Transforming growth factor β (TGF-β) Signaling Is Regulated by Electrical Activity in Skeletal Muscle Cells

BY MYOTUBE EXCITABILITY

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Transforming growth factor (TGF-β) is involved in several cellular processes such as cell proliferation, differentiation, and apoptosis. At the cell surface, TGF-β binds to serine-threonine kinase transmembrane receptors (type II and type I) to initiate Smad-dependent intracellular signaling cascades. During the early stages of skeletal muscle differentiation, myotubes start to evoke spontaneous electrical activity in association with contractions that arise following the maturation of the excitation-contraction apparatus. In this work, we report that TGF-β-dependent signaling is regulated by electrical activity in developing rat primary myotubes, as determined by Smad2 phosphorylation, Smad4 nuclear translocation, and p3TPLux reporter activity. This electrical activity-dependent regulation is associated with changes in TGF-β type I receptor (TβRI) levels, correlated with changes in transducing receptors at the cell membrane (measured through radiolabeling binding assays). The inhibition of electrical activity with tetrodotoxin (TTX) (13,14) primary myotube cultures after blocking spontaneous electrical activity modulation also occurred in adult skeletal muscles, suggesting that electrical activity-dependent regulation of TβRI also occurs in vivo. Additional results suggest that this activity-dependent regulation is mediated by myogenin. Altogether, these findings support the possibility for a novel regulatory mechanism acting on TGF-β signaling cascade in skeletal muscle cells.

Transforming growth factor β (TGF-β)3 is involved in several cellular processes, such as cell proliferation, differentiation, and apoptosis (1, 2). TGF-β signaling cascades initiate after ligand binding with the heterodimerization of type II (TβRII) and type I (TβRI) TGF-β receptors at the cell surface. This induces the phosphorylation of R-Smad proteins, such as Smad2 and Smad3, the association with co-Smads, such as Smad4, and their translocation to the nucleus in order to promote the transcription of target genes (3). Several regulatory mechanisms for these TGF-β signaling pathways have been described, including modification of transducing receptor turnover (induced by ligand binding, cell membrane distribution, and I-Smads) and cytoplasm-nuclear transport and half-life of Smad proteins (4).

In skeletal muscle cells, TGF-β acts as a strong inhibitor of myogenesis. It is known that TGF-β can inhibit myoblast differentiation in vitro, affecting the expression of muscle proteins such as myosin heavy chain and creatine kinase (5). Cell sensitivity to TGF-β-derived inhibition decreases during myoblast differentiation, and recent studies support a Smad3-mediated mechanism for this suppression (6). On the other hand, skeletal muscle cells develop excitability and contractile properties during the first stages of myogenesis. This phenotype has been associated to the onset of voltage-dependent channel protein expression (sodium and calcium channels) and to the maturation of the excitation-contraction apparatus (7–9). At more advanced developmental stages, muscle activity is controlled by motor innervation (10). Denervation has been the classical model to study the in vivo regulatory properties of electrical activity on muscle protein expression. Studies using this model have demonstrated that several myogenic factors, such as myogenin and MyoD, and ligand-activated and voltage-dependent channels, such as nicotinic acetylcholine receptor (nAChR) and sodium channels, are up-regulated after denervation (11, 12). Similar results, whereby voltage-dependent sodium channels and nAChR are up-regulated, have been reported for primary myotube cultures after blocking spontaneous electrical activity with tetrodotoxin (TTX) (13, 14).

The aim of the work presented here was to evaluate whether TGF-β signaling cascade could be modulated in skeletal muscle cells by an intrinsic property such as electrical activity. Our results demonstrate that such electrical activity modulation occurs in rat primary myotubes through the modification of TGF-β transducing receptor levels. TβRI at the cell surface was up-regulated following inhibition of spontaneous electrical activity involving transcriptional activation and down-regulated when this activity was promoted, pointing to a novel mechanism for the control of TGF-β signaling pathways in skeletal muscle cells. In vivo experiments demonstrated that this electrical activity-modulation also occurred in adult skeletal muscles after motor denervation, and additional analyses suggested a myogenin-dependent mechanism.

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3 The abbreviations used are: TGF-β, transforming growth factor β; TβRI, TGF-β type I receptor; TβRII, TGF-β type II receptor; nAChR, nicotinic acetylcholine receptor; TTX, tetrodotoxin; DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT, reverse transcription; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; bHLH, basic helix-loop-helix.
**EXPERIMENTAL PROCEDURES**

**Animals and Muscle Denervation**—2-month-old male Sprague-Dawley rats were anesthetized with xylazine/ketamine, and right legs were denervated by a sciatic section of 0.5 cm in the hip region of the hind limb as previously described (15). After 72 h, tibialis anterior muscle was excised for analysis. Muscles from contralateral legs were used as controls. For protein extracts, muscle tissue was frozen with liquid nitrogen, triturated, and homogenized in buffer containing 1% SDS, 1% Triton X-100, and protease inhibitors. Extracts were then centrifuged at 20,000 × g for 10 min and supernatants collected and analyzed for protein quantification.

**Cell Cultures**—Rat skeletal myoblasts were isolated from the hind legs of day 19 Sprague-Dawley rat fetuses. Briefly, muscle mass was dissected from bones, subjected to mechanic dissociation, and filtered through a 70-µm cell strainer. Cell suspensions were prepped to eliminate fibroblasts, and non-adherent cells were plated onto collagen-coated culture dishes at a density of 100,000 cells/ml. Myoblasts were grown at 37 °C with 8% CO2 in Dulbecco's modified Eagle's medium supplemented with 10% bovine serum and 10% horse serum. After 48 h, the medium was changed to Dulbecco's modified Eagle's medium consisting of 10% horse serum to induce myotube formation with 10 µM cytosine-β-d-arabinofuranoside to inhibit fibroblast proliferation. Under these conditions, primary myotubes showed spontaneous contractions as of day 2 in differentiation medium. To inhibit electrical activity, 1 µg/ml of TTX (Alomone, Jerusalem, Israel) was added to cell cultures at day 1 of differentiation.

**Electrical Stimulation**—Primary cultures were stimulated as described by Chahine et al. (16). Stimulation electrodes were immersed in culture medium, and myotubes were stimulated using a Grass stimulator with 0.4-ms 4-V pulses (threshold required to induce visible contractions) in 100-Hz trains lasting 1 s, applied once every 100 s for 12 h.

**DNA Transfections**—Primary myoblast cultures were transfected with 2 µg of p3TP-Lux or 2 µg of 6-47MEKLuc and 0.01 µg of pRLSV40 using the calcium phosphate DNA precipitation method (17, 18). After 6 h, cell cultures were subjected to a glyceral shock for 45 s and then induced to differentiate. Three days later, p3TPLux activity was determined by incubating cultures with 1 ng/ml of TGF-β1 for 12 h and then harvesting for luciferase assay.

**Western Blotting**—Proteins were extracted from primary cultures using phosphate-buffered saline containing 0.1% Triton X-100 plus protease inhibitors. Protein concentrations were determined using the Micro BCA™ protein assay kit (Pierce). Samples were electrophoresed in 10% polyacrylamide gels, transferred onto nitrocellulose membranes, and probed with one of the following antibodies: rabbit anti-TβRI (1:500) (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-pan-Na+, (1:200) (Alomone), rabbit anti-phospho-Smad2 (1:1000) (Calbiochem, La Jolla, CA), goat anti-Smad2 (1:250) (Santa Cruz Biotechnology), mouse anti-Smad4 (1:1000) (Santa Cruz Biotechnology), rabbit anti-myogenin (1:100) (Santa Cruz Biotechnology), mouse anti-GADPH (1:5000) (Chemicon, Temecula, CA). After incubation with horseradish peroxidase-conjugated secondary antibodies (1:10000) (Pierce), reactive proteins were visualized using chemiluminescent substrates (Pierce). For the detection of phospho-Smad2, proteins were extracted in radioimmune precipitation buffer plus protease inhibitors.

**Cross-linking Assays**—TGF-β1 was radiolabeled with [125I] using chloramine T, and affinity labeling was carried out for 4 h at 4 °C using disuccinimidyl sulfate as cross-linking reagent (Pierce). Samples were analyzed by 8 – 12% gradient SDS-PAGE as previously described (19).

**Immunocytochemistry Analysis**—Cells were fixed in 3% paraformaldehyde, permeabilized with 0.05% Triton X-100, and incubated with 1:100 monoclonal anti-Smad4 antibody (Santa Cruz Biotechnology). Bound antibodies were detected by incubating the cells with 1:100 affinity-purified fluorescein isothiocyanate-conjugated anti-mouse IgG (Pierce). After rinsing, the slides were viewed through a Nikon upright microscope equipped with epi-fluorescence.

**RESULTS**

**TGF-β-dependent Signaling Is Heightened in Inactive Primary Myotubes**—To determine whether electrical activity can modulate TGF-β cascade components in skeletal muscle cells undergoing differentiation, we analyzed the effect of inhibiting electrical activity in rat primary myotubes using TTX. These cells develop spontaneous electrical activity, together with visible contractions, early during in vitro differentiation. To begin with, the TGF-β-dependent intracellular cascade that triggers Smad-dependent pathways was examined by measuring the phosphorylation of Smad2 induced by TGF-β in control and TTX-treated primary myotubes. Fig. 1A shows that in TTX-treated cells the TGF-β-dependent phosphorylation of Smad2 was detectable at lower ligand concentrations than in control myotubes. In the same figure, the dose-response curves for this TGF-β-dependent phosphorylation are shown following band quantification.

Next, we determined whether the Smad-dependent downstream steps of the TGF-β signaling cascade were also affected by TTX treatment. For this, the nuclear translocation of Smad4 was analyzed by indirect immunolocalization. Fig. 1B shows that after TGF-β incubation the distribution of Smad4 in active primary myotubes remained cytosolic and excluded the nuclei, whereas in TTX-treated myotubes it concentrated in the nuclei, supporting the idea that in inactive myotubes the responsiveness to TGF-β is increased.

To evaluate whether the TGF-β-induced transcription of target genes was also affected in TTX-treated myotubes, we transfected primary cultures with the TGF-β-responsive reporter p3TPLux (21). Reporter activity increased ~4-fold in TTX-treated myotubes with respect to control cells (Fig. 1C), suggesting a more activated Smad-dependent TGF-β pathway in inactive myotubes than in control cells. Moreover, Fig. 1D shows that total Smad2 and Smad4 protein levels were not altered by TTX treatment, implying that the enhanced Smad2 phosphorylation and Smad4 nuclear translocation observed in inactive myotubes in response to TGF-β must be associated to an upstream variation in the signaling cascade.

**Total and Cell Surface TGF-β Type I Receptor Levels Are Increased in TTX-treated Myotubes**—To better understand the increased TGF-β responsiveness of inactive myotubes, we decided to measure protein levels of TβRI using Western blot analysis. A rise in TβRI protein levels was seen during primary myoblast differentiation (Fig. 2A), with a more notable increase observed upon inhibition of electrical activity at 48 or
72 h (cultures at day 4 or 5 of differentiation, respectively). To determine whether this augmentation in total TβRI levels corresponded to an increase in the number of cell surface receptors, we analyzed the receptor binding of radiolabeled 125I-TGF-β1 through affinity labeling experiments. Fig. 2B shows that labeling associated to TGF-β/TβRI complex was higher in TTX-treated myotubes than in control cells (153% ± 4.1 S.E., n = 3), suggesting that the rise in TβRI protein levels obtained after blocking electrical activity also corresponds to an increase in membrane receptors at the cell surface. Altogether, these results indicate that the heightened responsiveness to TGF-β of inactive myotubes as evaluated by determining the dose-response curves of Smad2 phosphorylation, Smad4 nuclear translocation, and p3TPLux reporter activity is consistent with the up-regulation of the transducing receptor TβRI as a result of TTX exposure.

**TTX-dependent Increase in TGF-β Type I Receptor Levels Involves Transcriptional Activation**—To determine whether the electrical activity-dependent up-regulation of TβRI occurs at a transductional and/or transcriptional level, at first we performed semi-quantitative RT-PCR analysis. Fig. 3A shows that PCR product corresponding to TβRI transcripts is increased in TTX-treated myotubes compared with control cultures (lanes 1 and 4). To estimate half-life values for TβRI transcripts in control and inactive cultures we analyzed the time-dependent decrease in PCR product after incubation with the RNA polymerase II inhibitor DRB (5,6-dichloro-1-β-d-ribofuranosylben-
Electrical Activity-dependent Regulation of TGF-β Signaling

To confirm that TTX-dependent increase in TβRI receptor levels requires transcription we studied the effect of DRB on receptor protein levels. Fig. 3B shows that TTX-induced TβRI up-regulation could be blocked by DRB, indicating that this activity-dependent up-regulation required transcription as a prior step. Furthermore, the induction of TβRI by chronic exposure to TTX (for 72 h) could also be progressively inhibited by DRB as seen in Fig. 3C. These results demonstrate that the onset and maintenance of TβRI up-regulation in paralyzed cultures is dependent on transcription.

On the other hand, to evaluate whether the increase in TβRI observed at the plasma membrane in TTX-treated myotubes could result from a lower receptor degradation rate, we analyzed the recovery of receptor levels using chloroquine (a lysosomal degradation inhibitor) in cycloheximide-treated cultures. Fig. 4 shows that the inhibition of the lysosomal pathway had a lesser effect on receptor recovery in TTX-treated cultures, pointing to the attenuation of this pathway despite the apparent decrease in receptor levels compared to controls. These results support the notion that the up-regulation of TβRI in inactive myotubes involves both a transcriptional activation and a reduction in the lysosome- and proteasome-dependent degradation of the receptor.

Myotube Hypereexcitability and Direct Stimulation Decrease TGF-β Type I Receptor Protein Levels—To test whether enhanced electrical activity in primary myotubes could negatively regulate TβRI protein levels, we analyzed the influence of cell excitability under two experimental conditions (Figs. 5 and 6). The action of TTX over voltage-dependent sodium channels is known to be reversible, and its inhibitory effect can be restored by rinsing the toxin (22). When rat primary myotubes chronically treated with TTX were washed to restore their electrical activity, this activity together with visible contractions was higher than in control cultures. This hypereexcitable phenotype is consistent with a TTX-dependent up-regulation of voltage-sensitive sodium channels (see below). Therefore, TβRI protein levels were determined under these conditions in which primary myotubes were allowed to recuperate their electrical activity. Fig. 5A shows that TβRI protein levels were significantly lower in myotubes treated with TTX, washed, and lysed 12 h later (w12) than in untreated (C, control) or unwashed (TTX) myotubes. The lower panel shows that myogenin protein levels followed the same pattern of electrical activity-dependent regulation. It is remarkable that after such a short period of time as 12 h the up-regulation of TβRI could be reverted and levels seen to decrease below those of controls. These findings indicate that receptor expression can be modulated in both directions (up- and down-regulated) by modifying the...
Electrical Activity-dependent Regulation of TGF-β Signaling

To confirm that lower TβRI levels in w12 myotubes correlated with a rise in electrical activity in these cultures, we transfected primary myotubes with the 6-47MEKLuc reporter plasmid in which a minimal promoter sequence of encephalin (MEK) is under the control of an enhancer sequence (47 bp) from the nAChR δ subunit. This enhancer sequence confers electrical activity-dependent regulatory properties to a heterologous promoter that result in the down-regulation of gene expression (23). As shown in Fig. 5D, w12 myotubes presented a lower reporter activity than control cells, denoting greater electrical activity in recovered myotubes than in untreated cultures. The increased reporter activity in TTX-treated cells is in concordance with the inhibition of spontaneous electrical activity as previously described for denervated muscles and inactive primary cultures (23, 24). Voltage-dependent sodium channels are required to generate action potentials, giving rise in cultured skeletal muscle cells to a linear relationship between ion channel levels and the frequency of action potentials (7). In addition, the up-regulation of sodium channels can be induced by the denervation of adult muscle as well as after blockade of electrical activity in muscle cell cultures by TTX (12, 13). Fig. 5E shows a progressive increase in sodium channel levels during the differentiation of primary myotubes, with a clear up-regulation in TTX-treated myotubes. Moreover, Fig. 5F shows that 12 h after rinsing off TTX (w12) sodium channel protein levels remained higher than in control myotubes. This result is consistent with a half-life >24 h for muscle sodium channels, estimated from experiments using DRB (data not shown). Together, these results show that w12 myotubes washed after chronic exposure to TTX exhibit enhanced electrical activity with regard to untreated cultures as measured through a reduction in 6-47MEKLuc reporter activity. This hyperexcitable phenotype, compatible with a higher sodium channel content, leads to a reduction in TβRI protein levels, suggesting that electrical activity negatively regulates this receptor in skeletal muscle cells.

The second approach used to study the electrical activity-dependent regulation of TβRI was to investigate whether direct electrical stimulation of primary myotubes could also negatively modulate receptor levels. Electrical activity was induced in primary myotubes by stimulating with extracellular electrodes. This method has been successfully employed in studying the electrical activity-dependent expression of several muscle proteins, such as nAChR and myogenin (25, 26). Fig. 6A shows that electrical stimulation for 12 h (STIM12) significantly decreased TβRI protein levels in primary myotubes, as expected. Fig. 6B shows that the electric stimulation protocol was also effective in down-regulating myogenin expression as described before (25, 26).

Electrical Activity-dependent Regulation of TGF-β Type I Receptor Occurs in Vivo in Adult Skeletal Muscle—The next question was to determine whether the activity-dependent regulation of TβRI observed in primary cultures also occurred in adult skeletal muscle in vivo. For this, we measured receptor protein levels in rat hind limb muscles after short-term motor denervation. Fig. 7 shows that 72 h after denervation, receptor levels increased in tibialis anterior compared with innervated contralateral muscles. Additionally, the middle panel shows that denervated muscles exhibited increased myogenin levels with respect to controls, as has been extensively documented (11). This result demonstrates that in adult skeletal muscles in vivo, TβRI protein levels are also modulated by electrical activity, supporting the notion that this control mechanism is maintained after differentiation of skeletal muscle cells.

Myogenin Is Required for Electrical Activity-dependent Regulation of TGF-β Type I Receptor—Considering that all changes in TβRI induced upon modification of the electrical activity of skeletal muscle cells were accompanied by equivalent changes in myogenin levels, as reproduced in Figs. 5, 6, and 7, and that the promoter sequences of mammalian TGF-β receptors contain E-boxes (binding sites for basic helix-loophelix (bHLH) transcription factors such as MyoD and myogenin, which are regulated by electrical activity) (11,14), we proceeded to evaluate the participation of myogenin in the electrical activity-dependent regulation of TβRI. For that purpose, we first analyzed the time courses and coincidence of myogenin and TβRI up-regulation after applying TTX to inhibit spontaneous electrical activity. Fig. 8A shows that TTX-dependent up-regulation of myogenin preceded the elevation observed in TβRI protein levels, suggesting that this bHLH myogenic factor possibly acts upstream in the intracellular cascade for the electrical activity-dependent regulation of TβRI. To examine the effects of myogenin on TβRI levels in primary myotubes, we next performed transient transfection experiments using an expression plasmid containing full-length myoge-
FIGURE 5. Heightened electrical activity decreases TβRI protein levels and suppresses TGF-β-dependent signaling. A, Western blot for TβRI in protein extracts taken from control untreated myotubes (C) at day 5 of differentiation, as well as TTX-treated cells (TTX) and cells treated with TTX, washed and lysed 12 h later (w12). GADPH immunostaining is shown as a loading control. The lower panel shows Western blotting for myogenin, using total extracts from myotube cells submitted to the same treatments as above. B, affinity labeling of myotubes at day 4 of differentiation using 100 pM 125I-TGF-β1. Phosphorimage shows total myotube cell extracts separated by SDS-PAGE. The migration position of the TβRI/TGF-β1 complex is indicated. Treatments applied to the cells are the following: Lane c, control myotubes (d4); lane TTX, TTX-treated myotubes; lane w12, TTX-treated myotubes, washed and lysed 12 h later; lane w24, TTX-treated myotubes, washed and lysed 24 h later; lane reTTX, TTX-treated myotubes, washed, reincubated with TTX 12 h later, and lysed after 12 h. C, p3TP-Lux reporter activity in control, TTX-treated, and w12 myotubes. Primary myoblasts were transiently co-transfected with p3TP-Lux and pRLSV40 to normalize transfection. At day 3 of differentiation, myotubes were incubated with 1 ng/ml of TGF-β1 for 12 h and then harvested at day 4 of differentiation (d4), after which dual luciferase activity was measured. Data are expressed as the means ± S.E. of three measurements from a representative experiment repeated twice. D, 4-7MEKLuc reporter activity in control, TTX-treated, and w12 myotubes at day 4 of differentiation. Primary myoblasts were transiently co-transfected with 4-7MEKLuc and pRLSV40 to normalize transfection. Data are expressed as the means ± S.E. of three measurements from a representative experiment repeated twice. E, Western blot for the α subunit of voltage-dependent sodium channels (anti-Pan-Na+) using total cell extracts of primary myotubes undergoing differentiation (day 2 to day 5; d2–d5). The last two lanes correspond to extracts from TTX-treated cultures. GAPDH immunostaining is shown as a loading control. F, Western blot for the α subunit of voltage-dependent sodium channels using total cell extracts from control, TTX-treated, and w12 myotubes at day 4 of differentiation. GAPDH immunostaining is shown as a loading control.
**Electrical Activity-dependent Regulation of TGF-β Signaling**

Results presented here demonstrate that the TGF-β signaling cascade is susceptible to myotube excitability when undergoing differentiation. The enhanced responsiveness to TGF-β exhibited by inactive myotubes was associated with the up-regulation of the TßRI. In contrast, transducing receptor was down-regulated when myotube electrical activity was promoted. This excitability-dependent regulation of TßRI also occurred in vivo in adult skeletal muscle after short-term motor denervation. Taken together, these findings point to a novel regulatory mechanism for TGF-β signaling in skeletal muscle cells.

The effects of electrical activity on TßRI levels in primary cultures (namely, TTX-dependent up-regulation and stimulation-dependent down-regulation) were significant even after time periods as short as 2 h, as estimated from Western blot and radiolabeling analyses in primary cultures treated with cycloheximide (data not shown), and also with short half-lives for TßRI detected in other cell types. For example, in pulse-chase experiments using CCL-64 lung epithelial cells, the half-life of TßRI was estimated to be ~12 h (28), whereas in osteoblasts, using transcription and protein synthesis inhibitors, it was estimated at 2 h for the protein and 7 h for the transcripts (29).

Semi-quantitative RT-PCR analysis showed that TßRI transcripts are increased in inactive myotubes without modification of RNA half-life, suggesting that TTX-induced up-regulation of TßRI involves transcriptional activation (Fig. 3A) and not increased transcript stability. Western blot analysis of the effect of DRB over TTX-induced up-regulation of TßRI protein levels confirmed that transcription is required for this regulation (Fig. 3, B and C). These results could be the consequence of a direct effect on the TßRI gene or on genes encoding transcription factors that act upstream in the cellular response to muscle inactivity. It is well known that bHLH factors, such as myogenin, are required for the up-regulation of nAChR subunits upon blocking of electrical activity in primary cultures or denervation (23, 24). Recent studies have shown that after denervation, myogenin up-regulation is associated with enhanced MEF2 transcriptional activity induced by the down-regulation of a histone deacetylase (30). Although the promoter sequences of TßRI and TßRII do not contain MEF2 binding sites, reports studying fibroblast myogenic conversion have provided evidence for a cooperative and synergic activation between MEF2 and myogenic bHLH factors in which MEF2 binding sites are not required (31).

Our results also indicate that up-regulation of TßRI in inactive myotubes involves, in addition to transcriptional activation, a reduction in the lysosome- and proteasome-dependent degradation rates of the receptor (Fig. 4). An unequal contribution of these two pathways to the degradation of TGF-β receptors has been observed in epithelial cells (32). On the other hand, there may also be an inactivity-induced redistribution of transducer receptors into different endocytic compartments at cell membrane, thereby decreasing TßRI turnover (33).

The effect on TßRI levels of increasing the electrical activity of primary myotubes was examined under two experimental conditions: after up-regulation of sodium channels induced by chronic inhibition of electrical activity with TTX and after direct electrical stimulation. In the first approach, the observed decrease in δ47-MEKLuc reporter activity pointed to a hyperexcitable phenotype associated with the up-regulation of sodium channels. Nevertheless, the possibility that chronic blockage of electrical activity can modify the gene expression of other ion channels, resulting in an equivalent phenotype, cannot be ruled out. A hyperexcitable state in denervated muscle associated to the up-regulation of SK3, a small conductance calcium-activated potassium channel, has indeed been described (34).
Moreover, another study demonstrated that the muscle chloride channel ClC-1 was down-regulated after denervation, leading to an increase in membrane resistance and excitability (35).

It is well known that TGF-β negatively regulates the early stage differentiation of skeletal muscle cells (5). Considering that TβRI is down-regulated by electrical activity in primary myotubes, it can be inferred that during muscle differentiation spontaneous electrical activity attenuates TGF-β-derived inhibition, allowing myogenesis to continue. In addition, the absence of this regulatory phenomenon in undifferentiated myoblasts when TGF-β acts as an activator of proliferation (36) can acquire relevance in long-term muscle denervation or regeneration processes after damage, when the proliferation of myoblasts in association with enhanced TGF-β signaling is required for the formation of new fibers. On the other hand, results obtained here using adult denervated

**FIGURE B. Myogenin is required for activity-dependent regulation of TβRI.** A, Western blot for myogenin and TβRI using the same protein extracts taken from myotubes incubated with TTX for different time periods between 0 and 9 h. Plot shows band quantification for myogenin and TβRI expression. B, Western blot for myogenin and TβRI using the same protein extracts obtained at day 3 of differentiation (d3) from control and TTX-treated myotubes as well as untransfected cells and myotubes transfected with the pEMSV-myogenin expression plasmid. Right panels show band quantification. C, Western blot for myogenin and TβRI using the same protein extracts taken from control myotubes and myotubes incubated for 24 h with TTX in the absence or presence of 5 mM sodium butyrate.
Electrical Activity-dependent Regulation of TGF-β Signaling

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