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Possible link of different slow calcium signals generated by membrane potential and hormones to differential gene expression in cultured muscle cells

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ABSTRACT

We studied the effect of IGF-I, insulin and testosterone on intracellular Ca^{2+} in cultured muscle cells. Insulin produced a fast (<1 s) and transient [Ca^{2+}] increase lasting less than 10 s. IGF-I induced a transient [Ca^{2+}] increase, reaching a fluorescence peak 6 s after stimulus, to return to basal values after 60 s. Testosterone induced delayed (35 s) and long lasting (100-200 s) signals, frequently associated with oscillations. IGF-I, testosterone and electrical stimulation-induced Ca^{2+} signals were shown to be dependent on IP_3 production. All of these Ca^{2+} signals were blocked by inhibitors of the IP_3 pathway. On the other hand, insulin-induced Ca^{2+} increase was dependent on ryanodine receptors and blocked by either nifedipine or ryanodine. The different intracellular Ca^{2+} patterns produced by electrical stimulation, testosterone, IGF-I and insulin, may help to understand the role of intracellular calcium kinetics in the regulation of gene expression by various stimuli in skeletal muscle cells.

Key terms: skeletal, transcription factors, insulin, IGF-I, testosterone

INTRODUCTION

Skeletal muscle is a highly plastic tissue, capable of suffering striking changes upon increased use or disuse, indicating that a very active system of regulation of gene expression must be present in muscle cells at all times. The tight association between muscle fibers and satellite muscle cells is at the basis of muscle hypertrophy and muscle fiber regeneration (Chargé and Rudnicki, 2004, see

Molgó et al., this issue). Muscle remodeling and repair following injury involves satellite cells and replication. Frequently, hypertrophy induced by hormonal stimuli is associated with an increase in satellite cell number and changes in satellite cell ultrastructure (Sinha-Hikim et al., 2003). Thus, fiber fate and differentiation processes cannot be completely dissociated from the continuous process of gene regulation that occurs upon muscle function. Furthermore, exercise induces several changes in mRNA levels and apparently there is a fine regulation of gene expression depending on frequency and duration of physical activity (Booth, 1998).

Exercise implies several changes in the environment of the muscle cell, i.e. hormone and neurotransmitter concentrations as well as electrical and mechanical stimuli. Repetitive electrical signals reaching the sarcolemma and transverse tubule membranes in a synchronous way produce a potential effect on neuromuscular transmission efficacy (Desaulniers et al., 2001). Several hormones have been reported to rise during exercise; namely testosterone, aldosterone, insulin and growth factors (Zoladz et al., 2002; Manetta et al., 2002; Kokalas et al., 2004). In addition, mechanical factors, temperature, ions and metabolite concentrations also change in the immediate surroundings of the fiber, making the study of factors that contribute to muscle gene regulation especially complex (Fluck and Hoppeler 2003). Myofibrillar protein content per whole muscle, myofibrillar protein synthesis, actin protein synthesis, actin mRNA and actin promoter activity have all been shown to increase in an animal overload model (reviewed in Booth, 2002), which leads to muscle hypertrophy.

As a further layer of complexity, the extrinsic micro-environment of the cell further governs the regulation of intracellular mediators such as AKT/PKB. For instance, insulin-like growth factor-I (IGF-I) is a well-documented activator of AKT/PKB kinase activity, via the activation of phosphatidylinositol-3-kinase (PI3-K) (for review, see Adams, 2002). Therefore, altering the extracellular concentrations of IGF-I via autocrine/paracrine action ultimately results in a marked phenotypic change.

Calcineurin (CaN) is a Ca^{2+}-calmodulin-dependent phosphatase that appears to be crucial in the signalling of functional overload-induced fibre hypertrophy. Dunn et al., (1999) demonstrated the importance of calcineurin in muscle fibre hypertrophy with various pharmacological inhibitors of calcineurin. Calcineurin is probably activated in overloaded muscles via the chronic increases in intra-cellular [Ca^{+2}] that occur under overloaded conditions as a result of a doubling of nerve-mediated muscle fibre activation and load-related increases in insulin-like growth factor (Dunn et al.,1999). Once activated, calcineurin may signal downstream genes involved in regulating muscle fibre size via dephosphorylation of its substrate transcription factors, nuclear factor of activated T cells (NFAT; Dunn et al., 1999). Various NFAT isoforms have been shown to be able to activate various genes, which have been implicated in the slow muscle fibre and muscle hypertrophy gene expression (Olson and Williams, 2000).

CALCIUM-DEPENDENT ACTIVATION OF TRANSCRIPTION FACTORS

A number of studies from different laboratories indicate that nuclear Ca^{+2} elevations activate enzymes that regulate transcription factors (Table I). Cyclic adenosine 3', 5'- monophosphate-responsive element binding protein (CREB) related transcription of genes has been shown to be dependent on nuclear Ca^{2+} (reviewed by Dolmetsch, 2003). In different cell models, Ca^{2+} dependence of CREB function has been identified at the level of mitogen_activated protein kinase (MAPK), Ca^{2+}-calmodulin (CaM)-activated kinase (CaMK) or Ca^{2+} dependent forms of PKC (Muthusamy and Leiden, 1998; Carrasco et al., 2003; Wu et al., 2001). Uncoupling of CRE-regulated gene expression has been shown to be dependent on the activity of protein phosphatases (Alberts et al., 1994; Genoux et al., 2002; Hagiwara et al., 1992). Calcium dependent CREB activation was shown to be an important mechanism in myotubes as well as in neurons (Carrasco et al., 2003; Carrasco et al., this issue).

Other Ca^{2+} activated transcription factors include Elk-1 (Choe et al., 2001), NFAT (Hogan et al., 2003; Dolmestch, 2003) and NFkB (Dolmestch et al., 1997). Due to the interplay between the phosphatase calcineurin (CaN) and NFAT kinases, the time course of nuclear Ca^{2+} rise will be critical for the transcription process to proceed (Dolmestch, 2003). The nucleus contains a high

concentration of CaM; this suggests that it has abundant CaM targets. CaM also translocates to the nucleus in response to a [Ca^{+2}] rise. CaM mediates transcriptional activation by the myocyte enhancer factor-2 (MEF-2), which recruits other Ca^{2+} sensitive transcriptional factors such as NFAT and transcriptional co-activators such as p-300 (Youn et al., 2000a). MEF-2 is also regulated by the CaN inhibitor protein CABIN1, which is another target of nuclear Ca^{2+} (Youn et al., 2002b).

Duration as well as location of the calcium transient could provide the necessary conditions for local activation of either a kinase or a phosphatase, allowing for a particular transcription factor to be activated, translocated to the nucleus, or both.

Signals transduced by kinases depend on the extent and duration of substrate phosphorylation. Calcium signals thus may give us the key to understanding the language the cell uses to transduce external stimuli into gene repression or activation.

It has been proposed that the kinetics of calcium signals plays a significant role in leading gene expression by use of alternative routes of signal transduction. One of the first hints into a time dependent mechanism for signaling cascades leading to gene expression was provided by Bito et al., (1996) who studied the signaling pathways by which synaptic inputs control the phosphorylation state of CREB and determine expression of CRE-regulated genes. Later work again called attention to the kinetics of Ca^{2+} signals by demonstrating that Ca^{+2} oscillation frequency determines gene expression (Li et al., 1998; Dolmetch et al., 1998). Recently, the elegant work of Violin et al., (2003) has provided insights into the possible mechanisms that link Ca^{+2} oscillations to the activity of a particular effector molecule, such as PKC. They generated genetically encoded fluorescent reporters for PKC activity that reversibly respond to stimuli activating PKC. In particular, phosphorylation of the reporter expressed in mammalian cells causes changes in fluorescence resonance energy transfer (FRET), allowing real time imaging of phosphorylation resulting from PKC activation. Targeting of the reporter to the plasma membrane, where PKC is activated, revealed oscillatory phosphorylation in HeLa cells in response to histamine; the oscillatory phosphorylation correlates with Ca^{2+} -controlled translocation of conventional PKC to the membrane without oscillations of PLC activity or diacylglycerol. In MDCK cells stimulated with ATP, however, PLC and diacylglycerol fluctuate together with Ca^{2+} and phosphorylation. Thus, specificity of PKC signalling depends on the local second messenger-controlled equilibrium between kinase and phosphatase activities, resulting in strict Ca^{2+}-controlled temporal regulation of substrate phosphorylation.

TABLE I

Examples of Ca^{2+} sensitive transcription factors and repressors

Transcription factor	Ca^{2+} sensitive event	Known mechanisms of Ca^{2+}dependence	Nuclear effect
NFAT	NFAT dephosphorylation CaN dependent	Nuclear translocation	CBP recruitment - enhances the transcriptional activity of MEF-2
NFkB	ikB dephosphorylation and degradation by CaM or CAMK II	Nuclear translocation	transcriptional activation
CREB	Phosphorylation by MAPK, CAMKII, CAMK IV, PKC, PKA	Nuclear translocation of kinases (ERK, PKA) and CaM	CBP recruitment - Binding to CRE transcriptional activation
		Removal of	

MEF-2	Inhibition by CABIN1 - activation by CaN	repressors and enabling bind to coactivators as p300	transcriptional activation
DREAM (transcription repressor)	DREAM release from DRE	Impairs recruitment of CBP by phospho CREB Block CREB	Block CREB- CBP transcription
Elk-1	Phosphorylation by MAPK, CAMK II or CAMK IV	ERK nuclear translocation	c-*fos* dependent transcription activation

CALCIUM SIGNALING PATTERNS IN MUSCLE CELLS

It is important then, to look for a cell model that will physiologically display a diversity of calcium signaling patterns and respond with different calcium signals to different stimuli. Such a model appears to be the cultured skeletal muscle cell. During the past several years our laboratory has studied both calcium signals and calcium dependent processes, using cultured muscle cells. We have been able to show that various stimuli, normally associated with physiological exercise, are capable of inducing slow calcium signals associated with the nuclei in cultured myotubes (Jaimovich et al., 2000; Estrada et al., 2000, Powell et al., 2001) (See Fig. 1) and in intact muscle fibers (Powell et al., 2003). These signals can be clearly dissociated from fast calcium transients involved in excitation-contraction coupling (Estrada et al., 2001, Araya et al., 2003) and appear to be involved in the regulation of gene expression (Jaimovich and Carrasco, 2002, Carrasco et al., 2003).

The dihydropyridine receptor (DHPR), normally a voltage dependent calcium channel, functions in skeletal muscle essentially as a voltage sensor, triggering intracellular calcium release for excitation-contraction coupling (Protasi, 2002). By means of this mechanism, a fast calcium release, via ryanodine receptor (RyR) channels after depolarization of skeletal myotubes occurs. Depolarization evokes a second, slow calcium wave unrelated to contraction that involves the cell nucleus (Jaimovich et al., 2000, Powell et al., 2001, Hidalgo et al., 2002). We have reported that DHPR may also be the voltage sensor for these slow calcium signals (Araya et al., 2003). Using DHPR inhibitors and the GLT cell line, which do not express the α_{1s} subunit of the DHPR, no Ca^{2+} transient of any type can be obtained following depolarization. After transfection of the α_{1s} DNA into the GLT cells, the slow calcium transient is recovered. Furthermore, we have shown that a G protein is involved in the onset of the slow calcium transient and the mechanism responsible for the calcium increase is the production of IP_3 after depolarization. In myotubes, depolarization produces an increase in gene expression. 10 μM nifedipine, but not ryanodine, inhibited c-*jun* and c-*fos* mRNA increase after K^+ depolarization. These results suggest a role for DHPR mediated calcium signals in regulation of early gene expression (Araya et al., 2003).

The fluorescent calcium signal from rat myotubes in culture was also monitored after field-stimulation with tetanic protocols (Eltit et al., 2004). During tetanus, calcium signals sensitive to ryanodine and associated to the EC coupling were evident; but several seconds later, a second long-lasting calcium signal, refractory to ryanodine, was consistently found. The onset kinetics of this slow signal was slightly modified in nominally calcium-free medium, as it was by both the frequency and number of pulses during tetanus. No signal was detected in the presence of TTX. The participation of the DHPR as the voltage sensor for this new signal was assessed by treatment with agonist and antagonist dihydropyridines (Bay K 8644 and nifedipine) showing an enhanced and inhibitory response, respectively. In the dysgenic GLT cell line, the signal was absent. Transfection of these cells with the α_{1s} subunit restored the slow signal. In myotubes, the IP_3 mass increase induced by a tetanus protocol temporally preceded the slow calcium signal. Both an IP_3R blocker and a PLC inhibitor (xestospongin C and U73122 respectively) dramatically inhibit this signal. Long-lasting, IP_3-generated slow calcium signals appear to be a physiological response to activity-related

fluctuations in membrane potential sensed by the DHPR.

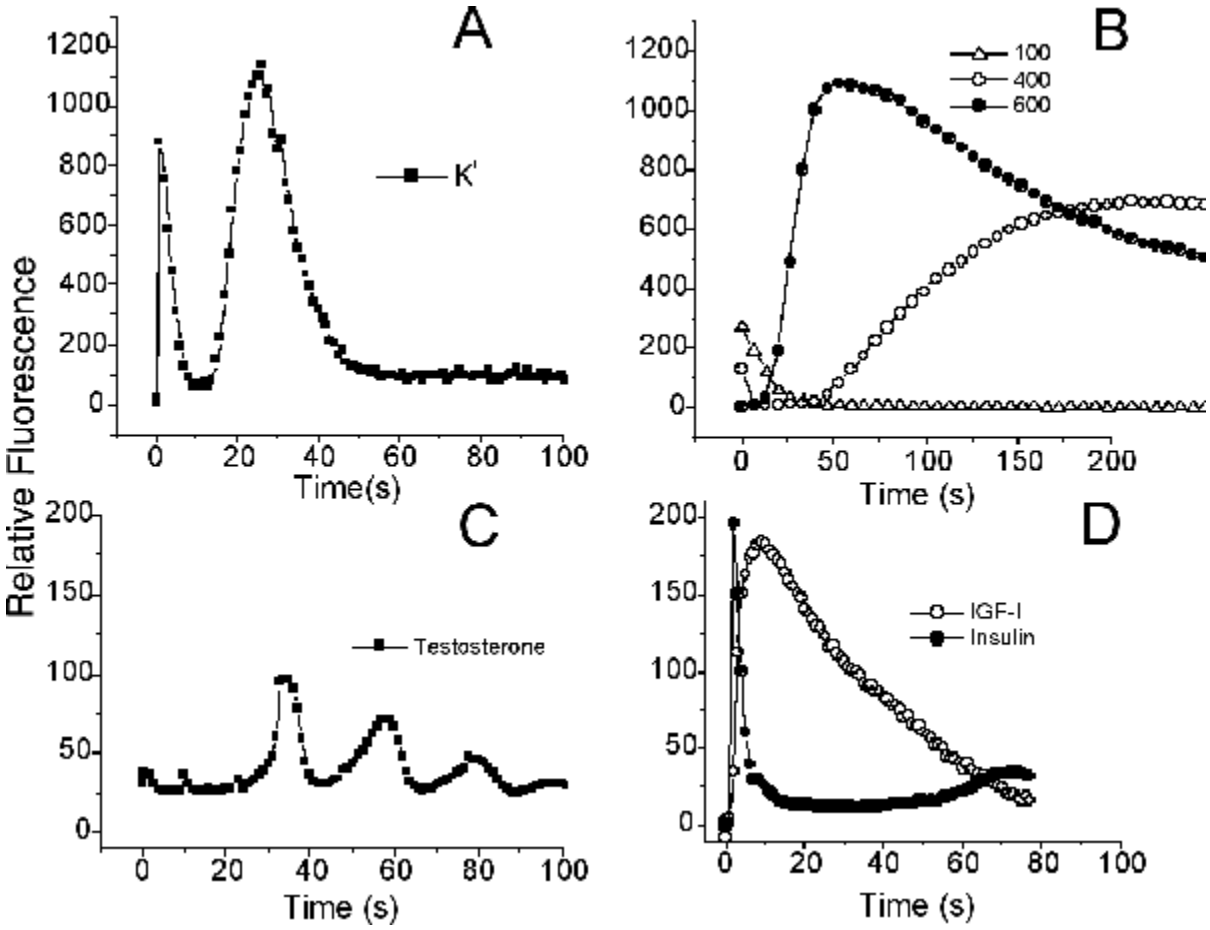

Figure 1. Calcium transients in myotubes evoked by different stimuli. Myotubes were cultured on glass coverslips until they reached 80% confluence. Calcium images were obtained from non-contracting myotubes pre-loaded with the fluorescence Ca^{2+} dye fluo 3 AM, using either an inverted confocal microscope or a fluorescence microscope, equipped with a cooled CCD camera and image acquisition system. Time series of images were recorded before and after cell stimulation. 80-100 images were recorded for each experiment, each series containing images recorded at time intervals of 1 -2 s and analyzed frame by frame with the image data acquisition program (Spectra-Source) of the equipment. Kinetics for Ca^{2+} increase in regions of interest (ROI) located within the cell are shown. A. Depolarization with 87 mM K^+, produce a fast calcium transient during the first seconds and a slower fluorescence increase. B. Electrical stimulations at 45 Hz during variable times totaling 100, 400 and 600 pulses. C. Testosterone (100 nM) induced calcium oscillations. D. IGF-I (10 nM) and insulin (50 nM) evoked different calcium signals.

The involvement of intracellular Ca^{2+} and extracellular signal-regulated kinases (ERK1/2) phosphorylation in the fast nongenomic effects of androgens in myotubes was also investigated (Estrada et al., 2000, 2003). Testosterone or nandrolone stimulation of myotubes produced a fast (less than 1 min) and transient Ca^{2+} increase, with an oscillatory pattern. Calcium signals were slightly reduced in Ca^{2+}-free medium but lack of oscillations was evident. Signals were blocked by U-73122 and xestospongin B, inhibitors of inositol 1,4,5-trisphosphate (IP_3) pathway. Furthermore, IP_3 increased transiently, 45 s after hormone addition. Cyproterone, an inhibitor of intracellular androgen receptor, neither affected the fast Ca^{2+} signal, nor the increase in IP_3. Calcium increases could also be induced by the impermeant testosterone conjugated to BSA (T-BSA) and the effect of testosterone was abolished in cells incubated with pertussis toxin. Stimulation of myotubes either with testosterone, nandrolone or T-BSA increased immunodetectable phosphorylation of ERK1/2 within 5 min and this effect was not inhibited by cyproterone. Phosphorylation was blocked by use

of inhibitors of the IP_3 pathway as well as by dominant negative Ras, MEK or the compound PD-98059, a MEK inhibitor. These results are consistent with a fast effect of testosterone, involving a G-protein linked receptor at the plasma membrane, IP_3-mediated Ca^{2+} signal and Ras/MEK/ERK pathway in muscle cells.

We studied the effect of both IGF-I and insulin on intracellular Ca^{2+} in primary cultured myotubes (Espinosa et al., 2004). IGF-I induced a transient Ca^{2+} increase reaching a peak around 8 s after the hormone addition and lasting about 60-80 s; this response was blocked by tyrosine kinase receptors inhibitors, genistein and AG538. IGF-I induced a fast IP_3 increase, kinetically similar to the Ca^{2+} rise and the Ca^{2+} signal was blocked by inhibitors of the IP_3 pathway. On the other hand, insulin produced a fast (<1 s) and transient Ca^{2+} increase. Insulin-induced Ca^{2+} increase was blocked in Ca^{2+} free medium and by either nifedipine or ryanodine. In the normal muscle NLT cell line, the Ca^{2+} signals induced by both hormones resemble those of primary myotubes. GLT cells, lacking α_{1s} subunit of DHPR, responded to IGF-I but not to insulin, while GLT cells transfected with α_{1s} subunit of DHPR did react to both hormones. Moreover, dyspedic muscle cells, lacking ryanodine receptors responded to IGF-I as NLT cells, however they show no insulin-induced calcium increase. Moreover, G protein inhibitors, PTX and GDPβS, blocked the insulin-induced Ca^{2+} increase without major modification of the response to IGF-I.

In cultured rat cardiomyocytes, IGF-I also induced a fast and transient increase in Ca^{2+} levels (Ibarra et al., 2004) apparent both in nucleus and cytosol, releasing this ion from intracellular stores through an IP_3-dependent signaling pathway. Ryanodine did not prevent the IGF-I-induced increase of Ca^{2+} levels but inhibited the basal and spontaneous Ca^{2+} oscillations observed when cardiac myocytes were incubated in Ca^{2+}-containing resting media. Signals are similar in both skeletal and cardia cmyocytes but spatial analysis of fluorescence images of IGF-I-stimulated cardiac myocytes incubated showed an early increase in Ca^{2+}, initially localized to the nucleus. Calcium imaging analysis suggested that part of the Ca^{2+} released by stimulation with IGF-I was initially contained in the perinuclear region. Unlike myotubes, in cardiomyocytes, pertussis toxin prevented the IGF-I-dependent mass increase of IP_3.

Table II summarizes the characteristics of the various calcium signals elicited by diverse stimuli in cultured myotubes. As detailed above, various stimuli produce strikingly different calcium signals due to a selective activation of IP_3 production with different kinetics. K^+ depolarization produces a complex Ca^{2+} response consisting in a very fast (less than 2 s) transient followed by a delayed, slower transient that starts 4-8 s after depolarization and lasts for 10-40 s (Fig. 1A). The fast Ca^{2+} transient depends on Ca^{2+} release through RyR while the slower transient depends on IP_3 receptors. Insulin induces a fast Ca^{2+} signal lasting 5-6 s and also depends on RyR. The signal evoked by

TABLE II

Features of Ca^{2+}-dependent cell signals induced by various stimuli in cultured myotubes

Stimulus	High K^+	45 Hz	Testosterone	IGF-I	Insulin
Receptor	DHPR	DHPR	Membrane Receptor?	IGF-IR	Ins-R
50% IP_3 rise time	< 2 s 10 s	75 s	30 s	7 s	No rise
Time to reach Ca^{2+} peak	10-30 s (transient)	100 s (long)	40 s (oscillation)	8 s (transient)	< 0.5 s (fast transient)

G-protein involved	Yes	ND	Yes	No?	Yes

IGF-I, on the other hand starts very fast and peaks in about 8 s, lasting about one minute (Fig. 1D). Testosterone is the only hormone that systematically induces a pattern of oscillations; the signals start normally with a slow rise and oscillations start at about 30-40 s with a frequency of about 0.1 Hz, lasting more than 2 min (Fig. 1C). Signals elicited by electrical stimulation vary in onset time and duration depending on both frequency and number of pulses (Fig. 1B). When stimulated with 100 pulses, a small and relatively fast transient similar to the one elicited by high K^+ was obtained. When the number of pulses was increased to 400, a delayed and very slow Ca^{2+} transient was obtained; if the number of pulses is further increased up to 600, the Ca^{2+} transients are faster and larger. All signals, with the exception of the fast component elicited by K^+ and the signal elicited by insulin, depend on IP_3 receptors and are associated with a rise in IP_3 concentration that peaks just before the Ca^{2+} rise.

As each of these stimuli will presumably affect expression of a particular set of genes, this system has the potential of providing us with clues on the mechanisms involved in the actual regulation of gene expression. In this context, calcium signals may be regarded as part of a code the cell uses to target the response to a given set of genes.

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