Depolarization-induced slow calcium transients activate early genes in skeletal muscle cells

Maria Angélica Carrasco, Nora Riveros, Juan Ríos, Marioly Müller, Francisco Torres, Jorge Pineda, Soledad Lantadilla, and Enrique Jaimovich

Instituto de Ciencias Biomédicas and Centro Fondo de Investigación Avanzada en Areas Prioritarias de Estudios Moleculares de la Célula, Facultad de Medicina, Universidad de Chile, Santiago 6530499, Chile

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Carrasco, Maria Angélica, Nora Riveros, Juan Ríos, Marioly Müller, Francisco Torres, Jorge Pineda, Soledad Lantadilla, and Enrique Jaimovich. Depolarization-induced slow calcium transients activate early genes in skeletal muscle cells. Am J Physiol Cell Physiol 284: C1438–C1447, 2003.—The signaling mechanisms by which skeletal muscle electrical activity leads to changes in gene expression remain largely undefined. We have reported that myotube depolarization induces calcium signals in the cytosol and nucleus via inositol 1,4,5-trisphosphate (IP3) and phosphorylation of both ERK1/2 and cAMP-response element-binding protein (CREB). We now describe the calcium dependence of P-CREB and P-ERK induction and of the increases in mRNA of the early genes c-fos, c-jun, and egr-1. Increased phosphorylation and early gene activation were maintained in the absence of extracellular calcium, while the increase in intracellular calcium induced by caffeine could mimic the depolarization stimulus. Depolarization performed either in the presence of the IP3 inhibitors 2-aminoethoxydiphenyl borate or xestospongin C or on cells loaded with BAPTA-AM, in which slow calcium signals were abolished, resulted in decreased activation of the early genes examined. Both early gene activation and CREB phosphorylation were inhibited by ERK phosphorylation blockade. These data suggest a role for calcium in the transcription-related events that follow membrane depolarization in muscle cells.

SKELETAL MUSCLE responds to exercise or to electrical stimuli with changes in gene expression at the level of structural proteins and energetic metabolism enzymes (19, 20, 27). In recent years, a number of studies on the early signaling mechanisms that might link skeletal muscle activity to biochemical and gene regulatory responses have been reported (24, 25). A major issue concerns the possible role of calcium in the early events that lead to changes in gene expression in muscle cells. In rat skeletal muscle cells in primary culture, decreased transcription of the nicotinic acetylcholine (ACh) receptor subunit RNAs was reported to occur after treatment with drugs that release calcium from the sarcoplasmic reticulum, thus arguing in favor of a role for intracellular calcium in activity-dependent gene expression in skeletal muscle (3). The effect of calcium influx through L-type channels induced by the agonist BAY K 8644, meanwhile, was found to reduce expression of the c-subunit of the nicotinic ACh receptor through posttranscriptional mechanisms (3). The role of calcium has also been approached by treating cultured skeletal muscle cells with the calcium ionophore A-23187. The increase in intracellular calcium following a prolonged exposure of primary culture to the ionophore induces a change in myosin from fast to slow isoforms (15). In L6 myotubes, cytochrome c gene expression is activated by intracellular calcium increase resulting from a 48-h incubation with A-23187 (10).

Although intracellular calcium in skeletal muscle cells has been thoroughly studied in relation to the fast process of muscle contraction, previous work in our laboratory (13, 14) has shown that the calcium increase in skeletal muscle cells induced by high-K+ depolarization is a complex event involving at least two components. After a very fast calcium transient related to excitation-contraction (E-C) coupling, there is a slower transient not related to contraction that lasts several seconds. Whereas the first component is associated with the ryanodine receptor, the second is inhibited by compounds that interfere with the inositol 1,4,5-trisphosphate (IP3) system, suggesting that these signals are mediated by IP3 receptors (7, 21). The dihydropyridine receptor that functions in skeletal muscle as a voltage sensor, and as such has a fundamental role in E-C coupling, is also a voltage sensor for IP3-mediated slow calcium signals in muscle cells (4).

We have determined that high K+ -induced depolarization brings about the stimulation of phosphorylation of ERK1/2 and of the transcription factor cAMP-response element-binding (CREB) protein (21). Furthermore, we have found that both responses are inhibited when the slow signal is blocked (21). These results suggested a signaling system mediated by Ca2+ and IP3 that could be involved in regulation of gene expression in skeletal muscle. In the present work, this study has been extended by examining early genes that...
are upregulated in skeletal muscle by either exercise or electrical stimulation (1, 5, 16, 17, 22). Experiments were also performed to study the contribution of the ERK and other pathways to both CREB phosphorylation and to early gene activation. We have found that the slow calcium transients elicited by K+ depolarization of myotubes are involved in transient increases of mRNA levels of the early genes c-fos, c-jun, and egr-1. We could also determine that the ERK pathway is involved in both CREB phosphorylation and c-fos, c-jun, and egr-1 activation. In addition, the inhibition of another MAPK, p38 MAPK, reduced P-CREB levels and c-fos and c-jun upregulation, whereas the pharmacological inhibition of CaMK only decreased c-fos mRNA levels. Results indicate that slow calcium transients in skeletal muscle cells are related to signaling pathways likely to be part of the early steps in transcriptional activation.

MATERIALS AND METHODS

Materials. Dulbecco’s modified Eagle’s medium/F-12 was from Sigma (St. Louis, MO). Fetal calf serum, calf serum, antibiotics, and antymycotic were from Life Technologies (Burlington, ON, Canada). Antibodies against dually phosphorylated forms of ERK-1 and ERK-2 (P-ERK) and CREB (P-CREB) were from Cell Signaling Technology (Beverly, MA). CREB antibody and anti-ERK2 were from UBI (Lake Placid, NY). Horseradish peroxidase (HRP)-conjugated anti-rabbit was purchased from Pierce (Rockford, IL), and HRP-conjugated anti-mouse was from Sigma. Enhanced chemiluminescence reagents were from Pierce or Amersham Pharmacia Biotech (Amersham, UK). The mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (MEK) inhibitor U-0126, the CaMK inhibitor KN-93, and the IP3 receptor blocker xestospongin C were from Calbiochem (San Diego, CA). AIP-Cell Physiol • VOL. 284 • JUNE 2003 • www.ajpcell.org

were submitted to the same bath changes to discard differentiation.

Western blot analysis. After treatment, cells were solubilized at 4°C in 0.1 ml of lysis buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 5 mM Na3VO4, 20 mM NaF, 0.2 mM 4-(2-aminophenyl)benzenesulfonyl fluoride, 1 mM benzamidine, 10 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 μM pepstatin. After incubation on ice for 20 min, cells were scraped from the dishes, sonicated for 1 min, and left on ice for 30 min. Nuclear and cellular debris were removed by microcentrifuge centrifugation at 17,000 g for 20 min. In determining protein concentration of the supernatants, BSA was used as standard. Antibodies against GAPDH were purchased from Fitzgerald (Haverhill, MA). Western blot analysis. Total cellular RNA was isolated by the guanidinium isothiocyanate method (6). Samples (15–20 μg) were electrophoresed on 1% agarose-formaldehyde gels, transferred by capillary blotting onto nylon membranes, and immobilized by photocross-linking. Blots were prehybridized for 1 h at 42°C in a buffer containing 50% deionized formamide, 5× SSPE (sodium chloride-sodium phosphate-EDTA/1% SDS, and 125 μg/ml salmon sperm DNA. Hybridizations with 1 × 106 cpm/ml [32P]ATP were carried out at 42°C overnight in the same solution. Membranes were stripped once with 2× SSPE/0.1% SDS solution for 5 min, once with 0.2× SSPE/0.1% SDS for 5 min, and twice with 0.1× SSPE/0.1% SDS at 68°C for 15 min before autoradiographic film exposure. After autoradiography, bands were quantified by densitometry using an NIH program. Ethidium bromide stain of gels before capillarity transfer and reprobing of blots with GAPDH confirmed the integrity of the RNA samples and documented equivalent loading of each lane in gels used for the analysis.

cDNA probes. Rat c-fos cDNA, 2.1 kb, subcloned into EcoRI sites of p-Sp65, and rat c-jun cDNA, 1.8 kb, subcloned into EcoRI of p-Gem-4, were propagated in electrocompetent Escherichia coli DH5α cells. Purified plasmids were digested with EcoRI, and the products were labeled with [α-32P]dATP by using the random primer/Klenow enzyme method. Plasmids were a kind gift of Dr. Tom Curran (Children’s Research Hospital, Memphis, TN). c-fos message was detected by using a 578-bp fragment prepared by RT-PCR from total RNA extracted from rat skeletal muscle cells in culture. The primers used were 5’-AGCTTCGCCGCCGCGCAAGAT-3’ and 5’-TAAAGAGAGGCGCTGGTTGGAAG-3’. Product was labeled as described for other probes.
Semiquantitative RT-PCR. cDNA was amplified by using c-fos, c-jun, or egr-1 primers, and the DNA concentration was normalized to GAPDH expression. PCR amplification was maintained in the exponential phase for each product. The c-fos primers used were 5'-AGGCCGACTCTTCCTCCGACAT-3' (sense) and 5'-CAGTAGCTGTCTCTACTTTGC-3' (antisense), corresponding to bases 235–533. The c-jun primers used were 5'-GCCGCCGCCGAGAACCTTCTGC-3'(sense) and 5'-CAGCTCAGGCGCCAGCAGCGCTTT-3' (antisense), corresponding to bases 577–1227.

Calcium measurement. Intracellular, ionized calcium images were obtained from rat myotubes with a fluorescence microscope (Olympus) equipped with a cooled charge-coupled device camera and an image acquisition system (Spectra Source MCD 600). Myotubes were washed three times with Krebs buffer (145 mM NaCl, 5 mM KCl, 2.6 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES-Na, and 5.6 mM glucose, pH 7.4) to remove serum and then loaded with 5.4 μM fluo-3 AM (from a stock in 20% Pluronic acid-DMSO) or, when indicated, with Oregon green BAPTA-5N, which was then deesterified in the cytoplasm for 30 min at room temperature. Cells were pre-incubated in resting solution (see below) containing the dye at a 5.4 μM concentration for 30 min at 25°C. Cells attached to coverslips were mounted in a 1-ml capacity perfusion chamber and placed in the microscope for fluorescence measurements after excitation with a filter system.

Fluorescent images were collected every 0.1–2.0 s and analyzed frame by frame with the data-acquisition program (Spectra Source) for the equipment. Cells were incubated in the Krebs buffer (see above) as a resting condition medium. Cells were exposed to high-K⁺ solutions (47 mM K⁺, replacing Na⁺) and depolarized by a fast (~1 s) change of solution using the perfusion system.

Statistics. Results are expressed as means ± SE, and the significance of differences was evaluated using Student’s t-test for paired data or ANOVA followed by Dunnett’s multiple comparison post test.

RESULTS

mRNA levels of the early genes c-fos, c-jun, and egr-1 in rat myotubes after depolarization. Time dependence studies of c-fos, c-jun, and egr-1 mRNA levels performed after the depolarization procedure revealed a transient twofold increase that peaked about 15 min after treatment for the three mRNAs (Fig. 1). This increase was significant for all three early genes. c-fos and egr-1 mRNA expression returned to basal levels after 60 min; c-jun mRNA levels, meanwhile, remained higher than basal at the end of this period.

An alternative technique used in this work to analyze early gene expression, semiquantitative RT-PCR, gave results similar to Northern blotting. An example for c-fos and c-jun is shown in Fig. 2A; the kinetics of mRNA increase were clearly demonstrated in this case. Values for eight experiments using RT-PCR and nine experiments using Northern blotting did not differ significantly. The values (means ± SE) for each gene, expressed as percentages with reference to a normalized 100% control, were, for c-fos, 230.5 ± 17.15% (n = 8) using RT-PCR and 196.7 ± 26.3% (n = 9) using Northern blotting; for c-jun, 215.5 ± 14.55% (n = 8) using RT-PCR and 183.9 ± 12.8% (n = 9) using Northern blotting; and for egr-1, 216.8 ± 9.89% (n = 8) using RT-PCR and 237.3 ± 49.76% (n = 3) using Northern blotting.

In rat myotubes in primary culture, changing the K⁺ concentration from 4.7 to either 47 or 84 mM resulted in depolarization, with a change in membrane potential from −42 mV to about −12 or +1 mV, respectively, inducing a similar calcium increase (13). Under these conditions, the depolarization-induced increase in intracellular calcium lasts for tens of seconds and then calcium levels return to basal (13, 14). According to these observations, we have compared the results obtained with two protocols that differ in the exposure time to elevated K⁺. In one protocol, myotubes were incubated in high-K⁺ medium for just 1 min, re-fed with resting condition medium, and collected for anal-

**Fig. 1.** K⁺ depolarization increases messenger RNAs of early genes c-fos, c-jun, and egr-1. Total RNAs were isolated from rat myotubes in primary culture depolarized by K⁺ at the times indicated, and c-fos, c-jun, and egr-1 mRNA levels were analyzed by Northern blot. The results were normalized to GAPDH expression and presented as percentages of untreated control cells (means ± SE); 5–10 experiments were analyzed for c-fos and c-jun, and 3 experiments were analyzed for egr-1. *P < 0.05, **P < 0.001, compared with untreated controls (ANOVA followed by Dunnett’s multiple comparison post test).
A

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\begin{array}{|c|c|c|c|c|c|c|}
\hline
K^+ (min) & 0 & 10 & 15 & 20 & 30 & 45 & 60 \\
\hline
\text{c-fos} & & & & & & & \\
\text{c-jun} & & & & & & & \\
\text{GAPDH} & & & & & & & \\
\hline
\end{array}
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B

![Graph showing RT-PCR analysis of c-fos and c-jun mRNA levels.](image)

Fig. 2. A: RT-PCR analysis of c-fos and c-jun mRNA levels. Results from a single experiment are shown to illustrate the mRNA kinetics following K⁺-induced depolarization. This technique was used for results shown and quantified in B and in Figs. 4 and 8. B: the increase in c-fos, c-jun, and egr-1 mRNA levels obtained after high K⁺-induced depolarization is equivalent to 2 different protocols. Myotubes were either incubated in high K⁺ for different times and collected for analysis, or exposed to high K⁺ for only 1 min, changed to resting condition medium, and collected for analysis at variable times. RT-PCR analysis of mRNA was performed. With both protocols, the maximal stimulation was obtained at 15 min (only time shown).

Analysis at various times. The other protocol consisted of continuous exposure of the rat myotubes to depolarizing K⁺ concentration; that is, after the initial depolarization-induced calcium release, the plasma membrane was kept depolarized for the duration of the experiment. The results obtained with both protocols at the 15 min phosphorylation peak were equivalent and are shown in Fig. 2B.

**Relationship between the slow calcium transient and ERK or CREB phosphorylation and early gene expression.** To link early gene activation with the slow calcium transient seen in myotubes (Fig. 3A), we first dissociated the fast calcium transient from the slow one, taking advantage of the difference in cytosolic calcium that each of them represents. It has been postulated that cytosolic calcium concentration must be very low during the slow transient, because no contraction was detected during this period (13) and the use of ratiometric calcium dyes so indicates (13, 14). To further stress this point, we used a calcium-sensitive dye (Oregon green BAPTA-5N) that has much lower affinity for calcium than fluo 3 (20 μM K₆ compared with 0.4 μM in the absence of Mg²⁺). Upon depolarization, the fast calcium transient, capable of reaching micromolar calcium concentrations, can be clearly seen in Oregon green-5 BAPTA-5N-loaded cells (Fig. 3B), but the slow calcium transient was not apparent. Taking advantage of this result, we tried calcium chelation with the cell-permeant chelator BAPTA-AM. When myotubes were preincubated with both fluo 3 and 100 μM BAPTA-AM for 30 min, the slow calcium transient was abolished, whereas the fast calcium transient was spared (Fig. 3C). Preincubation with BAPTA-AM also resulted in decreased levels of both P-ERK and P-CREB (Fig. 3D).

To directly relate the slow calcium transient to regulation of early gene expression, we conducted experiments in cells loaded with BAPTA-AM in the same conditions as those depicted in Fig. 3. A significant inhibition of the enhancement in mRNA expression after K⁺ depolarization was observed in BAPTA-AM-incubated cells compared with untreated controls for all three early genes tested (Fig. 4A). The same type of inhibition was obtained when cells were treated with the IP₃ inhibitor 2-APB (Fig. 4B), a drug already shown to be capable of inhibiting the slow calcium transient and both ERK and CREB phosphorylation (21). Xestospongin C, a toxin reported to block IP₃ receptors (7), also reduced enhancement of c-fos mRNA by 60% and c-jun mRNA by 67% (not shown).

In muscle cells treated with 10 μM ryanodine, a condition that inhibits the fast calcium transient, sparing the slow calcium signal, there was no significant change on the increase in ERK or CREB phosphorylation induced by depolarization. P-CREB mean values from two experiments performed with myotubes depolarized in the absence or presence of ryanodine, expressed as percentages with reference to a normalized 100% control, were 301.0 and 289.0% after 5 min of depolarization and 297.0 and 243.0% after 10 min of depolarization, respectively. P-ERK percentages (means ± SE, n = 3) in the absence or presence of ryanodine for myotubes exposed to 5 min of depolarization were 196.3 ± 21.7 and 197.0 ± 24.9% for P-ERK1, and 168.0 ± 11.1 and 166.7 ± 8.0% for P-ERK2.

**ERK and CREB phosphorylation and early gene mRNA levels in the absence of extracellular calcium.** Calcium transients arising from skeletal muscle cells in primary culture exposed to high K⁺ are normally independent of extracellular calcium (13). However, because calcium entry through either voltage-gated or store-operated channels is a possibility in these cells, it is important to assess whether in our experimental conditions calcium influx participates in the activation of ERKs, CREB, and early genes. Experiments conducted under resting and depolarization conditions with medium containing 0.5 mM EGTA and no added calcium showed that the effects of depolarization remained essentially the same in calcium-free conditions.
egr-1 mRNA levels were also increased by depolarization in the absence of extracellular calcium (not shown).

**Effect of increasing intracellular calcium with caffeine.** Results obtained in BAPTA-AM-loaded cells indicated that cytosolic calcium has a role in the effects induced by the depolarization treatment. To pharmacologically increase intracellular calcium, we incubated myotubes with 10 mM caffeine. Exposure to caffeine resulted in stimulation of ERK1/2 phosphorylation (Fig. 6A), CREB phosphorylation (Fig. 6B), and c-fos and c-jun mRNA levels (Fig. 6C). In Fig. 6C, the results from three independent experiments on myotubes exposed to either caffeine or high K⁺ are shown. The mRNA levels were very similar in both conditions. In one additional experiment (triplicate), the effect of caffeine on egr-1 mRNA levels also resulted in an increase similar to that for c-fos and c-jun (not shown).

In differentiated myotubes, caffeine generates long-lasting, massive calcium transients that give rise to propagated, slow calcium waves (9). To interpret the effects of caffeine, two questions must be answered. First, considering that caffeine normally acts through activation of ryanodine receptors, is the calcium pool involved in slow calcium signals also being depleted by caffeine? When myotubes were incubated with 1 μM thapsigargin, a slow calcium transient, product of calcium pump inhibition, could be seen in cells incubated in the absence of extracellular calcium (Fig. 7A). Under these conditions, caffeine did not elicit further calcium increase (Fig. 7A). Thapsigargin alone did induce an increase in ERK and CREB phosphorylation, but no further increase was evident upon treatment with both thapsigargin and caffeine (Fig. 7, B and C). In thapsigargin-treated cells, high K⁺-induced depolarization did not elicit a calcium transient, and neither induced any increase in P-ERK or P-CREB levels (not shown). Second, does caffeine or calcium released by caffeine also activate IP₃ receptors? We stimulated calcium release with caffeine in the presence of 2-APB, consid-

![Fig. 3. BAPTA-AM inhibits the depolarization-induced slow calcium transient and ERK and cAMP-response element-binding protein (CREB) phosphorylation. Relative fluorescence intensity from fluo 3-loaded myotubes is shown.](image-url)
Considering that in our system this compound inhibited the slow calcium component that is mediated by IP3 receptor activation. P-CREB levels were examined in myotubes exposed to 10 mM caffeine for 1 min in the presence of 50/μM 2-APB (myotubes were preincubated for 30 min with either 2-APB or vehicle). The values (means ± SE), expressed as percentages with reference to a normalized 100% control, were 258.3 ± 27.4% in myotubes exposed to caffeine, decreasing to 135.3 ± 8.9% in myotubes exposed to caffeine and 2-APB (n = 3, P < 0.05). The value (mean ± SE), expressed as a percentage of a normalized 100% control, was 234.3 ± 15.3% (n = 3) for c-fos and was reduced to 159.0 ± 15.0% (P < 0.001) in the presence of the inhibitor. c-jun mRNA levels changed from 213.0 ± 10.1% under control conditions (n = 3) to 162.0 ± 6.9% (P < 0.01) in the presence of the p38 MAPK inhibitor.

Effect of MAPK inhibition on CREB phosphorylation and early gene activation. Because depolarization of skeletal muscle cells in primary culture activates ERKs, the role of this kinase cascade on early gene expression and CREB phosphorylation was evaluated. To study the role of the ERK signaling cascade, we used U-0126, a specific MEK inhibitor described as a blocker of the phosphorylated and nonphosphorylated forms of MEK1 and MEK2 (8).

U-0126 (10 μM) completely blocked the increase in ERK1/2 phosphorylation (Fig. 8A). Basal P-ERK levels were also decreased by prior exposure to U-0126. As a consequence of this inhibition, P-CREB levels were diminished (Fig. 8B) to values ranging from 8 to 30% of controls as observed in four independent experiments. The c-fos, c-jun, and egr-1 mRNA levels (Fig. 8C) were also largely diminished under these conditions. These results support a role for the MEK-ERK cascade as a link between membrane potential-triggered signals and nuclear events.

SB-203580, a p38 MAPK inhibitor, was also tested. SB-203580 (10 μM) decreased P-CREB levels and c-fos and c-jun mRNA levels. P-CREB values (means ± SE) from three experiments performed with myotubes depolarized for 5 min in the absence or presence of the inhibitor, expressed as percentages of a normalized 100% control, were 260.5 ± 25.3 and 166.9 ± 21.41%, respectively (P < 0.05). The value (mean ± SE), expressed as a percentage of a normalized 100% control, was 234.3 ± 15.3% (n = 3) for c-fos and was reduced to 159.0 ± 15.0% (P < 0.001) in the presence of the inhibitor. c-jun mRNA levels changed from 213.0 ± 10.1% under control conditions (n = 3) to 162.0 ± 6.9% (P < 0.01) in the presence of the p38 MAPK inhibitor.

Fig. 4. Inhibition of the depolarization-induced slow calcium transient diminishes the increase in early gene mRNA levels. Myotubes were pretreated either with vehicle or 100 μM BAPTA-AM (A) or 50 μM 2-aminoethoxydiphenyl borate (2-APB; B) and depolarized with high K+. The mRNA levels were determined by semiquantitative RT-PCR. Results obtained at 15 min of exposure to high K+ in the control or experimental series were expressed as percentages of the corresponding control (no depolarization). Values are means ± SE.

*P < 0.05, **P < 0.001, compared with the increase obtained in control conditions (Student’s t-test for paired data).

Fig. 5. Absence of extracellular calcium inhibits neither ERK or CREB phosphorylation nor the increase in c-jun or c-fos mRNA levels. Depolarization was performed in either the presence of 3 mM calcium or the absence of calcium plus the addition of 0.5 mM EGTA.

A: Western blot of phosphorylated ERK1/2 and total ERK2. These results are representative of 3 independent experiments. B: CREB phosphorylation and total CREB as control for loading. Similar results were obtained in 3 experiments.

C: myotubes were depolarized in either the presence (+Ca) or absence (−Ca) of extracellular calcium. Values (means ± SE, n = 3) represent the maximal induction of c-fos and c-jun mRNA levels obtained by Northern blot.
CaMK inhibition decreases c-fos upregulation. KN-93 for CaMK inhibition was also tested. With 10 μM KN-93, there was no significant effect on the increase of P-CREB induced by high K+ (means ± SE), expressed as percentages of a normalized 100% control, were 227.0 ± 77.0% in control myotubes and 284.0 ± 81.0% in myotubes exposed to KN-93 (n = 3). Although c-jun mRNA levels were not affected (202.3 ± 3.5% for control and 215.0 ± 14.8% in the presence of KN-93, n = 4), there was a decrease in c-fos mRNA level from the control value of 224.8 ± 7.3 to 169.0 ± 4.6% (n = 4, P < 0.05). egr-1 levels were not assessed.

DISCUSSION

The present study gives further evidence for a link among membrane depolarization, the upregulation of c-fos, c-jun, and egr-1 mRNA levels, and P-ERK and P-CREB levels through a calcium- and IP3-mediated mechanism. We have demonstrated that after stimulation, there is a rapid and transient increase in early gene expression, that ERKs are involved in this upregulation as well as in CREB phosphorylation increase, and that the effects of depolarization are critically dependent on calcium released from IP3-sensitive intracellular stores. We have used cultured myotubes, which constitute a model system that has some of the elements of adult muscle fibers but is also a model for

**Fig. 6.** Caffeine stimulates ERK and CREB phosphorylation and induces an increase in c-fos and c-jun mRNAs. A: Western blot of phosphorylated ERKs from myotubes incubated with 10 mM caffeine. Total ERK2 is shown as control for loading. Results are representative of 3 experiments. B: Western blot of phosphorylated and total CREB. A representative experiment is shown. C: myotubes were either depolarized with K+ or treated with 10 mM caffeine. Northern blots were performed with samples obtained from 3 independent experiments. The results were obtained with either depolarization or caffeine at 15 min of stimulation and are presented as percentages (means ± SE) of the untreated control.

**Fig. 7.** Intracellular calcium store depletion by thapsigargin (TPG) prevents stimulation of ERK and CREB phosphorylation by caffeine. A: relative fluorescence intensity from fluo 3-loaded myotubes was determined as described in MATERIALS AND METHODS. The response to the sarco(endo)plasmic reticulum Ca2+-ATPase pump inhibitor TPG was determined in a medium containing no calcium plus the addition of 0.5 mM EGTA. Note the calcium transient elicited by TPG and the absence of calcium increase upon addition of caffeine. When external (10 mM) Ca2+ was added, a large calcium influx was apparent. B and C: rat myotubes were preincubated for 5 min in the absence of external calcium (medium containing 0.5 mM EGTA). TPG (1 μM) was added for 4 min, followed by caffeine or vehicle for 1 min. The effect of caffeine was assessed by incubating the myotubes for 1 min, also in resting medium containing no calcium. ERK phosphorylation (B) or P-CREB levels (C) were analyzed as described in MATERIALS AND METHODS. Data are reported as ERK phosphorylation or P-CREB immunoreactivity represented as average fold increases (means ± SE, n = 3) over basal level.
developing muscle cells. Under this scope, the signals we are studying could be interpreted as relevant for muscle cell development and differentiation; confirmation of their presence and role in adult muscle fibers awaits studies in a different system.

In recent years, several studies on the early signaling mechanisms that putatively link skeletal muscle activity to biochemical and gene regulatory responses have focused on early gene expression. Most of immediate early gene products are transcription factors that bind to promoter regulatory elements of a number of downstream genes, so they are likely to be involved in the adaptive responses induced by neural activity and contractile work in skeletal muscle. It has been shown that after exercise, human skeletal muscle upregulates the expression of most members of the fos and jun gene families (22). Upon electrical stimulation of the motor nerve, c-fos, c-jun, and egr-1 mRNAs increase in both rabbit and rat skeletal muscle (1, 5, 16, 17). egr-1 has also been reported to increase in C2C12 cells exposed to either the calcium ionophore A-23187 or the cholinergic agonist carbachol (2). The latter could be blocked by either ryanodine or dantrolene, indicating that calcium released from sarcoplasmic reticulum is involved.

The present data show that depolarization of rat myotubes in primary culture brings about a twofold transient increase in c-fos, c-jun, and egr-1 mRNA levels, with a maximum about 15 min after exposure to a high K⁺ concentration. It is interesting to note that a depolarization period of 1 min is enough to induce mRNA upregulation, still significant 30 min after stimulation, suggesting that within this minute the voltage sensors involved in the triggering of the cascades probably undergo a single activation process and that this activation is enough to produce the total effect. In fact, 1 min of depolarization was enough to trigger both ERK and CREB phosphorylation detected after 10–20 min (21).

The results obtained after either extracellular (or intracellular) calcium chelation indicate that calcium release from intracellular compartments is involved in the increase of the early genes examined. It is worth noting that calcium increases such as those produced by either thapsigargin or caffeine are able to mimic the effects of depolarization on intracellular signals. As previously indicated, in skeletal myotubes, the calcium increase induced by depolarization involves two components. There is a fast calcium transient, visualized in the whole myotube, and a slow, localized calcium transient that involves both the nuclei and the cytoplasm surrounding the nuclei (13). Whereas the fast component is antagonized by ryanodine, the slow transient is abolished by compounds that interfere with IP₃, such as 2-APB, an inhibitor of IP₃-induced calcium release, and U-73122, a PLC inhibitor (21). In a dyspedic (1B5)
cell line expressing no ryanodine receptors, only the slow calcium signal is induced by depolarization, and it is blocked by either 2-APB or U-73122 and also by the IP$_3$ receptor antagonist xestospongin-C (7). This evidence indicates that the calcium increase visualized at the nuclear level is induced by an IP$_3$-dependent mechanism and that this mechanism operates independently of the fast calcium transient. We have previously found that 2-APB blocked both the slow calcium transient and the increase in ERK and CREB phosphorylation obtained in skeletal muscle cells depolarized by K$^+$ (21). In this study, we have found that both 2-APB and xestospongin C also decreased the depolarization-induced c-fos, c-jun, and egr-1 mRNA increase. The same results have been obtained with the calcium chelator BAPTA-AM. Therefore, the reduction in both the slow calcium transient and the biochemical responses was obtained by two different procedures, both involving intracellular calcium. Results obtained using ryanodine point to the slow calcium signal as the one involving intracellular calcium. We have previously shown increased activity of isolated rat muscle (24, 25). In agreement with these observations, we have shown increased CREB regulation could be decreased both by MEK or p38 inhibitors, only c-fos was affected by the CAMK inhibitor KN-93. A scheme illustrating these findings is shown in Fig. 9.

Signal transduction pathways can be highly complex, and the cross talk between pathways can take place at several levels from the membrane to the nucleus (12). Undoubtedly, our results present a partial view of all the signaling events taking place in the cell; the focus was placed on some pathways that have been shown to respond to either exercise in skeletal muscle or calcium in other excitable cells.

The evidence provided in this study does suggest an important role for activity-induced slow intracellular calcium increase and underlines the need for further studies on calcium-dependent early signaling mechanisms in skeletal muscle cells.

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