NFAT activation by membrane potential follows a calcium pathway distinct from other activity-related transcription factors in skeletal muscle cells

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Depolarization of skeletal muscle cells triggers intracellular calcium signals mediated by ryanodine and IP3 receptors. We have reported that K^+-induced depolarization activates the transcriptional regulators ERKs, CREB, c-fos, c-jun and egr-1 through IP_3-dependent calcium release, whereas NFκB activation is elicited by both ryanodine and IP3 receptors-mediated calcium signals. We have further showed that field stimulation with electrical pulses results in an NFκB activation increase being it dependent of the amount of pulses and independent of their frequency.

In this work, we report the results obtained for NFAT-mediated transcription and translocation generated by both K^+ and electrical stimulation protocols in primary skeletal muscle cells and in C2C12 cells. The calcium source for NFAT activation is through release by ryanodine receptors and extracellular calcium entry. We found this activation to be independent on the number of pulses within a physiological range of stimulus frequency and enhanced by long-lasting low frequency stimulation. Therefore, activation of NFAT signaling pathway differs from that of NFκB and other transcription factors. Calcineurin enzyme activity correlates well with the relative activation of NFAT translocation and transcription using different stimulation protocols. Furthermore, both K^+-induced depolarization and electrical stimulation increases mRNA levels of type 1 IP3 receptor mediated by calcineurin activity, which suggests that depolarization may regulate IP3 receptor transcription.

These results confirm the presence of at least two independent pathways for excitation-transcription coupling in skeletal muscle cells, both dependent on calcium release and triggered by the same voltage sensor but activating different intracellular release channels.

Keywords: NFAT transcription, NFAT translocation, calcineurin, IP3 receptor, ryanodine receptor

INTRODUCTION

In skeletal muscle cells, depolarization by high K+ induces calcium release from intracellular stores mediated both by ryanodine (RyR) and by IP3 receptors (IP3R) (11, 18). The kinetics of the resulting calcium transients induced by these two mechanisms are different; while the RyR-mediated transient which drives contraction occurs within a second, there is a delayed long-lasting calcium transient, which lasts from several seconds to minutes, that is mediated by the IP3R (10, 18). The subcellular localization is also particular for each type of transient. The fast calcium increase can be seen in both cytoplasm, and nuclei; whereas the slower transient occurs most prominently in the nuclei with a minor component detected in the cytoplasm.

The transcriptional responses that arise from calcium signals, which may differ in their amplitude, kinetics, and subcellular localization, will have different outcomes (6, 8, 9, 12). We have already reported a highly specific activation of the transcriptional regulators ERKs, CREB, c-fos, c-jun, and egr-1 associated to K^+-depolarization-induced IP3R mediated-calcium release in skeletal muscle cells in primary culture (2, 4, 5, 31). Recently, we determined that both RyR and IP3R-mediated calcium transients regulate the increase of NFκB activity by depolarization in primary myotubes and in C2C12 cells (38). In addition, NFκB activity discriminates between the number of pulses in its response to field electrical stimulation of variable frequency and number of pulses. These precedents allow us to conclude that electrical activity of muscle cells depolarized either by high K^+ or by field electrical stimulation, which result in different patterns of calcium increase involving either RyR and/or IP3R (10, 18, 38), represent an adequate model to study calcium regulation of transcriptional responses. The characteristics of the calcium transients induced by stimulation with these different protocols are summarized in Table 1.

In this work, we study the regulation of the nuclear factor of activated T cells (NFAT) via calcium released by depolarization induced either by K^+ or by electrical stimulation in skeletal muscle

cells in culture. NFAT is highly dependent on calcium because calcineurin (CaN), a calcium/calmodulin-dependent serine/threonine phosphatase, regulates its activity (16). It is sensitive to dynamic changes in intracellular calcium as reported in lymphocytes (8, 9, 36) and also in striated muscle (26, 28, 29, 35, 37). NFAT is a multigene family composed of five proteins, NFAT1 (NFATc2), NFAT2 (NFATc1), NFAT3 (NFATc4), NFAT4 (NFATc3) and NFAT5 (TonEBP), of which NFAT1-4 proteins are regulated by calcium/CaN dependent signaling (16). In non-stimulated cells, NFAT proteins are normally located in the cytoplasm in a hyper-phosphorylated latent form. After an increase in the intracellular calcium concentration, cytoplasmic activated CaN directly dephosphorylates NFAT, inducing its nuclear accumulation and binding to their target promoter elements either alone or in combination with other transcription factors (16). Because CaN is present in the nucleus of stimulated cells, is able to maintain the dephosphorylated status and nuclear localization of NFAT. When nuclear calcium decreases, CaN activity is inhibited, NFAT is re-phosphorylated by kinases, exits the nucleus, and NFAT-dependent gene expression is terminated (16). Therefore, NFAT-dependent activation of transcription would require both cytoplasmic and nuclear calcium (8, 16).

In skeletal muscle cells, the expression of three NFAT isoforms (c1, c2, and c3) is at similar levels at the stage of myoblasts, nascent myotubes and mature myotubes (1). However, NFAT-mediated transcription increases with differentiation and besides, individual isoforms translocate to the nucleus at specific stages of the myogenesis in response to a calcium signal. In mature myotubes, the NFATc1 isoform responds to calcium increase (1). NFAT activation has been linked to various skeletal muscle processes like muscle growth and development (17), regulation of muscle hypertrophy (30, 33) and the switching from fast-to-slow phenotype (7, 26,28, 29, 37).

Concerning to the origin of the calcium involved, NFAT activation in response to 1 Hz stimulation of rabbit primary skeletal muscle culture would depend on RyR-mediated calcium release (26). K^+-depolarized C2C12 cells present the same dependence with an important role for extracellular

calcium as well (32). Other studies, performed in C2C12 cells and in cultured avian skeletal muscle fibers, have reported a role for both RyR and IP3R-mediated calcium release in NFAT activation (20, 21, 35).

Now, we report that both NFAT translocation and NFAT-mediated transcription induced by K^+ and electrical stimulation depends on calcium released by the RyR and not by the IP3R signaling pathway. In addition, there is a contribution of extracellular calcium. Field stimulation protocols varying in frequency and number of pulses, allowed us to study the specificity of NFAT response to these two parameters and to compare it with the NFκB response in the same model (38). We have also examined the IP3R1 mRNA level in primary myotubes depolarized by K^+ or electrically stimulated. We found a significant increase of the receptor messenger, inhibited by cyclosporin A (CsA), a CaN inhibitor, suggesting that depolarization may regulate the IP3R channel transcription.

MATERIALS AND METHODS

Reagents. CsA, U-73122 and A23187 were from BIOMOL Research Laboratories (Plymouth Meeting, PA). 2-aminoethoxydiphenyl borate (2-APB) was from Aldrich. Ryanodine and nifedipine were from Sigma. Anti-NFATc1 was from Affinity BioReagents (Golden, CO). β-actin antibody was from Sigma. Anti-histone 3 was from Santa Cruz Biotechnology. Secondary HRP-conjugated anti-rabbit and anti-mouse antibodies were from Pierce (Rockford, IL) and from Sigma, respectively. Cell culture media and reagents were from Sigma and from InVitrogen (Grand Island, NY).

Cell culture. Primary cultures of rat skeletal muscle cells were prepared from Sprague-Dawley neonates as previously reported (5, 38). Briefly, muscle tissue dissected from the hind limbs was minced and treated with collagenase for 15 min at 37º C and grown in medium composed of F12: DMEM (1: 1), 10% bovine serum, 2.5%FSB, 100 U/ml penicillin, and 10 mg/ml streptomycin in 60 mm plates. Two days after plating, fibroblast growth was inhibited with cytosine arabinoside (5 mM) maintained for 24 h. For differentiation, growth medium was changed to serum-free medium at day 4 of culture. 6-7 day-old cultures were employed for the experiments.

The C2C12 cell line (ATCC, Manassas, VA, USA) was grown in DMEM-F-12 (1:1) with 10% bovine serum and 2.5 % FCS. The serum was replaced by 5% horse serum in order to differentiate myoblasts into myotubes.

Plasmids reporter construction. NFAT transcriptional activity was mainly monitored with a NFAT-dependent reporter construct as described (29). Intron 3 segment from the human MCIP1 (DSCR1) gene was isolated by polymerase chain reaction (PCR) using genomic human DNA as templates and primers based on sequences information from NCBI databank. The PCR product, an 850 pb fragment containing 15 NFAT binding sites, was subcloned into a pGL3 luciferase reporter vector (Promega, Madison, WI) (Xho I site). The orientation of the insert was verified by PCR amplification

of the isolated DNA of the different clones. The other construct employed, obtained generously from Dr. Gerald Crabtree laboratory, consisted of a trimerized human distal IL2 NFAT site inserted into the IL2 minimal promoter, linked to luciferase (pGL3).

Cell transfection and luciferase reporter assay. Primary cells in culture were transiently transfected with FuGene 6 (Roche Diagnostics, Indianapolis, IN) as previously reported (38). Two days-old myoblasts were transfected with 3 µl FuGene 6 in 1.5 ml DMEM containing 0.9 µg of the reporter vector DNA and 0.1 µg of the *Renilla* phRL-TK vector (Promega, Madison, WI). The mixture was maintained for 12h, replaced by serum-free medium and the cells maintained until well differentiated. Cells were harvested and lysed 6 hours after the experiments. Each experimental condition was performed in duplicate. Luciferase activity was determined using a Dual-luciferase reporter assay system (Promega, Madison, WI) and luminescence was measured with a Berthold F12 luminometer. Results were normalized for transfection efficiency and expressed as the ratio of *Firefly* to *Renilla* luciferase. Transfection with the empty pGL3 luciferase plasmid as a control resulted in no increase in luciferase activity after stimulation. Transfection efficiency was 5% as previously reported in muscle cell primary cultures (38).

Cell treatment. Differentiated primary culture cells or differentiated C2C12 cells were washed with Ca^{+2} –Mg^{+2} free PBS and kept in Krebs-Ringer under resting conditions for 30 minutes (20 mM HEPES-Tris pH 7.4; 118 mM NaCl, 4.7 mM KCl, 3 mM CaCl2, 1.2 mM MgCl2 and 10 mM glucose). To induce depolarization by high K^+, NaCl was replaced isosmotically by KCl (84 mM). Resting or depolarizing saline without calcium contained 0.5 mM EGTA and 4.2 mM $MgCl_2$ (38).
Myotubes electrical stimulation was done as previously reported (38). 20-50 V amplitude, 1 ms supra-threshold voltage pulses were delivered through platinum wires from high current capacity stimulators. 400 and 1000 pulses delivered at frequencies of 1, 10 and 45 Hz were employed. Stimulation-induced myotube contraction was monitored under the microscope in each experiment in order to check for contraction as an index of both cell viability and stimulus intensity threshold.

Subcellular fractionation. Nuclear and cytosolic fractions from C2C12 cells were obtained according to Biswas et al., 1999 as reported (38). LAP 2 (BD Transduction Labs., Franklin Lakes, NJ) and IκBβ (Cell Signaling Technology, Beverly, MA) Western blots were performed to assess the purity of nuclear and cytosolic fractions, respectively.

Immunoblot analysis. Proteins were resolved by 10% SDS-PAGE, transferred to PVDF membranes (Millipore Corp, Bedford, MA), and blocked for 1 h at room temperature in TBS, 0.1% Tween-20 and 5% milk. Incubations with primary antibodies (1:1000) were performed at 4ºC overnight. After incubation for 1.5 h with HRP-conjugated secondary antibodies, enhanced chemiluminescence (ECL, Amersham Biosciences, UK) was performed. The films were scanned and the ImageJ program was employed for densitometric analysis of the bands. To correct for loading, membranes were stained with Coomassie Blue (BioRad, Hercules, CA) or stripped and blotted against β-actin or histone H3.

Immunocytochemistry. C2C12 myotubes grown on coverslips were fixed in ice-cold methanol, washed with TBS and permeabilized with 0.2% Triton X-100 for 5 minutes. Cells were blocked for 1 h in 5% PBS-BSA and incubated overnight with anti-NFATc1 antibody at 4°C. The cells were washed three times with PBS-BSA and incubated with secondary antibody for 90 minutes at room temperature. The coverslips were mounted in Vectashield (Vector Laboratories, Inc.) in order to retard photobleaching. The samples were evaluated with a confocal microscope (Carl Zeiss Axiovert 200M – LSM Pascal 5) and documented through computerized images.

Calcineurin phosphatase activity assays. Myotubes exposed to K^+ depolarization or electrical stimulation (45 Hz- 400 pulses) were lysed in 100 µl of calcineurin assay buffer (BIOMOL) at different times. Lysates were sonicated for 1 min, incubated on ice and centrifuged to 150,000 x g. The quantitative assay system was performed by using 3 µg of protein according to the manufacturer's procedure. Calcineurin phosphatase activity was measured spectrophotometrically by detecting free phosphate released from the calcineurin-specific RII phosphopeptide.

Semi-quantitative RT-PCR. Total RNA was extracted from primary skeletal muscle cells with TRIzol Reagent (InVitrogen, Carlsbad, CA) and cDNA was synthesized using SuperScript II reverse transcriptase (InVitrogen, Carlsbad, CA) and oligo dT primers. The cDNA was amplified by using IP3R1 primers (31) and the DNA concentration was normalized against β-actin. PCR amplification was maintained in the exponential phase for both products. The IP3R1 primers used were 5' GAA GAG AAA CTG TGC ATT 3' (sense) and 5' GGC AAT GGT CCA CTA TCA 3' (antisense). PCR conditions were: initial denaturation at 94 °C for 5 min, followed by 28 cycles at 94°C for 30 s, 55 °C for 30 s, 72 °C for 30 s) and final extension at 72ºC for 10 min, for both amplification of IP3R1 and β-actin cDNA. PCR products were resolved by electrophoresis on 1.8% agarose gel and stained with ethidium bromide. Bands were quantified by densitometric analysis with the ImageJ program.

Data analysis. Data are expressed as means ± S.E. One-way or two-way ANOVA followed by Bonferroni or Tukey post-test were employed to determine differences of means between groups.

RESULTS

High K^+ and electrical stimulation induce NFAT activation in primary myotubes and C2C12 cells. NFAT transcriptional activity measured in primary myotubes with the DSCR/MCIP1 reporter gene which contains 15 NFAT-binding sites (29) was significantly increased by high extracellular K^+ (84 mM, isosmotic exchange of NaCl by KCl) and by the field stimulation protocols (Fig. 1A). The NFAT-mediated transcription shows activation by the three sets of frequencies and amount of pulses used. At 45 and 10 Hz with 400 and 1000 pulses and at 1 Hz and 400 pulses statistically significant increases were found, and a much larger increase was observed with the electrical stimulation at 1 Hz and 1000 pulses (Fig.1A). These results were confirmed by the use of a weaker reporter that contains only three response elements to NFAT. In myotubes, it required three times the standard stimulation in order to get detectable activation (Fig. 1B). This verification was necessary to confirm that the

DSCR/MCIP1 reporter gene reflects NFAT transcriptional activity, since this construct contains other response elements in addition to the NFAT binding sites (29).

It has been reported that CsA blocks NFAT activation induced by electrical stimulation of adult muscle cells and of skeletal muscle cells (26, 28, 34), this action is mediated by CaN inhibition. We confirmed this finding in primary myotubes transfected with the DSCR/MCIP1 reporter gene and stimulated either by high K^+ or with electrical stimulation at 45 Hz with 1000 pulses, where the increase in NFAT transcriptional activity was completely inhibited by CsA (Fig. 1 C).

The NFAT translocation to the nucleus is a further confirmation of NFAT activation. To this purpose we studied NFATc1 translocation to the nucleus of C2C12 cells, because cytoplasmic and nuclear fractions from this cell line are much cleaner than those obtained from primary culture (38). C2C12 cells respond to high K^+-induced depolarization and to electrical stimulation with the same patterns of calcium transients as primary culture (11, 38). Four protocols were employed to analyse NFAT translocation, K^+-induced depolarization, and electrical stimulation with either, 45 Hz / 400 or 1000 pulses or 1 Hz / 1000 pulses. These protocols were selected considering the clear-cut differences in transcriptional activation determined with the strong reporter (Fig.1A). NFAT Western blots showed increased NFAT translocation in parallel to NFAT-mediated transcription obtained in response to these stimuli (Fig. 2A). As expected, nuclear NFAT lifetime was higher after 1 Hz /1000 pulses stimulation, followed by 45 Hz /1000 pulses, 45/400 pulses and by K^+-induced depolarization. Significant NFAT translocation was detected at 30-45, 15, 5-15 and 5 min, of the respective stimulation (Figure 2B).

We also performed immunocytochemistry after stimulation of C2C12 cells with high K^+ and electrical protocols. A slight NFATc1 translocation to the nuclei could be detected in stimulated cells, which was much smaller than the control NFAT translocation in response to the calcium ionophore A23187 that gave a significant translocation (not shown). These results did not allow quantification under the stimulation conditions employed but confirmed those obtained with subcellular fractions.

In order to better understand NFAT activation mechanisms by depolarization, we have also determined CaN activity by an enzymatic assay in primary myotubes stimulated by high K^+ and by the electrical protocol of 45 Hz / 400 pulses. CaN activity increase presented marked differences in kinetics and in the extent of activation comparing these two protocols (Fig. 3). The maximal increase in CaN activity after K^+ induced-depolarization was 2-fold at 1 min, while a maximal activation of 3 to 4-fold was determined 5 min after the electrical stimulation (Fig.3).

NFAT activation depends on RyR-mediated calcium release and on extracellular calcium entry. The role of calcium release from intracellular stores in NFAT activation was evaluated by measuring transcription and translocation. Experiments were performed with the 3X reporter in primary muscle cells exposed to high K^+ concentration, and with the 15X reporter in myotubes stimulated by high K^+ or by 45 Hz with 400 or 1000 pulses. In the presence of 50 μM ryanodine (receptor blocking concentration), a significant decrease of NFAT activation was found (Fig. 4 A-D). There was no blockade of activation by inhibiting the IP3R-mediated slow calcium transient with 50 μM 2-APB or with 30 μM U-73122, a PLC inhibitor (Fig. 4 A-F). In our hands, 2-APB abolishes the IP3R-dependent calcium transient, as does the PLC inhibitor (5, 11, 31). In C2C12 subcellular fractions, a significant decrease in NFATc1 translocation occurred mainly in the presence of ryanodine (Fig. 5 A, B, C). A small but significant effect of 2-APB was found only after 5 min of stimulation with 45 Hz/1000 pulses (Fig. 5B).

In the literature, NFAT increased translocation by high K^+-induced depolarization of C2C12 cells has been reported to depend on extracellular calcium (32, 35). We studied this aspect determining that NFAT-dependent transcription assessed by DSCR/MCIP1 reporter gene is partially dependent on extracellular calcium in myotubes stimulated with high K^+, and by the electrical stimulation protocols of 45 Hz /400 pulses and 1 Hz /1000 pulses (Fig. 6 A). Also, we found that NFATc1 translocation in the absence of extracellular calcium was significantly reduced compared to the results obtained in the

presence of extracellular calcium in myotubes stimulated with the 45 Hz /1000 pulses protocol (Fig 6B) and the 1 Hz /1000 pulses protocol (Fig. 6 C). The effect of calcium-free medium was not due to intracellular stores calcium depletion because myotubes challenged with 10 mM caffeine after 30 min in calcium-free saline presented calcium release at the same extent as controls in saline with 3 mM calcium (not shown).

We further examined NFAT –dependent transcription in experiments performed with the protocols high K^+, 45 Hz /400 pulses and 1 Hz /1000 pulses, now in the presence of 10 μM nifedipine. There was no decrease of activity except for a minor significant effect in cells exposed to high K^+ , suggesting that the requirement of extracellular calcium for NFAT activity does not depend on calcium influx through L-calcium channels (data not shown).

Depolarization induces a CaN-dependent increase in IP3R1 mRNA. In order to determine whether CaN activation and possibly NFAT activation by depolarization in skeletal muscle cells participate in the regulation of a late response gene, we have measured IP3R1 mRNA levels in primary myotubes depolarized by high K^+ or electrically stimulated.

Hippocampal cells depolarization induces NFATc4 activation and an increase in IP3R protein levels, both processes are CaN-dependent (14). Additionally, preliminary experiments in null mice for NFATc2 and c4 resulted in low levels of IP3R1 gene expression (14). Considering that the promoter region of the murine IP3R1 gene contains several candidate NFAT-binding sequences, these experiments suggest that the IP3R1 is a target of NFAT (14). Also in the hippocampus, exposure to BDNF results in activated NFAT transcription and in increased expression of IP3R1, both reduced by CaN inhibition (15). In cerebellar granule cells, IP3R1 expression is increased by depolarization-induced increase of intracellular calcium by L-channels or by NMDA receptors, and is inhibited by CaN pharmacologic blockade (13). The CaN dependence of IP3R1 expression has been corroborated in the same model analysing gene expression by microarrays (24).

We studied the IP3R1 mRNA levels in primary myotubes depolarized by high K^+. Data was collected up to 8 hours after K^+ exposure. There was a statistically significant increase at 4 h after the depolarization event (Fig. 7). As a control, cells were exposed to the same number of medium changes and maintained under resting conditions for 4 hours. Depolarization performed in the presence of 1 μM CsA, resulted in blockade of the effect. Very similar results were obtained in cells stimulated with the electrical protocol of 1 Hz and 1000 pulses. Five hours after the stimulus, there was a significant increase ($p < 0.05$) of IP3R1 mRNA with respect to cells maintained in resting conditions for 5 h, that was diminished in cells preincubated with 1 μM CsA. The values (mean ± SEM), expressed as fold induction of a normalized 100% control, were 217.3 ± 33.9 in myotubes exposed to the electrical protocol, and 123.6 ± 15.2 in cells stimulated in the presence of the CaN inhibitor ($n = 3$). These results indicate that CaN participates in the regulation of IP3R1 expression increased by depolarization, and suggest that NFAT activation might be involved in the signalling for IP3R1 synthesis.

DISCUSSION

Skeletal muscle cells in culture respond to depolarization induced by high K^+ and by different physiological patterns of electrical stimulation with two calcium transients, mediated by either RyR- or IP3R, with different kinetics and subcellular localization (10, 18, 38). The analysis of the activation of calcium-dependent transcription factors by these well-defined calcium transients might contribute to understand how calcium regulates excitation-transcription in skeletal muscle. A main result of this study is that in our system, NFAT presents a response to electrical stimulation that differs from that of the transcription factor NFκB (38). Furthermore, the NFAT activation requirement of RyR-mediated calcium increase is markedly different from that of NFκB activation, which depends on both RyR- and IP3R-calcium release (38). In addition, it is also different to other transcription factors that depend solely on the IP3R elicited calcium signal (2, 5, 22, 31, 38). The results on the calcium dependence of

different transcription factors were summarized in Table 2. Therefore, this work furthers the notion that transcription factors differentially sense and decode skeletal muscle calcium signals.

NFAT-mediated transcription increased in response to high K^+ (chronic depolarization) as well as to electrical stimulation (fluctuating depolarization). In our experimental setup, the electrical stimulation is the closest emulation to the physiological situation in the organism (10, 27). We used electrical protocols of frequencies from 1 to 45 Hz with 400 and 1000 pulses, which were all effective activating NFAT. However, the response to 1 Hz /1000 pulses was significantly higher than the response to the rest of the stimulation patterns used. The study of NFκB activation by the same stimuli meanwhile showed that the activation of this transcription factor discriminated between 400 and 1000 pulses at all frequencies (38).

Several reports have shown that a low frequency of long-lasting stimulation, mimicking slow-twitch fiber activity, activates NFAT both in in cultured cells and in adult fibers *in vitro* and *in vivo* (25, 26, 28, 29, 32, 37). When rabbit primary myotubes were treated with 1 and 10 Hz electrical stimulation, as a model to study fiber type transformation, NFAT was activated (25, 26). In addition, NFAT nuclear translocation in cultured adult mouse skeletal fibers in response to various patterns of electrical stimulation is strongly dependent on the temporal pattern of the stimuli (28). In this case, 10 Hz continuous or in trains stimulation resulted in NFAT translocation, while the application of 50 Hz trains, or the continuous stimulation at the low frequency of 1 Hz did not result in nuclear translocation (28). NFAT specific response to low frequency stimulation was also reported in rat adult denervated slow muscle (29), as well as in muscles of intact animals by stimulation of the motor nerve (32) and in living mice (37). In our rat myotube model, we found that NFAT is activated by low and high frequencies 3 to 4-fold over control levels. Only at 1 Hz with 1000 pulses, we obtained a 7-fold increase. These discrepancies may reflect that skeletal muscle cells at different developmental stages may present age-specific responses to the same temporal patterns of stimulation.

Low frequencies of stimulation tend to activate primarily fast calcium transients and no slow calcium transients (see Table 1) ; the fact that NFAT activation was similarly attained at a broad range of frequencies, supports the notion of a mechanism for activation based mainly on the fast calcium transient i.e. activation by RyR and not by IP3R signaling pathway. In our work, the 1 Hz stimulus does not result in a clear IP3R-dependent slow calcium transient (38), therefore, the blockade of the IP3 system should not affect the outcome. The rest of the protocols, high K^+ and stimulation frequencies at 45 and 10 Hz, show RyR- and IP3R-dependent calcium transients, but the inhibition of the IP3 system by 2-APB only showed a minor effect, significant only after 5 min of exposure to 45 Hz/1000 pulses in NFAT translocation measurements. Besides, we have obtained no differences when analyzing NFAT transcription in the presence of the phospholipase C inhibitor U-73122, supporting that the IP3 pathway is not regulating NFAT activation. The increase of NFAT-mediated transcription observed either with K^+ or with electrical stimulation at 45 Hz/400 or1000 pulses and 1 Hz/1000 pulses, is probably the result of NFAT longer nuclear lifetime. The RyR-dependent calcium increase with these stimuli lasts for 1, 9, 22 and 1000 s, respectively (see Table 1), and is visualized both in the cytoplasm and in the nuclei (10, 18, 38). Therefore, since NFAT activation depends on both cytoplasmic and nuclear calcium increase, these results suggest that RyR-induced calcium increase would contribute to a longer time window for translocation and thus for an augmented transcription. Because calcium detection with the fluorescent dye Fluo-3 AM gives information on the kinetics and localization of the calcium signals, but no information on calcium levels, we have assessed CaN activity directly. A recent study (35) in C2C12 cells has also reported that depolarization-induced NFATc1 nuclear entry depends on RyR-mediated calcium release by membrane depolarization. However in this process, there would be a contribution of the IP3-dependent calcium pool to NFAT signaling in myotubes by promoting NFAT nuclear exit. To activate the IP3 system the authors stimulate with UTP which mobilises IP3 and the combined stimulation of myotubes with KCl and UTP decreases NFAT translocation with respect to the level found with stimulation solely with KCl. They attribute the differences on NFAT trafficking to

spatial differences of the calcium response induced by RyR or IP3R activation, because UTP increases calcium only in the cytoplasm while mobilization of calcium by KCl shows increased calcium in the whole cell, including the nuclei (35). In our hands, K^+ or electrical stimulation also induce RyR-mediated calcium release in the whole cell, but the depolarization-induced IP3R-mediated slow calcium transient shows a well defined calcium increase in the nucleus apart from a smaller cytoplasmic component. If the IP3R-dependent calcium pool plays a role inhibiting NFAT nuclear exit, the inhibition of the IP3 system should increase NFAT translocation and transcription. In the present work, the stimulation with electrical protocols that do not induce IP3R-induced calcium release effectively results in higher NFAT activation as compared to those protocols that result in both fast and slow calcium signals. However, the stimulation with high K^+ and the 45 Hz pulses protocols in the presence of 2-APB, which results in inhibition of the slow calcium signal, was not significantly affected.

The regulation of NFAT-dependent transcription by calcium release mediated by the IP3R and the RyR signalling pathways has also been studied in avian skeletal muscle adult fibers in order to characterize the transcriptional regulation of the slow myosin heavy chain 2 MyHC2 promoter (19-21). Mutagenesis studies have indicated that both NFAT and MEF2 binding sites are required for innervation-induced MyHC2 promoter activity in slow muscle fibers (19). Basically, there is NFAT activation by RyR inhibition (20), while IP3R blockade results in the opposite effect (21). These results might reflect complex mechanisms of NFAT regulation in another developmental stage as well as differences with respect to the stimulatory pattern being used.

NFAT activation in C2C12 cells stimulated with K^+ depends on extracellular calcium (32, 35). Activation after 1 min KCl-induced depolarization in C2C12 myotubes resulted in NFATc1 nuclear localization in the absence of extracellular calcium. However, under the same conditions, when KCl exposure was increased from 1 to 10 min, nuclear NFATc1 was depleted from the nuclear fraction (32). The presence of extracellular calcium during the KCl exposure prolonged NFATc1 nuclear

localization, indicating that the SR calcium pool was important in initiating NFATc1 activation but that replenishment by extracellular calcium was required to maintain this activation. We have confirmed the extracellular calcium requirement for a fraction of NFAT activation by K^+ in C2C12 cells and extended it to primary myotubes, and in both cell type cultures in response to electrical stimulation. Besides, both IP3mRNA increase and NFAT activation were blocked by CaN inhibition, suggesting that NFAT could be involved in IP3R1 up-regulation. CaN is crucial in NFAT activation but also controls the activation of several other transcription factors. While in our experimental model NFAT activation requires extracellular calcium, the activation of other transcription factors we have studied is independent of this source of calcium. Thus, CREB, c-fos, c-jun and NFκB activation by depolarization was not affected by the absence of extracellular calcium (5, 38). As mentioned, extracellular calcium dependence of NFAT activation is another difference with respect to NFκB activation, which was found to be dependent on both RyR and IP3R- dependent calcium pools (38). In our experiments; the IP3 system inhibitor 2-APB had no significant effect on NFAT transcription or on translocation, this same inhibitor blocks about half of the NFκB response (38) and all of the CREB response (5). Different transcription factors then appear to be regulated by pharmacologically dissociable calcium transients (Table 2).

Our results indicate that IP3R1 mRNA levels increase in K^+-depolarized or electrically stimulated primary cells as occurs in hippocampal and cerebellar neurons (13, 14). Besides, both IP3mRNA increase and NFAT activation were blocked by CaN inhibition, suggesting that NFAT could be involved in IP3R1 up-regulation. CaN is crucial in NFAT activation but also controls the activation of several other transcription factors. Several genes involved in calcium regulation are activated by depolarization through a CaN-mediated mechanism in neurons, suggesting activity-dependent regulation of calcium signalling at the transcriptional level (see 24). In our work, NFAT activation in primary muscle cells was not dependent on IP3R-dependent calcium release. In adult fast fibers however, the inhibition of the IP_3R by 2-APB or by Xestospongin D resulted in reduction of NFAT-

dependent transcription and nuclear localization (21). NFAT activity regulation by IP_3R-dependent calcium signalling might have a significant role in a long-term muscle fiber remodelling but primary culture may not be the best model system to study this regulation. Since IP_3R-dependent calcium release participates in the regulation of transcription factors like early genes, CREB and NF-kB, the possibility is raised that IP_3R up-regulation might influence the activation of these factors. The results obtained in this work, with respect to CaN activation and its involvement in the IP3R1 increased expression with depolarization, open interesting questions with respect to the regulation of genes associated to calcium fluctuations by physiological activity in skeletal muscle.

We have demonstrated, using the same cell system, that membrane depolarization of muscle cells elicits highly selective mechanisms mediated by calcium that activate several transcriptional regulators (Fig. 8). In addition, the slow calcium signal in cultured muscle cells also regulates the expression of genes such as interleukin 6 (22) and troponin I (23); while other genes with calcium-dependent elements within their promoters such as α-actin 1 gene, appear not to be regulated by this particular calcium transient (23).

For skeletal muscle, both frequency and duration of electrical stimuli (or even lack of stimuli) have a meaning in terms of genes that will be up or down-regulated. For example, the frequency pattern is especially important for fiber type specification. Therefore, specific patterns of activity will generate specific calcium signals, which will be discriminated by the different genes. Our results suggest that low frequency stimulation activates a calcium-dependent pathway leading to maximal NFAT activation whilst maximal activation of other transcription factors as NFkB, CREB or AP1 needs an IP3-dependent calcium pathway that is activated only after high frequency stimulation (Fig. 8). These results and further studies on depolarization-induced activation of transcription factors and specific genes will contribute to understand the fine regulation of excitation-transcription coupling in skeletal muscle.

GRANTS

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FOOTNOTES

[1] Abbreviations

RyR, ryanodine receptor; IP3R, inositol-1,4,5-trisphosphate receptor; NFAT, nuclear factor of activated T cells; CaN, calcineurin; CsA, cyclosporin A; 2-APB, 2-aminoethoxydiphenyl borate

FIGURE LEGENDS

Fig.1. K^+-induced depolarization and electrical stimulation induce NFAT-dependent transcription in primary muscle cells. Seven day-old myotubes transfected with a NFAT reporter gene were incubated for 30 min in Krebs-Ringer under resting conditions prior to stimulation. Cells were either not stimulated (NS) or subjected to a 1min pulse with 84 mM KCl or exposed to different electrical stimulation protocols according to those described in "Materials and Methods," and harvested 6 hours after stimulation. Results were normalized for transfection efficiency and expressed as the ratio of *Firefly* to *Renilla* luciferase. Data are the means $\pm$ SE (error bars) of duplicates (n = 4-9 independent experiments), and expressed relative to that in not stimulated cells. Statistical analysis was performed by one-way ANOVA test followed by Bonferroni's post-test. Symbols: *, ** and *** correspond to $P < 0.05$, $P< 0.01$ and $P< 0.001$, respectively, with respect to the not stimulated control group.
A: DSCR/MCIP1 reporter gene, &&&, $P < 0.001$ with respect to K^+ stimulation and to single protocols of 10 and 45 Hz with 400 and 1000 pulses. **B:** 3 x NFAT reporter gene, cells transfected with this reporter gene were stimulated 3 times with each protocol. The activation of transcription by exposing cells to 10-7 M A23187 is shown for comparison. **C:** Cells transfected with the DSCR/MCIP1 reporter gene were pre-incubated with the vehicle or with 1 μM CsA for 30 min, stimulated in the absence or in the presence of CsA, and maintained for another 30 min with the inhibitor or the vehicle.

Fig.2. High K^+-induced depolarization and electrical stimulation induce NFATc1 translocation in C2C12 muscle cells. **A:** Western blots of NFATc1 in cytoplasmic (C) and nuclear (N) fractions obtained from C2C12 cells after exposure to 84 mM KCl for 1 min, to a single protocol of 45 Hz and 400 pulses, or at 45 Hz and 1000 pulses, or at 1 Hz and 1000 pulses. Results were normalized by

Coomassie blue stained membranes. **B:** nuclear NFATc1 densitometric analysis. Data are the means ± SE (error bars) of duplicates (n = 3 independent experiments), expressed relative to that in not stimulated cells (time 0). Statistical analysis was performed by two-way ANOVA test followed by Bonferroni's post-test. Symbols: *, ** and ***, correspond to $P < 0.05$, $P< 0.01$ and $P< 0.001$, respectively, with respect to the not stimulated control group.

Fig. 3. Calcineurin is activated by K^+-induced depolarization (**A**) and by electrical stimulation in primary skeletal muscle cells (**B**). Myotubes were stimulated by high K^+ or with a 45 Hz and 400 pulses protocol, and processed for measurement of CaN activity as described in "Materials and Methods." Data are the means ± SE (error bars) of duplicates (n = 3 independent experiments), expressed relative to that in not stimulated cells (time 0). Statistical analysis was performed by one-way ANOVA test followed by Bonferroni's post-test. * and ** , correspond to $P < 0.05$ and $P< 0.01$, respectively, with respect to the control group.

Fig.4. NFAT-mediated transcription induced by KCl and by electrical stimulation in primary myotubes depends on the RyR-induced fast calcium transient. NFAT-dependent transcription was evaluated in 7 days-old myotubes. Transfected myotubes were pre-incubated for 30 minutes with the vehicle or with 50 μM 2-APB , 50 μM ryanodine or 30 μM U73122, and stimulated by the protocols indicated in the Figure, in the absence or presence of these inhibitors. **A:** Myotubes transfected with the 3 x reporter gene were exposed to 3 cycles of high K^+ depolarization. The data in B, C and D was obtained in myotubes transfected with the DSCR/MCIP1 reporter gene. **B:** K^+ stimulation; **C:** 45 Hz /400 pulses; **D:** 45 Hz /1000 pulses; **E:** Myotubes transfected with the 3 x reporter gene were exposed to 3 cycles of 45 Hz/1000 pulses**; F:** Myotubes transfected with the 3 x reporter gene were exposed to 3 cycles of 1 Hz/1000 pulses. Data are the means ± SE (error bars) of duplicates, in 3 independent experiments. The data obtained with inhibitors is expressed with respect to the level of activation in stimulated cells in

the absence of inhibitors (control). Statistical analysis was performed by one-way ANOVA test followed by Tukey's post-test. Symbols: *, ** and ***, correspond to $P < 0.05$, $P< 0.01$ and $P< 0.001$, respectively, with respect to the control group.

Fig. 5. NFATc1 translocation induced by high K^+ and by electrical stimulation in C2C12 cells depends on the RyR-induced fast calcium transient. Cells were depolarized in the absence or in the presence of 50 μM 2-APB or of 50 μM ryanodine, and fractionated into cytoplasmic and nuclear fractions. Western blot of histone H3 was employed to correct for loading. **A:** High K^+ stimulation, left: representative Western blot, right: nuclear NFATc1 densitometric analysis; **B:** 45 Hz / 1000 pulses protocol; **C.** 1 Hz / 1000 pulses protocol. Results were obtained from 3 independent experiments performed in duplicate. Statistical analysis was performed two-way ANOVA test followed by Bonferroni's post-test. Symbols: *, ** and ***, correspond to $P < 0.05$, $P< 0.01$ and $P< 0.001$, respectively, with respect to the control group (time 0).

Fig 6. Extracellular calcium contributes to NFAT activation. Prior to stimulation, skeletal muscle cells were incubated for 30 min in resting conditions in the presence or absence of extracellular calcium and then exposed to high K^+ or to the 45 Hz /400 or 1 Hz /1000 pulses protocols. **A:** NFAT-dependent transcription in myotubes transfected with the DSCR/MCIP1 reporter gene. **B:** Nuclear NFATc1 in C2C12 cells stimulated with the 45 Hz /400 pulses protocol. **C:** Nuclear NFATc1 in C2C12 cells stimulated with the 1 Hz /1000 pulses protocol. Data are the means $\pm$ SE (error bars) of duplicates, in 3 independent experiments. Statistical analysis was performed by two-way ANOVA test followed by Bonferroni's post-test. Symbols: *, ** and ***, correspond to $P < 0.05$, $P< 0.01$ and $P< 0.001$, respectively, with respect to the not stimulated control group.

Fig 7. High K^+-induced depolarization stimulates a CaN-dependent increase in IP3R1 mRNA levels in primary myotubes. Cells were incubated for 30 min in resting conditions, exposed to high K^+ for 1 min and then maintained in resting conditions for 4 h. In parallel, cells from the same culture were preincubated and stimulated in the presence of 1 μM CsA. Controls for these experiments were obtained from not stimulated cells maintained for 30 min in resting conditions and processed (C 0 h), and cells that were further maintained in resting conditions for 4 h (C 4 h). **A:** IP3R1 and β-actin mRNA expression levels were tested by semi-quantitative RT-PCR. **B:** IP3R1 densitometric analysis. Data are the means $\pm$ SE (error bars) of 3 to 5 independent experiments. All the results were normalized with respect to β-actin. ANOVA test followed by Bonferroni's post-test was employed to analyze results with respect to C 4h. Symbol **, $P < 0.01$, cells stimulated with K^+ and analyzed after 4 h.

Fig 8. Schematic description of calcium pathways involved in NFAT and other transcriptional regulators activation. The figure is based on this work and on previous published data obtained in our laboratory. Membrane depolarization results in DHPR activation followed by RyR and IP3R-dependent calcium release and in calcium entry. Both RyR-mediated calcium signaling pathways and extracellular calcium contribute to NFAT activation. NFκB activation meanwhile, depends on both RyR- and IP3R-calcium pathways and does not require extracellular calcium (38). IP3R-dependent calcium release is involved in ERK and CREB phosphorylation (5, 31) and in early genes mRNA increase (2, 5). There is also PKC activation by the IP3R-dependent calcium release pathway (4).

Long-term low frequency stimulation will maximally activate NFAT pathway via RyR, while pathways dependent on IP3R will be activated only after high frequency stimulation (which will also activate the fast calcium release). On the other hand, transcription factors such as NFκB, will be partly activated by low frequency and maximally activated at high frequency through both pathways.

LEGEND Table 2

Consolidated results on the calcium dependence of myotubes exposed to high K^+ and /or to electrical stimulation protocols. Each transcription factor follows a particular pattern in response to calcium originated from different sources. CREB and early genes were studied in myotubes exposed to high K^+. NFκB and NFAT presented the same calcium dependence both in response to high K^+ and to electrical stimulation protocols.

Figure 1

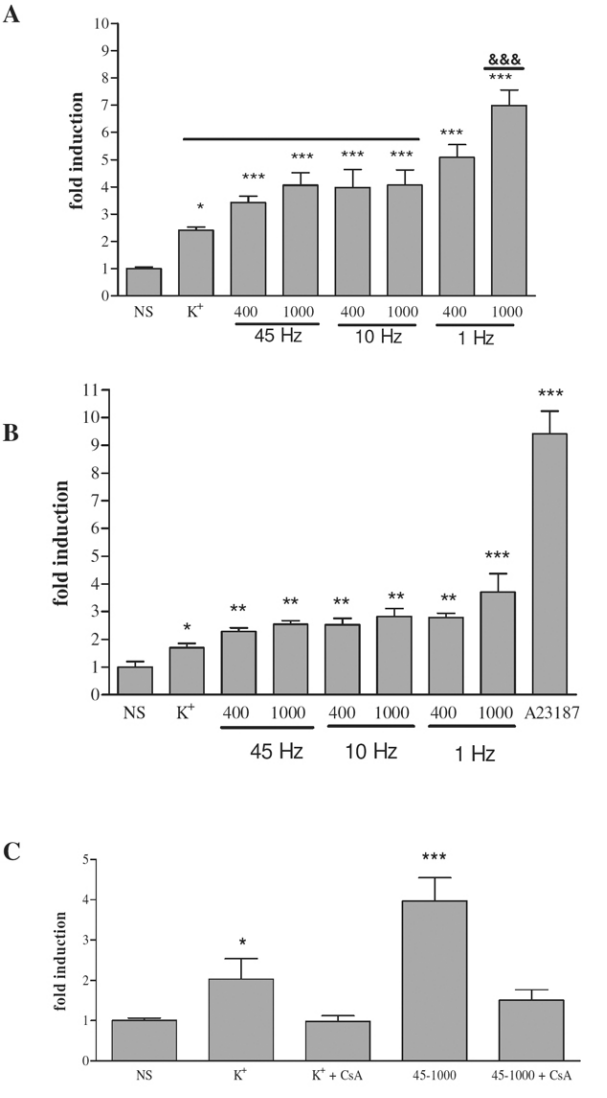
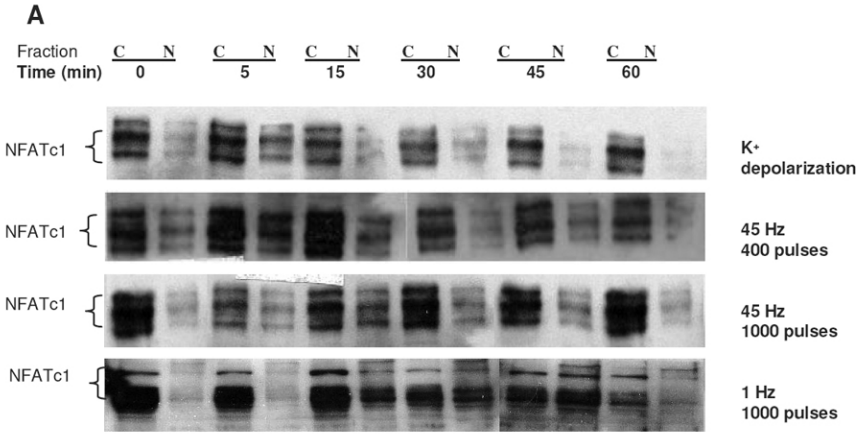

A
fold induction
NS
K+
400
1000
45 Hz
10 Hz
1 Hz
&&&
B
A23187
C
K+ + CsA
45-1000
45-1000 + CsA

Figure 2


A
Fraction C N C N C N C N C N C N
Time (min) 0 5 15 30 45 60
NFATc1
K+ depolarization
NFATc1
45 Hz 400 pulses
NFATc1
45 Hz 1000 pulses
NFATc1
1 Hz 1000 pulses

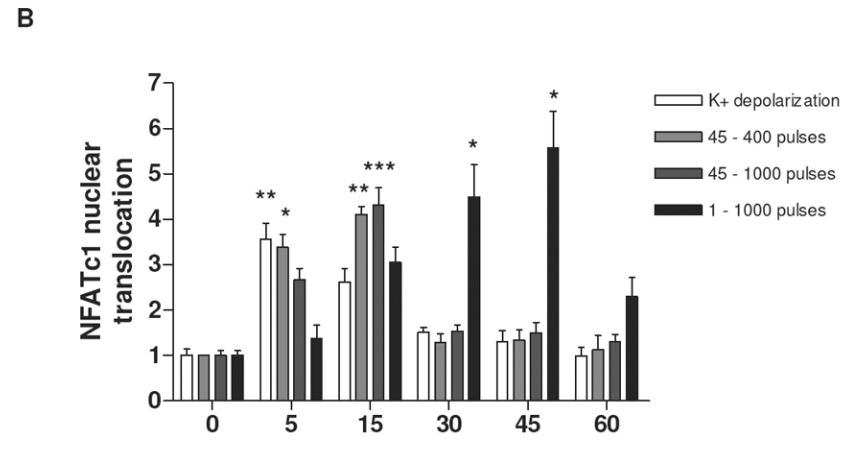
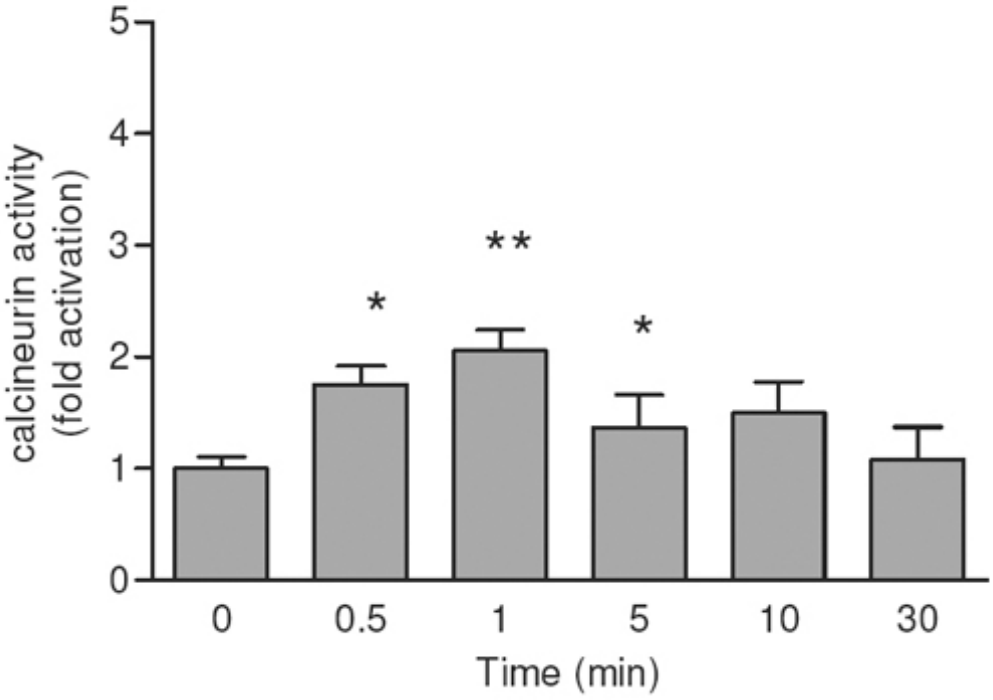

B
NFATc1 nuclear translocation
0
1
2
3
4
5
6
7
0
5
15
30
45
60
K+ depolarization
45 - 400 pulses
45 - 1000 pulses
1 - 1000 pulses

Figure 3

A


calcineurin activity
(fold activation)
5
4
3
2
1
0
*
**
*
0
0.5
1
5
10
30
Time (min)

B

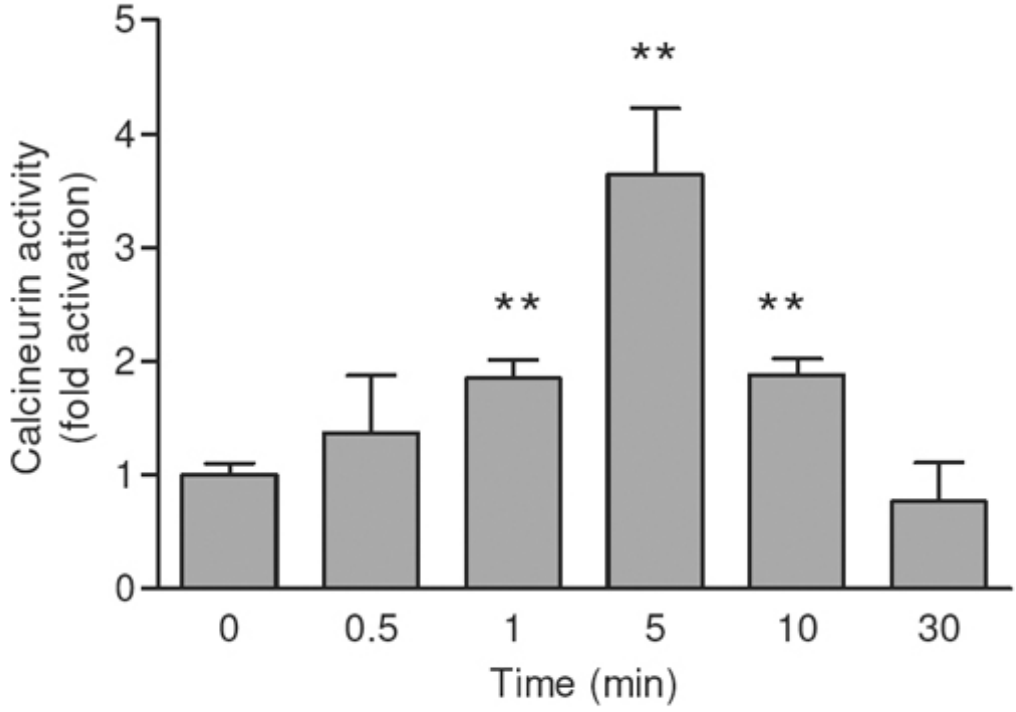

Calcineurin activity
(fold activation)
5
4
3
2
1
0
**
**
**
0
0.5
1
5
10
30
Time (min)

Figure 4

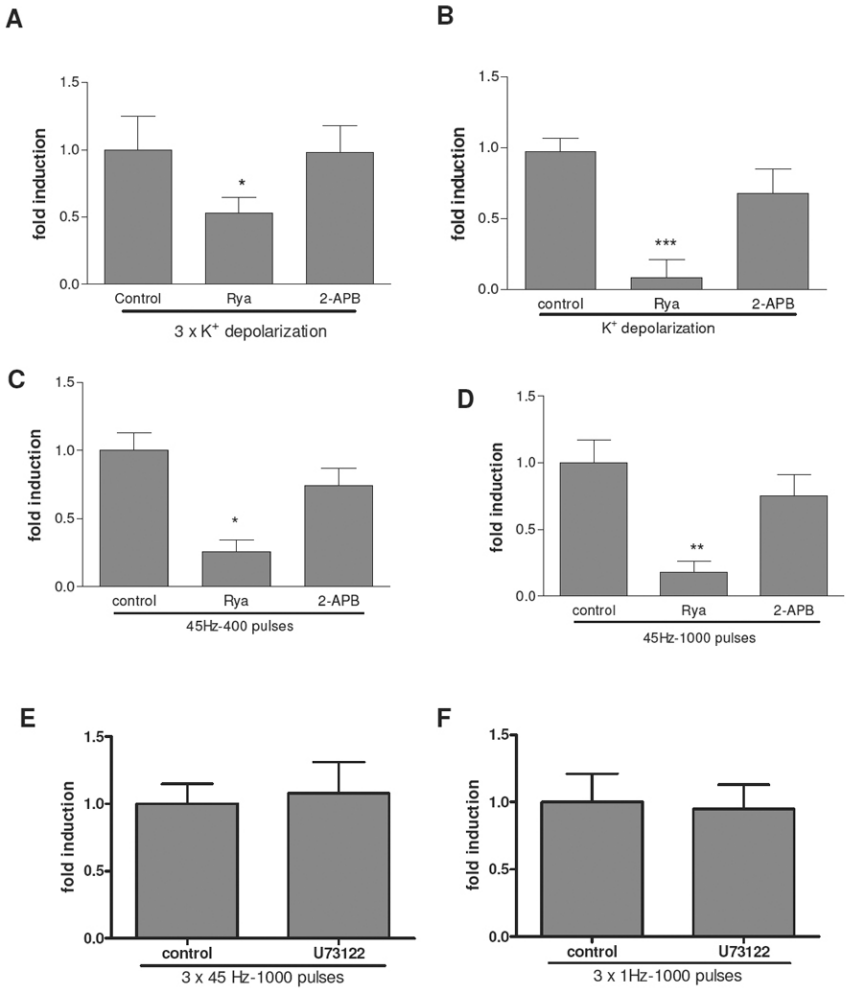

A
fold induction
1.5
1.0
0.5
0.0
*
Control
Rya
2-APB
3 x K+ depolarization
B
fold induction
1.5
1.0
0.5
0.0
***
control
Rya
2-APB
K+ depolarization
C
fold induction
1.5
1.0
0.5
0.0
*
control
Rya
2-APB
45Hz-400 pulses
D
fold induction
1.5
1.0
0.5
0.0
**
control
Rya
2-APB
45Hz-1000 pulses
E
fold induction
1.5
1.0
0.5
0.0
control
U73122
3 x 45 Hz-1000 pulses
F
fold induction
1.5
1.0
0.5
0.0
control
U73122
3 x 1Hz-1000 pulses

Figure 5

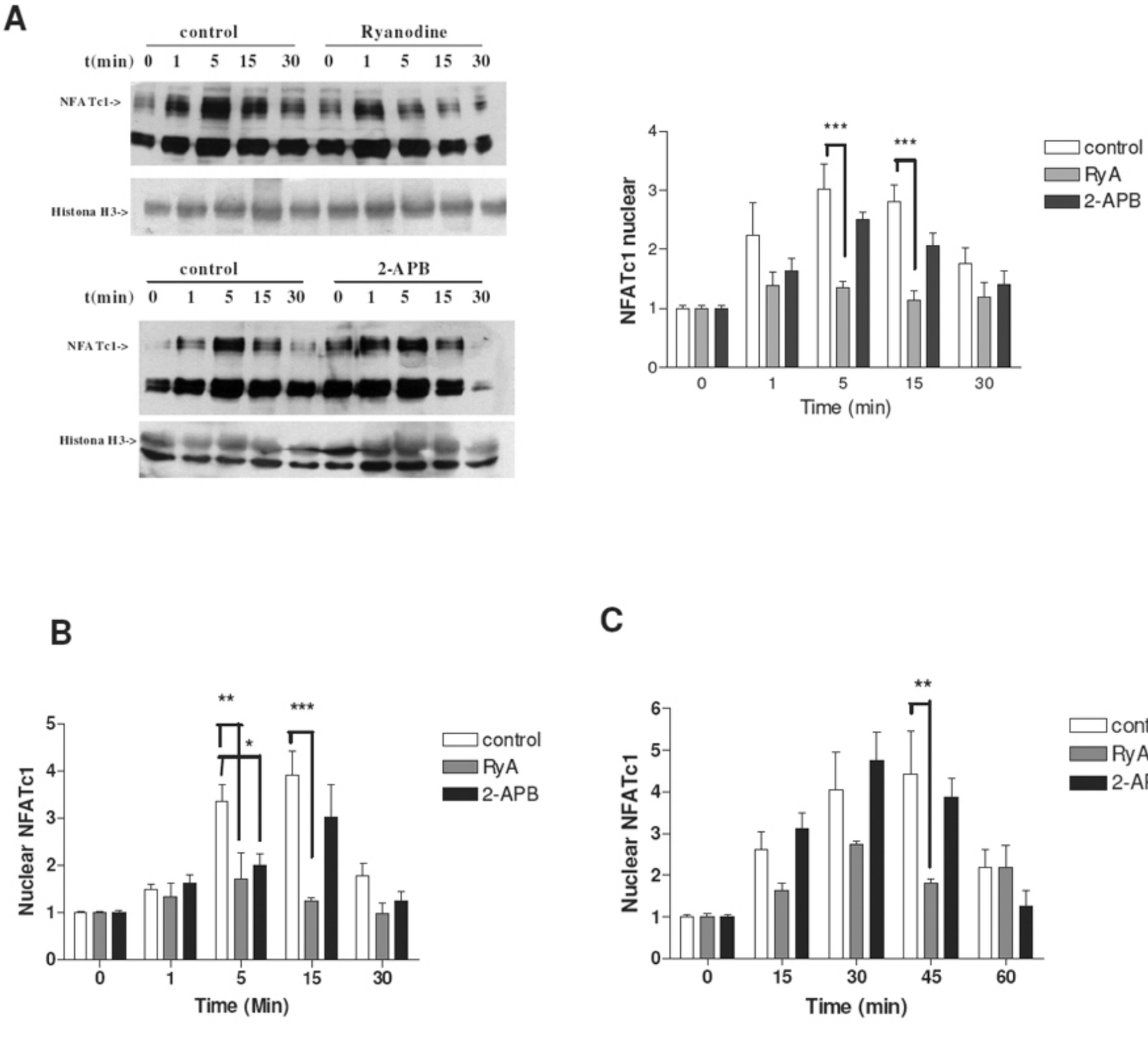

A
control
Ryanodine
t(min) 0 1 5 15 30 0 1 5 15 30
NFATc1->
Histona H3->
control
2-APB
t(min) 0 1 5 15 30 0 1 5 15 30
NFATc1->
Histona H3->
NFATc1 nuclear
Time (min)
control
RyA
2-APB
***
***
B
Nuclear NFATc1
Time (Min)
control
RyA
2-APB
**
*
***
C
Nuclear NFATc1
Time (min)
control
RyA
2-APB
**

Figure 6

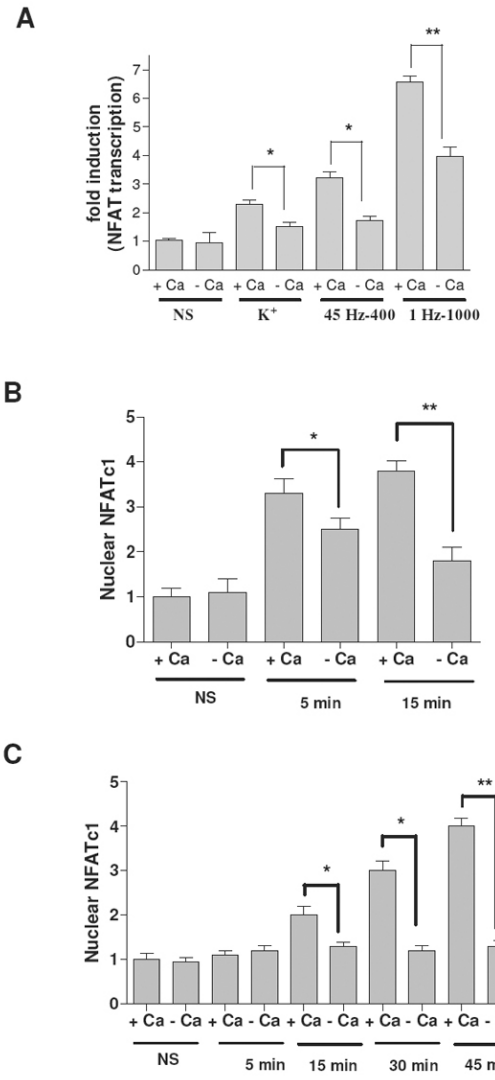
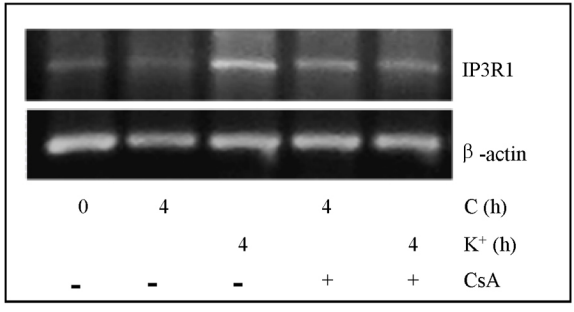

A
fold induction
(NFAT transcription)
0
1
2
3
4
5
6
7
*
*
**
+ Ca
- Ca
NS
K+
45 Hz-400
1 Hz-1000
B
Nuclear NFATc1
0
1
2
3
4
5
*
**
+ Ca
- Ca
NS
5 min
15 min
C
Nuclear NFATc1
0
1
2
3
4
5
*
*
**
+ Ca
- Ca
NS
5 min
15 min
30 min
45 min

Figure 7

A


IP3R1
β -actin
0
4
4
C (h)
4
4
K+ (h)
-
-
-
+
+
CsA

B

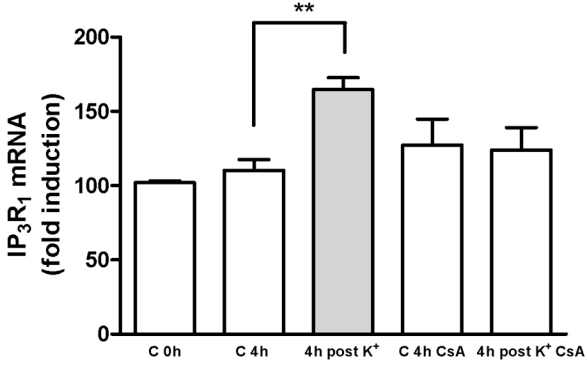

**
200
150
100
50
0
IP3R1 mRNA (fold induction)
C 0h
C 4h
4h post K+
C 4h CsA
4h post K+ CsA

Figure 8

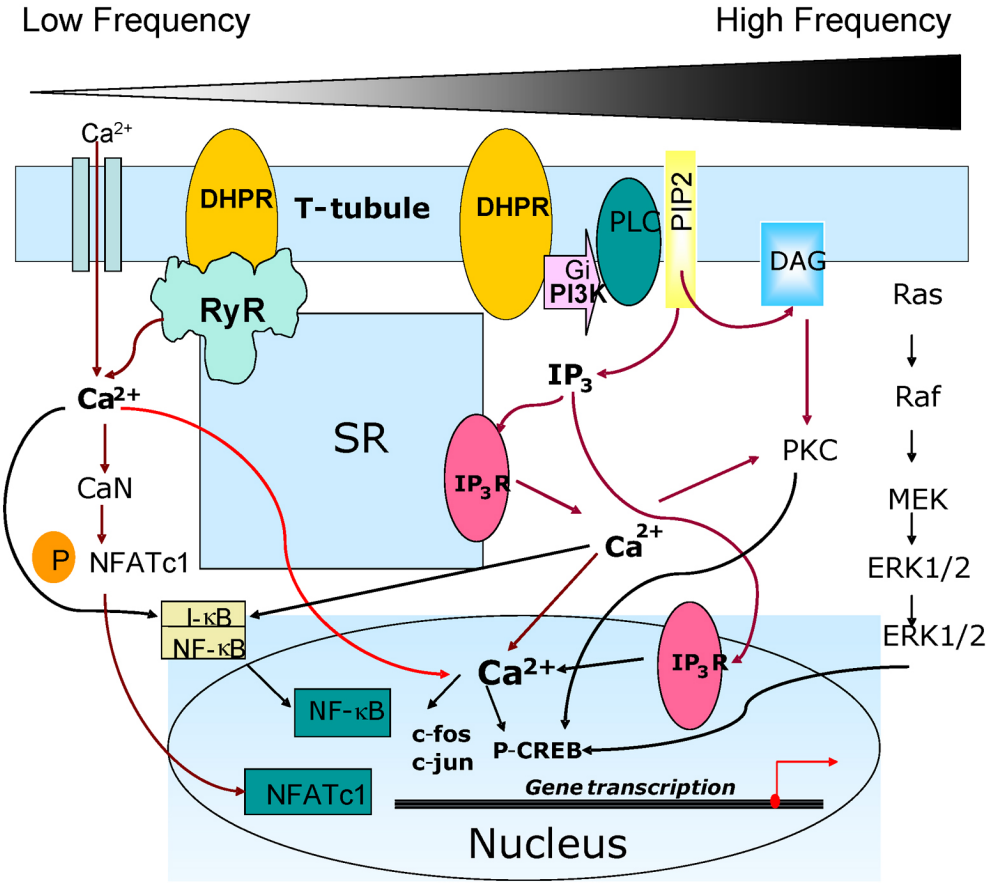

Low Frequency
High Frequency
Ca2+
DHPR
T-tubule
DHPR
PLC
PIP2
DAG
Gi
PI3K
RyR
Ras
Raf
MEK
ERK1/2
ERK1/2
Ca2+
IP3
SR
IP3R
PKC
CaN
P
NFATc1
Ca2+
I-κB
NF-κB
Ca2+
IP3R
NF-κB
c-fos
c-jun
P-CREB
NFATc1
Gene transcription
Nucleus

Table 1. *Characteristics of the calcium transients observed upon stimulation with the different protocols used in this work.*

Stimulation protocol	Stimulus total duration	Total calcium transient duration	RyR-dependent calcium transient duration	Identifiable components	References
45 Hz, 400p	9 s	1-2 min	9 s	2 (tetanus and slow)	(10)
45 Hz, 1000p	22 s	2-3 min	22 s	2 (tetanus and slow)	(37)
1 Hz, 1000p	1000 s	17 min	1000 s	1 (incomplete tetanus)	(37)
High K^+	30-300 s	< 1 min	1 s	2 (fast and slow)	(18)

Table 2. Source of calcium involved in the activation of the transcription factors CREB, NFκB, NFAT and early genes in cultured myotubes.

Transcription factor	Extracellular calcium	RyR-calcium store	IP3R –calcium store	References
Early genes c-fos, c-jun and egr-1	No	No	Yes	(2, 5)
CREB	No	No	Yes	(5, 30)
NFκB	No	Yes	Yes	(37)
NFAT	Yes	Yes	No	this work