Depolarization-induced slow Ca^{2+} transients stimulate transcription of IL-6 gene in skeletal muscle cells

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Juretić, Nevenka, Paola García-Huidobro, Juan Antonio Iturrieta, Enrique Jaimovich, and Nora Riveros. Depolarization-induced slow Ca^{2+} transients stimulate transcription of IL-6 gene in skeletal muscle cells. *Am J Physiol Cell Physiol* 290: C1428–C1436, 2006. First published December 28, 2005; doi:10.1152/ajpcell.00449.2005.—Contracting skeletal muscle produces and releases interleukin-6 (IL-6) in high amounts. Nevertheless, the mechanisms underlying IL-6 expression are not understood. Because inositol-1,4,5-trisphosphate (IP_3)-mediated slow Ca^{2+} signals evoked by depolarization of skeletal myotubes appears to play a role in the regulation of gene expression, we examined its involvement on IL-6 transcription. With the use of semiquantitative RT-PCR, we have shown that K^+ depolarization of myotubes induces a transient increase in IL-6 mRNA level, which peaks at 3–4 h and is independent of extracellular Ca^{2+}. Inhibitors of IP_3-dependent Ca^{2+} signals, like 2-aminoethoxydiphenyl borate (2-APB) and U-73122, decreased activation of IL-6 gene expression as did Ca^{2+} signals inhibitor BAPTA-AM, whereas ryanodine, a fast Ca^{2+} transient inhibitor, had no effect on IL-6 induction. Depolarization of myotubes transiently transfected with a reporter gene construct, containing 651 bp of IL-6 promoter, induced a twofold increase in promoter activity, which was abolished by either 2-APB or U-73122 and remained unaffected after ryanodine treatment. Site-directed mutagenesis of parental construct allowed us to identify activator protein-1 and NF-κB sequences as regulatory elements involved in IL-6 upregulation. Our results provide evidence for involvement of IP_3-mediated Ca^{2+} signals on IL-6 transcription in skeletal muscle cells.

myotubes; membrane potential; intracellular Ca^2; cytokines; gene expression

SKELETAL MUSCLE responds to exercise through quantitative and qualitative changes in gene expression that modify fiber characteristics and muscle mass (44, 45). Although the molecular mechanisms mediating cellular adaptation to exercise training remain undefined, Ca^{2+} involvement in both the activation of the hypertrophic response to increased workload and the expression of a slow contractile protein phenotype has been reported (20, 35, 36).

Interleukin-6 (IL-6) is one of the genes differentially expressed when skeletal muscle undergoes work overload (7). Although peritendonous tissue (30), subcutaneous fat (33), and the brain (38) produce IL-6 in response to exercise, several reports (15, 41) suggest that muscle fiber itself might be the main source of the large increase in plasma IL-6 concentration, observed after intense and prolonged training. Muscle contraction rapidly increases both mRNA expression (25, 29, 40, 53, 54) and nuclear transcriptional activity of IL-6 (29), and leads to a marked IL-6 protein release (54). Recent inmunohistochemical studies (43) give further evidence of IL-6 induction within skeletal muscle fibers after exercise.

Several roles for muscle IL-6 production have been proposed. It has been demonstrated that locally produced IL-6 is necessary for full differentiation of C_2C_{12} myoblasts (2) and that satellite cell proliferation is regulated by autocrine IL-6 secretion (4). Exogenously applied IL-6 enhances myoblast differentiation (39) and stimulates its proliferation. These evidences as a whole have led to the idea that IL-6 may be involved in hypertrophy during resistance exercise (42). In addition to its local action, muscle derived IL-6 has systemic effects. IL-6 stimulates lipolysis in adipose tissue and glicogenolysis in liver, releasing free fatty acids and glucose into circulation. Therefore, its action could be related to the maintenance of glucose homeostasis and energy supply during exercise (42).

Regardless of the evidence supporting essential roles for IL-6 production during exercise, the molecular mechanisms concerning its expression in skeletal muscle are poorly understood.

On the basis of the observation that ionomycin, a Ca^{2+} ionophore, induces IL-6 transcription in cell cultures from human skeletal muscle biopsies, increases in cytosolic Ca^{2+} level after muscle activity have been implicated as a signaling factor in the cascade that leads to increased IL-6 expression (15, 28). In addition, several consensus sequences described as Ca^{2+}-responsive regulatory element in other promoters have been identified within the IL-6 promoter region, including binding sites for the activator protein-1 (AP-1), NF-κB, and cAMP response element binding protein (CREB) (16, 51, 57, 58).

Previous work in our laboratory (1, 12, 23, 46) has shown that Ca^{2+} increase in skeletal muscle cells, induced by both high-K^+ depolarization or electrical stimulation, is a complex event involving at least two components with different kinetics. After a fast Ca^{2+} transient, known to be associated to the ryanodine receptor and related to excitation-contraction coupling, there is a slower transient, mainly associated to cell nuclei. Some studies suggest that depolarization induced slow Ca^{2+} signals are mediated by inositol-1,4,5-trisphosphate (IP_3) and are a consequence of IP_3 receptor (IP_3R) activation (1, 12, 13) because depolarization induces a transient increase on IP_3 concentration after a few seconds, preceding a slow Ca^{2+} signal (23). Moreover, preincubation of myotubes with com-

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pounds that interfere with the IP_3 system, such as 2-aminoethoxydiphenyl borate (2-APB), an inhibitor of IP_3-mediated Ca^{2+} release; xestospongin-C, an IP_3R blocker; or U-73122, a phospholipase C inhibitor, lead to the complete absence of slow Ca^{2+} waves (13, 23).

We have recently reported involvement of slow Ca^{2+} transients in the regulation of early steps that follow membrane depolarization and lead to gene expression (1, 6, 22, 46). Actually, IP_3-mediated Ca^{2+} signals induce both a transient activation of ERK mitogen-activated protein kinase (MAPK) and transcription factor CREB, and an increase in early genes *c-fos*, *c-jun*, and *egr-1* mRNA levels in depolarized skeletal muscle cells (6).

The goal of this study was to further explore the molecular mechanisms involved in the transcriptional activity of the IL-6 gene in skeletal muscle. We analyzed the involvement of depolarization induced Ca^{2+} signals on IL-6 expression using K^+-depolarized rat myotubes and C_2C_{12} cells as models and semiquantitative RT-PCR for IL-6 mRNA level determination. In addition, we studied the effect of intracellular Ca^{2+} signals on IL-6 transcription in depolarized myotubes transfected with a luciferase reporter plasmid containing a fragment of the IL-6 promoter. Our results give evidence for involvement of slow Ca^{2+} transients in the activation of IL-6 expression in skeletal muscle cells. This is the first study that directly demonstrates the role of depolarization-induced changes in intracellular Ca^{2+} levels, as a signaling factor for IL-6 expression in skeletal muscle cells.

MATERIALS AND METHODS

Materials. DMEM-F12 was purchased from Sigma (St. Louis, MO). Bovine serum and fetal bovine serum were purchased from GIBCO-BRL (Carlsbad, CA). Antibiotics and antimycotics were purchased from Life Technologies (Burlington, ONT, Canada). BAPTA-AM and Fluo 3-AM were from Molecular Probes (Eugene, OR). 2-APB was from Aldrich, xestospongin C was from Calbiochem (La Jolla, CA), and U-73122 and ryanodine were purchased from Biomol Research Laboratories (Plymouth Meeting, PA). All other reagents were from Sigma or Life Technologies.

Cell cultures. Rat skeletal muscle cells in primary culture were obtained by collagenase treatment of 21 fetal Sprague-Dawley rat hindlimb muscular tissue, essentially as previously described (24). Briefly, the tissue was mechanically dispersed and then treated with 0.2% (wt/vol) collagenase for 15 min at 37°C under mild agitation. The suspension was filtered through Nytex membranes, spun down at low speed, and preplated for 10–15 min for enrichment of myoblasts. Cells were plated on 60-mm culture dishes in a medium composed of DMEM-F12 (1:1), 10% bovine serum, 2.5% fetal bovine serum, antibiotics, and antimycotics. To eliminate remaining fibroblasts, 10 μM cytosine arabinoside were added when the myoblasts started to align. To induce differentiation, cells were cultured in serum-free medium. The experiments were performed in well-differentiated, 6 to 7 days of contracting myotubes.

C_2C_{12} myoblast line (American Type Culture Collection, Manassas, VA) were maintained in DMEM-F12 (1:1) supplemented with 10% bovine serum and 2.5% fetal bovine serum. To induce differentiation, the medium was changed to DMEM-F12 supplemented with 5% horse serum. Cells were studied 5–7 days after differentiation was initiated.

Depolarization. Cells were incubated with Krebs-Ringer medium containing (in mM) 4.7 KCl, 20 HEPES-Tris, pH 7.4, 118 NaCl, 4.7 KCl, 3 $CaCl_2$, 1.2 $MgCl_2$, and 10 glucose, under resting conditions for 30 min. Depolarization was induced by changing to a medium containing 84 mM KCl, whereas the Na^+ concentration was decreased proportionally to maintain the osmolarity of the solution. After a 5-min depolarization period, cells were incubated under resting conditions for different times. When pharmacological inhibitors were used, myotubes were preincubated for 30 min and afterward depolarized for 5 min in the presence of freshly prepared inhibitor solution. The inhibitors were not present during the subsequent incubation period.

All experiments were matched with vehicle-treated controls to discard pharmacological agents nonspecific effects. Both control and experimental cells were submitted to the same procedures.

Ca^{2+} measurement. For single cells, intracellular Ca^{2+} measurements, myoblasts were cultured to 80% confluence on glass coverslips, and then differentiated into myotubes. Ca^{2+} images were obtained from myotubes preloaded with Fluo 3-AM, a Ca^{2+}-sensitive fluorescent dye, by using an epifluorescence microscope (model T041; Olympus) coupled to a charge-coupled device-cooled camera and a computer with acquisition and image-processing software (Spectra Source MCD600 and Windbl, respectively).

Myotubes were washed three times with Krebs buffer composed of (in mM) 145 NaCl, 5 KCl, 2.6 $CaCl_2$, 1 $MgCl_2$, 10 HEPES-Na, and 5.6 glucose, pH 7.4, to remove serum, and loaded with 5.4 μM Fluo 3-AM for 30 min at 25°C. Cells attached to coverslips were mounted in a perfusion chamber and placed under the microscope for fluorescence measurements by excitation with a 488-nm wavelength argon laser beam. Initially fluorescence images were collected every 3.0 s for 2 min, and then every 30 s to complete 200 min, and analyzed frame by frame with the data-acquisition program of the equipment.

Cells were incubated in the Krebs buffer resting medium and thereafter depolarized during 5 min with high-K^+ solution (84 mM K^+) using a perfusion system. Intracellular Ca^{2+} was expressed as percentage of fluorescence intensity relative to basal fluorescence (a value stable for at least 5 min in resting conditions). The increase in fluorescence intensity of Fluo 3-AM is proportional to the rise in intracellular Ca^{2+} level (37).

Semiquantitative RT-PCR. Total RNA from skeletal muscle cells cultures was prepared by TRIzol Reagent (Invitrogen, Carlsbad, CA) extraction and reverse transcribed with the use of SuperScrip II, RNAse H-RT (Invitrogen). cDNA was amplified using rat-specific IL-6 primers for primary cultures and mouse IL-6 primers for C_2C_{12} cultures. DNA concentration was normalized to GAPDH expression, and both IL-6 and GAPDH were amplified simultaneously in a single PCR. Although GAPDH has been described as a housekeeping gene, we have previously checked that its expression was not affected either by the depolarizing stimuli or by the pharmacological inhibitors used (2-APB and U-73122).

The primers used were the following: rat IL-6 primers, 5′-ATGAAGTTTCTCTCCGCAAGAGACTTCCAGCCA-3′ (sense), 5′-CTAGGTTTGCCGAGTAGACCTCATCATAGTGACC-3′ (antisense); mouse IL-6 primers, 5′-ATGAAGTTTCTCTCCGCAAGAGACTTCCAGCCA-3′ (sense), 5′-CTAGGTTTGCCGAGTAGACCTCATAGTGAC-3′ (antisense); and GAPDH primers, 5′-TCCGCCCCTTCCGCTGATG-3′ (sense), 5′-CACGGAAGGCCATGCCAGTGA-3′ (antisense).

PCR amplification was maintained in the exponential phase for each product. PCR conditions were: one cycle of 95°C for 2 min, followed by 26 cycles of 95°C for 1 min, 58°C for 1 min, 72°C for 1 min, and a final cycle of 10 min at 72°C. PCR products were resolved by electrophoresis on 2% agarose gel and stained with ethidium bromide. Bands were quantified by densitometric analysis with the NIH Scion Image program.

Plasmids. Luciferase reporter plasmids were kindly provided by Dr. Oliver Eickelberg (Department of Medicine II, University of Giessen, Giessen, Germany). The parental plasmid, pIL6-Luc651, contained a 651-bp fragment of the human IL-6 gene promoter, located directly upstream of the transcriptional start site, subcloned into pGL3 basic luciferase reporter gene vector. pIL6-Luc651ΔAP1

and pIL6-Luc651ΔNF-κB have been obtained by site directed mutagenesis of AP-1 and NF-κB consensus sequences within pIL6-Luc651. The AP-1 consensus sequence 5′-TGAGTCAC-3′ (position −283 to −276) has been changed to 5′-TGCAGCAC-3′, and the NF-κB consensus sequence 5′-GGGATTTTCC-3′ (positions −72 to −63) to 5′-CTCATTTTCC-3′. These mutations have previously been shown to inactivate the described consensus sequences (10, 11, 34).

Cell culture and transfection. Rat myoblasts were seeded onto 60-mm-diameter culture dishes and grown in serum-free DMEM-F12 (1:1) to 70% confluence. Cell transfection was carried out using LipofectAmine Reagent (Invitrogen). One microgram of plasmid/well was incubated at room temperature with Lipofectamine in a 1:2 ratio for 30 min. The myotubes were then cotransfected with the DNA:lipid complex and a pCMV-βgal expression vector as a control for transfection efficiency. After 18 h of incubation at 37°C, cells were changed to differentiation medium and incubated for additional 48 h at 37°C.

Depolarization and luciferase assay. Transfected myotubes were depolarized with 84 mM K^+ for 5 min as previously described, and then incubated in medium DMEM-F12 (1:1) containing 10% bovine serum and 2.5% fetal bovine serum for 24 h at 37°C. When inhibitors were used, cells were preincubated for 30 min and then depolarized for 5 min in the presence of freshly prepared inhibitor solution. For luciferase assay, cells were treated with 200 μl of Glo Lysis buffer 1× (Promega, Madison, WI) for 5 min. Cell lysates (20 μl) were mixed with 30 μl of Luciferase Assay Substrate reagent (Promega) and luminescence of the samples was integrated over a time period of 10 s in a luminometer (Berthold FB12, Perkin Elmer, Gaithersburg, MD). Results were expressed as the percentage of increased relative light units/β-galactosidase ratio of depolarized myotubes compared with unstimulated myotubes (control, 100%).

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

RESULTS

Ca^{2+} levels in rat myotubes after depolarization. Because we wanted to study the role of Ca^{2+} signals on the regulation of IL-6 gene transcription, it was important to establish that we were dealing with essentially a single set of Ca^{2+} transients that lasts <60 s and that Ca^{2+} levels did not change afterward unless a new depolarization step was performed. Rat myotubes in primary cultures were loaded with Fluo 3-AM, incubated in high-K^+ concentration medium for 5 min and changed back to resting conditions. Fluorescence was recorded during the whole experiment (up to 180 min) and it is clear that depolarization produces a Ca^{2+} transient (Fig. 1) that lasts ~30 s. After this transient, which is composed of the slow component related to IP_3 receptors and involved in the regulation of gene expression (1, 6, 13, 22, 23, 46), Ca^{2+} levels returned to basal and remained without significant changes during at least 3 h (Fig. 1). Most of the previously described fast component, related to ryanodine receptors and excitation-contraction coupling (1, 13, 23, 46), was not seen due to the signal frequency.

K^+ depolarization increases IL-6 mRNA levels in rat myotubes and in differentiated C_2C_{12} cells. Rat myotubes in primary cultures or C_2C_{12} cells were incubated in high-K^+ medium for 5 min, changed to resting conditions, and collected for RT-PCR analysis at different times. Studies on primary cultures submitted to the depolarization procedure revealed a transient increase on IL-6 mRNA levels, compared with nondepolarized controls, reaching a 3.5-fold maximal stimulation

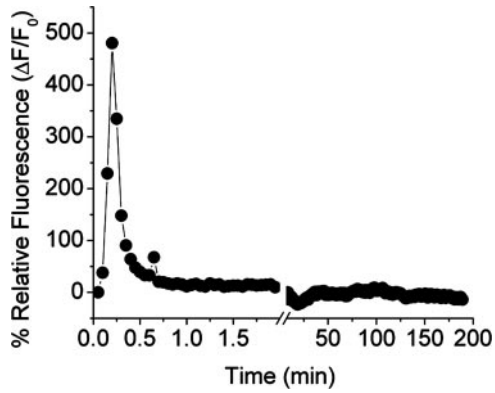

Fig. 1. Analysis of intracellular Ca^{2+} signals in rat myotubes. Fluorescence images of a representative experiment obtained from depolarized Fluo 3-AM-loaded myotubes. Fluorescence intensity was quantified from a selected region of the cell, using previously described software (13). Intracellular Ca^{2+} was expressed as the percentage of fluorescence intensity relative to basal fluorescence ($n = 5$).

3 h after treatment (357 ± 19%, $n = 3$) (Fig. 2*A*). Results were normalized to GAPDH expression. A significant increase in IL-6 mRNA level was already evident 30 min after the end of the stimulus (not shown).

Although basal IL-6 mRNA was undetectable in nondepolarized C_2C_{12} cultures, a progressively increased level was observed after 2 h and later, in high K^+-treated cells. Induction peaked about 4 h after stimulation (Fig. 2*B*) and returned to basal levels at 18 h (not shown). Values are expressed in arbitrary units that correspond to a percentage of the ratio between IL-6 and GAPDH expression at each time.

Ca^{2+} requirement for IL-6 induction: role of intracellular Ca^{2+}. Ca^{2+} transients evoked by exposure of myotubes to high K^+ concentration are normally independent of extracellular Ca^{2+} (23, 46). To rule out the participation of extracellular Ca^{2+} in our experimental conditions, rat myotubes were depolarized either in the presence of 3 mM Ca^{2+} or in a medium without Ca^{2+} containing 0.5 mM EGTA. Depolarization induced IL-6 mRNA levels were similar in both experimental conditions (Fig. 3*A*).

To demonstrate a more direct relationship between intracellular Ca^{2+} and IL-6 gene expression, we made use of the cell-permeable Ca^{2+} chelator BAPTA-AM. Preloading of primary cultures with 100 μM BAPTA-AM for 30 min under resting conditions, followed by 5-min exposure to high K^+ concentration resulted in a marked inhibition on IL-6 mRNA increased levels. Maximal induction obtained 3 h after stimulation in the absence of BAPTA-AM, 375 ± 5%, was reduced to 142 ± 21% in loaded cells ($n = 3$; Fig. 3*B*).

Depolarization-induced slow Ca^{2+} transients increase IL-6 mRNA levels in skeletal muscle cells. Ca^{2+} increase induced by high K^+ depolarization of skeletal muscle cells involves at least two components with different kinetics, the fast one is associated to ryanodine receptor and the slower transient is mediated by an intracellular IP_3 increase. To define which one participates on IL-6 induction, we used specific inhibitors that selectively abolish one of them.

Slow component of Ca^{2+} increase evoked by depolarization is blocked by compounds that interfere with the IP_3 system, such as 2-APB, U-73122, and xestospongin C (13, 46). We investigated the involvement of slow Ca^{2+} transients on IL-6

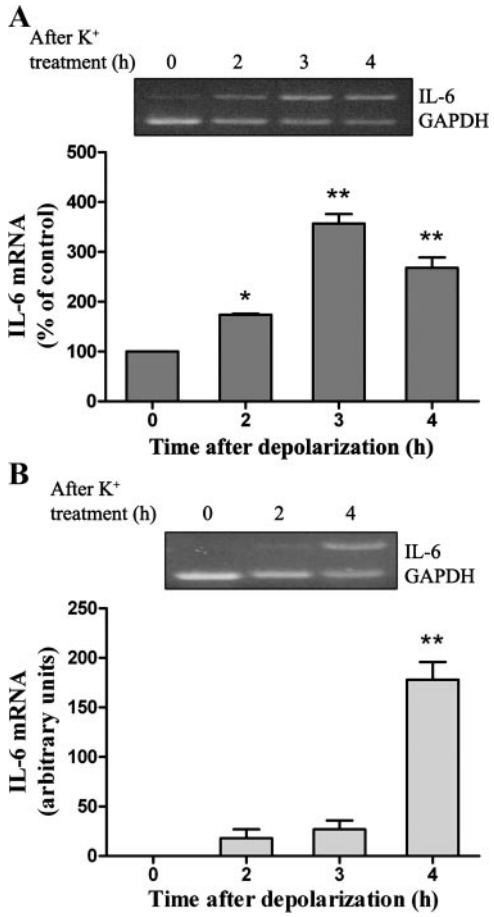

Fig. 2. K^+ depolarization increases IL-6 mRNA levels in both rat myotubes and differentiated C_2C_{12} cells. Total RNA was isolated from myotubes depolarized for 5 min with 84 mM K^+ and maintained in resting medium for the times indicated. IL-6 mRNA levels were analyzed by semiquantitative RT-PCR. *A*: IL-6 mRNA levels in rat myotubes in primary culture. *Top*, representative agarose gel of RT-PCR products from IL-6 and GAPDH mRNA amplification. *Bottom*, results normalized to GAPDH expression and presented as a percentage of untreated control cells (100%) (means ± SE; $n = 3$). *B*: IL-6 mRNA levels in C_2C_{12} myotubes. IL-6 induction is expressed as arbitrary units with respect to untreated control ($n = 3$). $*P < 0.05$, $**P < 0.01$ vs. ANOVA, followed by Dunnett's multiple-comparison test.

expression treating rat myotubes in primary culture with 50 μM 2-APB or 10 μM U-73122 before and during depolarization (Fig. 4*A*). Treatment of myotubes with these compounds resulted in the complete inhibition of maximal induced IL-6 expression at 3 h (381 ± 11% to 94 ± 6% for 2-APB, and 463 ± 3% to 138 ± 17% for U-73122, $n = 3$) (Fig. 4*B*).

Depolarization of myotubes performed in the presence of high concentrations of ryanodine eliminates the initial fast Ca^{2+} increase, whereas the slow Ca^{2+} transient is preserved (13). Exposure of rat myotubes to 25 μM ryanodine did not prevent IL-6 mRNA induction observed in nontreated depolarized cultures (Fig. 4*C*).

Depolarization of C_2C_{12} cells in a medium containing 50 μM 2-APB or 10 μM xestospongin C ended in a significant decrease in maximal induced IL-6 mRNA levels after 4 h in resting medium, with a percentage of inhibition of 86 ± 2% ($P < 0.05$, $n = 3$) and 67 ± 2% ($P < 0.001$, $n = 3$), respectively, with respect to depolarized control (100%, $n = 3$) (not shown).

As a whole, these experiments suggest that IP_3-mediated Ca^{2+} release, associated with the slow Ca^{2+} wave, is needed for triggering the cascade leading to IL-6 induction in skeletal muscle.

Depolarization increases IL-6 promoter transcriptional activity. To investigate whether depolarization is involved on IL-6 transcriptional activation, rat myotubes were transfected with a luciferase reporter gene construct containing nucleotides −651 to +1 of the human IL-6 promoter (pIL6-Luc651) (Fig. 5*A*).

Five-minute depolarization of transfected cells with 84 mM K^+ resulted in a twofold increase in luciferase expression, compared with nondepolarized transfected controls (206 ± 4% vs. 100 ± 3%; $n = 3$). Cultures transfected with the promoterless plasmid pGL3 basic ran in parallel served as negative controls. Increased IL-6 promoter transcriptional activity supports the role of depolarization in the regulation of IL-6 gene expression in skeletal muscle cells.

To further analyze the involvement of IP_3-mediated slow Ca^{2+} signals in IL-6 expression, transfected rat myotubes were pretreated, and then depolarized in a medium containing either 50 μM 2-APB or 10 μM U-73122. Inhibition of slow Ca^{2+} signals by two different mechanisms resulted in a significant decrease in depolarization mediated IL-6 promoter activation. Exposure of transfected myotubes to 25 μM ryanodine before and during K^+ treatment does not prevent increased IL-6 transcriptional activity (Fig. 5*B*).

These results are in agreement with preceding described observations obtained from RT-PCR analysis and support the

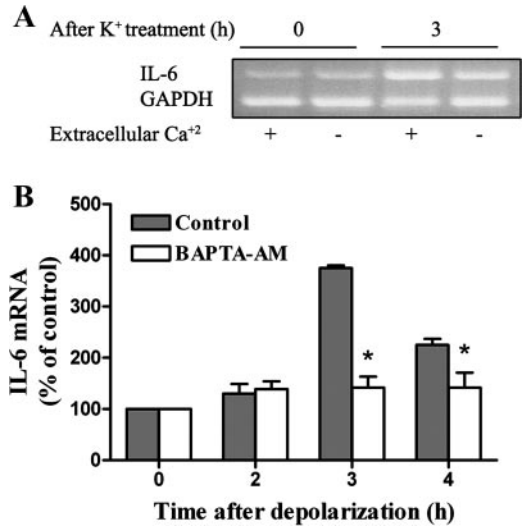

Fig. 3. Intracellular, but not extracellular, Ca^{2+} is needed to increase IL-6 mRNA levels in depolarized rat myotubes. *A*: absence of extracellular Ca^{2+} does not inhibit the increase in IL-6 mRNA levels. Characteristic agarose gel of semiquantitative RT-PCR products of IL-6 and GAPDH mRNA amplification from cells depolarized for 5 min with 84 mM K^+ and maintained in resting medium for 3 h. Depolarization was performed either in the presence of 3 mM Ca^{2+} or in the absence of Ca^{2+} plus the addition of 0.5 mM EGTA. *B*: BAPTA-AM inhibits IL-6 gene expression. Myotubes were pretreated for 30 min with vehicle or 100 μM BAPTA-AM under resting conditions, depolarized for 5 min with 84 mM K^+ in the absence or presence of BAPTA-AM, and maintained in resting medium for the times indicated. IL-6 mRNAs levels were determined by RT-PCR. Results were normalized to GAPDH expression and presented as means ± SE ($n = 3$) of the relative-induction effect of depolarization with respect to the control. $*P < 0.05$ vs. high-K^+ conditions at correspondent time, evaluated by *t*-test for paired data.

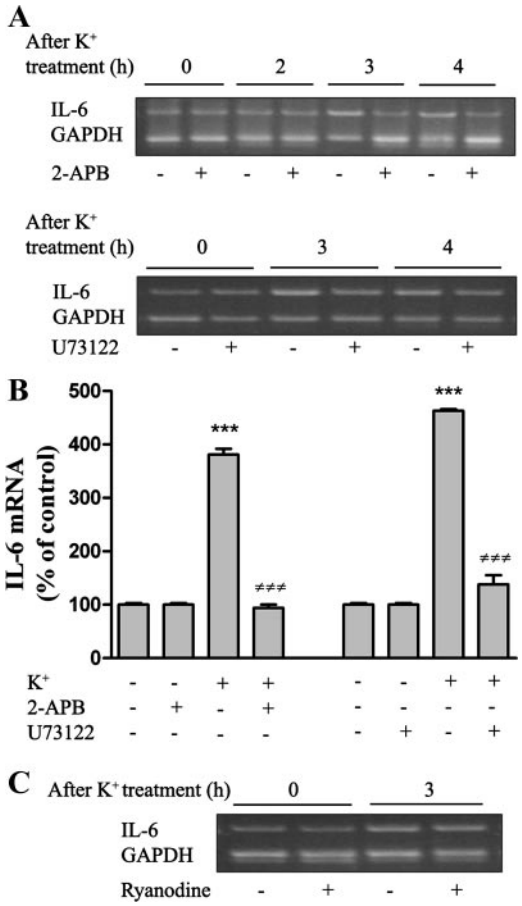

Fig. 4. Inhibition of depolarization-induced slow Ca^{2+} transient diminishes increased IL-6 mRNA levels in rat myotubes in primary culture. After a 30-min preincubation period with or without pharmacological agents, myotubes were depolarized for 5 min with 84 mM K^+ and maintained in resting medium for the times indicated. Inhibitors were present during the whole 5-min depolarization period. Total RNA was isolated and IL-6 levels were analyzed by semiquantitative RT-PCR. *A*: representative agarose gel of RT-PCR products from cells depolarized in the presence of 50 μM 2-aminoethoxydiphenyl borate (2-APB), 10 μM U-73122, or vehicle [ethanol (EtOH) and DMSO, respectively]. *B*: IL-6 mRNA levels 3 h after depolarization. The results were normalized to GAPDH expression and expressed as a percentage of the corresponding non depolarized control (100%). Bars represent means ± SE ($n = 3$). ***$P < 0.001$ vs. correspondent nondepolarized control, ≠≠≠$P < 0.001$ vs. high-K^+ conditions, evaluated by Bonferroni's test. *C*: ryanodine does not affect increased IL-6 mRNA levels. Characteristic agarose gel of RT-PCR products from cells depolarized in the presence of 25 μM ryanodine or EtOH and maintained in resting medium for 3 h.

involvement of depolarization induced slow Ca^{2+} signal on IL-6 gene transcription in skeletal muscle cells.

Regulatory sequences associated with depolarization IL-6 gene activation: involvement of AP-1 and NF-κB. IL-6 production is regulated by transcription factors AP-1 or NF-κB in other cell types exposed to different stimuli (11, 32, 34). To identify *cis*-regulatory sequences associated with depolarization induced IL-6 gene activation in skeletal muscle, rat myotubes were transfected with luciferase reporter plasmids, in which either AP-1 or NF-κB binding sites has been modified by site directed mutagenesis (pIL6-Luc651ΔAP1 and pIL6-Luc651ΔNF-κB, respectively). Depolarization of transfected cells resulted in a significant decrease of luciferase activity (Fig. 6), providing support for the role of both AP-1 and NF-κB responsive elements in the regulation of IL-6 gene expression.

DISCUSSION

We provide evidence here for a role of depolarization induced slow Ca^{2+} transients, not related to contraction, as an early signal leading to IL-6 transcriptional activation in skeletal muscle cells. We demonstrated that K^+ depolarization increases IL-6 mRNA levels in both differentiated C_2C_{12} and rat skeletal muscle cells in primary culture, and that the effect is critically dependent on Ca^{2+} released from IP_3-sensitive intracellular stores. Transfection of myotubes with a reporter gene containing a fragment of IL-6 promoter evidenced that the transcription process was activated after depolarization. Site-specific mutagenesis of regulatory *cis*-elements in the promoter region allowed us to identify that AP-1 and NF-κB are required for maximal induced IL-6 expression. Our results give additional support to previously reported IL-6 production by skeletal muscle contractile activity.

It has been demonstrated that contracting skeletal muscle synthesizes IL-6 in high amounts (55). Locally produced IL-6 is involved in myoblast proliferation and satellite cell recruitment, both processes tightly associated to muscle hypertrophy and muscle fibers regeneration after injury (17, 26, 27). Repet-

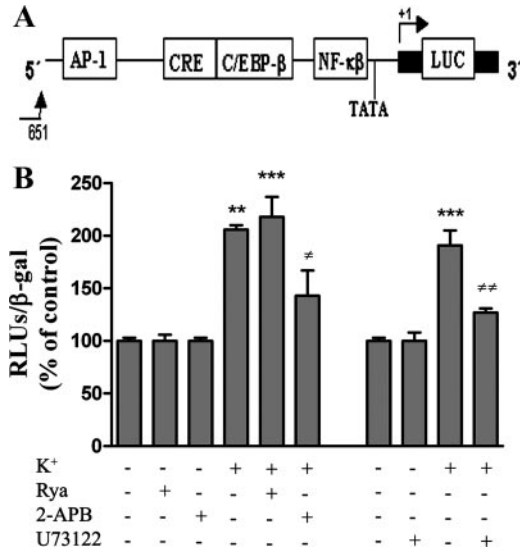

Fig. 5. *A*: luciferase reporter gene pIL6-Luc651. Scheme of consensus elements contained into the fragment spanning 651 bp directly upstream of the transcriptional start site of the IL-6 promoter fragment gene, fused to the luciferase (Luc) coding region of plasmid pGL3 basic (11). AP-1, activator protein-1; CRE, cAMP-responsive element; C/EBP-β, CCAAT/enhancer binding protein-β; NF-κB, nuclear factor-κB. *B*: depolarization-induced slow Ca^{2+} transients are involved on IL-6 promoter transcriptional activity. Rat myoblasts were cotransfected with pIL6-Luc651 and pCMV-βgal by lipofection. After cell differentiation, myotubes were stimulated with 84 mM K^+ for 5 min in the presence of either 25 μM ryanodine, 50 μM 2-APB, and 10 μM U-73122, or the corresponding vehicle (EtOH for Rya and 2-APB; DMSO for U-73122). Cells were harvested at 24 h and processed for luciferase assays. Results were expressed as percentage of increased relative light units (RLUs)/β-galactosidase ratio of depolarized myotubes in absence or presence of the drugs compared with non stimulated myotubes (control, 100%). RLU represents luciferase activity >10 s measured in a luminometer. Results are expressed as means ± SE ($n = 3$). **$P < 0.01$, ***$P < 0.001$ vs. correspondent nondepolarized control, ≠$P < 0.05$, ≠≠$P < 0.01$ vs. high K^+ conditions, evaluated by Bonferroni's test.

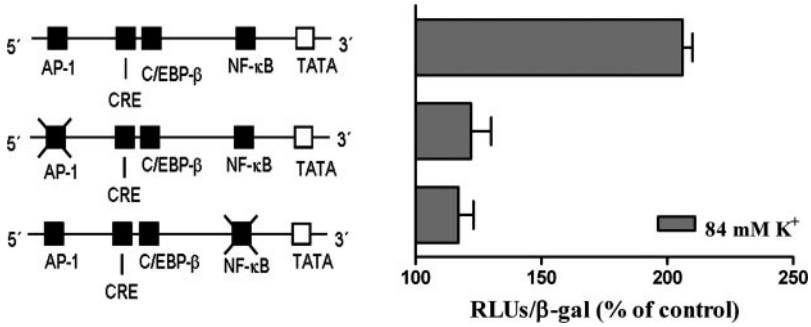

Fig. 6. Effect of AP-1 and NF-κB mutants on IL-6 promoter transcriptional activity induced by depolarization. Cells were transfected with a luciferase reporter plasmid containing a wild-type IL-6 promoter construct (pIL6-luc651) or an IL-6 promoter construct, in which either the AP-1 or NF-κB binding site has been mutated (pIL6-luc651ΔAP1 or pIL6-luc651ΔNF-κB, respectively). Myotubes were stimulated with 84 mM K^+ for 5 min, changed to full medium, and analyzed after 24 h. Induction of promoter activity by 84 mM K^+ was assessed for the respective construct, corrected for transfection efficiency, and expressed as percentage of induction compared with unstimulated cells (control, 100%). Results are expressed as means ± SE ($n = 3$).

itive muscle contraction rapidly increases intracellular IL-6 mRNA levels (15, 29, 53, 54) and nuclear transcriptional activity (29), leading to marked IL-6 protein release (54, 55). Although IL-6 expression in muscle tissue has been intensely investigated, the molecular mechanisms concerning IL-6 gene expression are poorly understood.

Ca^{2+} involvement in signaling pathways that lead to changes in gene expression is a well-established phenomenon in different cell types (3, 19). Depolarization of skeletal muscle cells, by both high-K^+ and electrical stimulation, induces at least two identifiable Ca^{2+} signals (1, 12, 23). In addition to a fast Ca^{2+} transient mediated by ryanodine receptor channels and associated with muscle contraction, there is a second IP_3-mediated slow Ca^{2+} wave, mainly localized in the nuclei, that have been related to transcriptional events that follow membrane depolarization (1, 6, 22).

Because ionomycin, a Ca^{2+} ionophore that induces an important rise in intracellular Ca^{2+} levels, leads to a progressive increase in IL-6 mRNA levels in muscle cells (21, 28), we focused our work to further study the mechanisms that link membrane depolarization to gene expression and particularly the involvement of slow Ca^{2+} transients in IL-6 transcriptional activation. Ca^{2+} participation on IL-6 transcriptional activity has been previously described in depolarized primary cortical cells and PC-12 cells (48).

We have found that depolarization of rat myotubes in primary culture brings about a transient 3.5-fold increase in IL-6 mRNA level, with a maximum about 3 h after exposure to a high K^+ concentration (Fig. 2*A*), whereas depolarization of differentiated C_2C_{12} cells results in a clearly increased level 4 h after stimulation (Fig. 2*B*). It is interesting to note that a 5-min depolarization period is enough to induce upregulation of IL-6 mRNA, an effect that lasts for several hours.

Although the time course of increased IL-6 mRNA expression in both rat myotubes and C_2C_{12} cells is similar to that previously reported in electrically stimulated rat skeletal muscle and in human skeletal muscle biopsies after exercise, the magnitude of IL-6 upregulation obtained in those studies is higher than the one reported here (25, 29, 40, 53, 54). These quantitative differences may be explained because both the type of stimulus and the models used in each study were different. High K^+ depolarization, in contrast to tetanic electrical stimulation, clamps the membrane voltage to a depolarized condition (24), thus K^+-induced depolarization represents basically a single synchronous stimulus for all the cells in the plate, after which the voltage sensors should inactivate, a condition that probably represents a mild stimulus compared with an important train of stimuli coming from the nerve.

Although previous evidence indicates that Ca^{2+} transients evoked by exposure of myotubes to high K^+ concentration are independent of extracellular Ca^{2+} (23) it was important to assess whether in our experimental conditions, Ca^{2+} influx through either voltage-gated or store-operated channels was implicated on IL-6 induction. Similar values in IL-6 mRNA induced levels were observed in depolarized cells incubated either with Ca^{2+} or in a Ca^{2+}-free medium (Fig. 3*A*), meaning that extracellular Ca^{2+} is not involved on IL-6 gene activation.

Experiments carried out in BAPTA-AM preloaded myotubes cells, resulted in reduced induction of IL-6 mRNA levels (Fig. 3*B*) and allow us to establish a more direct relationship between Ca^{2+} release from intracellular compartments and the early events that lead to increased IL-6 expression.

As has been pointed out, depolarization-induced intracellular Ca^{2+} release in skeletal muscle cells involves two components. The slow one, mediated by IP_3R, is abolished by compounds that interfere with the IP_3 system, such as 2-APB, xestospongin C, and U-73122, whereas the fast Ca^{2+} transient is antagonized by ryanodine (13). We observed that depolarization stimulated IL-6 expression was significantly inhibited by the slow Ca^{2+} blockers mentioned above (Fig. 4, *A* and *B*), but was unaffected by ryanodine treatment (Fig. 4*C*). These results strongly suggest involvement of IP_3-mediated Ca^{2+} release in the signaling cascade leading to IL-6 induction in skeletal muscle.

It seems particularly important to establish whether increased IL-6 mRNA levels were followed by actual enhancement in IL-6 transcriptional activity. We have clearly demonstrated that depolarization of myotubes transfected with a luciferase reporter gene containing nucleotides (−651 to +1) of the human IL-6 promoter (pIL6-Luc651) (Fig. 5*A*) increases luciferase expression. Experiments performed in the presence of either 2-APB or U-73122 showed a decrease in induced promoter activity. Ryanodine treatment at concentrations that block only the fast Ca^{2+} transient does not prevent K^+-evoked transcriptional response (Fig. 5*B*).

Taken together, our results suggest that depolarization induced slow Ca^{2+} signals stimulate transcription of IL-6 in muscle cells and support previous proposals about the implication of IP_3 receptors in the control of both nucleoplasmic Ca^{2+} levels and the transcription-related events that follow membrane depolarization (1, 5, 6, 22, 23, 46). To our knowledge, this is the first report describing the involvement of IP_3-mediated slow Ca^{2+} signals on IL-6 transcriptional activity in skeletal muscle cells.

There have been few reports of transcription factors involved in the regulation of IL-6 gene expression in skeletal

muscle; nevertheless, at least three known consensus sequences within its promoter region, AP-1, NF-κB, and CRE sites, have been involved on the regulation of IL-6 in different cell types (16, 50, 51, 57, 58). The regulatory role of each one varies depending on the cell system and the type of stimuli used (57). It has been demonstrated that AP-1 is required for IL-6 induction in transforming growth factor-β_1-stimulated fibroblasts (11). In addition, JunD, an important component of AP-1 dimers, regulates IL-6 gene transcription in activated hepatic stellate cells (52). On the other hand, NF-κB involvement on IL-6 induced expression has been reported in C_2C_{12} differentiating myoblasts (2), in palmitate-treated human myotubes (59), in IL-1β stimulated skeletal muscle cells in culture (32) and in mouse myoblasts and skeletal muscle after lipopolysaccharide exposure (17). Concerning the CRE regulatory site, its involvement has been demonstrated in thrombin (56) and angiotensin (18) control of IL-6 expression in vascular smooth muscle cells.

To identify regulatory sequences that are responsible for depolarization induced IL-6 upregulation in skeletal muscle cells, we made use of mutants of the parental pIL6-luc651 construct. Site-directed mutagenesis of either AP-1 or NF-κB regulatory elements, within the IL-6 promoter, almost completely abolished increased luciferase expression in depolarized transfected myotubes (Fig. 6).

Our results indicate that AP-1 and NF-κB sites, present in the IL-6 promoter, are required for depolarization induced IL-6 transcription. Because membrane depolarization in skeletal muscle cells leads to a rise in the intracellular Ca^{2+} concentration and both AP-1 and NF-κB sequences have been described as Ca^{2+} responsive regulatory elements in other genes (16, 50, 51, 57), we suggested that Ca^{2+} plays an important role in mediating the signaling pathways that trigger their activation and leads to induced IL-6 expression. Abolishment of luciferase reporter gene expression in experiments performed in the presence of 2-APB or U-73122, both of which interfere with the IP_3 signaling cascade, and the lack of effect of ryanodine, strongly supports slow Ca^{2+}/IP_3-mediated signal involvement in depolarization-induced IL-6 transcriptional activity.

Signaling pathways leading to the activation of NF-κB elicited by KCl depolarization has been characterized in primary cultures of neonatal cerebellar granule neurons (31). It has been demonstrated that the rise of intracellular Ca^{2+} through opening of L-voltage-sensitive Ca^{2+} channels at the plasma membrane and indirect opening of IP_3Rs associated with the intracellular Ca^{2+} stores are responsible for NF-κB activity in these cells (31). In addition, intracellular Ca^{2+} rise induces IL-6 expression through activation of NF-κB transcription factor in astrocytes (49).

AP-1 activity might be controlled by depolarization-dependent signaling pathways on several levels. AP-1 transcription factor consists of c-Fos/c-Jun proteins heterodimer, therefore its activation could be related to transcriptional induction of members of the *fos* and *jun* gene families. We have recently reported that K^+ depolarization of muscle cells leads to a transient increase of *c-fos* and *c-jun* mRNA levels, which occurs in the absence of extracellular Ca^{2+}, in the presence of high concentrations of ryanodine, but is significantly reduced by slow IP_3-mediated Ca^{2+} transient blockage (6). Thus induction of *c-fos* and *c-jun* expression, observed in depolarized myotubes, could be related to slow Ca^{2+} signaling pathways leading to IL-6 transcription.

In addition, transcription factor AP-1 could interact synergistically with Ca^{2+}-activated nuclear factor of activated T cells, as has been described for Ca^{2+}-dependent transcriptional activity of certain genes in lymphocytes and cardiomyocytes (8).

Although our results indicate that the activity of both AP-1 and NF-κB binding sites are required for maximal IL-6 gene induction, we cannot rule out the possibility that other regulatory sequences in the promoter participate in the regulation of IL-6 production in skeletal muscle. Previous work from our laboratory show that K^+ depolarization induces a transient activation of transcription factor CREB, in primary rat skeletal muscle cells (5, 6, 46). Because CRE consensus sequence is present within IL-6 promoter (50, 51), we can assume that induced IL-6 expression could be modulated either by a direct interaction between activated CREB and the CRE site or through interactions with other proteins like Fos/Jun, components of the AP-1 transcription factor, as has been described in other systems (9, 47). Further experiments on the subject should help to clarify this point.

Our findings could be inserted in the mechanistic model, recently presented by our group, depicting the pathways that link membrane depolarization and intracellular Ca^{2+} increase to early gene expression in skeletal muscle cells (1). This model includes receptors and pathways known to be involved

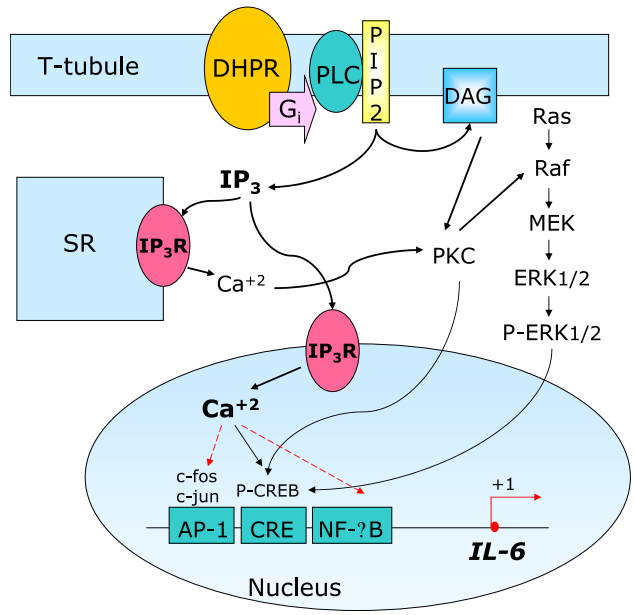

Fig. 7. Proposed pathways that link intracellular Ca^{2+} increase to IL-6 gene regulation in skeletal muscle cells. Membrane depolarization, through activation of dihydropyridine receptor (DHPR), results in IP_3-induced Ca^{2+} release. IP_3-mediated Ca^{2+} signaling pathways lead to subsequent activation of ERK, CRE binding protein (CREB), and both *c-fos* and *c-jun* gene expression (1). Our findings that indicate that IL-6 luciferase reporter gene expression is abolished by agents that interfere with IP_3-mediated Ca^{2+}-signaling pathways, and that both AP-1 and NF-κB sites, present in the IL-6 promoter, are required for maximal IL-6 transcription, allow us to suggest that the serial activation of some of these components might be involved in either AP-1 or NF-κB increased activity leading to the pronounced IL-6 response (broken arrows). This figure has been adapted from published data obtained in our laboratory (1). IP_3R, IP_3 receptor; PIP_2, phosphatidylinositol 4,5-bisphosphate; DAG, diacylglycerol.

in IP_3-generated Ca^{2+} signals. The signaling pathway begins at the dihydropyridine receptor, which activates phospholipase C by an unknown mechanism to produce IP_3 (1). IP_3 diffuses into the cytosol and reach IP_3 receptors located both at the sarcoplasmic reticulum membrane and at the nuclear envelope, inducing Ca^{2+} release. Several Ca^{2+}-dependent mechanisms are activated, including ERKs1/2 MAPK, CREB transcription factor, p38 MAPK, and early gene c-*fos* and c-*jun* expression (1, 6). It is possible that the serial activation of some of these components give rise to either AP-1 or NF-κB increased activity leading to the pronounced IL-6 response (Fig. 7).

Considering the multiple important biological effects of IL-6, increased knowledge on the regulation of muscle IL-6 production has significant clinical implications. In addition to its local effects on myogenesis (2) and tissue repair after injury (17, 26, 27), several lines of evidence suggest that IL-6 works in a hormone-like fashion, exerting an effect on the liver, thereby regulating glucose homeostasis and playing a key role in adipose tissue lipolysis to achieve enough energy in periods of high metabolic demand (15, 42). Moreover, IL-$6^{-/-}$ mice have reduced endurance and energy expenditure during exercise, suggesting that IL-6 is necessary for normal exercise capacity (14). Our results point to an important role for the slow Ca^{2+} transient evoked by depolarization in the activation of pathways that likely link excitation to IL-6 gene transcription. Understanding the molecular basis underlying the adaptive mechanisms of skeletal muscle in response to depolarization will provide valuable insight into the various physiological processes that control skeletal muscle mass both during development and under physiological and pathological conditions.

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