR, Sturek M, Holloszy JO. (2002) Regulation of GLUT4 biogenesis in muscle: evidence for involvement of AMPK and Ca(2+). Am J Physiol Endocrinol Metab 282, 1008-1013.

Park S, Uesugi M, Verdine GL. (2000) A second calcineurin binding site on the NFAT regulatory domain. Proc Natl Acad Sci U S A 97, 7130-7135.

Parker D, Jhala US, Radhakrishnan I, Yaffe MB, Reyes C, Shulman AI, Cantley LC, Wright PE, Montminy M. (1998) Analysis of an activator:coactivator complex reveals an essential role for secondary structure in transcriptional activation. Mol Cell 2, 353-359.

Parsons SA, Wilkins BJ, Bueno OF, Molkentin JD. (2003) Altered skeletal muscle phenotypes in calcineurin Aalpha and Abeta gene-targeted mice. Mol Cell Biol 23, 4331-4343.

Passier R, Zeng H, Frey N, Naya FJ, Nicol RL, McKinsey TA, Overbeek P, Richardson JA, Grant SR, Olson EN. (2000) CaM kinase signaling induces cardiac hypertrophy and activates the MEF2 transcription factor in vivo. J Clin Invest 105, 1395-1406.

Pette D and Staron RS. (1997) Mammalian skeletal muscle fiber type transitions. Int Rev Cytol 170, 143-223.

Pette D and Staron RS. (2001) Transitions of muscle fiber phenotypic profiles. Histochem Cell Biol 115, 359-372.

Pette D and Vrbova G. (1992) Adaptation of mammalian skeletal muscle fibers to chronic electrical stimulation. Rev Physiol Biochem Pharmacol 120, 115-202.

Pette D. (2001) Historical perspectives: plasticity of mammalian skeletal muscle. J Appl Physiol 90, 1119-1124.

Pilegaard H, Saltin B, Neufer PD. (2003) Exercise induces transient transcriptional activation of the PGC-1alpha gene in human skeletal muscle. J Physiol 546, 851-858.

Potthoff MJ and Olson EN. (2002) MEF2: a central regulator of diverse developmental programs. Development 134, 4131-4140.

Potthoff MJ, Wu H, Arnold MA, Shelton JM, Backs J, McAnally J, Richardson JA, Bassel-Duby R, Olson EN. (2007) Histone deacetylase degradation and MEF2 activation promote the formation of slow-twitch myofibers. J Clin Invest 117, 2459-24567.

Putkey JA and Waxham MN. (1996) A peptide model for calmodulin trapping by calcium/calmodulin-dependent protein kinase II. J Biol Chem 271, 29619-29623.

Rao A, Luo C, Hogan PG. (1997) Transcription factors of the NFAT family: regulation and function. Ann Rev Immunol 15, 707-747.

Rich RC and Schulman H. (1998) Substrate-directed function of calmodulin in autophosphorylation of Ca2+/calmodulin-dependent protein kinase II. J Biol Chem 273, 28424-28429.

Rios E and Pizarro G. (1991) Voltage sensor of excitation-contraction coupling in skeletal muscle. Physiol Rev 71, 849-908.

Röckl KS, Witczak CA, Goodyear LJ. (2008) Signaling mechanisms in skeletal muscle: acute responses and chronic adaptations to exercise. IUBMB Life 60, 145-153.

Rose AJ and Hargreaves M. (2003) Exercise increases Ca2+-calmodulin-dependent protein kinase II activity in human skeletal muscle. J Physiol 553, 303-309.

Rose AJ, Alsted TJ, Kobberø JB, Richter EA. (2007*a*) Regulation and function of Ca2+-calmodulin-dependent protein kinase II of fast-twitch rat skeletal muscle. J Physiol 580, 993-1005.

Rose AJ, Frøsig C, Kiens B, Wojtaszewski JF, Richter EA. (2007*b*) Effect of endurance exercise training on Ca2+ calmodulin-dependent protein kinase II expression and signalling in skeletal muscle of humans. J Physiol 583, 785-795.

Rose AJ, Kiens B, Richter EA. (2006) Ca2+-calmodulin-dependent protein kinase expression and signalling in skeletal muscle during exercise. J Physiol 574, 889-903.

Rose AJ, Michell BJ, Kemp BE, Hargreaves M. (2004) Effect of exercise on protein kinase C activity and localization in human skeletal muscle. J Physiol 561, 861-870.

Roth SY, Denu JM, Allis CD. (2001) Histone acetyltransferases. Annu Rev Biochem 70, 81-120.

Rustin P, Munnich A, Rötig A. (2002) Succinate dehydrogenase and human diseases: new insights into a well-known enzyme. Eur J Hum Genet 10, 289-291.

Ryder JW, Bassel-Duby R, Olson EN, Zierath JR. (2003) Skeletal muscle reprogramming by activation of calcineurin improves insulin action on metabolic pathways. J Biol Chem 278, 44298-44304.

Sahyoun N, LeVine H 3rd, Bronson D, Cuatrecasas P. (1984) Ca2+-calmodulin-dependent protein kinase in neuronal nuclei. J Biol Chem 259(15):9341-9344.

Santalucía T, Camps M, Castelló A, Muñoz P, Nuel A, Testar X, Palacin M, Zorzano A. (1992) Developmental regulation of GLUT-1 (erythroid/Hep G2) and GLUT-4 (muscle/fat) glucose transporter expression in rat heart, skeletal muscle, and brown adipose tissue. Endocrinology 130, 837-846.

Santalucía T, Moreno H, Palacín M, Yacoub MH, Brand NJ, Zorzano A. (2001) A novel functional co-operation between MyoD, MEF2 and TRalpha1 is sufficient for the induction of GLUT4 gene transcription. J Mol Biol 314, 195-204.

Sato K, Suematsu A, Nakashima T, Takemoto-Kimura S, Aoki K, Morishita Y, Asahara H, Ohya K, Yamaguchi A, Takai T, Kodama T, Chatila TA, Bito H, Takayanagi H. (2006) Regulation of osteoclast differentiation and function by the CaMK-CREB pathway. Nat Med 12, 1410-1416.

Schiaffino S, Gorza L, Sartore S, Saggin L, Ausoni S, Vianello M, Gundersen K, Lomo T. (1989). Three myosin heavy chain isoforms in type 2 skeletal muscle fibres. J Muscle Res Cell Motil 10, 197-205.

Schiaffino S, Sandri M, Murgia M. (2007) Activity-dependent signaling pathways controlling muscle diversity and plasticity. Physiology (Bethesda) 22, 269-278.

Schneider MF and Chandler WK. (1987) Voltage dependent charge movement of skeletal muscle: a possible step in excitation-contraction coupling. Nature 242, 244-246.

Serfling E, Berberich-Siebelt F, Avots A, Chuvpilo S, Klein-Hessling S, Jha MK, Kondo E, Pagel P, Schulze-Luehrmann J, Palmetshofer A. (2004) NFAT and NF-kappaB factors-the distant relatives. Int J Biochem Cell Biol 36, 1166-1170.

Serrano AL, Murgia M, Pallafacchina G, Calabria E, Coniglio P, Lomo T, Schiaffino S. (2001) Calcineurin controls nerve activity-dependent specification of slow skeletal muscle fibers but not muscle growth. Proc Natl Acad Sci U S A 98, 13108-13113.

Shadel GS and Clayton DA. (1997) Mitochondrial DNA maintenance in vertebrates. Annu Rev Biochem 66, 409-435.

Shaw JP, Utz PJ, Durand DB, Toole JJ, Emmel EA, Crabtree GR. (1988) Identification of a putative regulator of early T cell activation genes. Science 241, 202-205.

Sheng M, Thompson MA, Greenberg ME. (1991) CREB: a Ca(2+)-regulated transcription factor phosphorylated by calmodulin-dependent kinases. Science 252, 1427-1430.

Simmerman HK and Jones LR. (1988) Phospholamban: protein structure, mechanism of action, and role in cardiac function. Physiol Rev 78, 921-947.

Smith JA, Collins M, Grobler LA, Magee CJ, Ojuka EO. (2006) Exercise and CaMK activation both increase the binding of MEF2A to the Glut4 promoter in skeletal muscle in vivo. Am J Physiol Endocrinol Metab 292, 413-420.

Smith JA, Kohn TA, Chetty AK, Ojuka EO. (2008) CaMK activation during exercise is required for histone hyper-acetylation and MEF2A binding at the MEF2 site on the Glut4 gene. Am J Physiol Endocrinol Metab 295, 698-704.

Soderling TR and Stull JT. (2001) Structure and regulation of calcium/calmodulin-dependent protein kinases. Chem Rev 101, 2341-2352.

Soderling TR, Chang B, Brickey D. (2001) Cellular signaling through multifunctional Ca2+/calmodulin-dependent protein kinase II. J Biol Chem 276, 3719-3722.

Soderling TR. (1996) Structure and regulation of calcium/calmodulin-dependent protein kinases II and IV. Biochim Biophs Acta 1297, 131-138.

Sparrow DB, Miska EA, Langley E, Reynaud-Deonauth S, Kotecha S, Towers N, Spohr G, Kouzarides T, Mohun TJ. (1999) MEF-2 function is modified by a novel co-repressor, MITR. EMBO J 18, 5085-5098.

Spector SA. (1985*a*) Effects of elimination of activity on contractile and histochemical properties of rat soleus muscle. J Neurosci 5, 2177-2188.

Spector SA. (1985*b*) Trophic effects on the contractile and histochemical properties of rat soleus muscle. J Neurosci 5, 2189-2196.

Srinivasan M, Edman CF, Schulman H. (1994) Alternative splicing introduces a nuclear localization signal that targets multifunctional CaM kinase to the nucleus. J Cell Biol 12, 839-852.

Sun P, Enslen H, Myung PS, Maurer RA. (1994) Differential activation of CREB by Ca2+/calmodulin-dependent protein kinases type II and type IV involves phosphorylation of a site that negatively regulates activity. Genes Dev 8, 2527-2539.

Sussman MA, Lim HW, Gude N, Taigen T, Olson EN, Robbins J, Colbert MC, Gualberto A, Wieczorek DF, Molkentin JD. (1998) Prevention of cardiac hypertrophy in mice by calcineurin inhibition. Science 281, 1690-1693.

Swoap SJ. (1998) In vivo analysis of the myosin heavy chain IIB promoter region. Am J Physiol 274, 681-687.

Swoap, SJ, Hunter RB, Stevenson EJ, Felton HM, Kansagra NV, Lang JM, Esser KA, Kandarian SC. (2000) The calcineurin-NFAT pathway and muscle fiber-type gene expression. Am J Physiol Cell Physiol 279, C915-924.

Thai MV, Guruswamy S, Cao KT, Pessin JE, Olson AL. (1998) Myocyte enhancer factor 2 (MEF2)-binding site is required for GLUT4 gene expression in transgenic mice. Regulation of MEF2 DNA binding activity in insulin-deficient diabetes. J Biol Chem 273, 14285-14292.

Tobimatsu T and Fujisawa H. (1989) Tissue-specific expression of four types of rat calmodulin-dependent protein kinase II mRNAs. J boil Chem 264, 17907-17012.

Tokumitsu H and Soderling TR. (1995) Requirements for calcium and calmodulin in the calmodulin kinase activation cascade. J Biol Chem 271, 5617-5622.

Tokumitsu H, Wayman GA, Muramatsu M, Soderling TR. (1997) Calcium/calmodulin-dependent protein kinase kinase: identification of regulatory domains. Biochemistry 36, 12823-12827.

Torgan CE and Daniels MP. (2001) Regulation of myosin heavy chain expression during rat skeletal muscle development in vitro. Mol Biol Cell 12, 1499-1508.

Tothova J, Blaauw B, Pallafacchina G, Rudolf R, Argentini C, Reggiani C, Schiaffino S. (2006). NFATc1 nucleocytoplasmic shuttling is controlled by nerve activity in skeletal muscle. J Cell Sci 119, 1604-1611.

Tozzo E, Gnudi L, Kahn BB. (1997) Amelioration of insulin resistance in streptozotocin diabetic mice by transgenic overexpression of GLUT4 driven by an adipose-specific promoter. Endocrinology 138, 1604-1611.

Vega RB, Harrison BC, Meadows E, Roberts CR, Papst PJ, Olson EN, McKinsey TA. (2004) Protein kinases C and D mediate agonist-dependent cardiac hypertrophy through nuclear export of histone deacetylase 5. Mol Cell Biol 24, 8374-8385.

Vega RB, Huss JM, Kelly DP. (2000) The coactivator PGC-1 cooperates with peroxisome proliferator-activated receptor alpha in transcriptional control of nuclear genes encoding mitochondrial fatty acid oxidation enzymes. Mol Cell Biol 20, 1868-1876.

Verdin E, Dequiedt F, Kasler HG. (2003) Class II histone deacetylases: versatile regulators. Trend Genet 19, 286-293.

Virbasius JV and Scarpulla RC. (1994) Activation of the human mitochondrial transcription factor A gene by nuclear respiratory factors: a potential regulatory link between nuclear and mitochondrial gene expression in organelle biogenesis. Proc Natl Acad Sci U S A 91, 1309-1313.

Vrbova G. (1963) The effects of motoneurone activity on the speed of contraction of striated muscle. J Physiol (London) 169, 313-526.

Wang HG, Pathan N, Ethell IM, Krajewski S, Yamaguchi Y, Shibasaki F, McKeon F, Bobo T, Franke TF, Reed JC. (1999) Ca2+-induced apoptosis through calcineurin dephosphorylation of BAD. Science 284, 339-343.

Wang J and Best PM. (1992) Inactivation of the sarcoplasmic reticulum calcium channel by protein kinase. Nature 359, 739-741.

Wiegand G and Remington SJ. (1986) Citrate synthase: structure, control, and mechanism. Annu Rev Biophys Biophys Chem 15, 97-117.

Witcher DR, Kovacs RJ, Schulman H, Cefali DC, Jones LR. (1991) Unique phosphorylation site on the cardiac ryanodine receptor regulates calcium channel activity. J Biol Chem 266, 11144-11152.

Wright DC, Geiger PC, Holloszy JO, Han DH. (2005) Contraction- and hypoxia-stimulated glucose transport is mediated by a Ca2+-dependent mechanism in slow-twitch rat soleus muscle. Am J Physiol Endocrinol Metab 288, 1062-1066.

Wright DC, Hucker KA, Holloszy JA, Han DH. (2004) Ca2+ and AMPK both mediate stimulation of glucose transport by muscle contractions. Diabetes 53, 330-335.

Wright DC. (2007) Mechanisms of calcium-induced mitochondrial biogenesis and GLUT4 synthesis. Appl Physiol Nutr Metab, 840-845.

Wu H, Kanatous SB, Thurmond FA, Gallardo T, Isotani E, Bassel-Duby R, Williams RS. (2002) Regulation of mitochondrial biogenesis in skeletal muscle by CaMK. Science 296, 349-352.

Wu H, Naya FJ, McKinsey TA, Mercer B, Shelton JM, Chin ER, Simard AR, Michel RN, Bassel-Duby R, Olson EN, Williams RS. (2000) MEF2 responds to multiple calcium-regulated signals in the control of skeletal muscle fiber type. EMBO J 19, 1963-1973.

Wu X and McMurray CT. (2001) Calmodulin kinase II attenuation of gene transcription by preventing cAMP response element-binding protein (CREB) dimerization and binding of the CREB-binding protein. J Biol Chem 276, 1735-1741.

Wu Z, Puigserver P, Andersson U, Zhang C, Adelmant G, Mootha V, Troy A, Cinti S, Lowell B, Scarpulla RC, Spiegelman BM. (1999) Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. Cell 98, 115-124.

Yang SN, Tang YG, Zucker RS. (1999) Selective induction of LTP and LTD by postsynaptic [Ca2+]i elevation. J Neurophysiol 81, 781-787.

Youn HD, Chatila TA, Liu JO. (2000) Integration of calcineurin and MEF2 signals by the coactivator p300 during T-cell apoptosis. EMBO J 19, 4323-4331.

Zalk R, Lehnart SE, Marks AR. (2007) Modulation of the ryanodine receptor and intracellular calcium. Annu Rev Biochem 76, 367-385.

Zhu J and McKeon F. (1999) NF-AT activation requires suppression of Crm1-dependent export by calcineurin. Nature 398, 256-260.

Zierath JR, He L, Gumà A, Odegoard Wahlström E, Klip A, Wallberg-Henriksson H. (1996) Insulin action on glucose transport and plasma membrane GLUT4 content in skeletal muscle from patients with NIDDM. Diabetologia 39, 1180-1189.

Zorzano A, Palacín M, Gumà A. (2005) Mechanisms regulating GLUT4 glucose transporter expression and glucose transport in skeletal muscle. Acta Physiol Scand 183, 43-58.