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

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Valdés JA, Gaggero E, Hidalgo J, Leal N, Jaimovich E, Carrasco MA. NFAT activation by membrane potential follows a calcium pathway distinct from other activity-related transcription factors in skeletal muscle cells. Am J Physiol Cell Physiol 294: C715–C725, 2008. First published January 9, 2008; doi:10.1152/ajpcell.00195.2007.—Depolarization of skeletal muscle cells triggers intracellular Ca2+ signals mediated by ryanodine and inositol 1,4,5-trisphosphate (IP3) receptors. Previously, we have reported that K+-induced depolarization activates transcriptional regulators ERK, cAMP response element-binding protein, c-fos, c-jun, and egr-1 through IP3-dependent Ca2+ release, whereas NF-κB activation is elicited by both ryanodine and IP3 receptor-mediated Ca2+ signals. We have further shown that field stimulation with electrical pulses results in an NF-κB activation increase dependent of the amount of pulses and independent of their frequency. In this work, we report the results obtained for nuclear factor of activated T cells (NFAT)-mediated transcription and translocation generated by both K+- and electrical stimulation protocols in primary skeletal muscle cells and C2C12 cells. The Ca2+ source for NFAT activation is through release by ryanodine receptors and extracellular Ca2+ entry. We found this activation to be independent of the number of pulses within a physiological range of stimulus frequency and enhanced by long-lasting low-frequency stimulation. Therefore, activation of the NFAT signaling pathway differs from that of NF-κB and other transcription factors. Calcineurin enzyme activity correlated well with the relative activation of NFAT translocation and transcription using different stimulation protocols. Furthermore, both K+-induced depolarization and electrical stimulation increased mRNA levels of the 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 Ca2+ release and triggered by the same voltage sensor but activating different intracellular release channels.

nuclear factor of activated T cells transcription; nuclear factor of activated T cells translocation; calcineurin; inositol 1,4,5-trisphosphate receptor; ryanodine receptor

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and NFAT-mediated transcription induced by K$^{+}$21, 35). The presence of Ca$^{2+}$ increases with differentiation and, in addition, individual isoforms (NFATc1, NFATc2, and NFATc3) is at similar levels at the stages of myoblasts, nascent myotubes, and mature isoforms (1). However, NFAT-mediated transcription in response to a Ca$^{2+}$ signal. In mature myotubes, the NFATc1 isoform responds to Ca$^{2+}$ increases (1). NFAT activation has been linked with various skeletal muscle processes like muscle growth and development (17), regulation of muscle hypertrophy (30, 33), and the switching from the fast to slow phenotype (7, 26, 28, 29, 37).

Concerning the origin of the Ca$^{2+}$ involved, NFAT activation in response to 1-Hz stimulation of rabbit primary skeletal muscle cultures would depend on RyR-mediated Ca$^{2+}$ release (26). K$^{+}$-depolarized C2C12 cells present the same dependence, with an important role for extracellular Ca$^{2+}$ as well (32). Other studies, performed in C2C12 cells and cultured avian skeletal muscle fibers, have reported a role for both RyR and IP$_{3}$R-mediated Ca$^{2+}$ release in NFAT activation (20, 21, 35).

In the present study, we report that both NFAT translocation and NFAT-mediated transcription induced by K$^{+}$ and electrical stimulation depends on Ca$^{2+}$ release by the RyR and not by the IP$_{3}$R signaling pathway. In addition, there was a contribution of extracellular Ca$^{2+}$.

Field stimulation protocols varying in frequencies and numbers of pulses allowed us to study the specificity of the NFAT response to these two parameters and to compare it with the NF-$\kappa$B response in the same model (38). We also examined the type 1 IP$_{3}$R (IP$_{3}$R1) mRNA level in primary myotubes depolarized by K$^{+}$ or electrical stimulation. We found a significant increase of the receptor messenger, which was inhibited by cyclopasin A (CsA), a CaN inhibitor, suggesting that depolarization may regulate IP$_{3}$R channel transcription.

**MATERIALS AND METHODS**

**Reagents.** CsA, U-73122, and A-23187 were from BioMol Research Laboratories (Plymouth Meeting, PA). 2-Aminooxydiphenyl 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 H$_{4}$ was from Santa Cruz Biotechnology. Secondary horseradish peroxidase-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 InVitrogen (Grand Island, NY).

**Cell culture.** The experimental protocol complied with the “Guiding Principles for Research Involving Animals and Human Beings” of the American Physiological Society and was approved by the Bioethics Committee for Investigation in Animals of the Facultad de Medicina, Universidad de Chile. Primary cultures of rat skeletal muscle cells were prepared from Sprague-Dawley neonates as previously reported (5, 38). Briefly, muscle tissue dissected from the hindlimbs was minced and treated with collagenase for 15 min at 37°C and grown in medium composed of F-12 DMEM (1:1), 10% BSA, 2.5% FBS, 100 U/ml penicillin, and 10 mg/ml streptomycin in 60-mm plates. Two days after cells had been plated, fibroblast growth was inhibited with cytosine arabinoside (5 mM) and maintained for 24 h. For differentiation, growth medium was changed to serum-free medium at day 4 of culture. Six- to seven-day-old cultures were used for the experiments.

The C2C12 cell line (American Type Culture Collection, Manassas, VA) was grown in DMEM-F-12 (1:1) with 10% BSA and 2.5% FCS. The serum was replaced by 5% horse serum to differentiate myoblasts into myotubes.

**Plasmid reporter construction.** NFAT transcriptional activity was mainly monitored with a NFAT-dependent reporter construct as previously described (29). The intron 3 segment from the human Down syndrome critical region homolog (DSCR)/myocyte-enriched CaN-interacting protein 1 (MCIP1) gene was isolated by PCR using genomic human DNA as the template and primers based on sequence information from the National Center for Biotechnology Information databank. The PCR product, an 850-bp fragment containing 15 NFAT binding sites, was subcloned into a pGL3 luciferase reporter vector (Xhol site) (Promega, Madison, WI). 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 the laboratory of Dr. Gerald Crabtree, consisted of a trimered human distal IL-2 NFAT site inserted into the IL-2 minimal promoter and 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-day-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). The mixture was maintained for 12 h, media were replaced by serum-free media, and cells were maintained until well differentiated. Cells were harvested and lysed 6 h after the experiments. Each experimental condition was performed in duplicate. Luciferase activity was determined using a dual-luciferase reporter assay system (Promega), 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 cells had been stimulated. The 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\(^{2+}\)- and Mg\(^{2+}\)-free PBS and kept in Krebs-Ringer buffer under resting conditions for 30 min [20 mM HEPES-Tris (pH 7.4), 118 mM NaCl, 4.7 mM KCl, 3 mM CaCl\(_2\), 1.2 mM MgCl\(_2\), and 10 mM glucose]. To induce depolarization by high K\(^{+}\), NaCl was replaced isosmotically by KCl (84 mM). Resting or depolarizing saline without Ca\(^{2+}\) contained 0.5 mM EGTA and 4.2 mM MgCl\(_2\) (38).

Myotube electrical stimulation was done as previously reported (38). In brief, 20- to 50-V amplitude, 1-ms suprathreshold voltage pulses were delivered through platinum wires from high-current capacity stimulators; 400 and 1,000 pulses delivered at frequencies of 1, 10, and 45 Hz were employed. Stimulation-induced myotube contraction was monitored under a microscope in each experiment 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. (38) as previously reported. Western blots for lamin-associated polypeptide 2 (LAP2) (BD Transduction Laboratories, Franklin Lakes, NJ) and IκB-β (Cell Signaling Technology, Beverly, MA) were performed to assess the purity of the nuclear and cytosolic fractions, respectively.

Immunoblot analysis. Proteins were resolved by 10% SDS-PAGE, transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA), and blocked for 1 h at room temperature in Tris-buffered saline (TBS), 0.1% Tween 20, and 5% milk. Incubations with primary antibodies (1:1,000) were performed at 4°C overnight. After an incubation for 1.5 h with horseradish peroxidase-conjugated secondary antibodies, enhanced chemiluminescence (Amersham Biosciences) was performed. 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 (Bio-Rad, 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 min. Cells were blocked for 1 h in 5% PBS-BSA and incubated overnight with anti-NFATc1 antibody at 4°C. Cells were then washed three times with PBS-BSA and incubated with secondary antibody for 90 min at room temperature. Coverslips were mounted in Vectashield (Vector Laboratories) to retard photobleaching. Samples were evaluated with a confocal microscope (Carl Zeiss Axiovert 200M-LSM Pascal 5) and documented through computerized images.

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

Semi-quantitative RT-PCR. Total RNA was extracted from primary skeletal muscle cells with TRIzol Reagent (Invitrogen), and cDNA was synthesized using SuperScript II reverse transcriptase (Invitrogen) and oligo dT primers. cDNA was amplified using IP\(_3\)R\(_1\) primers (31), and the DNA concentration was normalized against β-actin. PCR amplification was maintained in the exponential phase for both products. The IP\(_3\)R\(_1\) primers used were 5’-GAAGAGAACTGTG-3’ (sense) and 5’-GCATGTCCATCTACA-3’ (antisense). PCR conditions were as follows: initial denaturation at 94°C for 5 min, followed by 28 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, and final extension at 72°C for 10 min for both amplification of IP\(_3\)R\(_1\) and β-actin cDNA. PCR products were resolved by electrophoresis on a 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 ± SE. One-way or two-way ANOVA followed by Bonferroni or Tukey post tests 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 field stimulation protocols (Fig. 1A). The NFAT-mediated transcription showed activation by the three sets of frequencies and amounts of pulses used. At 45 and 10 Hz with 400 and 1,000 pulses and at 1 Hz and 400 pulses statistically significant increases were found, and a much larger increase was observed with electrical stimulation at 1 Hz and 1,000 pulses (Fig. 1A). These results were confirmed with the use of a weaker reporter that contained only three response elements to NFAT. In myotubes, it required three times the standard stimulus 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 NFAT-binding sites (29).

It has been reported that CsA blocks NFAT activation induced by electrical stimulation of adult muscle cells and 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 1,000 pulses, where the increase in NFAT transcriptional activity was completely inhibited by CsA (Fig. 1C).

NFAT translocation to the nucleus is a further confirmation of NFAT activation. For 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 electrical stimulation with the same patterns of Ca²⁺ transients as in primary culture (11, 38). Four protocols were employed to analyze NFAT transcription, K⁺-induced depolarization, and electrical stimulation at either 45 Hz with 400 or 1,000 pulses or 1 Hz with 1,000 pulses. These protocols were selected considering the clear-cut differences in transcriptional activation determined with the strong reporter (Fig. 1A). Western blots for NFAT showed increased NFAT transcription in parallel to NFAT-mediated transcription obtained in response to these stimuli (Fig. 2A). As expected, the nuclear NFAT lifetime was higher after stimulation at 1 Hz with 1,000 pulses, followed by 45 Hz with 1,000 pulses, 45 Hz with 400 pulses, and K⁺-induced depolarization. Significant NFAT translocation was detected at 30–45, 15, 5–15, and 5 min, of the respective stimulation (Fig. 2B).

We also performed immunocytochemistry after stimulation of C2C12 cells with high K⁺ and electrical protocols. A slight NFATc1 translocation to nuclei could be detected in

![Fig. 2. High K⁺-induced depolarization and electrical stimulation induce NFATc1 translocation in C2C12 muscle cells. A: Western blots of NFATc1 in the cytoplasmic (C) and nuclear (N) fractions obtained from C2C12 cells after an exposure to 84 mM KCl for 1 min or to a single protocol of stimulation at 45 Hz with 400 pulses (45-400), 45 Hz with 1,000 pulses (45-1000), or 1 Hz with 1,000 pulses (1-1000). Results were normalized using Coomassie blue-stained membranes. B: nuclear NFATc1 densitometric analysis. Data are means ± SE (error bars) of duplicates (n = 3 independent experiments) and are expressed relative to values in NS cells (time 0). Statistical analysis was performed by two-way ANOVA followed by Bonferroni’s post test. *P < 0.05, **P < 0.01, and ***P < 0.001 with respect to the control NS group.](image)
NFAT ACTIVATION IN CULTURED SKELETAL MUSCLE

stimulated cells, which was much smaller than the control NFAT translocation in response to Ca\(^{2+}\) ionophore A-23187, which gave significant translocation (not shown). These results did not allow quantification under the stimulation conditions employed but confirmed those obtained with subcellular fractions.

To better understand NFAT activation mechanisms by depolarization, we also determined CaN activity by an enzymatic assay in primary myotubes stimulated by high K\(^{+}\) and the electrical protocol of stimulation at 45 Hz with 400 pulses. The CaN activity increase presented marked differences in kinetics and in the extent of activation when these two protocols were compared (Fig. 3). The maximal increase in CaN activity after K\(^{+}\)-induced depolarization was twofold at 1 min, whereas maximal activation of three- to fourfold was determined 5 min after the electrical stimulation (Fig. 3).

NFAT activation depends on RyR-mediated Ca\(^{2+}\) release and extracellular Ca\(^{2+}\) entry. The role of Ca\(^{2+}\) release from intracellular stores in NFAT activation was evaluated by measuring transcription and translocation. Experiments were performed with the 3× reporter in primary muscle cells exposed to a high K\(^{+}\) concentration and with 15× reporter in myotubes stimulated by high K\(^{+}\) or 45 Hz with 400 or 1,000 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 with inhibition of the IP\(_{3}\)R-mediated slow Ca\(^{2+}\) transient using 50 μM 2-APB or 30 μM U-73122, a PLC inhibitor (Fig. 4, A–F). 2-APB abolished the IP\(_{3}\)R-dependent Ca\(^{2+}\) transient, as did 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–C). A small but significant effect of 2-APB was found after only 5 min of stimulation at 45 Hz with 1,000 pulses (Fig. 5B).

In the literature, increased NFAT translocation by high K\(^{+}\)-induced depolarization of C2C12 cells has been reported to depend on extracellular Ca\(^{2+}\) (32, 35). We studied this aspect and determined that NFAT-dependent transcription as assessed by the DSCR/MCIP1 reporter gene was partially dependent on extracellular Ca\(^{2+}\) in myotubes stimulated with high K\(^{+}\) and by electrical stimulation protocols at 45 Hz with 400 pulses and 1 Hz with 1,000 pulses (Fig. 6A). Also, we found that NFATc1 translocation in the absence of extracellular Ca\(^{2+}\) was significantly reduced compared with results obtained in the presence of extracellular Ca\(^{2+}\) in myotubes stimulated at 45 Hz with 1,000 pulses (Fig. 6B) and 1 Hz with 1,000 pulses (Fig. 6C). The effect of Ca\(^{2+}\)-free medium was not due to intracellular Ca\(^{2+}\) depletion because myotubes challenged with 10 mM caffeine after 30 min in Ca\(^{2+}\)-free saline presented Ca\(^{2+}\) release to the same extent as controls in saline with 3 mM Ca\(^{2+}\) (not shown).

We further examined NFAT-dependent transcription in experiments performed with the protocols of high K\(^{+}\) and stimulation at 45 Hz with 400 pulses and 1 Hz with 1,000 pulses in the presence of 10 μM nifedipine. There were no decreases of activity except for a minor significant effect in cells exposed to high K\(^{+}\), suggesting that the requirement of extracellular Ca\(^{2+}\) for NFAT activity does not depend on Ca\(^{2+}\) influx through L-type Ca\(^{2+}\) channels (data not shown).

Depolarization induces a CaN-dependent increase in IP\(_{3}\)R1 mRNA. 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 measured IP\(_{3}\)R1 mRNA levels in primary myotubes depolarized by high K\(^{+}\) or electrical stimulation.

Hippocampal cell depolarization induces NFATc4 activation and an increase in IP\(_{3}\)R protein levels, and both processes are CaN-dependent (14). Additionally, preliminary experiments in mice null for NFATc2 and NFATc4 resulted in low levels of IP\(_{3}\)R1 gene expression (14). Considering that the promoter region of the murine IP\(_{3}\)R1 gene contains several candidate NFAT-binding sequences, these experiments suggested that IP\(_{3}\)R1 is a target of NFAT (14). Also in the hippocampus, exposure to brain-derived neurotrophic factor resulted in activated NFAT transcription and increased expression of IP\(_{3}\)R1, and both were reduced by CaN inhibition (15). In cerebellar granule cells, IP\(_{3}\)R1 expression was increased by depolarization-induced increase of intracellular Ca\(^{2+}\) by L-type Ca\(^{2+}\) channels or by N-methyl-D-aspartate receptors and was inhibited by CaN pharmacological blockade (13). The CaN dependence of IP\(_{3}\)R1 expression has been corroborated in the same model by analysis of gene expression by microarrays (24).

We studied IP\(_{3}\)R1 mRNA levels in primary myotubes depolarized by high K\(^{+}\). Data were collected up to 8 h after K\(^{+}\) exposure. There was a statistically significant increase at 4 h after the depolarization event (Fig. 7). As a control, cells were

![Fig. 3. CaN is activated by K\(^{+}\)-induced depolarization (A) and electrical stimulation (B) in primary skeletal muscle cells. Myotubes were stimulated by high K\(^{+}\) or stimulation at 45 Hz with 400 pulses and then processed for measurements of CaN activity as described in MATERIALS AND METHODS. Data are means ± SE (error bars) of duplicates (n = 3 independent experiments) and are expressed relative to values in NS cells (time 0). Statistical analysis was performed by one-way ANOVA followed by Bonferroni’s post test. *P < 0.05 and **P < 0.01 with respect to the control group.](image)
Fig. 4. NFAT-mediated transcription induced by KCl and electrical stimulation in primary myotubes depends on the ryanodine receptor (RyR)-induced fast Ca$^{2+}$ transient. NFAT-dependent transcription was evaluated in 7-day-old myotubes. Transfected myotubes were preincubated for 30 min with vehicle, 50 μM 2-aminoethoxydiphenyl borate (2-APB), 50 μM ryanodine (Rya), or 30 μM U-73122 and then stimulated by the indicated protocols in the absence or presence of these inhibitors. A: myotubes transfected with 3X reporter gene were exposed to 3 cycles of high K$^+$ depolarization. Data in B–D were obtained in myotubes transfected with the DSCR/MCIP1 reporter gene. B: K$^+$ stimulation; C: 45 Hz with 400 pulses; D: 45 Hz with 1,000 pulses. E: myotubes transfected with 3X reporter gene were exposed to 3 cycles of 45 Hz with 1,000 pulses. F: myotubes transfected with 3X reporter gene were exposed to 3 cycles of 1 Hz with 1,000 pulses. Data are means ± SE (error bars) of duplicates of 3 independent experiments. Data obtained with inhibitors are 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 followed by Tukey’s post test. *P < 0.05, **P < 0.01, and ***P < 0.001 with respect to the control group.
transcription factor discriminated between 400 and 1,000 pulses at all frequencies (38).

Several reports have shown that a low frequency of long-lasting stimulation, mimicking slow-twitch fiber activity, activates NFAT in both cultured cells and adult fibers in vitro and in vivo (25, 26, 28, 29, 32, 37). When rabbit primary myotubes were treated with electrical stimulation at 1 and 10 Hz, 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, stimulation at 10 Hz, with either continuous or train stimulation, resulted in NFAT translocation, whereas the application of 50-Hz trains or continuous stimulation at a low frequency of 1 Hz did not result in nuclear translocation (28). The 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 was activated by low and high frequencies at three- to fourfold over control levels. We obtained a sevenfold increase only with electrical stimulation at 1 and 10 Hz, 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, stimulation at 10 Hz, with either continuous or train stimulation, resulted in NFAT translocation, whereas the application of 50-Hz trains or continuous stimulation at a low frequency of 1 Hz did not result in nuclear translocation (28). The 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 was activated by low and high frequencies at three- to fourfold over control levels. We obtained a sevenfold increase only with stimulation at 1 Hz with 1,000 pulses. 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 Ca\(^{2+}\) transients and no slow Ca\(^{2+}\) 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 Ca\(^{2+}\) transient, i.e., activation by RyRs and not by the IP\(_3\)R signaling pathway. In our work, the 1-Hz stimulus did not result in a clear IP\(_3\)R-dependent slow Ca\(^{2+}\) transient (38); therefore, the blockade of the IP\(_3\) system should not have affected the outcome. The rest of the protocols (high K\(^+\) and stimulation frequencies at 45 and 10 Hz) showed RyR- and IP\(_3\)R-dependent Ca\(^{2+}\) transients, but the inhibition of the IP\(_3\) system by 2-APB only showed a minor effect, significant only after 5 min of exposure to 45 Hz with 1,000 pulses, in NFAT translocation measurements. In addition, we obtained no differences when we analyzed NFAT transcription in the presence of the PLC inhibitor U-73122, supporting that the IP\(_3\) pathway is not regulating NFAT activation. The increase of NFAT-mediated transcription observed with either high K\(^+\) or electrical stimulation at 45 Hz with 400 or 1,000 pulses and 1 Hz with 1,000 pulses is probably the result of the NFAT longer nuclear lifetime. RyR-dependent Ca\(^{2+}\) increases with these stimuli lasted for 1, 9, 22, and 1,000 s, respectively (see Table 1) and was visualized in both the cytoplasm and nucleus (10, 18, 38). Therefore, since NFAT activation depends on both cytoplasmic and nuclear Ca\(^{2+}\) increases, these results suggest that the RyR-induced Ca\(^{2+}\) increase would contribute to a longer time window for translocation and thus for augmented transcription. Because Ca\(^{2+}\) detection with the fluorescent dye fluo-3 AM gives information on the kinetics and localization of Ca\(^{2+}\) signals but no information on Ca\(^{2+}\) levels, we assessed CaN activity directly. A recent study (35) in C2C12 cells has also reported that depolarization-induced NFATc1 nuclear entry depends on RyR-mediated Ca\(^{2+}\) release by membrane depolarization. However, in this process, there would be a contribution of the IP\(_3\)-dependent Ca\(^{2+}\) pool to NFAT signaling in myotubes by promoting NFAT nuclear exit. To activate the IP\(_3\) system, the authors stimulated cells with UTP, which mobilizes IP\(_3\), 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 attributed the differences in NFAT trafficking to spatial differences of the Ca\(^{2+}\) response induced by RyR or IP3R activation, because UTP increases Ca\(^{2+}\) only in the cytoplasm, whereas mobilization of Ca\(^{2+}\) by KCl shows increased Ca\(^{2+}\) in the whole cell, including the nucleus (35). In our hands, K\(^+\) or electrical stimulation also induced RyR-mediated Ca\(^{2+}\) release in the whole cell, but the depolarization-induced IP3R-mediated slow Ca\(^{2+}\) transient showed a well-defined Ca\(^{2+}\) increase in the nucleus apart from a smaller cytoplasmic component. If the IP3R-dependent Ca\(^{2+}\) 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 did not induce IP3R-induced Ca\(^{2+}\) release effectively resulted in higher NFAT activation compared with those protocols that resulted in both fast and slow Ca\(^{2+}\) signals. However, the stimulation using protocols of high K\(^+\) and 45-Hz pulses in the presence of 2-APB, which results in inhibition of the slow Ca\(^{2+}\) signal, was not significantly affected.

The regulation of NFAT-dependent transcription by Ca\(^{2+}\) release mediated by IP3R and RyR signaling pathways has also been studied in avian skeletal muscle adult fibers to characterize the transcriptional regulation of the slow myosin heavy chain 2 (MyHC2) promoter (19–21). A mutagenesis study (19) has indicated that both NFAT and MEF2 binding sites are required for innervation-induced MyHC2 promoter activity in slow muscle fibers. Basically, there is NFAT activation by RyR inhibition (20), whereas IP3R blockade results in the opposite effect (21). These results might reflect complex mechanisms of

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**Fig. 6.** Extracellular Ca\(^{2+}\) contributes to NFAT activation. Before being stimulated, skeletal muscle cells were incubated for 30 min under resting conditions in the presence or absence of extracellular Ca\(^{2+}\) and then exposed to high K\(^+\) or stimulation at 45 Hz with 400 pulses or 1 Hz with 1,000 pulses. **A:** NFAT-dependent transcription in myotubes transfected with the DSCR/MCIP1 reporter gene. **B:** nuclear NFATc1 in C2C12 cells stimulated at 45 Hz with 400 pulses. **C:** nuclear NFATc1 in C2C12 cells stimulated at 1 Hz with 1,000 pulses. Data are means ± SE (error bars) of duplicates of 3 independent experiments. Statistical analysis was performed by two-way ANOVA followed by Bonferroni’s post test. *P < 0.05 and **P < 0.01 with respect to the control NS group.

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**Fig. 7.** High K\(^+\)-induced depolarization stimulates a CaN-dependent increase in type 1 inositol 1,4,5-trisphosphate (IP3) receptor (IP3R1) mRNA levels in primary myotubes. Cells were incubated for 30 min under resting conditions, exposed to high K\(^+\) for 1 min, and then maintained under resting conditions for 4 h. In parallel, cells from the same culture were preincubated and stimulated in the presence of 1 \(\mu\)M CsA. Controls for these experiments were obtained from NS cells maintained for 30 min under resting conditions and then processed (C 0 h) and cells that were further maintained under resting conditions for 4 h (C 4 h). **A:** IP3R1 and β-actin mRNA expression levels were tested by semiquantitative RT-PCR. **B:** IP3R1 densitometric analysis. Data are means ± SE (error bars) of 3–5 independent experiments. All results were normalized with respect to β-actin. ANOVA test followed by Bonferroni’s post test was used to analyze the results with respect to C 4 h. *P < 0.05 and **P < 0.01 with respect to the control NS group.
NFAT regulation in another developmental stage as well as differences with respect to the stimulatory pattern used.

NFAT activation in C2C12 cells stimulated with K⁺ depends on extracellular Ca²⁺ (32, 35). Activation after 1 min of KCl-induced depolarization in C2C12 myotubes resulted in NFATc1 nuclear localization in the absence of extracellular Ca²⁺. 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 Ca²⁺ during the KCl exposure prolonged NFATc1 nuclear localization, indicating that the sarcoplasmic reticulum Ca²⁺ pool was important in initiating NFATc1 activation but that replenishment by extracellular Ca²⁺ was required to maintain this activation. We confirmed the extracellular Ca²⁺ requirement for a fraction of NFAT activation by K⁺ in C2C12 cells and extended it to primary myotubes, and in both cell types in cultures in response to electrical stimulation. While in our experimental model NFAT activation requires extracellular Ca²⁺, the activation of other transcription factors we have studied is independent of this source of Ca²⁺. Thus, CREB, c-fos, c-jun, and NF-κB activation by depolarization were not affected by the absence of extracellular Ca²⁺ (5, 38). As mentioned above, the extracellular Ca²⁺ dependence of NFAT activation is another difference with respect to NF-κB activation, which was found to be dependent on both RyR- and IP₃R-dependent Ca²⁺ pools (38). In our experiments, the IP₃ system inhibitor 2-APB had no significant effect on NFAT transcription or translocation; this same inhibitor blocks about half of the NF-κB response (38) and all of the CREB response (5). Different transcription factors thus appear to be regulated by pharmacologically dissociable Ca²⁺ transients (Table 2).

Our results indicated that IP₃R₁ mRNA levels increase in K⁺-depolarized or electrically stimulated primary cells, as occurs in hippocampal and cerebellar neurons (13, 14). In addition, both the IP₃ mRNA increase and NFAT activation were blocked by CaN inhibition, suggesting that NFAT could be involved in IP₃R₁ upregulation. CaN is crucial in NFAT activation but also controls the activation of several other transcription factors. Several genes involved in Ca²⁺ regulation are activated by depolarization through a CaN-mediated mechanism in neurons, suggesting activity-dependent regulation of Ca²⁺ signaling at the transcriptional level (see Ref. 24). In our work, NFAT activation in primary muscle cells was not dependent on IP₃R-dependent Ca²⁺ release. In adult fast-twitch fibers, however, the inhibition of the IP₃R by 2-APB or by xestospongin D resulted in a reduction of NFAT-dependent transcription and nuclear localization (21). NFAT activity regulation by IP₃R-dependent Ca²⁺ signaling might have a significant role in long-term muscle fiber remodeling, but primary culture may not be the best model system in which to study this regulation. Since IP₃R-dependent Ca²⁺ release participates in the regulation of transcription factors like early genes, CREB, and NF-κB, the possibility is raised that IP₃R upregulation might influence the activation of these factors. The results obtained in this work, with respect to CaN activation and its involvement in IP₃R₁ increased expression with depolarization, open interesting questions with respect to the regulation of genes associated with Ca²⁺ fluctuations by physiological activity in skeletal muscle.

![Fig. 8. Schematic description of Ca²⁺ pathways involved in NFAT and other transcriptional regulator activation. The diagram is based on this work and on previous published data obtained in our laboratory. Membrane depolarization results in dihydropyridine receptor (DHPR) activation followed by RyR and IP₃R-dependent Ca²⁺ release and Ca²⁺ entry. Both RyR-mediated Ca²⁺ signaling pathways and extracellular Ca²⁺ contribute to NFAT activation. NF-κB activation, however, depends on both RyR- and IP₃R-dependent Ca²⁺ pathways and does not require extracellular Ca²⁺ (38). IP₃R-dependent Ca²⁺ release is involved in ERK and cAMP response element-binding protein (CREB) phosphorylation (5, 31) and in early gene mRNA increases (2, 5). There is also PKC activation by the IP₃R-dependent Ca²⁺-release pathway (4). Long-term low-frequency stimulation will maximally activate NFAT pathway via RyRs, whereas pathways dependent on IP₃Rs will be activated only after high-frequency stimulation (which will also activate fast Ca²⁺ release). On the other hand, transcription factors as NF-κB will be partly activated by low-frequency stimulation and maximally activated at high-frequency stimulation through both pathways. Gi, G protein; PI3K, phosphatidylinositol 3-kinase; PI₃P₂, phosphatidylinositol 4,5-bisphosphate; DAG, diacylglycerol; SR, sarcoplasmic reticulum.](image-url)
We have demonstrated, using the same cell system, that membrane depolarization of muscle cells elicits highly selective mechanisms mediated by Ca^{2+} that activate several transcriptional regulators (Fig. 8). In addition, the slow Ca^{2+} signal in cultured muscle cells also regulates the expression of genes such as IL-6 (22) and troponin I (23), whereas other genes with Ca^{2+}-dependent elements within their promoters, such as the α-actin 1 gene, appear not to be regulated by this particular Ca^{2+} transient (23).

For skeletal muscle, both the frequency and duration of electrical stimuli (or even lack of stimuli) have a meaning in terms of genes that will be upregulated or downregulated. For example, the frequency pattern is especially important for fiber type specification. Therefore, specific patterns of activity will generate specific Ca^{2+} signals, which will be discriminated by the different genes. Our results suggest that low-frequency stimulation activates a Ca^{2+}-dependent pathway leading to maximal NFAT activation, whereas maximal activation of other transcription factors, such as NF-κB, CREB, or activator protein-1, need an IP3-dependent Ca^{2+} pathway, which 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 understanding the fine regulation of excitation-transcription coupling in skeletal muscle.

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