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

Nevenka Juretić, Paola García-Huidobro, Juan Antonio Iturrieta, Enrique Jaimovich, and Nora Riveros

Centro de Estudios Moleculares de la Célula, Instituto de Ciencias Biomédicas, Facultad de Medicina, Universidad de Chile, Santiago, Chile

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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 (IP3)-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 IP3-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-$\kappa$B sequences as regulatory elements involved in IL-6 upregulation. Our results provide evidence for involvement of IP3-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 peritendinous 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 immunohistochemical 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 C2C12 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 evidence 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 glycogenolysis 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-$\kappa$B, 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 (IP3) and are a consequence of IP3 receptor (IP3R) activation (1, 12, 13) because depolarization induces a transient increase on IP3 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-amino-ethoxydiphenyl borate (2-APB), an inhibitor of IP_3_-mediated Ca^{2+} release; xestospongin-C, an IP_3_R 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 Fluoro-3-AM were from Molecular Probes (Eugene, OR). 2-ABP was from Aldrich, xestospongin C was from Calbiochem (La Jolla, CA), 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. The suspension was 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 Fluor 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 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 SuperScrp 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'-AT-GAA-TTTCATCTCCGCAAGAGACTTTCCAGCCA-3' (sense), 5'-CTAGTTTGGCGGAGTAGCCATCACATGAGC-3' (antisense); mouse IL-6 primers, 5'-ATGAAAGTTTCTCTCCGCAAGAGCTTTCCAGCCA-3' (sense), 5'-CTAAGTTTGCAGATGACATCATCAGTGACC-3' (antisense); and GAPDH primers, 5'-CCCTGCCTCCTGCGGTAT-3' (sense), 5'-ACCGAGGGCGATGTCC-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, plcL6-Luc651, contained a 651-bp fragment of the human IL-6 gene promoter, located directly upstream of the transcriptional start site, subcloned into pG3 basic luciferase reporter gene vector. plcL6-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′-TGCAAGCAC-3′, and the NF-κB consensus sequence 5′-GGGATTTC-3′ (positions −72 to −63) to 5′-CTCAGTTTCC-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 (1:1) to 70% confluence. Cell transfection was carried out using 60-mm-diameter culture dishes and grown in serum-free DMEM-F12.

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

Ca2+ levels in rat myotubes after depolarization. Because we wanted to study the role of Ca2+ signals on the regulation of IL-6 gene transcription, it was important to establish that we were dealing with essentially a single set of Ca2+ transients that lasts <60 s and that Ca2+ 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 Ca2+ transient (Fig. 1) that lasts ~30 s. After this transient, which is composed of the slow component related to IP3 receptors and involved in the regulation of gene expression (1, 6, 13, 22, 23, 46), Ca2+ 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 C2C12 cells. Rat myotubes in primary cultures or C2C12 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 3 h after treatment (357 ± 19% of control, n = 3) (Fig. 2A). 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 C2C12 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. 2B) 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.

Ca2+ requirement for IL-6 induction: role of intracellular Ca2+. Ca2+ transients evoked by exposure of myotubes to high K+ concentration are normally independent of extracellular Ca2+ (23, 46). To rule out the participation of extracellular Ca2+ in our experimental conditions, rat myotubes were depolarized either in the presence of 3 mM Ca2+ or in a medium without Ca2+ concentration of 0.5 mM EGTA. Depolarization induced IL-6 mRNA levels were similar in both experimental conditions (Fig. 3A).

To demonstrate a more direct relationship between intracellular Ca2+ and IL-6 gene expression, we made use of the cell-permeable Ca2+ 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. 3B).

Depolarization-induced slow Ca2+ transients increase IL-6 mRNA levels in skeletal muscle cells. Ca2+ 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 IP3 increase. To define which one participates on IL-6 induction, we used specific inhibitors that selectively abolish one of them.

Slow component of Ca2+ increase evoked by depolarization is blocked by compounds that interfere with the IP3 system, such as 2-APB, U-73122, and xestospongin C (13, 46). We investigated the involvement of slow Ca2+ transients on IL-6

Fig. 1. Analysis of intracellular Ca2+ 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 Ca2+ was expressed as the percentage of fluorescence intensity relative to basal fluorescence (n = 5).
Ca2+ high concentrations of ryanodine eliminates the initial fast/hyperpolarized for 5 min with 84 mM K+ resulted in the complete inhibition of maximal induced IL-6 gene expression at 3 h (381 vs. 463 arbitrary units, **P < 0.01 vs. ANOVA, follow by Dunnett’s multiple-comparison test). Treatment of myotubes with these compounds prevented IL-6 mRNA induction observed in nontreated depolarized cultures (Fig. 4B). Expression treating rat myotubes in primary culture with 50 μM 2-APB or 10 μM U-73122. Inhibition of slow Ca2+ signals by two different mechanisms resulted in a significant decrease in depolarization mediated IL-6 promoter activity. Exposure of transfected myotubes to 25 μM ryanodine before and during K+ treatment does not prevent increased IL-6 transcriptional activity (Fig. 5B).

As a whole, these experiments suggest that IP3-mediated slow Ca2+ release, associated with the slow Ca2+ 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. 5A).

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 IP3-mediated slow Ca2+ 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 Ca2+ 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. 5B).

These results are in agreement with preceding described observations obtained from RT-PCR analysis and support the
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-Luc651 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 C2C12 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-
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. 2A), whereas depolarization of differentiated C\(_2\)C\(_12\) cells results in a clearly increased level 4 h after stimulation (Fig. 2B). 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 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. 3A), meaning that extracellular Ca\(^{2+}\) is not involved in IL-6 gene activation.

Experiments carried out in BAPTA-AM preloaded myotubes cells, resulted in reduced induction of IL-6 mRNA levels (Fig. 3B) 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\(_3\)-R, 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. 4C). 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. 5A) 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. 5B).

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 muscles.
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-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 C2C12 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 thombin (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 Ca²⁺ concentration and both AP-1 and NF-κB sequences have been described as Ca²⁺ responsive regulatory elements in other genes (16, 50, 51, 57), we suggested that Ca²⁺ 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 IP₃ signaling cascade, and the lack of effect of ryanodine, strongly supports slow Ca²⁺/IP₃-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 Ca²⁺ through opening of l-voltage-sensitive Ca²⁺ channels at the plasma membrane and indirect opening of IP₃Rs associated with the intracellular Ca²⁺ stores is responsible for NF-κB activity in these cells (31). In addition, intracellular Ca²⁺ 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 Ca²⁺, in the presence of high concentrations of ryanodine, but is significantly reduced by slow IP₃-mediated Ca²⁺ transient blockage (6). Thus induction of c-fos and c-jun expression, observed in depolarized myotubes, could be related to slow Ca²⁺ signaling pathways leading to IL-6 transcription.

In addition, transcription factor AP-1 could interact synergistically with Ca²⁺-activated nuclear factor of activated T cells, as has been described for Ca²⁺-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 Ca²⁺ increase to early gene expression in skeletal muscle cells (1). This model includes receptors and pathways known to be involved

![Diagram](http://ajpcell.physiology.org/)

Fig. 7. Proposed pathways that link intracellular Ca²⁺ increase to IL-6 gene regulation in skeletal muscle cells. Membrane depolarization, through activation of dihydropyridine receptor (DHPR), results in IP₃-induced Ca²⁺ release. IP₃-mediated Ca²⁺ 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 IP₃-mediated Ca²⁺-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). IP₃R, IP₃ receptor; PIP₂, phosphatidylinositol 4,5-bisphosphate; DAG, diacylglycerol.
in IP3-generated Ca²⁺ signals. The signaling pathway begins at the dihydroypridine receptor, which activates phospholipase C by an unknown mechanism to produce IP3 (1). IP3 diffuses into the cytosol and reach IP3 receptors located both at the sarcoplasmic reticulum membrane and at the nuclear envelope, inducing Ca²⁺ release. Several Ca²⁺-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-kB 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 Ca²⁺ 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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REFERENCES

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