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IGF-1 Induces IP_3-Dependent Calcium Signal Involved in the Regulation of Myostatin Gene Expression Mediated by NFAT During Myoblast Differentiation

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Skeletal muscle differentiation is a complex and highly regulated process characterized by cell cycle arrest, which is associated with morphological changes including myoblast alignment, elongation, and fusion into multinucleated myotubes. This is a balanced process dynamically coordinated by positive and negative signals such as the insulin-like growth factor I (IGF-1) and myostatin (MSTN), respectively. In this study, we report that the stimulation of skeletal myoblasts during differentiation with IGF-1 induces a rapid and transient calcium increase from intracellular stores, which are principally mediated through the phospholipase C gamma (PLC γ)/inositol 1,4,5-triphosphate (IP_3)-dependent signaling pathways. This response was completely blocked when myoblasts were incubated with LY294002 or transfected with the dominant-negative p110 gamma, suggesting a fundamental role of phosphatidylinositol 3-kinase (PI3K) in PLCγ activation. Additionally, we show that calcium released via IP_3 and induced by IGF-1 stimulates NFAT-dependent gene transcription and nuclear translocation of the GFP-labeled NFATc3 isoform. This activation was independent of extracellular calcium influx and calcium release mediated by ryanodine receptor (RyR). Finally, we examined *mstn* mRNA levels and *mstn* promoter activity in myoblasts stimulated with IGF-1. We found a significant increase in mRNA contents and in reporter activity, which was inhibited by cyclosporin A, 11R-VIVIT, and by inhibitors of the PI3Kγ, PLCγ, and IP_3 receptor. Our results strongly suggest that IGF-1 regulates myostatin transcription through the activation of the NFAT transcription factor in an IP_3/calcium-dependent manner. This is the first study to demonstrate a role of calcium-dependent signaling pathways in the mRNA expression of myostatin.

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Skeletal muscle differentiation is a complex and highly regulated process, characterized by cell cycle arrest, which is associated with morphological changes that include myoblast alignment, elongation, and fusion into multinucleated myotubes (Andrés and Walsh, 1996). This process largely depends on the actions of the myoblast determination factor (MyoD) family, collectively referred to as myogenic regulatory factors (MRFs), which act in coordination with transcription factors, such as myocyte-specific enhancer (MEF) and nuclear factor of activated T cells (NFAT), to activate the expression of genes required for muscle differentiation (Molkentin and Olson, 1996; Armand et al., 2008).

Several growth factors regulate myoblast differentiation in an autocrine/paracrine fashion; however, two of the most important coordinators of this process are the positive regulator, insulin-like growth factor I (IGF-1), and the negative modulator, myostatin (MSTN). IGF-1 is a member of insulin-related peptides which play a fundamental role during myogenesis, stimulating both proliferation and differentiation of muscle cells (Coleman et al., 1995). On the other hand, myostatin, also known as growth differentiation factor 8 (GDF-8), is a member of the TGF-β superfamily of growth factors and is a negative regulator of skeletal muscle proliferation and differentiation (Ríos et al., 2002).

Mature IGF-1 binds to the membrane type I IGF-1 receptor (IGF-1R), inducing its autophosphorylation of the tyrosine residues serving as a docking sites for adaptor molecules, such as insulin receptor substrate proteins (IRS; Glass, 2005; Philippou et al., 2007). Through these effectors, IGF-1 activates two main signaling pathways, the Ras-Raf-mitogen activated protein kinase (MEK)-extracellular signal-regulated kinase (ERK) pathway and the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (PKB/Akt) pathway (Glass, 2005; Noguchi, 2005). However, several recent studies in rat myotubes and cardiomyocytes have demonstrated that IGF-1 binding to its receptor also activates phospholipase C gamma (PLCγ) mainly through the PI3K pathway, which enhances IP_3 synthesis and promotes calcium release from intracellular stores (Ibarra et al., 2004; Espinosa et al., 2004). It has been proposed that calcium release induced by IP_3 is responsible for the regulation of

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calcium-dependent transcription factors involved in the modification of gene expression (Ibarra et al., 2004; Espinosa et al., 2004; Juretić et al., 2006; Valdés et al., 2007; Jorquera et al., 2009; Casas et al., 2010).

The nuclear factor of activated T cells (NFAT) is a transcription factor regulated by calcium/calcineurin-dependent signaling (Hogan et al., 2003; Valdés et al., 2008). After the rise of intracellular calcium, the activated, calcium-dependent phosphatase calcineurin directly dephosphorylates NFAT, inducing its nuclear accumulation and transcriptional activity by binding to their target promoter elements (Hogan et al., 2003). The inactivation and exporting back to the cytoplasm of NFAT depends on re-phosphorylation by cellular kinases. Primary myoblast cells express three isoforms of NFAT- NFATc1, NFATc2, and NFATc3, whose expression is present throughout all stages of myogenesis (Abbott et al., 1998). However, each isoform undergoes calcium-induced nuclear translocation at specific stages of muscle differentiation; NFATc3 is active during myoblast differentiation; NFATc2 is active during myotube formation, and NFATc1 is active in mature myotubes (Abbott et al., 1998; Delling et al., 2000).

IGF-1 induces the transcription of several muscle-specific genes involved in the positive regulation of myoblast differentiation (Palmer et al., 1997; Kuninger et al., 2004; Bhasker and Friedmann, 2008). However, recent studies report that IGF-1 also stimulates the expression of myostatin in a negative myogenic auto-regulatory loop (Shyu et al., 2005; Yang et al., 2007; Kurokawa et al., 2009). Also, in C2C12 myoblasts, myostatin is up-regulated following IGF-1 treatment (Yang et al., 2007). However, it is unknown which signaling pathways mediate MSTN expression induced by IGF-1, but in silico analyses have shown that the murine MSTN gene promoter contains several NFAT consensus binding sites, suggesting that calcium-dependent signaling pathways may regulate myostatin gene expression (Valdes and Molina, 2008). Moreover, it has been recently reported that myostatin reduces IGF-1-induced Akt/TORC1/p70S6K signaling, inhibiting myoblast differentiation and myotube size, thus suggesting that both signaling pathways are interacting in order to regulate muscle differentiation (Trendelenburg et al., 2009).

We therefore hypothesized that calcium release induced by IGF-1 during myoblast differentiation might stimulate *mstn* transcription through a calcineurin/NFAT-dependent signaling pathway. It was found that stimulation of skeletal myoblasts with IGF-1 induced a fast and transient calcium increase from intracellular stores, mainly mediated through a PI3Kγ/PLCγ/IP_3-dependent signaling pathway. Furthermore we found that IGF-1 regulates *mstn* transcription through the activation of the NFAT transcription factor in an IP_3/calcium-dependent manner.

Materials and Methods

Reagents

Recombinant rat IGF-1 was obtained from R&D System (Minneaolis, MN). Cyclosporin A, U73122, LY294002 were obtained from Biomol Research Laboratories (Plymouth Meeting, PA). Genistein, picropodophyllin (PPP), xestospongin C, and 11R-VIVIT were obtained from Calbiochem (La Jolla, CA). Antibodies against pPLC gamma 1, PLC gamma 1, phosphotyrosine, IP_3 receptor 1, pIGF-1Rβ, IGF-1Rβ, β actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against Akt and phospho-Akt were purchased from Cell Signalling (Beverly, MA). IP_3R1 siRNA (sc-42476) was obtained from Santa Cruz Biotechnology. Ryanodine and nifedipine were obtained from Sigma (St. Louis, MO). Secondary HRP-conjugated anti-rabbit antibody was obtained from Pierce (Rockford, IL). Fluo-3 AM and Hoechst were obtained from Molecular Probes (Eugene, OR). NFAT-pGL4 and pGL4. 10 reporter plasmids were obtained from Promega (Madison, WI).

Cell cultures

Primary cultures of rat skeletal myoblasts were prepared from Sprague–Dawley neonatal hindlimbs. The muscle tissue was dissected, minced, and treated with collagenase for 15 min at 37°C. Growth medium was composed of DMEM-F-12 (1:1 mixture), 10% bovine serum, 2.5% FBS, 100 U/ml penicillin, and 10 mg/ml streptomycin. To arrest fibroblast growth, we added cytosine arabinoside (5 mM) 2 days after plating for 24 h. At Day 3, cultures were differentiated in serum-free medium. The experiments were performed at Day 4.

Phospholipase Cγ activity assay

Myoblast in 100 mm dishes were washed twice with PBS and then incubated with RIPA lysis buffer (50 mM HEPES, pH 7.4, 1% Triton X-100, 0,1% SDS, 150 mM NaCl, 1 mM EDTA and Calbiochem protease and phosphatase inhibitors cocktail) for 5 min. Cells were scraped and incubated at 4°C on a rotator for 1 h. Lysates were centrifuged at 5,000*g*, and the supernatants were collected and incubated with 2 μg polyclonal PLCγ1 antibodies at 4°C overnight and then 20 μl of Ultralink immobilised protein G (Pierce Chemical) at 4°C for 1 h. After centrifugation (1,000*g*, 5 min), the pellet was resuspended in 500 μl of reaction buffer (25 mM HEPES, 80 mM KCl, 3 mM EGTA, 0,5 mM DTT, pH 7,0). 50 μl of suspension was incubated with 10 μM [^{3}H]PIP2 (6.5 Ci/mmol; PerkinElmer, Boston, MA), phosphatidylcholine and phosphatidylserine in a molar ratio of 1:3:3 in the reaction buffer. The reaction was ended by adding 200 μl of 10% TCA at 5 min and then 200 μl of 10% BSA. The radioactivity was determined by scintillation counter.

Calcium detection

Myoblasts were preloaded with 5.4 μM fluo-3 AM for 30 min at 37°C in a resting solution (in mM: 145 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 Na-HEPES, and 5.6 glucose, pH 7.4); cleavage by intracellular esterases leaves the active form fluo-3 trapped inside the cell. Myoblasts were transferred to the recording chamber and mounted on an inverted fluorescence microscope. Fluorescence was detected using excitation at 488 nm and emission at 510–540 nm: Images were collected every 2 sec and regions of interest were analyzed with the Image J program (NIH). The calcium signals are presented as a percentage of fluorescence intensity relative to basal fluorescence.

Myostatin reporter vector construction

Myostatin reporter transcriptional activity was mainly monitored with a luciferase reporter gene (pGL4) construct containing nucleotides from −1,880 to +124 of murine myostatin promoter. The myostatin promoter segment was isolated by PCR using genomic rat DNA as the template and primers based on sequence information from the National Center for Biotechnology Information databank. The PCR product, an 2,004-bp fragment containing five NFAT-binding sites, was cloned in pGEMT-easy vector and subcloned into a pGL4 luciferase reporter vector (*Xho*I site; Promega). The orientation of the insert was verified by PCR amplification of the isolated DNA of the different clones and sequencing.

Cell transfection and luciferase reporter assay

Primary cells in culture were transiently transfected with Lipofectamine 2000. Briefly, 3-day-old myoblasts were transfected with 2 μl Lipofectamine 2000 in 1 ml of DMEM-F12 containing 0.9 μg of the reporter vector DNA and 0.1 μg of the Renilla phRL-TK vector (Promega). The mixture was maintained for 4 h, media were replaced by serum-media, and cells were maintained until 4th day. Type 1 IP_3R mRNA was knocked down using 100 nM of siRNA (sc-42476; Santa Cruz Biotechnology) with 2 μl Lipofectamine

2000 in 1 ml of DMEM-F12. In luciferase reporter assay, cells were harvested and lysed 12 h after the experiments. Each experimental condition was performed in duplicates. Luciferase activity was determined using a dual-luciferase reporter assay system (Promega), and luminescence was measured with a Berthold F12 luminometer. Results were normalized for transfection efficiency and expressed as the ratio of firefly to Renilla luciferase. Transfection with the empty pGL4 luciferase plasmid as a control resulted in no increase in luciferase activity after cells had been stimulated.

Cell solubilization and immunoblot analysis

Cells were solubilized in 0.1 ml of lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40) and a protease and phosphatase inhibitors cocktails (Calbiochem), and then centrifuged at 17,000*g* for 15 min at 4°C to remove debris. In determining protein concentration of the supernatants, BSA was used as standard. Proteins extracts were resolved by 10% SDS–PAGE, transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA), and blocked for 1 h at room temperature in Tris-buffered saline (TBS), 0.1% Tween 20, and 5% milk. Incubations with primary antibodies (1:1,000) were performed at 4°C overnight. After incubation for 1.5 h with HRP-conjugated secondary antibodies, membranes were developed by enhanced chemiluminescence (Amersham Biosciences, Amersham, UK). The films were scanned, and the ImageJ program was employed for densitometric analysis of the bands. To correct for loading, membranes were stripped and blotted against total phospholipase C gamma.

Immunoprecipitation

Myoblast in 100 mm dishes were washed twice with PBS and then incubated with RIPA lysis buffer (50 mM HEPES, pH 7.4, 1% Triton X-100, 0,1% SDS, 150 mM NaCl, 1 mM EDTA and a Calbiochem protease and phosphatase inhibitors cocktails) for 5 min. Cells were scraped and incubated at 4°C on a rotator by 1 h. Lysates were centrugated at 5,000*g*, and the supernatants were collected and incubated with 2 μg polyclonal PLCγ1 antibodies at 4°C overnight and then 20 μl of Ultralink immovilized protein G (Pierce Chemical) at 4°C for 1 h. After centrifugation (1,000*g*, 5 min), the pellet was resuspended in 20 μl of lysis buffer and then analyzed by immunoblot analysis as described above.

Semiquantitative RT-PCR

Total RNA was extracted from primary skeletal myoblast with TRIzol Reagent (Invitrogen, Grand Island, NY), and cDNA was synthesized using SuperScript II reverse transcriptase (Invitrogen) and oligo dT primers. cDNA was amplified using MSTN primers, and the DNA concentration was normalized against β-actin. PCR amplification was stopped in the exponential phase for both products. The MSTN primers used were 5′-GAAGAGAAACTGTGCATT-3′ (sense) and 5′ GGCAATGGTCCACTATCA-3′ (antisense). PCR conditions were as follows: initial denaturation at 94°C for 5 min, followed by 28 cycles at 94°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec, and final extension at 72°C for 10 min for both amplification of myostatin and β-actin cDNA. PCR products were resolved by electrophoresis on a 1.8% agarose gel and stained with ethidium bromide. Bands were quantified by densitometric analysis with the ImageJ program.

NFATc3-eGFP localization and quantification in skeletal myoblast

Primary skeletal myoblasts were grown on coverslips and transiently transfected with Lipofectamine 2000 (InVitrogen) according to the manufacturer's specifications. Briefly, 3-day-old myoblasts plated in 60-mm culture dishes were transfected with 1 μg NFATc3-eGFP vector plus 2 μl of Lipofectamine 2000 in 1 ml of DMEM-F12. After 6 h, the medium–DNA complexes were replaced by serum-free medium. Four-day-old myoblasts transfected with the NFATc3-eGFP plasmid (Aramburu et al., 1999) were stimulated with IGF-1 (10 nM) in the presence or absence of cyclosporin A (50 μM), U73122 (50 μM), xestospongin C (50 μM), and LY294002 (50 μM). After 120 min treatment, myoblasts were stained with 5 μg/ml Hoechst 33342 (InVitrogen) and fixed in ice-cold methanol. Coverslips were mounted and recorded by fluorescence microscopy (Olympus IX-81, Center Valley, PA) and documented through computerized images. Subcellular distribution of NFATc3-GFP was quantified using the program Image J (NIH, Bethesda, MD) taking a region of interest (ROI) that covered the same numbers of pixels in nucleous and cytoplasm, and expressed as the ratio between NFATc3Nuc/NFATc3 Cyt.

Statistical analysis

Data are expressed as mean ± SE. Differences in means between groups were determined using one-way ANOVA followed by Bonferroni's post-test. Data accepted as significant if a value of $P < 0.05$.

Results

IGF-1 activates PLCγ and induces calcium release from intracellular stores in skeletal myoblasts

In order to determine whether IGF-1 activates PLCγ in skeletal myoblasts, we monitored the activation of the enzyme by measuring IP_3 generated after 10 nM IGF-1 adding. The PLCγ activity began to increase 15 sec after IGF-1 treatment, was maximal after 30 sec and returned to basal level 60 sec after stimulation (Fig. 1A). We found that pre-treatment of myoblasts with the PI3K inhibitor LY294002 (50 μM) significantly inhibited the IGF-1 induced IP_3 production (Fig. 1A). To corroborate the participation of the PI3K in PLCγ activation, myoblasts were transfected with a kinase inactive version of the PI3K gamma (PI3γ DN) and stimulated with IGF-1 reducing the maximal activity of PLCγ in a 40% (Fig. 1B). In addition, we tested PLCγ tyrosine phosphorylation induced by IGF-1, by PLCγ immunoprecipitation and subsequent immunoblotting, revealing that PLCγ showed a similar phosphorylation kinetics pattern to IP_3 production (Fig. 1C,D). Interestingly, we found that LY294002 did not significantly inhibit the phosphorylation of PLCγ, suggesting that PI3K may regulate PLCγ activity by an independent tyrosine phosphorylation mechanism.

PLCγ hydrolyzes phosphatidylinositol-4,5-biphosphate to generate inositol-1,4,5-triphosphate which induces calcium release from intracellular stores. We visualized intracellular calcium rise induced by IGF-1 in myoblasts loaded with Fluo3-AM. The addition of 10 nM IGF-1 to rat skeletal myoblasts induced a fast and transient increase in intracellular calcium concentrations. The calcium signals reached the peak about 20 sec after IGF-1 treatment and returned near basal values after 60 sec (Fig. 2A). Region of interest (ROI) analyses showed that the calcium increase took place in nuclei and cytoplasm with the same magnitude and kinetics (data not shown). In order to evaluate whether or not the IGF-1 induced calcium increase was due to calcium influx, we examined the effect of nifedipine (50 μM) and extracellular calcium absence (EGTA 0.5 mM; Figs. 2B and 2C, respectively). In the presence of both conditions, the IGF-1-induced calcium response was similar to control suggesting that extracellular calcium does not have a role in the IGF-1 induced calcium increase in skeletal myoblast. To determine whether IGF-1 receptor activation is involved in the calcium signals induced by IGF-1 we used genistein (100 μM) and cyclolignan picropodophyllin (PPP, 5 μM), a general tyrosine kinase inhibitor and IGF-1 receptor tyrosine kinase inhibitor, respectively. The Addition of genistein and PPP

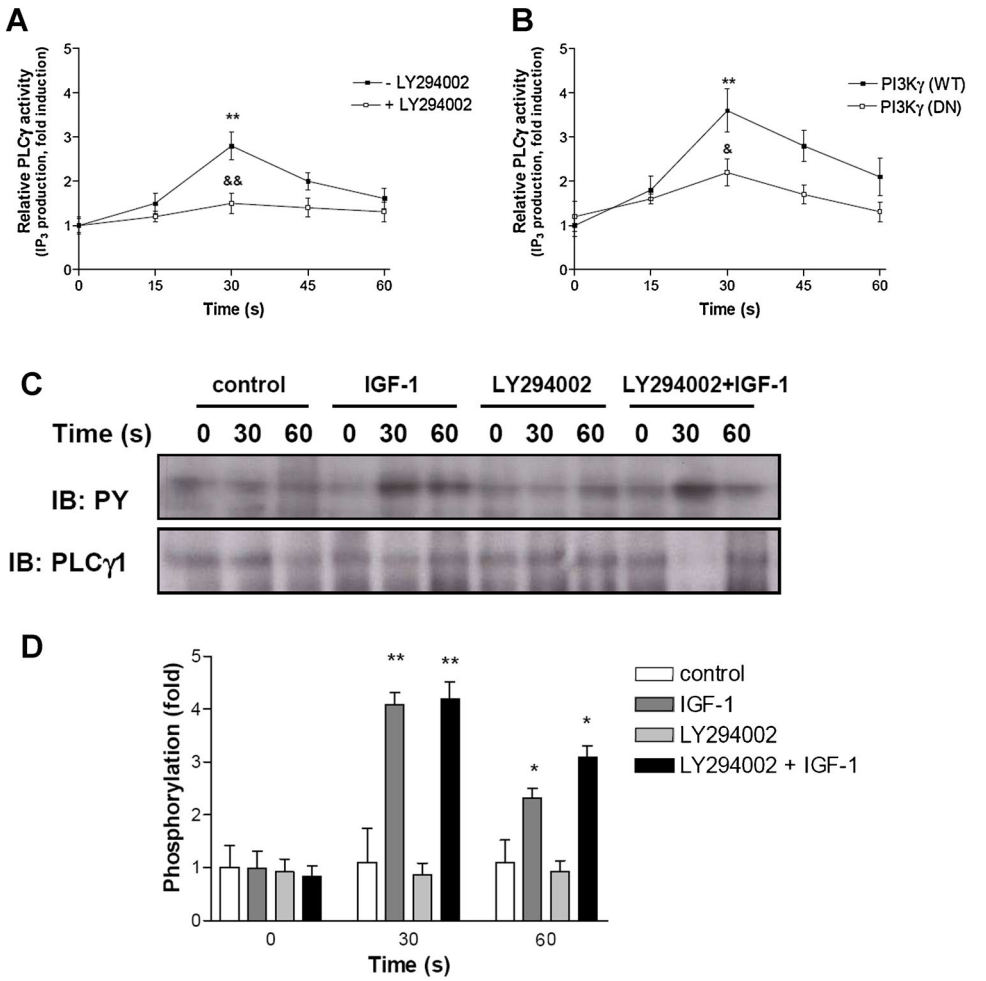

Fig. 1. IGF-1 activates PLC gamma in skeletal myoblasts: (A) Time course of PLCγ activity: myoblasts incubated in the absence or presence of 50 μM LY294002 for 30 min, and then stimulated with IGF-1 (10 nM) for the indicated times. PLCγ activity was assayed as described in Materials and Methods Section. Results are expressed as percentage of the basal IP3 as mean ± SEM of values obtained of duplicates of three independent experiments. B: Time course of PLCγ activity: myoblasts transfected with WT PI3Kγ or DN PI3Kγ were stimulated with IGF-1 (10 nM) for the indicated times. PLCγ activity was assayed as described in Materials and Methods Section. Results are expressed as percentage of the basal IP3 as mean ± SEM of values obtained of duplicates of three independent experiments. C: Representative immunoblots of PLCγ phosphorylation: PLCγ was immunoprecipitated from myoblast treated with IGF-1 (10 nM) in the absence or presence of LY294002 (50 μM), and then immunoblotted with anti-phosphotyrosine antibody. D: Densitometric analysis of immunoblots from (C). Date obtained at 0, 30, and 60 sec as mean ± SEM of duplicates of three independent experiments. Statistical analysis was performed by one-way ANOVA followed by Bonferroni's post test. $^{*}P < 0.05$ and $^{**}P < 0.01$ with respect to control group (0 sec); &$P < 0.05$ and &&$P < 0.01$ versus maximal PLCγ activity (30 sec).

completely blocked the IGF-1 induced calcium increase (Figs. 2D and 2E, respectively). Immunoblot analysis revealed that the preincubation of myoblasts with genistein or PPP inhibited the phosphorylation of the IGF-1Rβ induced by IGF-1 (Supplementary On line Material, Fig. 8). These data suggest that the increase in intracellular calcium induced by IGF-1 depends on the activation of the IGF-1 receptor.

The main mechanisms of calcium release from sarco/endoplasmic reticulum are mediated by ryanodine receptor (RyR) and IP_3 receptor (IP_3R; Stiber et al., 2005). To investigate the participation of RyR in IGF-1 induced calcium transient, skeletal myoblasts were preincubated with 20 μM ryanodine and stimulated with IGF-1 (Fig. 2F). In presence of ryanodine, the IGF-1 induced calcium response was similar to control condition. In contrast the preincubation of myoblast with PLC inhibitor (U73122, 50 μM) or the transfection with a dominant-negative form of PLCγ1 (PLCγ1 DN) completely blocked the calcium increases induced by IGF-1 (Figs. 2G and 2H, respectively). Complementary the preincubation of myoblasts with IP_3 receptor inhibitor xestospongin C (Xpg C, 50 μM) or the transfection with siRNA IP_3R1 ($siIP_3R1$) diminished the calcium increases induced by IGF-1 (Figs. 2I and 2J, respectively). Immunoblot analysis revealed that the transfection of myoblast with siIP3R1 diminished the expression of IP3R1 (Supplementary On line Material, Fig. 9). These results suggest that IGF-1 induced calcium release from internal stores is a PLCγ1-mediated/IP_3-dependent process.

A known upstream activator of PLCγ is PI3K (Maffucci and Falasca, 2007), we then tested the effect of LY294002 (50 μM). Treatment with LY294002 strongly blocked the calcium signals induced by IGF-1 (Fig. 2K), a similar results were obtained when myoblasts were transfected with PI3Kγ DN (Fig. 2L). Immunoblots of pAkt/Akt, a downstream target of PI3Kγ, revealed an important inhibition of the phosphorylation of Akt

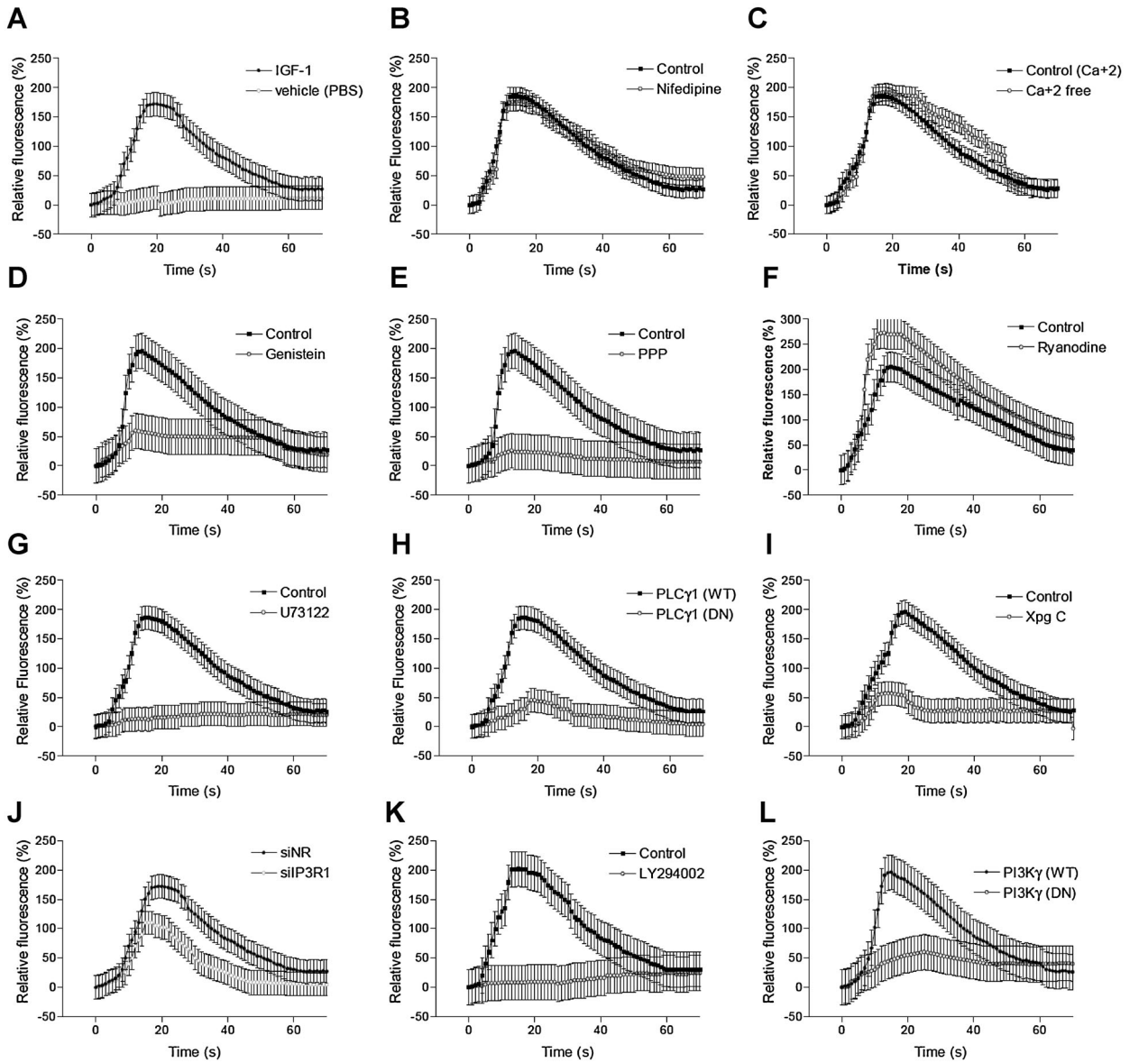

Fig. 2. IGF-1 induces intracellular calcium increase in myoblasts mediated through a PLCγ/IP3-dependent signaling pathway: Myoblasts loaded with fluo3-AM were preincubated for 30 min with pharmacological inhibitors and then stimulated with 10 nM IGF-1. Representative traces are shown for the time course of calcium signals detected from selected regions of interest from confocal images. The signal was normalized to the basal fluorescence. Fluorescence time course of myoblasts stimulated with (A) IGF-1 (10 nM)or vehicle (PBS). B: IGF-1 in the presence or absence of 50 μM nifedipine. C: IGF-1 in the presence or absence of extracellular calcium (EGTA 0.5 mM). D: IGF-1 in the presence or absence of 100 μM genistein. E: IGF-1 in the presence or absence of 5 μM PPP. F: IGF-1 in the presence or absence of 20 μM ryanodine. G: IGF-1 in the presence or absence of 50 μM U73122. H: Myoblast transfected with an inactive version of the PLC gamma (PLCγ1 DN) or wild type PLCγ1 and stimulated with IGF-1. I: IGF-1 in the presence or absence of 50 μM xestospongin C. J: Myoblast transfected with siRNA IP3R1 (siIP3R1) or non related siRNA (siNR) and stimulated with IGF-1. K: IGF-1 in the presence or absence of 50 μM LY294002. L: Myoblast transfected with a kinase inactive version of the PI3K gamma (PI3Kγ DN) or wild-type PI3K gamma and stimulated with IGF-1.

in the presence of LY294002 or in myoblasts transfected with PI3Kγ DN (Supplementary On line Material, Fig. 10). These results suggest that the signaling cascade induced via the IGF-1 receptor requires the combined participation of PI3Kγ and PLCγ to induce a calcium transient.

NFAT activation depends on IP_3-mediated calcium release induced by IGF-1

In the literature, increased NFAT-dependent transcription induced by IGF-1 has been reported in adult skeletal muscle and myotubes (Musarò et al., 1999). NFAT transcriptional activity was measured in skeletal myoblasts transiently transfected with NFAT-pGL4 reporter vector. The NFAT-mediated transcription showed 3.5-fold activation after stimulation with 10 nM IGF-1 (Fig. 3A). The classic description for NFAT activation shows that the phosphatase calcineurin dephosphorylates NFAT exposing nuclear localization sequences, inducing NFAT translocation. The preincubation of myoblasts with cyclosporin A (CsA, 50 μM), a calcineurin inhibitor, and 11R-VIVIT (2 μM), a NFAT inhibitor, completely blocked NFAT transcriptional activity (Figs. 3A and 3E,

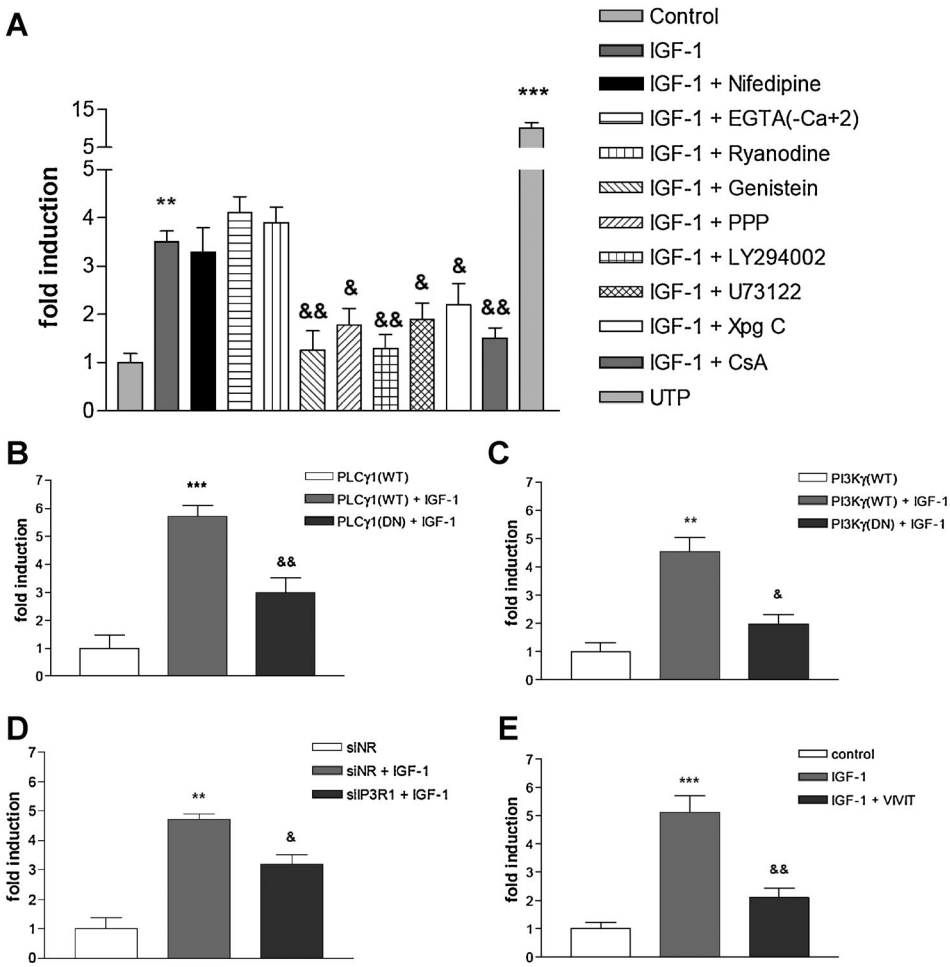

Fig. 3. IGF-1 induces NFAT-dependent transcription, influenced by intracellular calcium released through IP3 receptor calcium channels and mediated by PLCγ and PI3Kγ. A: Co-transfected myoblasts with NFATpGL4 reporter vector and phRL-TK normalizer vector were pre-incubated for 30 min with vehicle, 50 μM nifedipine, 0.5 mM EGTA, 20 μM ryanodine, 100 μM genistein, 5 μM PPP, 50 μM LY294002, 50 μM U73122, 50 μM Xestospongin C, 50 μM cyclosporine A and then stimulated with 10 nM IGF-1. Additionally myoblasts were treated with 10 nM UTP. B: Co-transfected myoblasts with NFAT-pGL4 reporter vector, phRL-TK normalizer vector and PLCγ1 (WT or DN) were stimulated with 10 nM IGF-1. C: Co-transfected myoblasts with NFAT-pGL4 reporter vector, phRL-TK normalizer vector and PI3Kγ (WT or DN) were stimulated with 10 nM IGF-1. D: Co-transfected myoblasts with NFAT-pGL4 reporter vector, phRL-TK normalizer vector and siRNA (NR or IP3R1) were stimulated with 10 nM IGF-1. E: Co-transfected myoblasts with NFAT-pGL4 reporter vector and phRL-TK normalizer vector were pre-incubated for 60 min with vehicle or 2 μM 11RVIVIT and then stimulated with 10 nM IGF-1. Results were normalized for transfection efficiency and expressed as the ratio of firefly to Renilla luciferase. Data are means ± SEM of duplicates (three independent experiments) and are expressed relative to values in control cells. Statistical analysis was performed by oneway ANOVA followed by Bonferroni's post-test. $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to the control group. &$P < 0.05$ and &&$P < 0.01$ compared to IGF-1 treated group.

respectively). It has been reported in skeletal myotubes that NFAT activation induced by electrical stimulation is through ryanodine receptors (RyR) calcium release and extracellular calcium entry (Valdés et al., 2008). We studied this aspect in transfected myoblasts with NFAT reporter vector preincubated in extracellular calcium absence (EGTA) or in the presence of nifedipine or ryanodine, and then stimulated with IGF-1 (10 nM). In these conditions the IGF-1 induced NFAT transcriptional activity was similar to myoblasts stimulated with IGF-1 alone (Fig. 3A), indicating that extracellular calcium influx and RyR calcium do not have a significant participation in the IGF-1-induced NFAT activation in myoblasts.

In adult cardiac myocytes, neonatal rat ventricular myocytes and C2C12 myoblasts, it has been reported that NFAT activity is dependent upon IP_3 receptor activation (Stiber et al., 2005; Higazi et al., 2009; Rinne and Blatter, 2010). The role of calcium release from intracellular stores in NFAT activation mediated by the axis IGF1R-PI3Kγ-PLCγ-IP_3R was also evaluated by NFAT reporter assays. The presence of the inhibitors genistein, PPP, LY294002, U73122, or Xpg C significantly blocked the NFAT-dependent transcriptional activity (Fig. 3A). These results were corroborated in myoblasts transfected with dominant negative forms of PLCγ, PI3Kγ, or siIP_3R1 and stimulated with IGF-1, showing a significant inhibition of NFAT activity (Figs. 3B, 3C, and 3D respectively). The treatment of myoblasts with UTP (10 nM), an IP_3 generating agonist, stimulated 10.3-fold NFAT transcriptional activity (Fig. 3A).

Considering that the isoform NFATc3 is involved in myoblast differentiation, we have studied GFP-labeled NFATc3 translocation to the nucleus in myoblasts counterstained with Hoechst. Under basal conditions, NFATc3 was detected in both cytosol and nucleus (Fig. 4A,B). Translocation to the nucleus was evident 120 min after IGF-1 stimulation (Fig. 4C,D). The addition of CsA, LY294002, U73122, and Xpg C completely

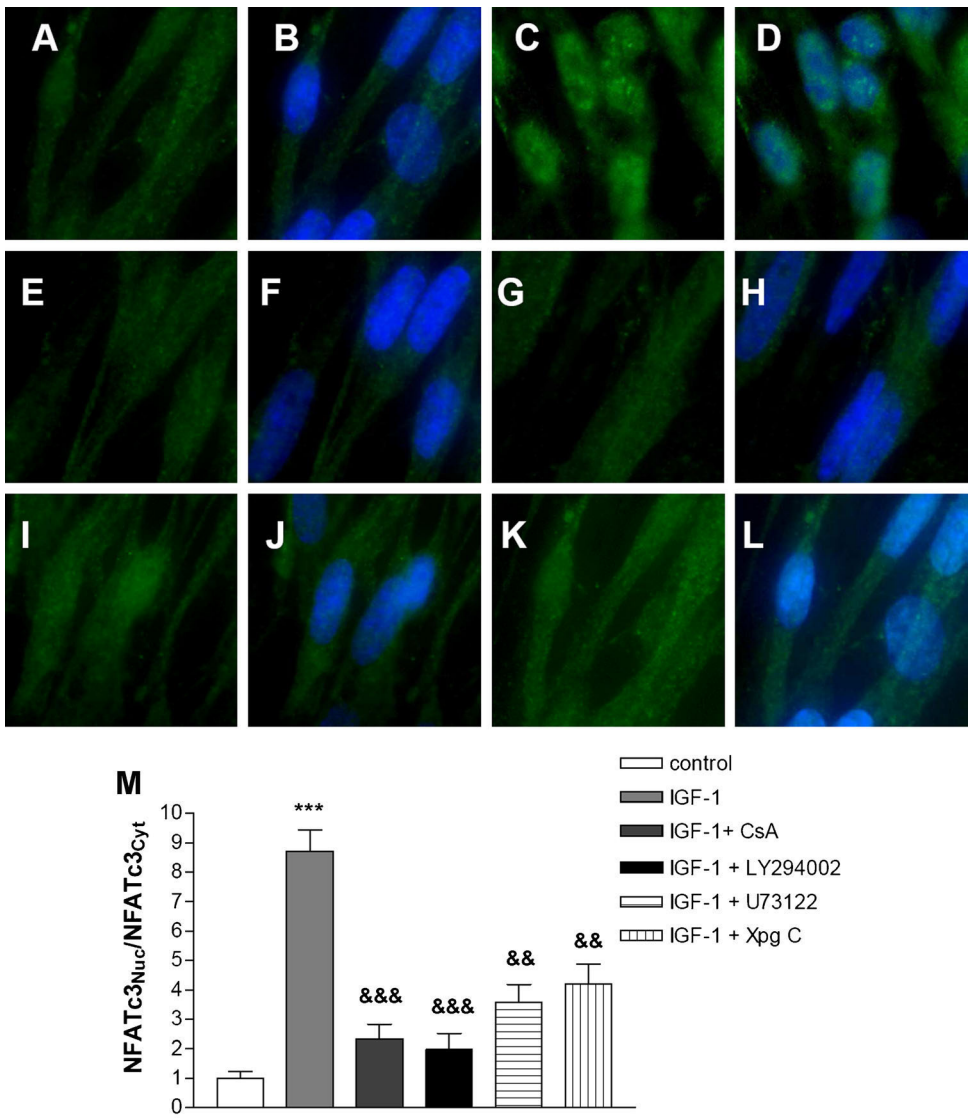

Fig. 4. Calcium release induced by IP3 and mediated by PLCγ and PI3Kγ participates in NFATc3 translocation in myoblasts stimulated with IGF-1. Four-day myoblasts transfected with the NFATc3-enhanced green fluorescent protein (eGFP) plasmid were incubated under resting conditions before stimulation with IGF-1. Nuclei were stained with Hoechst and myoblasts were fixed 120 min after stimulation and visualized by fluorescence microscopy. For each condition, images of NFATc3-eGFP and merge of NFATc3-eGFP with Hoechst are presented. Six independent experiments were performed for each condition, detailed as follows: (A,B) control condition. C,D: 10 nM IGF-1. E,F: Preincubation with Ciclospirine A (50 μM) and stimulated with IGF-1. G,H: Pre-incubated with LY294002 (50 μM) and stimulated with IGF-1. I,J: Preincubation with U73122 (50 μM) and stimulated with IGF-1. K,L: Preincubation with Xestospongine C (50 = M) and stimulated with IGF-1. M: Quantitative data of NFATc3-GFP translocation as described in Materials and Methods Section. Statistical analysis was performed by one-way ANOVA followed by Bonferroni's post-test. $^{***}P < 0.001$ compared to the control group. &&$P < 0.01$ and &&&$P < 0.001$ compared to IGF-1 treated group.

inhibited the NFAT translocation (Figs. 4E,F, 4G,H, 4I,J, and 4K-L respectively). Quantitative data of NFATc3-GFP translocation are shown in Figure 4M.

These combined results show that NFAT activation as measured by reporter gene assays and NFATc3-GFP translocation induced by IGF-1 requires calcium release through IP_3 receptor mediated by the PI3Kγ and PLCγ.

IGF-1 stimulation induces a NFAT-dependent increase in myostatin mRNA and myostatin promoter transcriptional activity

It has been previously reported that myostatin mRNA and proteins levels are up-regulated following IGF-1 treatment in C2C12 myoblasts and cardiomyocytes (Shyu et al., 2005; Yang et al., 2007). We confirmed this finding in skeletal myoblasts incubated with IGF-1 and maintained in resting conditions for different times before RT-PCR analysis. We observed a significant increase in myostatin mRNA levels, observing 4.5-fold higher expressions after 4 h of stimulation (Fig. 5A,B). Considering that the promoter region of the murine myostatin gene contains several candidates of NFAT-binding sites, we analyzed myostatin mRNA expression in the presence of calcineurin-NFAT inhibitors and IP_3 axis inhibitors. IGF-1 stimulation performed in the presence of CsA (Fig. 5C,D) or 11R-VIVIT (Fig. 5K,L) partially blocked myostatin expression, indicating that calcineurin-NFAT and other calcineurin independent signaling pathways may regulate myostatin

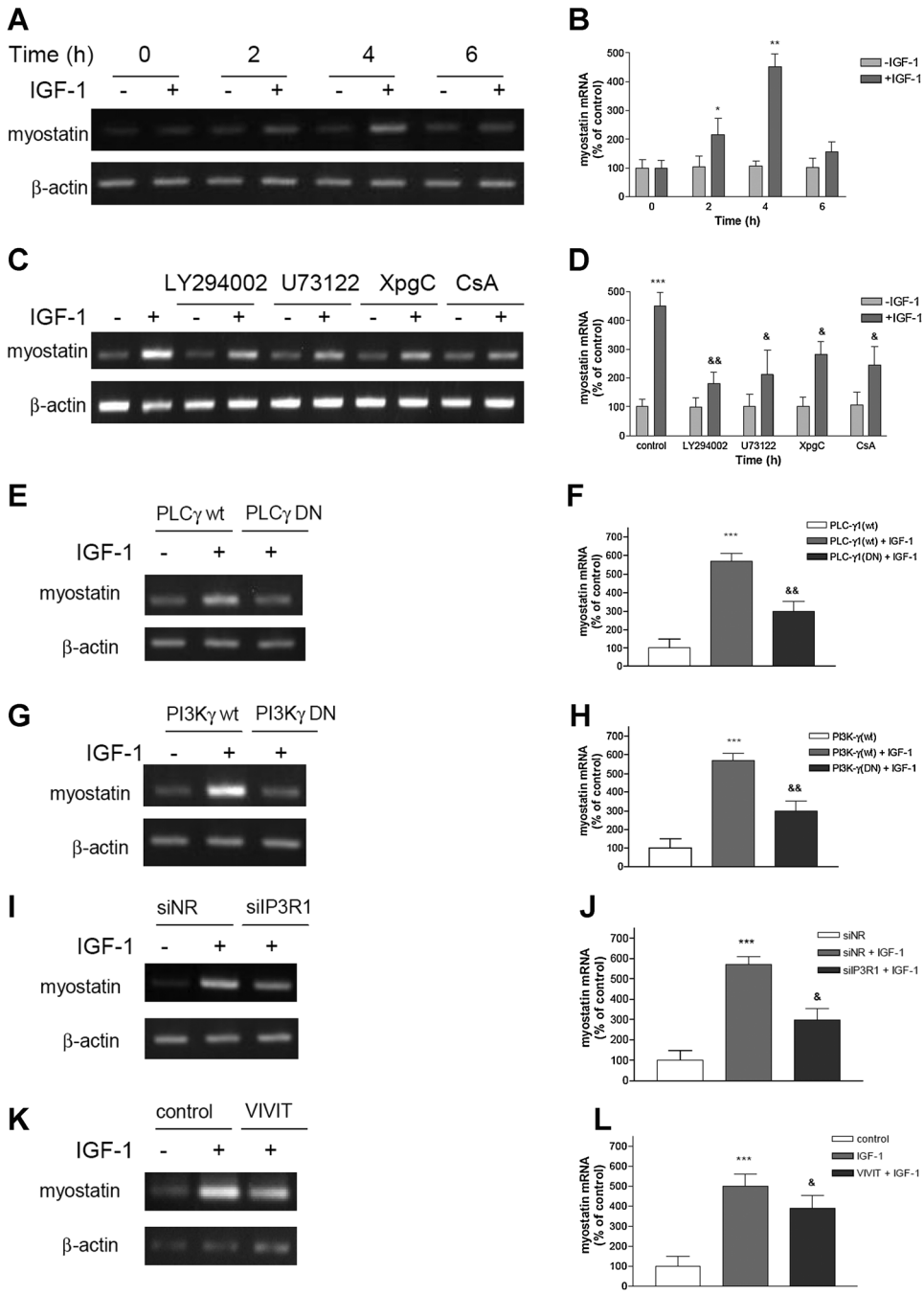

Fig. 5. IGF-1 stimulates a calcium-dependent increase in myostatin mRNA levels in myoblasts. A,B: Total RNA was isolated from myoblasts stimulated with 10 nM IGF-1 and maintained in resting medium for the times indicated. Myostatin mRNA levels were analyzed by semiquantitative RT-PCR: (A) representative agarose gel of RT-PCR products from myostatin and beta actin mRNA amplification (B) Myostatin and beta actin densitometric analysis. C,D: Total RNA was isolated from myoblast stimulated with 10 nM IGF-1, preincubated in the presence or absence of cyclosporin A (50 μM), LY294002 (50 μM), U73122 (50 μM), xestospongin C (50 μM), and maintained in resting medium for 4 h. Myostatin mRNA levels were analyzed by semiquantitative RT-PCR: (C) Representative agarose gel of RT-PCR products from myostatin and beta actin mRNA amplification (D) myostatin and beta actin densitometric analysis. E,F: Total RNA was isolated from myoblasts transfected with PLCγ WT or PLCγ DN, stimulated with 10 nM IGF-1 and maintained in resting medium for 4 h. Myostatin mRNA levels were analyzed by semiquantitative RT-PCR: (E) Representative agarose gel of RT-PCR products from myostatin and beta actin mRNA amplification. F: Myostatin and beta actin densitometric analysis. G,H: Total RNA was isolated from myoblasts transfected with PI3Kγ WT or PI3Kγ DN, stimulated with 10 nM IGF-1 and maintained in resting medium for 4 h. Myostatin mRNA levels were analyzed by semiquantitative RT-PCR: (G) Representative agarose gel of RTPCR products from myostatin and beta actin mRNA amplification (H) myostatin and beta actin densitometric analysis. I,J: Total RNA was isolated from myoblasts transfected with siRNA NR or siRNA IP3R1, stimulated with 10 nM IGF-1 and maintained in resting medium for 4 h. Myostatin mRNA levels were analyzed by semiquantitative RT-PCR: (I) Representative agarose gel of RT-PCR products from myostatin and beta actin mRNA amplification (J) Myostatin and beta actin densitometric analysis. K,L: Total RNA was isolated from myoblasts stimulated with 10 nM IGF-1, pre-incubated in the presence or absence of 11R-VIVIT (2 μM) and maintained in resting medium for 4 h. Myostatin mRNA levels were analyzed by semiquantitative RTPCR: (K) Representative agarose gel of RT-PCR products from myostatin and beta actin mRNA amplification (L) myostatin and beta actin densitometric analysis. Data are means ± SEM of duplicates (three independent experiments) and are expressed relative to values in control cells. Statistical analysis was performed by oneway ANOVA followed by Bonferroni's post-test. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ compared to the control group. &$P < 0.05$ and &&$P < 0.01$ compared to IGF-1 treated group.

transcription. IGF-1 stimulation performed in the presence of LY294002, XpgC, or U73122 resulted in blockage of the effect (Fig. 5C,D). These results were corroborated by transfection of myoblasts with dominant negative forms of PLCγ, PI3Kγ, or siIP3R1 stimulated with IGF-1, showing a significant inhibition of myostatin mRNA expression (Figs. 5E,F, 5G,H, and 5I,J, respectively). These results indicate that calcium release through IP_3 receptors, mediated by PI3Kγ and PLCγ participates in the regulation of myostatin expression.

To investigate whether IGF-1 is involved on myostatin transcriptional activation, rat myoblasts were transfected with a luciferase reporter gene construct containing nucleotides from −1,880 to +124 of murine myostatin promoter (Fig. 6A). IGF-1 stimulated luciferase expression 10.5-fold compared with non stimulated transfected cells (Fig. 6B). Cultures transfected with the promoter pGL4 plasmid was used as a negative control. Preincubation with CsA and 11R-VIVIT partially blocked the transcriptional activity of myostatin promoter (Figs. 6B and 6F, respectively). To further analyze the involvement of IP_3-dependent calcium signals in myostatin expression, transfected rat myoblasts were pretreated with the inhibitors LY294002, XpgC or U73122, and stimulated with IGF-1. Inhibition of any component of the PI3Kγ-PLCγ-IP_3R axis resulted in a significant reduction in IGF-1 mediated myostatin promoter activation (Fig. 6B). Myoblasts transfected with dominant negative forms of PLCγ, PI3Kγ, or siIP3R1 and stimulated with IGF-1, showed a significant inhibition of transcriptional activity of myostatin promoter (Figs. 6C, 6D, and 6E, respectively). These results agree with myostatin RT-PCR experiments and support the involvement of IP_3-dependent calcium signals in myostatin expression.

Discussion

Muscle differentiation constitutes a complex and highly regulated process, coordinated by a fine balance between positive and negative signals; nevertheless, studies of the interaction between the regulatory signals of differentiation in muscle have been very limited (Trendelenburg et al., 2009). In the present study, we start to elucidate this subject, showing

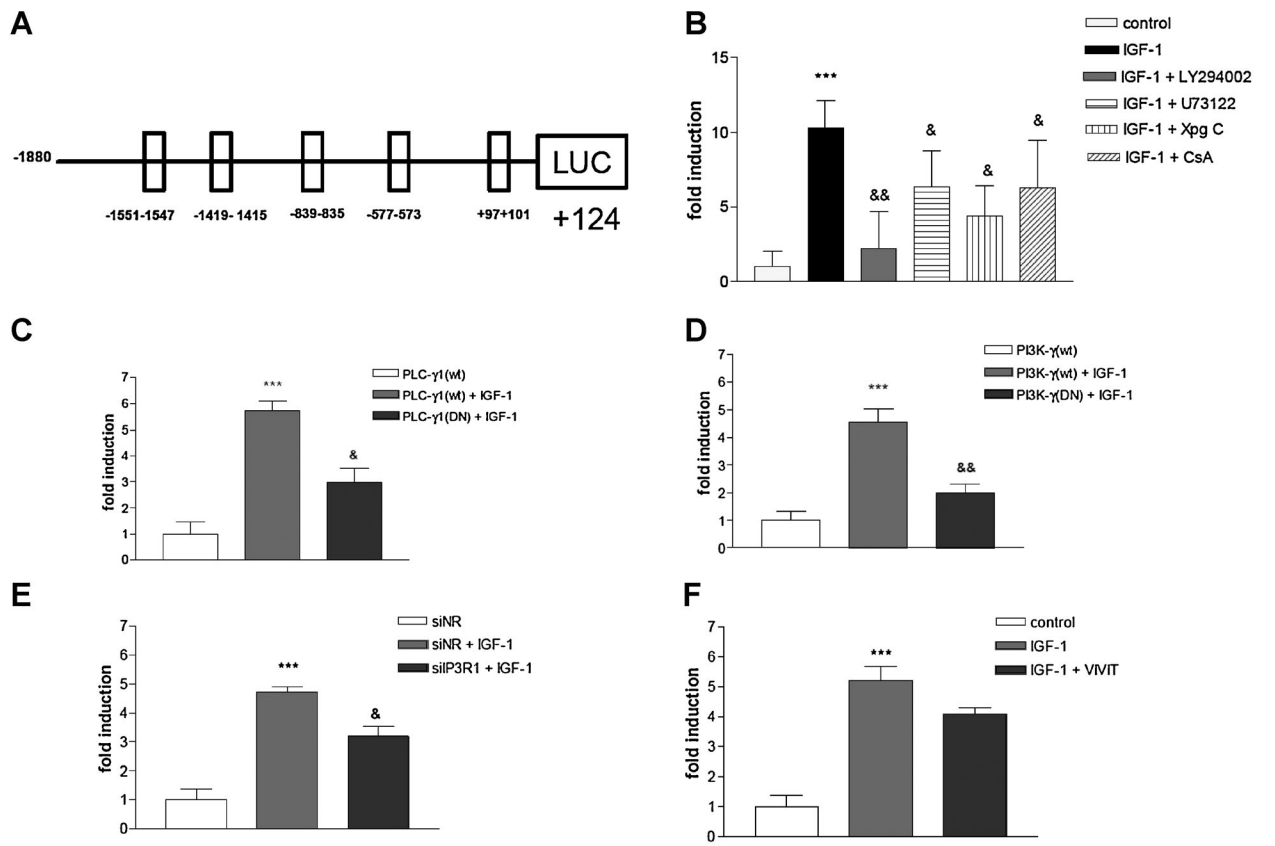

Fig. 6. IGF-1 induces myostatin promoter transcriptional activity in myoblasts. A: Scheme of NFAT consensus element contained into the fragment spanning 2,000 bp upstream of the transcriptional start site of the myostatin promoter fragment gene, fused to the luciferase coding region of pGL4 plasmid. B: Cotransfected myoblasts with myostatin-pGL4 reporter vector and phRL-TK normalizer vector were preincubated for 30 min with vehicle, 50 μM LY294002, 50 4M U73122, 50 μM xestospongin C or 50 μM cyclosporin A and then stimulated with 10 nM IGF-1. Results were normalized for transfection efficiency and expressed as the ratio of firefly to Renilla luciferase. C: Co-transfected myoblasts with myostatin-pGL4 reporter vector, phRL-TK normalizer vector and PLCγ1 (WT or DN) were stimulated with 10 nM IGF-1. D) Co-transfected myoblasts with myostatin-pGL4 reporter vector, phRL-TK normalizer vector and PI3Kγ (WT or DN) were stimulated with 10 nM IGF-1. E: Co-transfected myoblasts with myostatin-pGL4 reporter vector, phRL-TK normalizer vector and siRNA (NR or IP3R1) were stimulated with 10 nM IGF-1. Results were normalized for transfection efficiency and expressed as the ratio of firefly to Renilla luciferase. F: Cotransfected myoblasts with myostatin-pGL4 reporter vector and phRL-TK normalizer vector were preincubated for 60 min with vehicle or 11R-VIVIT (2 μM) and then stimulated with 10 nM IGF-1. Results were normalized for transfection efficiency and expressed as the ratio of firefly to Renilla luciferase. Data are means ± SEM of duplicates (three independent experiments) and are expressed relative to values in control cells. Statistical analysis was performed by one-way ANOVA followed by Bonferroni's post test. ***$P < 0.001$ compared to the control group. &$P < 0.05$ and &&$P < 0.01$ compared to IGF-1 treated group.

for the first time three important findings: (1) IGF-1 induces a fast and transient calcium release from intracellular stores that is mainly mediated by the PI3Kγ, PLCγ, and IP_3 receptor pathways in differentiated myoblasts. (2) IGF-1 activates the NFATc3 transcription factor via IP_3 in a calcium-dependent fashion. (3) Calcium-dependent signaling pathways mediated by the NFAT transcription factor regulate myostatin gene expression, which constitutes the most important observation of this study.

IGF-1 in muscle has a dual role during myogenesis; the first is to stimulate the proliferation of myoblasts and the second is to stimulate differentiation. One of the pathways activated by IGF-1 involves Ras-Raf signaling to extracellular response kinases (ERKs) which can activate a number of transcription factors as well as other protein kinases. In muscle cell cultures, this pathway has been shown to promote increased cell proliferation. A second pathway involves the activation of PI3K, generating phosphatidylinositol (3,4,5)-triphosphate (PIP_3) from phosphatidylinositol (4,5)-bisphosphate (PIP_2). PIP_3 in turn acts as a docking site for Akt kinase, leading to Akt activation. This pathway has been shown to promote myoblast differentiation and hypertrophy in skeletal muscle. Although Akt is certainly the most characterized downstream target of PI3K, many other proteins, including PLCγ, have been proposed as targets. In the present study, we show that the stimulation of myoblasts with physiological concentrations of IGF-1 during differentiation induces the tyrosine phosphorylation of PLCγ, however, optimal PLCγ activity requires the activation of PI3Kγ. This experimental observation has been previously reported in cardiac myoblasts (Hong et al., 2001), where the authors reported that IGF-1 activates PLCγ1 through a tyrosine phosphorylation-dependent mechanism and also through a tyrosine phosphorylation-independent mechanism mediated by PI3Kγ. The main mechanism of PLCγ activation is via the RTK receptor, tyrosine phosphorylation, and recruitment to the receptor through its two SH2 domains. Nevertheless, it has been described that tyrosine phosphorylation is not sufficient for full activation (Maffucci and Falasca, 2007). Here, we report that PI3K inhibition did not significantly inhibit the phosphorylation of PLCγ, supporting the hypothesis that the N terminal PH domain of PLCγ interacts with PIP_3 and might provide a means to localize PLCγ to the plasma membrane where it catalyzes IP_3 formation (Maffucci and Falasca, 2007).

Complementarily, we show that IGF-1 induces a fast and transient increase of calcium in cultured rat myoblasts. This calcium was released from intracellular stores through an IP_3-dependent mechanism, essentially fully dependent upon of receptor tyrosine kinase (RTK) activity, PI3Kγ, and PLCγ. This experimental observation has been reported in other cellular models like myotubes (Espinosa et al., 2004), cardiomyoblasts (Ibarra et al., 2004), porcine thyroid cells (Takasu et al., 1989), and chondrocytes (Poiraudeau et al., 1997). To characterize additional mechanisms activated by IGF-1 that could be involved in an intracellular calcium rise, we performed measurements of calcium signals in myoblasts preincubated with nifedipine, ryanodine, and extracellular calcium absence. Our results also indicate that the effect of IGF-1 on calcium increase was independent of RyR or calcium influx. In skeletal rat myotubes, it has been reported that IGF-1 induces an intracellular calcium increase involving at least two processes; the main part of the calcium signals appear to be due to calcium release from intracellular stores through IP_3 receptors, similar to our finding, however, an early component dependent on the dihydropyridine receptor (DHPR) and RyR was also detected (Espinosa et al., 2004), but which was not present in our observations. We assume that during myoblast proliferation the components of the excitation-contraction system (DHPR and RyR) are still immature or non-functional. Stiber et al. (2005) determined that undifferentiated myoblasts express inositol triphosphate receptors (IP_3Rs) and transient receptor potential channels (TRPC3) while differentiated myotubes functionally express RyR and DHPR. Agonist (UTP) stimulation of the G protein coupled receptor P2Y2 in myoblasts induces calcium release from IP_3R calcium channels and activates calcium influx via receptor operated channels (Stiber et al., 2005). Similarly in cultured rat cardiac myocytes, it has been found that IGF-1 increases calcium levels through a Gβγ subunit, the protein G-PI3K-PLC signaling pathway that involves the participation of IP_3. However, they determined that Gβγ subunits dissociated from Gi protein is a critical component of IGF-1-induced calcium transient in cardiac myocites (Ibarra et al., 2004). In our case, preliminary results show that the preincubation of myoblasts with pertussis toxin (PTX) did not modify the magnitude and kinetics of calcium release induced by IGF-1 (data not shown), suggesting that PLCγ activation is directly mediated by PI3Kγ. Altogether, these results suggest that IGF-1 induces calcium release from intracellular stores mediated by PI3Kγ and PLCγ.

IGF-1-induced calcium release activates the transcription factor NFAT, which is completely calcineurin dependent. NFAT is a calcium-dependent transcription factor, composed of the NFAT1–4 protein, regulated by calcium-calcineurin phosphatase. In non-stimulated cells, NFAT is located in the cytosol in a hyperphosphorylated state, and, after an increase in calcium concentrations, calcineurin directly dephosphorylates NFAT thus inducing its nuclear accumulation. It has been previously demonstrated in skeletal muscle cells that IGF-1 treatment induces a transient activation of the phosphatase calcineurin that relates to muscle differentiation and hypertrophy (Musarò et al., 1999; Semsarian et al., 1999; Friday et al., 2000). However, the underlying molecular mechanism by which endogenous calcineurin is activated by IGF-1 is unknown. We postulate that calcium release induced by IP_3 in skeletal myoblasts is responsible for the transient activation of calcineurin and subsequent dephosphorylation/nuclear NFAT accumulation. This hypothesis is supported by NFAT reporter activity measurements, where the preincubation of myoblasts with CsA blocked NFAT-dependent transcription activity, similar to the preincubation with the PI3Kγ and PLCγ inhibitors. Additionally, we demonstrated that myoblast treated with IGF-1 increased nuclear accumulation of NFATc3. We chose to study the isoform c3 because this isoform has been linked with myoblast differentiation (Abbott et al., 1998; Delling et al., 2000); however, the current study does not exclude regulation of other NFAT isoforms. It has been described that cardiac cells respond to mobilization of the IP_3R calcium pool with an increase in nuclear NFATc1 protein (Rinne & Blatter, 2010).

The last and most important observation here is that calcium-dependent signaling pathways mediated by the transcription factor NFAT may regulate myostatin gene expression. Myostatin, also known as growth differentiation factor 8 (GDF-8), is a member of the TGF-β superfamily involved in the negative regulation of skeletal muscle mass. In vitro studies have shown that myostatin can block the differentiation of myoblasts into myotubes by down-regulating the expression of MyoD and myogenin (Thomas et al., 2000; Langley et al., 2002; Ríos et al., 2002; Joulia et al., 2003). We have found that myoblast stimulation with IGF-1 brings about a transient 4.5-fold increase in myostatin mRNA level, with a maximum at about 4 h after exposure to IGF-1. A similar result was reported in C2C12 and chicken myoblasts treated with IGF-1 (Yang et al., 2007; Kurokawa et al., 2009). Also in the study of Yang et al. (2007), IGF-1 has the potential to induce myostatin expression via the PI3K/Akt pathways (Yang et al., 2007). The inhibition of PLCγ, IP3R, and calcineurin in myoblast partially reduced the myostatin mRNA levels, suggesting that

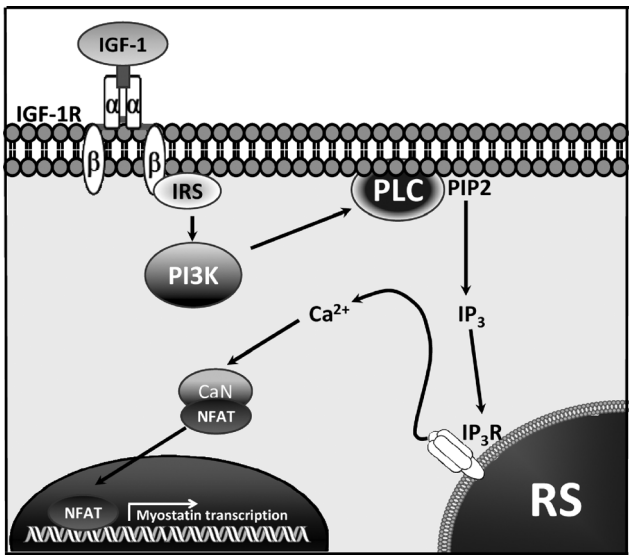

Fig. 7. Schematic description of calcium signaling pathways involved in NFAT transcriptional activation. IGF-1 binding to its receptor results in IGF-1 receptor activation and phosphorylation of PI3Kγ and PLCγ to produce IP3. IP3 diffuses into the cytosol and reaches IP3 receptors, inducing calcium release. This calcium signals induce the transient calcineurin phosphatase activation which induces NFATc3 nuclear translocation and transcriptional activation by binding to myostatin promoter, where regulates myostatin mRNA expression.

other calcium–calcineurin-independent transcription factors may regulate myostatin expression. It is interesting to note that the preincubation of myoblasts with the PI3K inhibitor (LY294002) or transfection with PI3Kγ DN completely blocks the myostatin mRNA expression induced by IGF-1, corroborating the pivotal role of this protein in the IGF-1 signaling pathways.

It seems particularly important to establish whether or not the increase in myostatin mRNA levels was followed by actual enhancement in myostatin promoter activity. We have demonstrated that stimulation with IGF-1 of myoblast transfected with a luciferase reporter gene, containing nucleotides (−1,880 to +124) of murine myostatin promoter, increases luciferase expression. Several studies have analyzed the 5′ upstream regulatory sequences of myostatin genes from human, bovine, porcine, and murine, showing considerable sequence homology and sharing many functional transcription factor binding motifs: MyoD, MEF2, CREB, Smad, FoxO (Ma et al., 2001; Spiller et al., 2002; Salerno et al., 2004; Allen and Unterman, 2007; Allen and Du, 2008). In silico analysis shows that the myostatin gene promoter contains several NFAT consensus binding sites. Experiments performed in the presence of the calcineurin and NFAT inhibitors corroborated the participation of calcium-calcineurin in myostatin expression. Moreover, myostatin has been proposed as a possible downstream gene target of calcineurin. It has been found that myostatin mRNA levels are higher in transgenic mice with enhanced calcineurin signaling, compared to wild-type counterparts (Michel et al., 2004, 2007). Furthermore, the administration of CsA results in lower muscle myostatin mRNA levels. Although our results indicate that both calcium and calcineurin are required for maximal myostatin gene induction, we do not rule out the possibility that other sequences in the promoter can participate in the regulation of myostatin production in myoblasts.

In conclusion, we summarize our findings in the following mechanistic model (Fig. 7): The stimulation of myoblasts with IGF-1 activates both PI3Kγ and PLCγ to produce IP_3. IP_3 diffuses into the cytosol and reaches IP_3 receptors, inducing calcium release. These calcium signals induce the transient activation of calcineurin phosphatase, which induces NFATc3 nuclear translocation and transcriptional activation by binding to the myostatin promoter, regulating its expression. To our knowledge this is the first study to demonstrate a role of calcium-dependent signaling pathways in the mRNA expression of myostatin, a negative regulator of skeletal muscle growth. Deep understanding of the signaling pathways involved in myostatin expression represents a fundamental condition in the promising development of treatment for muscle wasting diseases.

Acknowledgments

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