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NF-κB activation by depolarization of skeletal muscle cells depends on ryanodine and IP_3 receptor-mediated calcium signals

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Valdés JA, Hidalgo J, Galaz JL, Puentes N, Silva M, Jaimovich E, Carrasco MA. NF-κB activation by depolarization of skeletal muscle cells depends on ryanodine and IP_3 receptor-mediated calcium signals. *Am J Physiol Cell Physiol* 292: C1960–C1970, 2007. First published January 10, 2007; doi:10.1152/ajpcell.00320.2006.—Depolarization of skeletal muscle cells by either high external K^+ or repetitive extracellular field potential pulses induces calcium release from internal stores. The two components of this release are mediated by either ryanodine receptors or inositol 1,4,5-trisphosphate (IP_3) receptors and show differences in kinetics, amplitude, and subcellular localization. We have reported that the transcriptional regulators including ERKs, cAMP/Ca^{2+}-response element binding protein, c-*fos*, c-*jun*, and *egr*-1 are activated by K^+-induced depolarization and that their activation requires IP_3-dependent calcium release. We presently describe the activation of the nuclear transcription factor NF-κB in response to depolarization by either high K^+ (chronic) or electrical pulses (fluctuating). Calcium transients of relative short duration activate an NF-κB reporter gene to an intermediate level, whereas long-lasting calcium increases obtained by prolonged electrical stimulation protocols of various frequencies induce maximal activation of NF-κB. This activation is independent of extracellular calcium, whereas calcium release mediated by either ryanodine or IP_3 receptors contribute in all conditions tested. NF-κB activation is mediated by IκBα degradation and p65 translocation to the nucleus. Partial blockade by *N*-acetyl-L-cysteine, a general antioxidant, suggests the participation of reactive oxygen species. Calcium-dependent signaling pathways such as those linked to calcineurin and PKC also contribute to NF-κB activation by depolarization, as assessed by blockade through pharmacological agents. These results suggest that NF-κB activation in skeletal muscle cells is linked to membrane depolarization and depends on the duration of elevated intracellular calcium. It can be regulated by sequential activation of calcium release mediated by the ryanodine and by IP_3 receptors.

electrical stimulation; transcription; intracellular calcium stores

CALCIUM IONS PLAY a central role in transcriptional regulation (7, 12, 15, 22, 45). The calcium-related transcription factors involve cytoplasmic and/or nuclear signaling pathways (19, 22, 52). In addition, nuclear calcium increase can directly modify transcription factors or modulate the chromatin structure as well as other elements that play a role in the regulation of the general transcription machinery (44). Transcriptional responses will depend on the particular nature of the stimulus-induced calcium transients, which may vary in their amplitude, kinetics, and spatial properties (17, 20–22, 26).

Skeletal muscle cells provide a unique environment where regulation of transcription must occur within a background of strong oscillatory calcium levels. Our laboratory has reported the presence of a complex pattern of calcium increase induced by depolarization, related to excitation-contraction and to excitation-transcription signaling in cultured muscle cells. In addition to the fast calcium transient mediated by the ryanodine receptor (RyR) channels, which drives muscle contraction, there is an inositol 1,4,5-trisphosphate (IP_3) receptor (IP_3R)-mediated calcium release that generates long-lasting calcium transients (4, 23, 25, 36, 37). This IP_3-induced calcium signal, which appears most prominently in the nuclei as well as faintly in the cytoplasm surrounding the nuclei, is not related to muscle contraction.

Our group has reported a role for this signal in the regulation of several transcription-related events that follow membrane depolarization induced by K^+ (4, 13, 14, 16, 37, 46). In primary culture of rat skeletal muscle cells, K^+ depolarization induces transient activation of ERK MAPK and of the transcription factor cAMP/Ca^{2+}-response element binding protein (CREB), as well as an increase in the mRNAs of early genes c-*fos*, c-*jun*, and *egr*-1 (13, 15, 16, 37, 46). The activation of these transcriptional regulators occurs in the absence of extracellular calcium or in the presence of high concentrations of ryanodine, which are inhibitory of the RyR response, but is significantly reduced by inhibitors of the IP_3R system that block the generation of the slow calcium transient (4, 13, 16, 46). CREB phosphorylation in this model depends on the activation of both ERKs and PKCα, most likely driven by the slow calcium transient induced by the IP_3 system (13, 16). On the other hand, a role for RyR-mediated calcium release in the regulation of transcription has been reported for NFAT (nuclear factors of activated T cells) activation induced by K^+ in C_2C_{12} cells (49).

The present work aimed to expand the study on transcription factors as downstream targets of depolarization-induced calcium signaling in skeletal muscle cells, comparing their outcome for the chronic depolarization by K^+ with the more physiological stimulation induced by electrical pulses delivered in different amounts and frequencies. We focused on NF-κB, which is activated by physical exercise and contraction of isolated muscles (2, 31, 32, 38), by mechanical stretch (41), and by elevated calcium concentration induced by mitochondrial stress in cultured muscle cells (9). Also, an important

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activation of this factor is seen in response to muscle disuse and in cachexia (28, 35).

In general, in nonstimulated cells, the predominantly cytoplasmic NF-κB proteins are associated with the inhibitory proteins IκB. Upon stimulation, these IκB proteins are phosphorylated by IKK, ubiquinated, and degraded, thus allowing the translocation to the nucleus of the NF-κB complex (30). In addition, other regulatory steps may occur within the nucleus such as posttranslational modifications of NF-κB proteins and chromatin remodeling (18). Besides skeletal muscle, calcium appears to be involved in the induction of NF-κB activity in several cell types. In neurons, activation of NF-κB is triggered by a rise of intracellular calcium induced by either glutamate or depolarization (27, 29, 39, 42, 43). Depolarization-induced calcium increase also activates NF-κB in pancreatic β-cells (6). In cerebellar granule neurons, NF-κB activation by depolarization requires calcium for both nuclear translocation and the stimulation of the NF-κB p65 protein transactivating activity by phosphorylation (42). Studies on NF-κB activation by well-defined calcium signals in terms of spatial and temporal properties have been performed in lymphocytes. Although a single spike of calcium is sufficient to trigger IκB degradation and NF-κB translocation to the nucleus (20), transcription efficiency is increased by high-frequency calcium elevations and is maximal with a sustained intracellular calcium elevation, probably reflecting an increased calcium level in the nucleus (21, 22).

In this work, we report NF-κB activation upon depolarization with both types of stimulation (high K^+ and pulses) in rat primary skeletal muscle cells and the C_2C_{12} cell line. This activation requires an increased intracellular calcium level dependent on both RyR- and IP_3R-mediated calcium transients, which become more or less represented depending on the nature of the stimulus. Calcium transients of relative short duration activate an NF-κB reporter gene to an intermediate level, whereas long-lasting calcium increases obtained by prolonged electrical stimulation protocols of various frequencies induce maximal activation of NF-κB. The use of pharmacological inhibitors suggests that reactive oxygen species (ROS), calcineurin (CaN), and PKC are involved in NF-κB activation, whereas the ERK contribution appears to be minor. These results provide new information and insight as to how chronic and transient stimulation are integrated to activate the NF-κB response by the complex calcium signals that are evoked.

MATERIALS AND METHODS

Reagents. PKC inhibitor Gö-6976, *N*-acetyl-L-cysteine (NAC), and cyclosporin A (CsA) were obtained from Biomol Research Laboratories. Bisindoleylmaleimide I (BIM I), U0126, and KN-93 were obtained from Calbiochem (La Jolla, CA). 2-Aminoethoxydiphenyl borate (2-APB) was obtained from Aldrich. Antibodies against IκBα, IκBβ, and p65 were obtained from Cell Signaling Technology (Beverly, MA). Anti-LAP2 was obtained from BD Transduction (Lexington, KY). Anti-histone H3 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Ryanodine, β-actin antibody, and horseradish peroxidase (HRP)-conjugated anti-mouse antibody were obtained from Sigma. Secondary HRP-conjugated anti-rabbit antibody was obtained from Pierce (Rockford, IL). Fluo-3 AM, Alexa Fluor 546 goat anti-rabbit IgG, and Alexa Fluor 488 goat anti-mouse IgG were obtained from Molecular Probes (Eugene, OR).

Cell cultures. Primary cultures of rat skeletal muscle cells were prepared from Sprague-Dawley neonatal hindlimbs as previously reported (16). Briefly, the muscle tissue was dissected, minced, and treated with collagenase for 15 min at 37°C. Growth medium was composed of DMEM-F-12 (1:1 mixture), 10% bovine serum, 2.5% FBS, 100 U/ml penicillin, and 10 mg/ml streptomycin. To arrest fibroblast growth, we added cytosine arabinoside (5 mM) 2 days after plating for 24 h. At *day 4*, cultures were differentiated in serum-free medium. The experiments were performed after 6–7 days in culture.

Myoblasts of the C_2C_{12} cell line (ATCC, Manassas, VA) were cultivated in DMEM-F-12 (1:1 mixture) with 10% bovine serum and 2.5% FCS. For differentiation, the serum was replaced by 5% horse serum. Cells were studied 5–7 days after differentiation was initiated. Cell culture media and reagents were obtained from Sigma and Invitrogen (Grand Island, NY).

Plasmid construct. A plasmid containing six tandem repeats of NF-κB binding sites linked to a luciferase reporter (pGL3; Promega, Madison, WI) was constructed. Briefly, the plasmid was produced using six copies of consensus sequences inserted immediately upstream of the pGL3 promoter (*Xho*I site). The orientation of the insert was verified by PCR amplification of the isolated DNA of the different clones.

Transient transfection and luciferase reporter assay. Primary culture cells were transiently transfected with FuGene 6 (Roche Applied Science) according to the manufacturer's specifications. Briefly, 2-day-old myoblasts plated in 60-mm culture dishes were transfected with 0.9 μg of the reporter vector DNA (6× NFκB-pGL3) and 0.1 μg of the *Renilla* phRL-TK vector (Promega) plus 3 μl of FuGene 6 in 1.5 ml of DMEM. After 12 h, the medium-DNA complexes were replaced by serum-free medium. Luciferase activity was determined using a dual-luciferase reporter assay system (Promega), and light detection was carried out in 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 resulted in no increase in luciferase activity after stimulation. Transfection efficiency was 5%, evaluated using an enhanced green fluorescent protein (eGFP) expression vector (Clontech), and was calculated as the percentage of the fluorescence-emitting cells in the total number of cells. The duration of the reporter expression was assessed by the analysis of luciferase activity at different times (3, 6, 12, and 24 h) after the stimuli. At 6 h the maximum response was obtained; therefore, cells were lysed 6 h after the stimulus.

Cell depolarization treatment. Differentiated primary culture cells or differentiated C_2C_{12} cells were washed with Ca^{2+}/Mg^{2+}-free PBS and maintained in Krebs-Ringer 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). The exchange to a medium containing 84 mM KCl, while the osmolarity was maintained by decreasing the NaCl concentration, induced depolarization by high K^+.

The electrical field stimulation to elicit action potentials of either a single cell or a whole dish of cells was done through platinum wires with the application of 1-ms suprathreshold voltage pulses delivered from high-current-capacity stimulators. Voltage amplitudes were 8–10 V for individual cells when the wire separation was no more than 1 mm and 20–50 V for whole dish stimulation with the electrodes separated by 5–6 mm. The efficiency of this stimulation was monitored under the microscope to verify the presence of myotube contraction and fluo-3 transients through the epi-illumination of the dish with a 100-W mercury lamp with the proper filters for excitation and emission of the fluorophore. The frequency and number of pulses delivered was controlled by software for each experimental condition. Most commonly, 400 or 1,000 pulses were delivered at 1, 10, or 45 Hz.

Subcellular fractionation and cell solubilization. Nuclear and cytosolic fractions from C_2C_{12} cells were obtained according to standard procedures (8). Cells were homogenized in 0.3 M sucrose, 10 mM Tris · HCl, pH 8.0, 10 mM NaCl, 3 mM $MgCl_2$, 0.2 mM EDTA, 5 mM Na_3VO_4, 20 mM NaF, 10 mM sodium pyrophosphate, and a protease inhibitor cocktail (Calbiochem) and then centrifuged at 1,500 *g* for 5 min at 4°C. The cytoplasmic fraction was obtained after further

centrifugation of the supernatant at 10,000 *g* for 10 min at 4°C. The nuclear fraction was obtained after washing the original nuclear pellet with the homogenization buffer containing 0.5% Nonidet P-40 (NP-40), followed by lysis with a hypotonic buffer and centrifugation at 17,000 *g* for 15 min to remove the chromatin fraction. LAP2 and IκBβ Western blots were performed to verify the quality of the fractions. LAP2 labeled the nuclear fraction, and IκBβ was used to assess the purity of both fractions because in C_2C_{12} cells, this protein is only cytosolic (9).

Cells were solubilized in 0.1 ml of lysis buffer (50 mM Tris·HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 5 mM Na_3VO_4, 20 mM NaF, and 10 mM sodium pyrophosphate) and a protease inhibitor cocktail (Calbiochem), sonicated, incubated on ice for 20 min, and centrifuged to remove debris.

Immunoblot analysis. Whole cell lysates or subcellular fractions 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 incubation for 1.5 h with HRP-conjugated secondary antibodies, membranes were developed by enhanced chemiluminescence (Amersham Biosciences, Amersham, UK). 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 (Bio-Rad, Hercules, CA) or stripped and blotted against β-actin (whole lysate) or histone H3 (nuclear fraction lysate).

Immunostaining and confocal imaging. Primary skeletal muscle cells 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-p65 antibody at 4°C. The cells were washed three times with PBS-BSA and incubated with secondary antibody for 90 min at room temperature. The coverslips were mounted in Vectashield (Vector Laboratories) to diminish photobleaching. The samples were evaluated with a confocal microscope (Zeiss Axiovert 200M-LSM Pascal 5) and documented through computerized images.

Calcium detection. Myotubes were preloaded with 5.4 μM fluo-3 AM for 30 min at 37°C in a resting solution (in mM: 145 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 Na-HEPES, and 5.6 glucose, pH 7.4); cleavage by intracellular esterases leaves the active form fluo-3 trapped inside the cell. The cells, grown on 25-mm glass coverslips, form the bottom of a 1-ml capacity chamber and were excited with a 488-nm wavelength argon laser beam attenuated 1–6% of its full intensity. The detector gain and offset were optimized for each data acquisition, whereas the pinhole was kept constant to gather a 7-μm optical slice in the *z*-axis. A fraction of the field of view with a ×20 Plan-Apochromat objective was used to obtain time series of images sampled at rates from 0.2 to 7 s per frame. For each time series the mean fluorescence from selected regions of interest covering a nuclear section of individual cells was tabulated and plotted against time as the normalized difference with the minimum fluorescence ($\Delta F/F_{min}$). Cells were exposed to high KCl solutions and depolarized by a fast (~1 s) change of solution by using the perifusion system, or they were electrically stimulated as described above. All experiments were carried out at room temperature (23°C).

Statistical analysis. Data are expressed as means ± SE. Differences in means between groups were determined using one-way ANOVA followed by Bonferroni's posttest.

RESULTS

Previous reports from our laboratory (23, 36) have shown that K^+ depolarization as well as the electrically induced depolarization with tetanic protocols in skeletal muscle cells in primary culture elicit a dual-phase calcium transient with a ryanodine and an IP_3-sensitive component. The calcium response of C_2C_{12} to K^+ follows the same pattern as in primary myotubes (25). In this report we have explored the patterned electrical stimulation at 1, 10, or 45 Hz with either 400 or 1,000 pulses to obtain a closer physiological representation of the in vivo condition of NF-κB activation. We have extended the study on calcium signals induced by electrical stimulation in primary culture to this wider range of frequencies and amount of pulses delivered, and we have determined the calcium signals induced in C_2C_{12} cells in response to these stimuli.

Characterization of C_2C_{12} and primary myotubes calcium signals by confocal imaging. Figure 1 depicts representative time-course fluorescence traces of fluo-3 for the intracellular calcium signal in electrically stimulated myotubes and in differentiated cells from the C_2C_{12} cell line. The signals were obtained from a group of cells under confocal microscope observation as described in MATERIALS AND METHODS. Figure 1*A*, *left*, shows the fast and slow calcium increases in a myotube during and after a 45-Hz, 400-pulse protocol, which is the general behavior for a prototypical tetanic stimulation that already has been reported by our laboratory

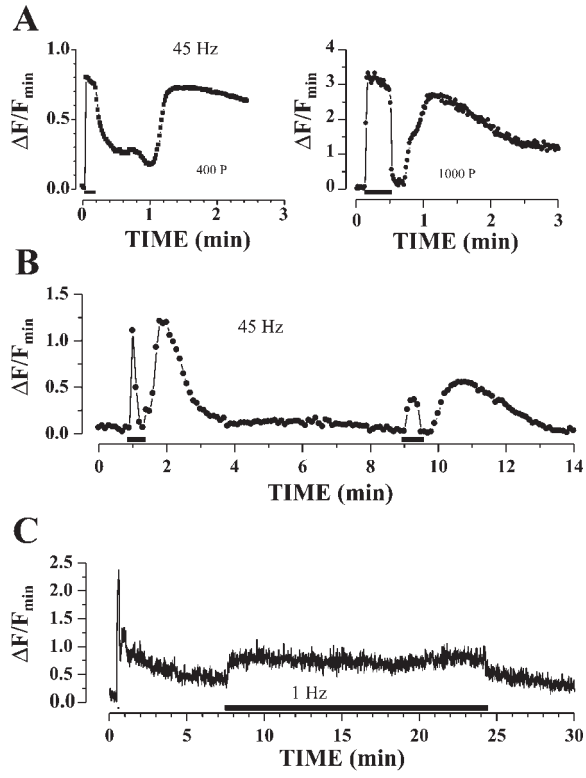

Fig. 1. Calcium transients induced by depolarization in primary culture and in C_2C_{12} cells. Representative traces are shown for the time course of calcium signals detected by fluo-3 fluorescence from selected regions of interest from confocal images of myotubes and C_2C_{12} cells. Electrical field stimulation was generated by 1-ms pulses of amplitude sufficient to generate the fast transient, which was monitored visually. The signal was normalized to the basal fluorescence by the evaluation of $[F(t) - F_{min}]/F_{min}$ ($\Delta F/F_{min}$). *A*: fast and slow signals elicited by the 45-Hz, 400-pulse (P) protocol (*left*) and another cell stimulated at 45 Hz and 1,000 pulses (*right*). Images were acquired at 0.5 s/frame every 1 s. *B*: trace from a C_2C_{12} cell subjected to the same protocols as in *A*. Images were acquired at 0.33 s/frame every 6 s. *C*: trace from a myotube response to the 45-Hz, 400-pulse protocol as a control and then to a 1-Hz, 1,000-pulse test protocol. Images were acquired at 0.42 s/frame every 1 s. It is clear that a sustained increase in calcium is present during the long-lasting stimulation.

(23). Figure 1*A*, *right*, shows that when at 45 Hz the amount of pulses is increased to 1,000, a similar pattern develops, although this time there is a shorter delay in the onset of the slow calcium signal. Figure 1*B* shows results obtained for C_2C_{12} cells. In this case both protocols, 400 and 1,000 pulses at 45 Hz, were applied sequentially to the same cell, and the fluorescence sampling was performed at a lower rate to prevent probe photobleaching over the long time of light exposure. This lower sampling rate explains the spike-like signal observed during the stimulation with 400 pulses. The fast response in the 400- and 1,000-pulse protocols is followed by long-lasting slow calcium transient as in primary myotubes. Figure 1*C* shows the calcium signals for a myotube stimulated with a 45-Hz, 400-pulse protocol followed by a 1-Hz, 1,000-pulse protocol. At this low frequency of stimulation, a partly fused tetanic response of the fast calcium signal was apparent and was maintained during the whole length of the stimulation protocol (>16 min long).

Both K^+ and electrical stimulation induce NF-κB reporter activity in primary myotubes. To measure transcription induction, we transiently transfected primary myotubes with a construct containing six tandem repeats of a consensus NF-κB binding site linked to a luciferase reporter plasmid. There was NF-κB activation in cells depolarized with K^+ or exposed to electrical stimulation (Fig. 2). A twofold activation was found in cells depolarized with K^+ as well as in cells exposed to 400 pulses of electrical stimulation at either 45 or 10 Hz. Decreasing the frequency to 1 Hz resulted in no statistically significant increase in NF-κB activity compared with controls in four experiments performed in duplicate. The trend toward increasing activation by using 400 pulses at the different frequencies tested was not enough to be statistically significant. Meanwhile, increasing the stimuli to 1,000 pulses showed an overall increase in NF-κB activation without significant differences between the frequencies.

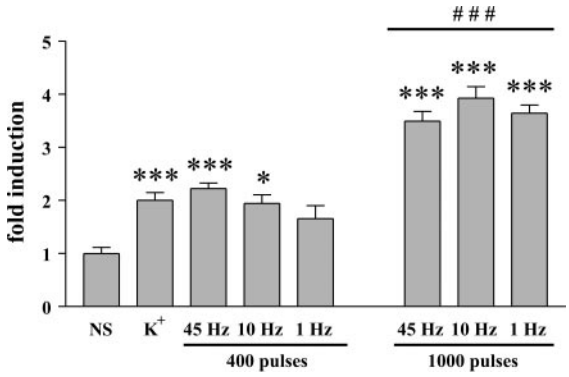

Fig. 2. Effect of KCl and electrical stimulation on NF-κB-dependent transcription in primary muscle cells. Seven-day-old myotubes transfected with a NF-κB reporter gene were incubated for 30 min in Krebs-Ringer under resting conditions before stimulation. Cells were either not stimulated (NS) or were subjected to a 1-min pulse with 84 mM KCl or exposed to different electrical stimulation protocols, according to the protocols described in MATERIALS AND METHODS, and harvested 6 h after stimulation. Results were normalized for transfection efficiency and expressed as the ratio of firefly to *Renilla* luciferase. Data are means ± SE (error bars) of duplicates (n = 3–9 independent experiments), expressed relative to results in NS cells. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's posttest. $^*P < 0.05$; $^{**}P < 0.01$, $^{***}P < 0.001$ with respect to the control group. $^{\#\#\#}P < 0.001$ with respect to K^+ stimulation and to 400-pulse protocols.

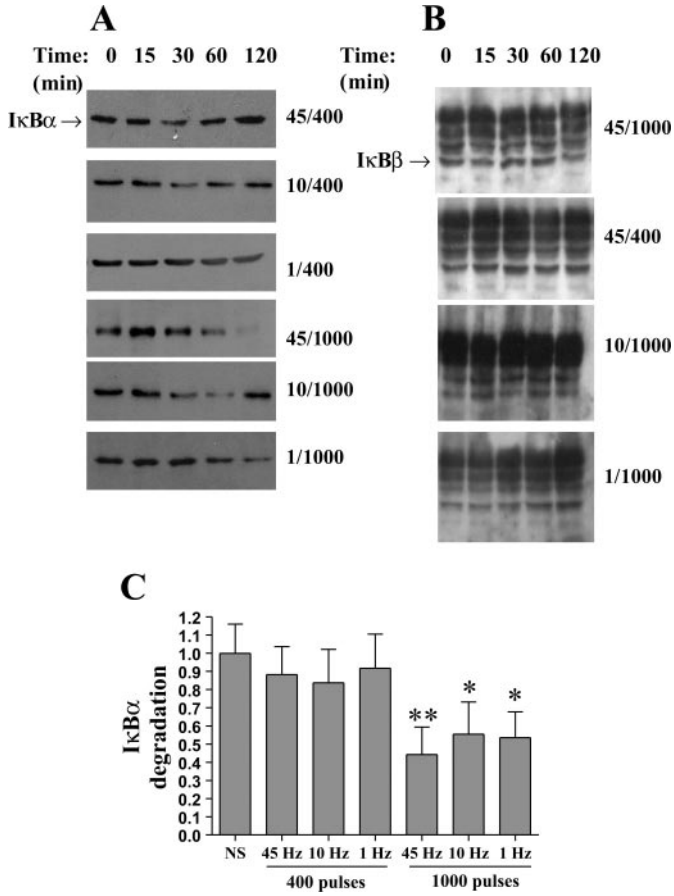

Fig. 3. Electrical stimulation of primary muscle cells induces IκBα degradation. Whole cell lysates from electrically stimulated primary myotubes were analyzed. *A*: Western blots of IκBα. *B*: Western blots of IκBβ. *C*: quantification of IκBα by image analysis (ImageJ) of the bands at times between 30 min and 1 h according to IκBα maximal degradation in each protocol. The results are expressed with respect to the NS cells. Coomassie blue-stained membranes or β-actin bands were analyzed as control for loading. Data are means ± SE (error bars) of duplicates from 4 independent experiments. $^*P < 0.05$, $^{**}P < 0.01$; determined using one-way ANOVA followed by Bonferroni's posttest.

These results indicate that NF-κB activation responds to a wide range of frequencies, although the most relevant aspect appears to be the time lapse during which the calcium level remains elevated.

NF-κB activation is mediated by IκBα degradation and p65 translocation to the nucleus. In the classic NF-κB activation pathway, signaling pathways activate IKKβ, which then phosphorylates IκB proteins, with subsequent degradation of the inhibitory protein. In the alternative pathway, there is activation of a NF-κB-inducing kinase and IKKα-dependent p100 phosphorylation (30). We have investigated whether the IκB proteins α and β are degraded in our model. Western blotting was performed in total lysate of primary myotubes exposed to the different electrical stimulation protocols. As shown in Fig. 3*A*, maximal degradation peak varied according to the stimulation protocol. The level of IκBα degradation detected was higher in response to 1,000- than to 400-pulse protocols (Fig. 3, *A* and *C*). There were no changes in IκBβ under these conditions (Fig. 3*B*). Considering that IκBα masks the nuclear localization sequence of

p65, a prototypical NF-κB protein, and that the predominant species in many cell types is a p65:p50 heterodimer, we have studied p65 translocation to the nucleus, using Western blotting, in cytoplasmic and nuclear fractions of C_2C_{12} cells depolarized with K^+ or exposed to electrical stimulation protocols. This cell line was employed because the subcellular fractions obtained were cleaner than those prepared from primary myotubes. Following the protocol described in Materials And Methods (8), immunoreactivity for the nuclear membrane marker LAP2 was only found in the nuclear fraction, whereas IκBβ immunoreactivity was predominantly found in the cytoplasmic fraction (IκBβ used as a marker of cytoplasm; see MATERIALS AND METHODS). As previously reported (25) and complemented in this work (Fig. 1), calcium signals in this cell line present the same behavior as those obtained in primary culture. Under basal conditions, p65 protein was mainly detected in the cytosolic fraction (Fig. 4*A*). Translocation to the nuclear fraction was clearly evident 90 and 120 min after stimulation with both K^+ and the electrical protocols employed. A tendency toward a higher degree of translocation with the 45-Hz, 1,000-pulse protocol compared with the 45-Hz, 400-pulse protocol could be observed (Fig. 4*B*). Translocation of p65 also was assessed using immunocytochemistry in primary myotubes stimulated at 45 Hz with 1,000 pulses (Fig. 4*C*). In the absence of stimulation, p65 was not detected in the nuclei. There was a marked increase in p65 level at 90 min after stimulation. We can conclude that both IκBα degradation and p65 translocation to the nucleus are involved in the mechanisms leading to NF-κB activation.

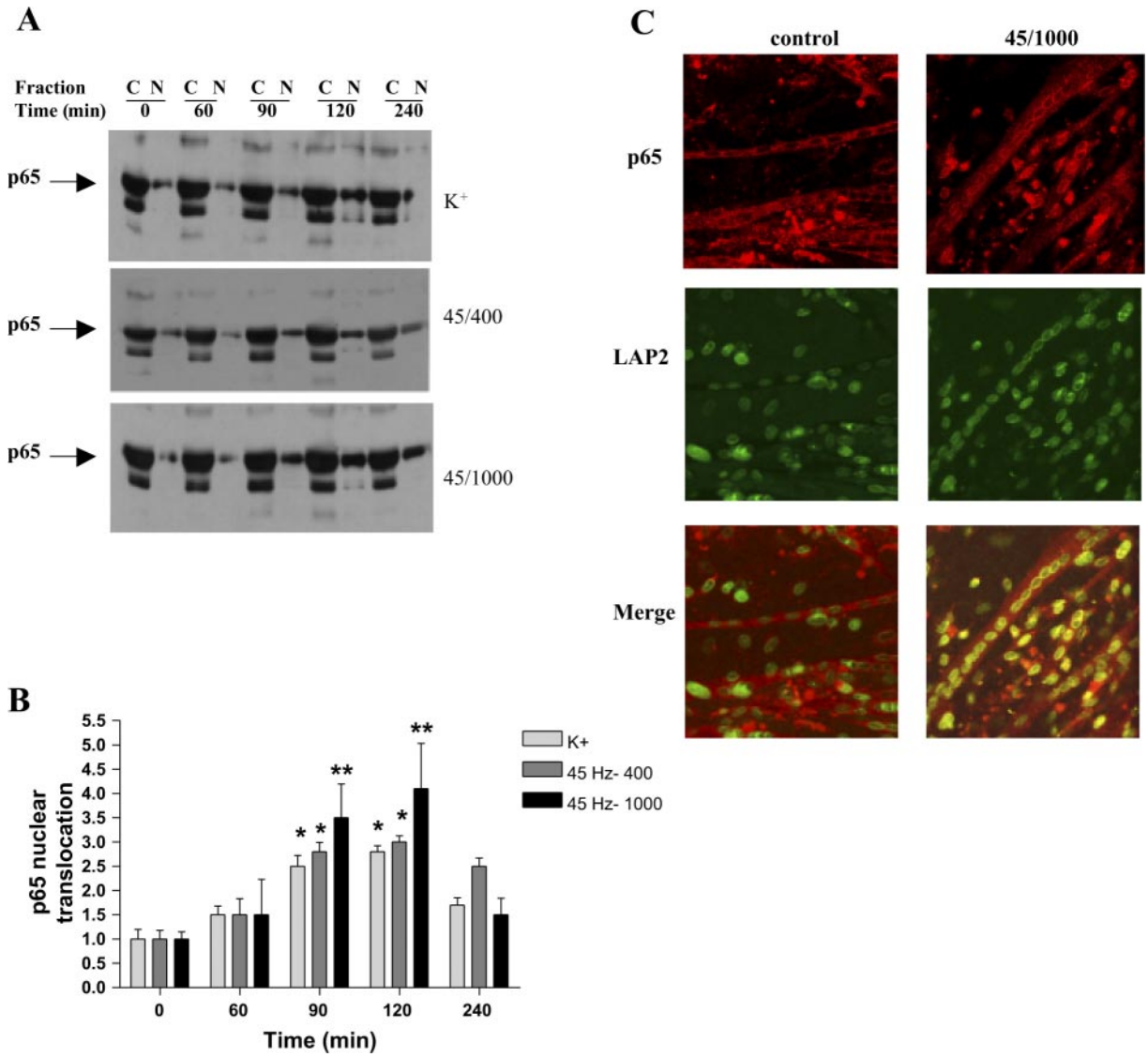

Fig. 4. KCl and electrical stimulation of C_2C_{12} muscle cells induce p65 translocation. *A*: Western blots of p65 in cytoplasmic (C) and nuclear (N) fractions obtained from C_2C_{12} cells after exposure to 84 mM KCl for 1 min or to 45-Hz, 400-pulse or 45-Hz, 1,000-pulse protocols. Coomassie blue-stained membranes or β-actin and histone H3 bands were analyzed as control for loading. *B*: densitometric analysis of p65 Western blots of C_2C_{12} nuclear fractions in NS cells or after exposure of cells to high K^+ or to different electrical protocols. Data obtained at 60, 90, 120, and 240 min are means ± SE (error bars) of duplicates from 3 independent experiments. $^*P < 0.05$, $^{**}P < 0.01$ with respect to NS cells; determined using one-way ANOVA test followed by Bonferroni's posttest. *C*: immunocytochemistry of p65 in primary muscle cells analyzed under control conditions or 90 min after exposure to a 45-Hz, 1,000-pulse protocol. Primary muscle cells nuclei are shown labeled by LAP2 antibodies.

Calcium involved in NF-κB activation arises from intracellular stores in both fast and slow calcium transients. To determine whether NF-κB activation requires extracellular calcium, we analyzed transcription in primary myotubes stimulated by high K^+ or by electrical stimulation in calcium-free conditions. To this purpose, calcium-free Krebs-Ringer was supplemented with 0.5 mM EGTA and 3 mM $MgCl_2$ (total $MgCl_2$ = 4.2 mM). In the absence of extracellular calcium, NF-κB activation did not show significant differences with respect to the results obtained in the presence of calcium in four independent experiments (Fig. 5*A*).

To investigate the participation of the fast and the slow calcium transients that are induced by both K^+ and electrical stimulation, we performed reporter gene and p65 translocation experiments in the presence of specific inhibitors to these calcium signals. The use of 50 μM ryanodine (inhibitory of the RyR) blocks the fast calcium transient, and with 2-APB, which blocks calcium release mediated by the IP_3 system, there is inhibition of the slow calcium transient (13, 16, 25, 36, 37, 46). Reporter gene experiments in primary myotubes depolarized by K^+ resulted in decreased NF-κB activation with both ryanodine and 2-APB; the decrease was higher when cells were depolarized in the presence of the two blockers (Fig. 5*B*). An important decrease in the simultaneous presence of these inhibitors also was observed in cells exposed to the 45-Hz, 400-pulse protocol (Fig. 5*C*). The contribution of RyR- and IP_3R-dependent calcium release also was significant but lower in cells stimulated with 1,000 pulses (Fig. 5*D*). The same overall results were obtained in experiments analyzing C_2C_{12} p65 translocation to the nucleus (Fig. 6, *A* and *B*). The control values for the electrical protocols of 45 Hz and 400 and 1,000 pulses show the same tendency that was already indicated for Fig. 4*B*, i.e., that is, there was higher translocation at a higher number of pulses delivered. Together, these data indicate that both RyR- and IP_3R-mediated calcium transients participate in NF-κB activation.

Study of signaling pathways involved in NF-κB activation. NF-κB activation can be regulated by multiple signaling pathways, at the level of both IKK activation and transcriptional activity (30). Since calcium plays an important role in NF-κB activation, the involvement of cellular sensors of calcium levels in subsequent steps of signal transduction was evaluated. We have measured global NF-κB activity using a reporter gene in primary myotubes depolarized by K^+ (Fig. 7*A*) or by stimulation protocols of 45 Hz with 400 (Fig. 7*B*) or 1,000 pulses (Fig. 7*C*) in the presence of pharmacological inhibitors of different signaling pathways. Considering the calcium dependence of the serine/threonine phosphatase CaN and that constitutively active CaN activates NF-κB in C_2C_{12} cells (3), we assessed the effect of exposing primary myotubes to depolarization by K^+ or electrical protocols in the presence of 10 μM CsA, a CaN inhibitor. There was a significant decrease in NF-κB activation with all protocols under this condition. We next asked whether CaMKs were part of the NF-κB activation pathway. Pharmacological and molecular experiments in neurons have shown that CaMKII, in particular, is required for NF-κB activation by calcium (42, 43). Also, our group has already reported that CaMKs are activated by K^+-induced depolarization, a condition in which a significant decrease by KN-93 in c-*fos* mRNA levels (16) was reported. KN-93 (10

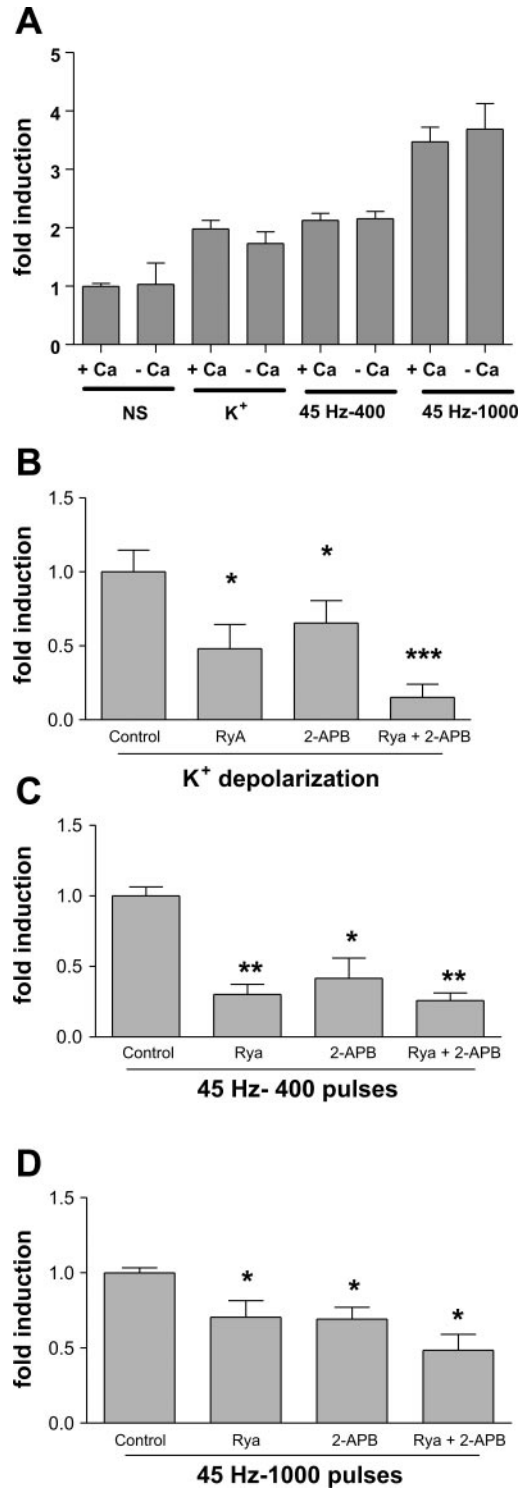

Fig. 5. NF-κB activity induced by KCl and by electrical stimulation in primary myotubes depends on calcium from intracellular stores. NF-κB-dependent transcription was evaluated in 7-day-old myotubes. *A*: myotubes were incubated for 30 min in resting conditions in the presence or absence of extracellular calcium before stimulation with high K^+ or with several electrical protocols under the same conditions. *B–D*: myotubes were preincubated for 30 min with the vehicle or with the inhibitors 50 μM 2-aminoethoxydiphenyl borate (2-APB), 50 μM ryanodine (Rya), or both and stimulated as indicated in the absence or presence of the inhibitors. Data are means ± SE (error bars) of duplicates from 4 independent experiments. The data obtained with inhibitors are expressed with respect to the level of activation in stimulated cells in the absence of inhibitors (control). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$; determined using one-way ANOVA followed by Bonferroni's posttest.

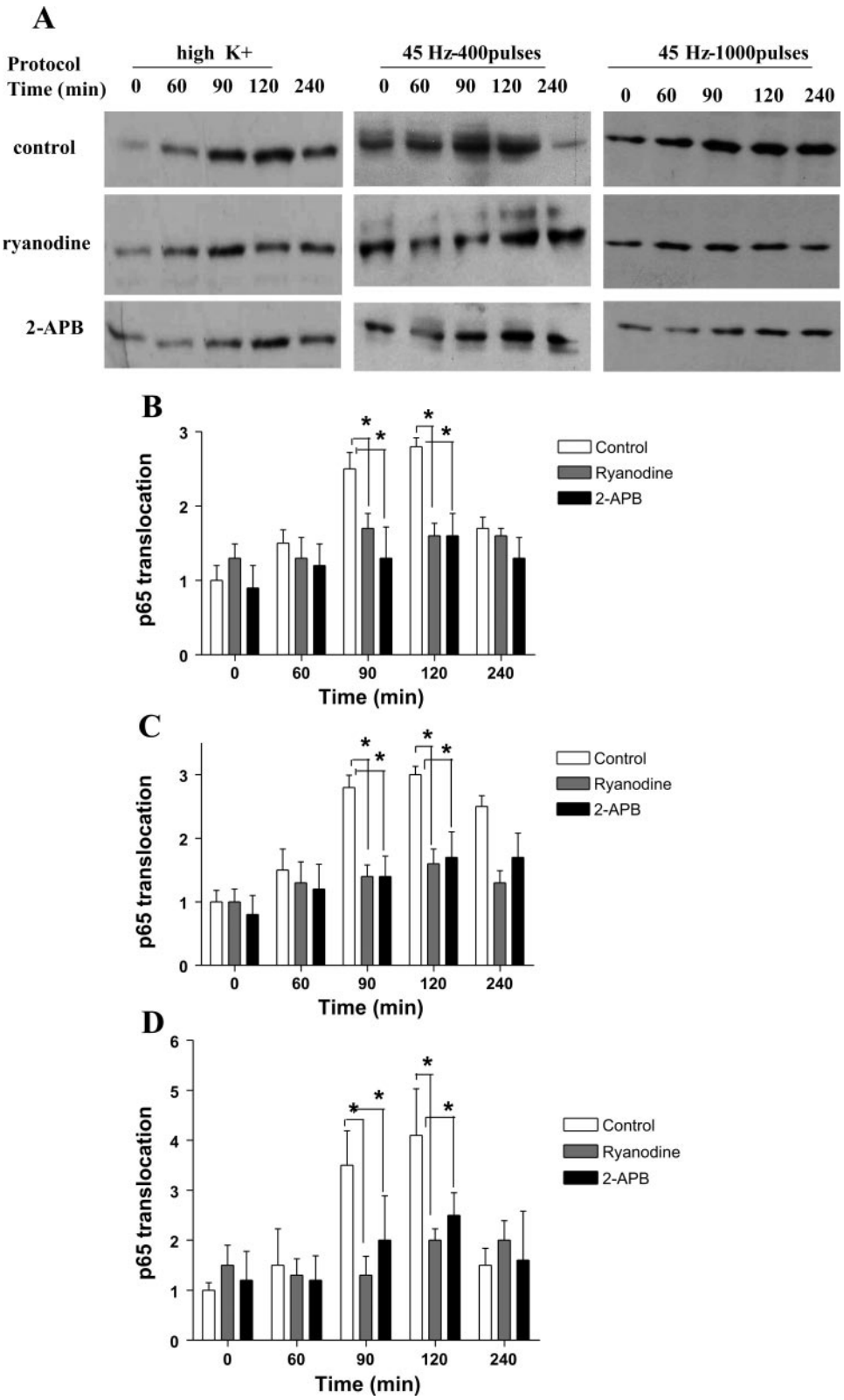

Fig. 6. p65 translocation induced by KCl and by electrical stimulation in C_2C_{12} cells depends on calcium from intracellular stores. C_2C_{12} cells were depolarized in the absence or presence of 50 μM 2-APB or 50 μM ryanodine and fractionated into cytoplasmic and nuclear fractions. *A*: Western blot of p65 in nuclear fractions of C_2C_{12} exposed to high-K^+ depolarization or electrical stimulation protocols. Coomassie blue-stained membranes or histone H3 bands were analyzed as control for loading. *B*: densitometric analysis of nuclear p65 Western blots. C_2C_{12} cells were either not stimulated or exposed to a 1-min pulse of 84 mM KCl. *C*: densitometric analysis of nuclear p65 Western blots. C_2C_{12} cells were either not stimulated or exposed to 45 Hz and 400 pulses. *D*: densitometric analysis of nuclear p65 Western blots. C_2C_{12} cells were either not stimulated or exposed to 45 Hz and 1,000 pulses. The data obtained with inhibitors are expressed with respect to the level of activation in stimulated cells in the absence of inhibitors. Data are means ± SE (error bars) of duplicates from 3 independent experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$; determined using one-way ANOVA followed by Bonferroni's posttest.

μM) was unable to modify the level of NF-κB activity in any of the protocols we used.

In a previous work, our group (13) reported the IP_3R calcium release-dependent activation of PKCα in K^+-depolarized primary myotubes. To study the participation of PKC in NF-κB activation, we exposed cells to BIM I (2.5 μM), a specific inhibitor of most PKC isoforms, and to Gö-6976 (1 μM), a specific inhibitor of calcium-responsive PKCs. There was a significant decrease in NF-κB activation with both inhibitors in cells stimulated with high K^+ and with the 45-Hz,

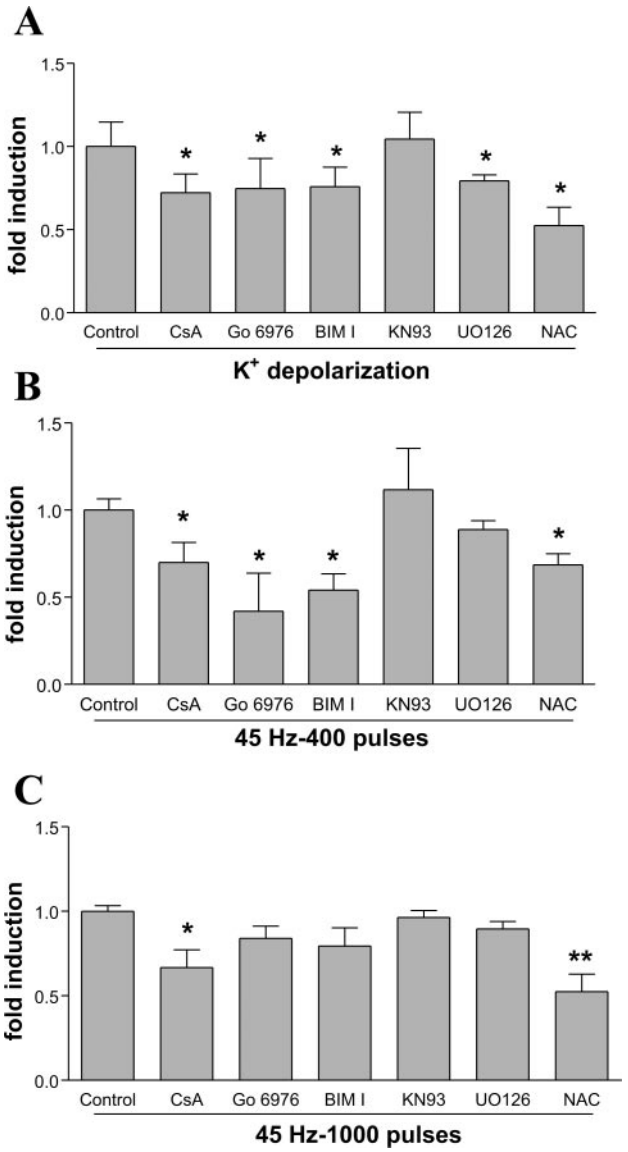

Fig. 7. Calcineurin (CaN), PKC, ERKs, and reactive oxygen species (ROS) are involved in NF-κB activation. Transfected primary myotubes were pretreated by 30 min with the following inhibitors: 10 μM cyclosporine A (CsA), 10 μM Gö-6976, 2.5 μM bisindoleylmaleimide I (BIM I), 10 μM KN-93, 10 μM U0126, and 30 mM *N*-acetyl-L-cysteine (NAC). Stimulation by K^+ or by different electrical protocols was performed in the presence of the inhibitors. *A*: stimulation by high K^+ concentration. *B*: electrical stimulation at 45 Hz and 400 pulses. *C*: electrical stimulation at 45 Hz and 1,000 pulses. Data are means ± SE (error bars) of duplicates from 4 independent experiments. The data obtained with inhibitors are expressed with respect to the level of activation in stimulated cells in the absence of inhibitors (control). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$; determined using one-way ANOVA followed by Bonferroni's posttest.

400-pulse protocol. There was no effect in cells stimulated with the 45-Hz, 1,000-pulse protocol.

Recently, NF-κB induction by contraction of skeletal muscle in vitro was reported to be significantly decreased by a combination of ERKs and p38 inhibitors (31). Our group has already reported that both ERK phosphorylation and the correspondent IP3R-dependent calcium release are stimulated by K^+-induced depolarization in primary myotubes (16, 46). Now we have studied whether ERKs phosphorylation is increased in cells electrically stimulated at 45 Hz with 400 pulses. A transient increase in ERK1 and ERK2 phosphorylation was already detected 2 min after stimulation, recovering to basal levels after 10 min. The maximal activation values (means ± SE) from four experiments, obtained at 5 min and normalized with respect to the nonstimulated control, were 2.25 ± 0.36 for ERK1 and 2.20 ± 0.50 for ERK2 ($P < 0.05$, ANOVA and Bonferroni). Based on these results, U0126, a selective MEK inhibitor that blocks the MEK-ERK pathway inhibiting ERK phosphorylation, was employed to determine whether ERKs are involved in NF-κB induction. There was a significant inhibition of NF-κB activation in cells exposed to high K^+ and no effect with the other protocols examined (Fig. 7).

We also have tested the effect of the general antioxidant NAC on NF-κB activation. Skeletal muscle activity is accompanied by production of reactive oxygen species (ROS) (50), and the activation of NF-κB by exercise has been proposed to be mediated by ROS (38). Recently, our group reported ROS formation in primary skeletal muscle cells in response to K^+ and to 45 Hz with 400 pulses (24). The addition of 30 mM NAC significantly decreased NF-κB activation in all protocols, suggesting involvement of ROS in this process.

DISCUSSION

Skeletal muscle cells are capable of changing both their structure and their function in response to activity. Calcium signaling has a crucial role in this adaptive process, but little is known about how the properties of calcium signals originating through different sources can regulate transcription (15). We have chosen to study the transcription factor NF-κB, considering the reports on its activation by physiological and pathological stimuli in skeletal muscle (28, 31, 35, 38, 41) and its regulation by calcium described in several systems (6, 27, 29, 39, 42, 43).

NF-κB activation is involved in both proliferation and differentiation of myotubes (5). In adult muscle, activation can be induced by exercise, contraction in vitro, mechanical stretching, and muscle wasting (28, 31, 35, 38, 41). In transgenic animals specifically expressing IKKβ in muscle, there is a sustained NF-κB activation that induces profound muscle wasting (10). The effects of the transient NF-κB activation induced by exercise are, nevertheless, not clear. It has been proposed that it could play physiological roles such as regulating normal repair and regeneration after muscle damage induced by high-intensity exercise (31). We investigated in the present study the relationship between different patterns of intracellular calcium transients and NF-κB activation in cultured skeletal muscle cells. We found that NF-κB is activated by both high extracellular K^+ (brief chronic depolarization) and by electrical stimulation (fluctuating depolarization). Provided that the stimulus duration remains relatively short, a fast and a slow calcium signal can be identified, and both signals appear to be involved in NF-κB activation. Long-lasting electrical stimulation induces both fast and slow calcium transients in such a way that the distinction between them becomes less apparent, resulting in a fused, continuous high calcium level that maximally activates NF-κB. Our work in skeletal muscle cells in culture stimulated by high K^+ and by electrical protocols has allowed us to characterize the early events linking membrane depolarization to NF-κB activation.

In previous studies, our group has determined that K^+ depolarization (1 min in our experiments) induces a fast calcium transient that lasts for <2 s and a slow calcium transient that lasts for tens of seconds (36, 46). The NF-κB activation obtained by K^+ and by 9- and 40-s protocols of electrical stimulation (45 Hz, 400 pulses and 10 Hz, 400 pulses, respectively) is of the same magnitude. Our interpretation is that once a threshold of calcium level is attained, NF-κB is similarly activated independently of how the calcium transient was generated. When 1,000 pulses electrical protocols are used, there is a higher overall NF-κB response independent of the length of the stimulation (22–1,000 s) as if it senses that calcium is already above a necessary threshold for its activation. We consider this behavior to represent a coarse mechanism of activation by different calcium levels. The question whether different calcium patterns are responsible for differentially encoding the specificity of cellular responses has been studied for NF-κB in nonexcitable cell systems. In B lymphocytes, the amplitude and duration of calcium signals control differential activation of the transcriptional regulators NFAT, NF-κB, and JNK (20). Physiological stimulation in this model results in an initial rise of calcium followed by a low, sustained plateau. The exposure of cells to ionomycin in conditions that mimic the fast calcium rise activates both NF-κB and JNK. Otherwise, mimicking the slow calcium plateau activates NFAT but not NF-κB or JNK. The role of calcium oscillations with different amplitudes and frequencies also has been investigated. Homogeneous and synchronous receptor-independent calcium oscillations in Jurkat T cells generated using a calcium-clamp technique were used as artificial stimulus to investigate the regulation of transcription of NFAT, NF-κB, and Oct/OAP (21). These authors found that frequent oscillations stimulated all three transcription factors, whereas infrequent oscillations activated only NF-κB; therefore, this transcription factor in particular could be activated by a wide range of oscillatory frequencies. On the other hand, in human aortic endothelial cells stimulated with the agonist histamine, NF-κB activation increased with histamine-induced oscillations of increasing frequency (33). In a skeletal muscle cell, it is the calcium transient overall duration, more than frequency, that will have physiological meaning. Our results suggest that NF-κB activation occurs once a calcium threshold is attained and then mostly discriminates between short-duration and long-term calcium rises; a relatively short tetanic stimulation will originate both a high calcium-fused signal (the tetanus) as well as a long-lasting slow calcium transient. Both signals appear to potentiate NF-κB activation. A long-lasting stimulation of high or even relatively low frequency will elicit a long-lasting calcium rise, producing maximal NF-κB activation.

The electrical stimulation patterns used in our experiments induced degradation of IκBα and translocation of the prototypical NF-κB family member p65 to the nucleus, in parallel with the degree of activation of the NF-κB reporter gene, whereas there was no effect on the inhibitory protein IκBβ. NF-κB activation induced by exercise in rat muscle (38) and by CaN overexpression in C_2C_{12} cells (3) is mediated by IκBα. Mechanical stretch of diaphragm muscles results in nuclear translocation of p65 and p50 associated to IκBα degradation (41). Different pathways, meanwhile, can be activated by mitochondrial stress or during disuse atrophy (9, 34). On the other hand, the IκBβ activation and subsequent release of p50 and cRel proteins for nuclear translocation has been reported in C_2C_{12} muscle cells subjected to mitochondrial stress (9). In unloaded rat soleus muscles, the nuclear level of p50 but not of p65 is increased (34). Also, in the latter model there is an increase of Bcl-3, a nuclear IκB family member.

Depolarization and the ensuing calcium increase resulting in activation of NF-κB have been reported in other excitable cells (6, 42, 43). In cerebellar granule neurons, NF-κB activity is proportional to the concentration of extracellular K^+, involving activity of L-type calcium channels and IP_3R (42). NF-κB activity in hippocampal cells would require extracellular calcium and a local increase within 1–2 μm of the influx point (43). In pancreatic β-cells, NF-κB activation mainly depends on calcium influx from the extracellular compartment through L-type calcium channels; the participation of calcium from intracellular stores was not evaluated (6). In the present work, there were no significant differences in NF-κB activation in the absence or the presence of external calcium with all protocols employed. Calcium influx also was reported not to participate in NF-κB activation induced by axial mechanical stretch of the diaphragm muscle (41). We have determined that the high K^+-induced activation of the transcriptional regulators ERKs, CREB, and the early genes c-*fos* and c-*jun* also is also modified in the absence of extracellular calcium (16). Skeletal muscle cells present L-type calcium channels that mainly behave as voltage sensors (47), and calcium entry through these channels is slow and minor; however, a significant calcium entry has been reported in muscle fibers with tetanic stimulation (11). The involvement of calcium entry in skeletal muscle cells has been reported as a signal for the expression of nicotinic ACh receptor subunits (1, 51). Recently, calcium entry through non-voltage-dependent calcium channels has been suggested to participate in NFAT activation induced by high K^+ in C_2C_{12} cells (49).

In this work we have shown that both the RyR- and IP3R-mediated calcium release from intracellular stores contributes to NF-κB activation. The use of the blockers ryanodine and 2-APB, separately or combined, resulted in a decrease in NF-κB activation, as evidenced with both reporter gene and p65 translocation experiments. The higher decrease in response to the simultaneous addition of both inhibitors, with both high K^+ and the electrical protocol of 45 Hz and 400 pulses, can be interpreted as an independent stimulation of NF-κB activation by the two calcium signaling pathways. The relative decrease in NF-κB activation induced by the protocol of 45 Hz and 1,000 pulses with both ryanodine and 2-APB was smaller than that obtained with 400 pulses. These results could mean that with longer stimulation, mechanisms such as a long-term increase in calcium, independent of the release, as well as calcium-independent mechanisms and/or phenomena like ROS production (24), can take place under these conditions. Although the action of 2-APB has been reported as nonspecific, under experimental conditions our group has clearly shown it to have an effect over the IP_3 cascade (16, 46). Any possible effect of 2-APB on calcium entry through transient receptor potential (TPR) channels does not apply in our system, since NF-κB activation does not require external calcium. An important aspect to consider is that activation of ERKs, CREB, and the early genes c-*fos*, c-*jun*, and *egr*-1 induced by depolarization rely solely on the slow calcium signal (16, 46).

Furthermore, calcium released by the RyR pathway also participates in regulation of transcription in depolarized skeletal muscle cells, as has been reported for NFAT (49). Therefore, the existence of redundant mechanisms for NF-κB activation may imply a possible regulatory system for this transcription factor depending on whether the stimulus triggers one or both calcium signals.

To characterize the calcium-dependent signaling pathways that could be involved in NF-κB activation by K^+-induced depolarization and electrical stimulation protocols, we studied the involvement of calcium sensors as CaN, calcium-dependent PKCs, CaMKs, and ERKs. Kinases and phosphatases are involved in NF-κB activation at both the level of nuclear translocation and for stimulation of the NF-κB p65 protein transactivating activity (18, 30, 42).

NF-κB activation by either high K^+ or 45-Hz, 400-pulse electrical stimulus was similarly decreased by most of the inhibitors, whereas increasing the number of pulses to 1,000 resulted in an activation affected only by CsA and NAC. This result might reflect the fact that this electrical stimulus results in NF-κB activation, which appears to be less dependent on calcium release. A role for CaN in NF-κB activation has been reported in skeletal muscle cells and in other cell systems. In Jurkat cells, activation by calcium of NF-κB was completely suppressed by the CaN inhibitor CsA (21), whereas basal NF-κB activity in granular cerebellar cells was partially mediated by CaN (42). In C_2C_{12} cells expressing constitutively active CaN, there is a strong enhancement of NF-κB activity that is decreased by pharmacological and genetic CaN inhibition (3). In our case, the inhibition was not complete, suggesting the participation of other signaling pathways as well. Mitochondrial stress-induced NF-κB activation, which is mediated by IκBβ dephosphorylation, also involves calcium-mediated increase in CaN activity (9). Therefore, CaN could participate in the regulation of both inhibitory proteins. Other important downstream calcium-dependent pathways are the CaMKs II and IV. CaMKII is activated by exercise in human muscle (48). The results obtained with the pharmacological inhibitor KN-93 were negative for all stimulation protocols used. Concerning ERKs, only with high K^+ stimulation does there seem to be some dependency of NF-κB activation on this signaling pathway. The involvement of this pathway was studied by considering the recent data of Ho et al. (31), where combined ERK and p38 inhibition resulted in an important decrease in IKK activation induced by muscle contraction. Also, in pancreatic β-cells, ERK mediates NF-κB activation induced by depolarization, and not by TNF-α (6). There was a partial contribution of PKC to NF-κB activation determined in cells depolarized by K^+ and by the electrical protocol of 45 Hz and 400 pulses, as evidenced with the inhibitors. In primary skeletal myotubes depolarized by K^+, we have reported the activation of PKCα but not the other calcium-dependent PKC isoforms βI and βII (13). Therefore, it can be suggested that PKCα participates at least in NF-κB activation by K^+ depolarization. The involvement of non-calcium-dependent PKC isoforms, however, cannot be discarded.

Treatment of primary cells with NAC (30 mM), a ROS inhibitor, reduced NF-κB reporter gene activation induced by K^+ and by electrical stimulation to ~50% of control values. We have determined that both high K^+ and electrical stimulation of 45 Hz and 400 pulses induce ROS increase (24); therefore, a situation can be expected that is similar to results of the other protocols employed in this work. In skeletal muscle of rats exercised until exhaustion, NF-κB binding in the nuclear fraction was partially blocked by the antioxidant pyrrolidine dithiocarbamate (PDTC), whereas the accompanying events, IκBα degradation and phosphorylation, as well as elevation of p50 nuclear content, were almost abolished by this antioxidant (38). Also, NF-κB binding activity in diaphragm induced by axial mechanical stretch was blocked by 30 mM NAC, suggesting ROS involvement in this model (41). The effect of high micromolar concentrations of H_2O_2 on NF-κB activity has been reported in skeletal muscle cells (2, 40). In L6 cells, H_2O_2 increases p65 translocation (2), and in C2C12 cells, H_2O_2 induces an increase in NF-κB-dependent promoter activity and IκBα phosphorylation and degradation (40).

The results reported in this work provide a new insight into how information encoded in the temporal pattern of action potentials is transmitted and integrated in the cell to control the activation of the calcium-dependent transcription factor NF-κB. The fact that the calcium signal originating from two different sources can be integrated to elicit the transcription factor maximal activation suggests a possible regulatory mechanism, considering that a short number of pulses will only elicit short-lived, ryanodine-sensitive calcium transients (23), whereas increases in either frequency or number of pulses will trigger the slow calcium transient, providing a long-lasting calcium increase and thus significantly increasing NF-κB activity.

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