Prostaglandin F\textsubscript{2\alpha} stimulates growth of skeletal muscle cells via an NFATC2-dependent pathway

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Skeletal muscle growth requires multiple steps to form large multinucleated muscle cells. Molecules that stimulate muscle growth may be therapeutic for muscle loss associated with aging, injury, or disease. However, few factors are known to increase muscle cell size. We demonstrate that prostaglandin F\textsubscript{2\alpha} (PGF\textsubscript{2\alpha}) as well as two analogues augment muscle cell size in vitro. This increased myotube size is not due to PGF\textsubscript{2\alpha}-enhancing cell fusion that initially forms myotubes, but rather to PGF\textsubscript{2\alpha} recruiting the fusion of cells with preexisting multinucleated cells. This growth is mediated through the PGF\textsubscript{2\alpha} receptor (FP receptor). As the FP receptor can increase levels of intracellular calcium, the involvement of the calcium-regulated transcription factor nuclear factor of activated T cells (NFAT) in mediating PGF\textsubscript{2\alpha}-enhanced cell growth was examined. We show that NFAT is activated by PGF\textsubscript{2\alpha} and the isoform NFATC2 is required for PGF\textsubscript{2\alpha}-induced muscle cell growth and nuclear accretion, demonstrating the first intersection between prostaglandin receptor activation and NFAT signaling. Given this novel role for PGF\textsubscript{2\alpha} in skeletal muscle cell growth, these studies raise caution that extended use of drugs that inhibit PG production, such as nonsteroidal anti-inflammatory drugs, may be deleterious for muscle growth.

Introduction

Skeletal myogenesis follows an ordered set of cellular events involving cell cycle exit of myoblasts, their subsequent differentiation, and fusion to form multinucleated myofibers in vivo or myotubes in vitro. In most cases, mammalian muscle growth requires the fusion of differentiated muscle cells with the growing multinucleated muscle cell (Darr and Schultz, 1989; Rosenblatt and Parry, 1992; Phelan and Gonyea, 1997; Barton-Davis et al., 1999; Horsley et al., 2001; Mitchell and Pavlath, 2001). By adding additional nuclei to muscle cells during growth, an increased number of nuclei are contained within one cytoplasm, allowing each nucleus to regulate more cytoplasm (Allen et al., 1999). These fusion events allow increased protein synthesis and increases in cell size. Understanding the molecular pathways that regulate muscle growth are important for treating muscle disorders and loss of muscle mass during aging. However, few molecules are known to stimulate increased muscle cell fusion and skeletal muscle growth.

Prostaglandins (PGs)* are paracrine signaling molecules that are synthesized from arachidonic acid in response to cytokines, cell injury, or growth factors (Funk, 2001). The synthesis of PGs involves the metabolism of arachidonic acid by cyclooxygenase enzymes into an intermediate PG. Specific PG synthases convert this intermediate PG into the primary PG molecules (PGE\textsubscript{2}, PGF\textsubscript{2\alpha}, PGI\textsubscript{2}, and PGD\textsubscript{2}). Once produced, PGs are secreted and mediate signaling through G protein–coupled receptors that are distinct for each PG. Activation of PG receptors leads to an array of effects in a range of cell and tissue types, including skeletal muscle.

PGs have been implicated in skeletal muscle growth. For skeletal muscle to grow, a population of myoblasts must be available to differentiate and fuse with the myofiber. Different PGs can control proliferation (Zalin, 1987), differentiation (Schutzle et al., 1984), as well as fusion of myoblasts (Zalin, 1977; David and Higginbotham, 1981; Entwistle et al., 1986; Rossi et al., 1989). Once myotubes are formed, muscle cell size continues to increase through enhanced protein synthesis. PGs regulate this stage of growth by altering both protein degradation and protein synthesis within myotubes (Rodemann and Goldberg, 1982; Palmer, 1990; Vandeburgh et al., 1990). Consistent with a general role for PGs in skeletal muscle growth, inhibition of PG production blocks growth.
of myofibers in vivo (Templeton et al., 1986; McLennan, 1987). These data suggest that PGs regulate muscle growth by influencing multiple steps of myogenesis.

Signaling pathways that are activated by calcium are important for skeletal muscle growth (Abbott et al., 1998; Dunn et al., 1999; Musaro et al., 1999; Sensarri et al., 1999; Delling et al., 2000; Friday et al., 2000; Horsley et al., 2001; Mitchell et al., 2002). PGs have been shown to activate increases in intracellular calcium within a variety of muscle cell types (Asboth et al., 1996; Chen et al., 1997; Yew et al., 1998; Yousufzai and Abdel-Latif, 1998). Specifically, PGE_{2a} and PGE_{2} can activate increases in intracellular calcium through their receptors, PGE_{2a} receptor (FP) and EP_{2}/EP_{3}, respectively (Breyer et al., 2001). One calcium-regulated pathway involved in skeletal muscle growth is the family of transcription factors, nuclear factor of activated T cells (NFAT; Horsley and Pavlath, 2002). Several NFAT isoforms are expressed in skeletal muscle, and the regulation of individual NFAT isoforms appears to occur at the level of nuclear translocation (Abbott et al., 1998). For instance, the NFATC2 isoform is activated only in newly formed or nascent myotubes but not at other stages of myogenesis (Abbott et al., 1998). Previously, we have shown that the NFATC2 isoform is important for skeletal muscle growth (Horsley et al., 2001), but upstream activators of this pathway have not been elucidated.

Because PGE_{2a} can increase intracellular calcium and calcium signaling pathways are important for numerous stages of myogenesis that contribute to muscle growth, we hypothesized that PGE_{2a} may regulate skeletal muscle growth. Although PGE_{2a} can regulate the final stages of muscle growth by inducing protein synthesis (Vandenburgh et al., 1990), we sought to investigate the role of PGE_{2} in other steps of myogenesis that require calcium, such as differentiation (Shainberg et al., 1969; Morris and Cole, 1979) and fusion (Shainberg et al., 1969; Knudsen and Horwitz, 1977). We show that PGE_{2a} enhances myonuclear accretion after the initial formation of myotubes, leading to increases in myotube size. Furthermore, the growth induced by PGE_{2a} occurs through an NFAT-dependent pathway. These data implicate not only a novel function for PGE_{2a} in skeletal muscle growth but also a novel intersection between prostaglandin and NFAT signaling pathways.

**Results**

**PGE_{2a} increases muscle cell size and nuclear number**

To test the hypothesis that PGE_{2a} has a role in the growth of skeletal muscle cells, differentiating primary muscle cultures were treated with different doses of PGE_{2a} or with a stable synthetic analogue of PGE_{2a}, 17-phenyl trinor PGF_{2a} (17-phPGF_{2a}). After 24 h, the majority of cells are differentiated and have formed a few multinucleated cells. Although no difference is apparent between the vehicle- and drug-treated groups at 24 h, after 48 h, drug-treated myotubes are larger in size as compared with vehicle (Fig. 1 A), suggesting that PGE_{2a} can regulate muscle growth.

The formation of a multinucleated cell requires multiple cellular processes including the formation of an adequate number of myoblasts through cell proliferation, their differentiation, and subsequent membrane fusion. To determine if PGE_{2a} increases cell proliferation and/or cell survival in our assay, the DNA content was quantified. No difference exists in the DNA content between PGE_{2a} and vehicle-treated cells (Fig. 1 B). Differentiation was assessed in vehicle and PGF_{2a}-treated cultures at 24 and 48 h by immunostaining the cultures with embryonic myosin heavy chain (EMyHC), a marker of differentiation, and counting the number of nuclei contained within EMyHC-positive cells. The percentage of differentiated cells does not differ be-
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Yew et al., 1998; Katsuyama et al., 2002). Other doses of
ams et al., 1996; Griffin et al., 1998; Kunapuli et al., 1998;
effects in assays using cardiac and smooth muscle cells (Ad-
in Fig. 1 E. When PGF$_{2a}$ is administered at the onset of dif-
percentage of myotubes are present with two to four nuclei as
as to existing myotubes, the number of nuclei in individual
were treated with vehicle treated culture (Fig. 1 G). However, when
myonuclear number to the same extent as PGF$_{2a}$, does not affect myoblast proliferation or survival, differentiation, or fusion to lead to muscle growth.

After the initial fusion of myoblasts that forms a multinu-
cell fusion that contributes to muscle growth can be determined. To determine if PGF$_{2a}$ increases muscle cell size by enhancing addition of myonu-
cell to existing myotubes, the number of nuclei in individual
were treated with a specific FP agonist (fluprostenol) that
increases muscle cell size by enhancing addition of myonu-
cellular number to the same extent as PGF$_{2a}$, but at lower
treatment is the strongest inducer of NFAT activation and increases muscle cell size, as well as an increase in myonuclear number to the same extent as PGF$_{2a}$, at similar doses (Fig. 2 B).

To further study the effect of PGF$_{2a}$ on increases in myonu-
treated with PGF$_{2a}$ at different stages of fusion. To determine if
PGF$_{2a}$ can act at the initial stages of cell fusion, cells were
were only treated with PGF$_{2a}$ at the onset of differentiation at 0 h in
differentiation media (DM). To determine if PGF$_{2a}$ acts
during later fusion events, cells were only treated at 24 h, a
time when cells are beginning to fuse and a few multinucleated
cells are present (Fig. 1 A). In both cases, the nu-
clear number of individual myotubes was analyzed at 48 h, as
in Fig. 1 E. When PGF$_{2a}$ is administered at the onset of dif-
ferentiation, no significant difference exists in the per-
centage of myotubes with five or more nuclei as compared with
vehicle-treated cultures (Fig. 1 G). However, when
PGF$_{2a}$ is administered at 24 h, the percentage of myotubes
with five or more nuclei is significantly higher than vehicle-
treated cells. This difference is comparable to the increase in
nuclear number when PGF$_{2a}$ is added at both 0 and 24 h. These data further confirm that PGF$_{2a}$ acts at later stages of
muscle cell fusion to allow an increase in muscle cell size.

Activation of the FP receptor induces cell growth
PGF$_{2a}$ primarily mediates its cellular effects by binding with
high affinity ($K_i = 3.4$ nM) to the FP prostanoid receptor
(Breyer et al., 2001). However, PGF$_{2a}$ can also bind with
lower affinity to EP$_1$ ($K_i = 1,300$ nM) and EP$_3$ ($K_i = 75$
NMs) receptors. To determine if muscle growth induced by
the addition of PGF$_{2a}$ occurs through the FP receptor, cells were
treated with a specific FP agonist (fluprostenol) that
has a similar affinity for the FP receptor as PGF$_{2a}$ ($K_i = 3.8$
MNs) but does not bind to other prostanoid receptors (Breyer et al., 2001). Fluprostenol induces an increase in muscle cell
size (Fig. 2 A) as well as an increase in myonuclear number to the same extent as PGF$_{2a}$, at similar doses (Fig. 2 B).

To determine if endogenous PGF$_{2a}$ regulates myonuclear
accretion and acts through the FP receptor, cells were
treated with a selective, competitive FP antagonist, AL-8810
($K_i = 426$ nM; Griffin et al., 1998, 1999), after 24 h in
DM corresponding to the later stages of fusion. In the presence of $10^{-7} M$ AL-8810, myotube cultures contain few
cells with five or more nuclei (Fig. 2 C). Together, these
data suggest that PGF$_{2a}$-induced muscle growth is mediated
through the FP receptor and that endogenous PGF$_{2a}$ is re-
quired for muscle growth.

NFAT activity is required for muscle growth by PGF$_{2a}$
PGF$_{2a}$ signaling is known to increase levels of intracellular
calcium in a variety of cell types including smooth muscle
and cardiac muscle (Yew et al., 1998; Yousufzai and Abdel-
Latif, 1998). NFAT is a family of calcium-regulated tran-
scription factors that has been implicated in skeletal muscle
growth (Musaro et al., 1999; Horsley et al., 2001; Kegley et
al., 2001). To investigate whether NFAT is involved in
PGF$_{2a}$-induced skeletal muscle growth, cells were infected
with a retrovirus encoding VIVIT, a specific peptide inhibi-
tor of NFAT activation. VIVIT acts by preventing the inter-
action between NFAT and calcineurin but not between cal-
icineurin and other substrates (Aramburu et al., 1999; Friday
et al., 2000; Friday and Pavlath, 2001). Primary muscle cells
infected with control retrovirus exhibit an increase in cell
size when treated with $10^{-6} M$ PGF$_{2a}$ (Fig. 3 A), similar to
uninfected cells (Fig. 1 A). In contrast, cells infected with VIVIT retrovirus do not increase cell size when treated with PGF2α/H9251. To quantify these observations, we analyzed the cultures with the nuclear number assay used in Fig. 1. Cells infected with control retrovirus and treated with PGF2α/H9251 show a significant increase in the percentage of myotubes with five or more nuclei. However, cells infected with the VIVIT retrovirus and treated with PGF2α/H9251 are similar in nuclear number to nontreated cells. These data implicate that a calcineurin- and NFAT-dependent signaling pathway is involved in skeletal muscle growth induced by PGF2α.

**PGF2α activates nuclear translocation and transcriptional activity of NFAT**

Given the requirement of NFAT in PGF2α-induced muscle growth, direct activation of NFAT signaling by PGF2α/H9251 was analyzed in muscle cells. First, cells were transiently transfected with an NFATC2-GFP fusion construct and treated with 10−6 M PGF2α after 24 h in DM. Without treatment, cells expressing NFATC2-GFP exhibit GFP throughout the cell (Fig. 4 A). Stimulation with PGF2α results in translocation and accumulation of NFAT in nuclei of myotubes. This nuclear translocation of NFAT is blocked by treatment with cyclosporine A (CsA), suggesting that calcineurin is activated by PGF2α. In addition, PGF2α-induced nuclear translocation of NFATC2 is inhibited by cotreatment with the FP antagonist, AL-8810. Thus, stimulation of the FP receptor is responsible for NFAT activation and not a nonspecific increase in intracellular calcium at this dose of PGF2α. Transcriptional activity of NFAT was also analyzed in cells containing a NFAT responsive luciferase reporter construct (Fig. 5 B). Luciferase activity is increased in cells treated with 10−6 M PGF by approximately ninefold. Together, these data indicate that PGF2α activates NFAT signaling in skeletal muscle.

**PGF2α affects myotube size through NFATC2**

Three isoforms of NFAT are expressed in skeletal muscle cells (Abbott et al., 1998). Because the NFATC2 isoform regulates skeletal muscle growth (Horsley et al., 2001) and is activated by PGF2α (Fig. 4), we investigated whether NFATC2 is required for cell growth induced by PGF2α by examining NFATC2−/− muscle cells treated with PGF2α. NFATC2−/− cells differentiate and initially fuse normally but have a defect in further myonuclear accretion that prevents muscle cell growth (Horsley et al., 2001). Addition of PGF2α to NFATC2−/− cultures does not increase myotube size (Fig. 5...
NFATC2 is not necessary for the expression of the FP receptor in these mutant cells. To determine if NFATC2 regulates the expression of the FP receptor, mRNA levels of the FP receptor were examined in wild-type and NFATC2-/- cells using RT-PCR. As shown in Fig. 5 C, the FP receptor mRNA expression was examined by RT-PCR after 24 h in DM in wild-type and NFATC2-/- muscle cells. Representative ethidium bromide staining of acrylamide gel is shown with 18S rRNA as an internal control.

Figure 5. PGF2alpha does not induce myotube growth or fusion of NFATC2-/- muscle cells. (A) NFATC2-/- myoblasts were induced to differentiate and treated with vehicle or 10^-6 M PGF2alpha for 48 h, followed by immunostaining for EMyHC. Bar, 60 μm. (B) Nuclear number assays were performed as in Fig. 1 E on NFATC2-/- cultures treated with vehicle or PGF2alpha at a range of doses. Data are the mean ± SEM of three independent cell isolates. (C) FP receptor mRNA expression was examined by RT-PCR after 24 h in DM in wild-type and NFATC2-/- muscle cells. Representative ethidium bromide staining of acrylamide gel is shown with 18S rRNA as an internal control.

To further test whether PGF2alpha requires NFATC2 to induce muscle growth, we determined if expression of a recombinant NFATC2 in NFATC2-/- muscle cells could rescue the inability of these cells to increase in cell size and nuclear number in response to PGF2alpha. When treated with 10^-6 M PGF2alpha, NFATC2-/- muscle cells infected with control retrovirus do not increase in size (Fig. 6 A) or in the percentage of myotubes with five or more nuclei (Fig. 6 B). Consistent with previous results (Horsley et al., 2001), expression of a recombinant NFATC2 in NFATC2-/- muscle cells restores myotube growth. However, NFATC2-/- cells expressing a recombinant NFATC2 and treated with PGF2alpha exhibit a greatly enhanced cell size relative to similarly treated wild-type cells that is associated with an increase in nuclear number. These data further demonstrate that PGF2alpha-induced muscle growth is mediated through NFATC2-dependent pathways.

Discussion

The majority of molecules known to regulate muscle cell fusion in mammals mediate the initial formation of a multinucleated muscle cell (Knudsen, 1992; Yagami-Hiromasa et al., 1995; Barnoy et al., 1996; Gorza and Vitadello, 2000). Recent work in Drosophila (Rau et al., 2001) and in mammals (Horsley et al., 2001) has identified genes that regulate initial fusion events that form a myotube distinctly from the cell fusion that occurs with an existing myotube. These data suggest that the formation of a large multinucleated muscle cell involves two stages of fusion. Thus, initially, a subset of mononucleated muscle cells fuse with each other to form small nascent myotubes containing several nuclei. Subsequently, additional differentiated muscle cells fuse with the nascent myotube and muscle growth occurs. In this paper, we show that PGF2alpha does not act on the initial fusion of muscle cells that forms myotubes because PGF2alpha does not affect the percentage of nuclei in myotubes (Fig. 1 D). Rather, PGF2alpha is a novel regulator of the second stage of muscle cell fusion as it increases the number of nuclei within myotubes (Fig. 1, E and F). Treatment of muscle cells after the formation of myotubes has begun is sufficient to induce this increase in myonuclear number (Fig. 1 G). Thus, in contrast to other molecules, PGF2alpha mediates muscle cell fusion in skeletal muscle cells.
growth by enhancing myonuclear accretion in the nascent myotube. Because myoblast fusion requires multiple steps (Wakelam, 1985), PGF₂α can enhance myonuclear accretion by effecting cell motility, alignment, recognition, adhesion, or membrane union.

PGs are a large family of molecules and only a few specific PGs have been studied in myogenesis. Previously, the effect of PGF₂α on muscle cell fusion was examined with doses of PGF₂α higher than 10⁻⁶ M (Rossi et al., 1989). No effect was seen with these doses, which is consistent with our results in Fig. 1 E. Other PGs have been shown to regulate muscle cell fusion. PGE₂ and PGE₁ regulate fusion by inhibiting and increasing the initial fusion of myoblasts, respectively (Zalin, 1977; Entwistle et al., 1986; Rossi et al., 1989). Consistent with a general role for PGs in muscle cell fusion and growth, inhibition of PG synthesis with inhibitors of the cyclooxygenase enzymes inhibits myoblast fusion (Zalin, 1977; David and Higginbotham, 1981; Entwistle et al., 1986) and the growth of myofibers in vivo (Templeton et al., 1986; McLennan, 1987). Given the differential effect of individual PG molecules on skeletal muscle cells, the synthesis of PG molecules is likely coordinated to control multiple steps of muscle growth.

What is the signal transduction pathway by which PGF₂α induces muscle growth? Our data are consistent with a role for the PGF₂α receptor, FP. Treatment of cells with fluprostanol, a specific FP receptor agonist, can activate increases in myotube size and nuclear number, similar to PGF₂α (Fig. 2). Muscle growth is inhibited by AL-8810, a specific FP receptor antagonist. In addition, the FP receptor is expressed in skeletal muscle cells at the time of growth and fusion (Fig. 5 C). Several lines of evidence indicate that downstream of FP receptor activation a calcineurin/NFAT-dependent pathway is the key requirement for PGF₂α-induced muscle growth. Expression of a specific inhibitor of NFAT activation by calcineurin completely abrogates the effects of PGF₂α on cell growth. In addition, PGF₂α induces nuclear translocation of NFATC2 in a calcineurin and FP receptor-dependent manner. Furthermore, increased cell size or myonuclear number does not occur in response to PGF₂α in NFATC2⁻/⁻ cells, but is restored when recombinant NFATC2 is introduced into the cells. Indirectly, the time in myogenesis in which PGF₂α is effective also supports a role for the involvement of NFAT. The timing of PGF₂α action on myotube growth (Fig. 1 G) concurs with the timing of NFATC2 activation in skeletal muscle cells (Abbott et al., 1998), and is consistent with a role for NFATC2 during the second stage of cell fusion during myotube growth (Horsley et al., 2001).

Together, these data strongly support a requirement for NFATC2 in PGF₂α-induced skeletal muscle growth, and implicate a calcium signaling pathway downstream of PGF₂α in skeletal muscle growth. The mechanism by which NFATC2 regulates muscle growth is unknown. Few NFAT-regulated genes are known in cell types outside of the immune system (Horsley and Pavlath, 2002). The identification of the genes regulated by the PGF₂α-NFATC2 signaling pathway in skeletal muscle may reveal novel mechanisms of muscle growth.

Activity is a potent stimulus for muscle growth. Such muscle stimulation leads to release of PGF₂α (Vandenburgh et al., 1995; Trappe et al., 2001) as well as activation of transcription factors such as NFAT (Liu et al., 2001; Kubis et al., 2002). The PGF₂α-NFATC2 pathway described here may contribute to regulating muscle growth in vivo. PGF₂α stimulation of cells overexpressing recombinant NFATC2 leads to enhanced cell fusion, and dramatic increases in cell size or hypertrophy (Fig. 6). PGF₂α can induce hypertrophy of other muscle cell types. Hypertrophic growth of cardiac myocytes in vitro is stimulated by PGF₂α (Adams et al., 1996; Lai et al., 1996; Kunapuli et al., 1998). In addition, exogenous PGF₂α can stimulate vascular smooth muscle hypertrophy (Dorn et al., 1992). Because cardiac and smooth muscle are mononucleated cells, hypertrophy of these cell types does not require cell fusion but involves protein accumulation and thus differs from hypertrophy of skeletal muscle cells, which involves both cell fusion and increased protein accumulation. Other studies have demonstrated that PGF₂α does increase protein synthesis in skeletal muscle cells (Vandenburgh et al., 1990). By activating both cell fusion and protein synthesis, PGF₂α may regulate hypertrophy of skeletal muscle in vivo.

The function of the PGF₂α-NFATC2 pathway in skeletal muscle may be clinically relevant. Nonsteroidal antiinflammatory drugs such as celebrex and ibuprofen are a widespread treatment for inflammation and pain relief. Often these drugs are prescribed after surgery or muscle injury. As these drugs are inhibitors of cyclooxygenase enzyme activity, PG synthesis may be decreased and lead to deleterious effects in skeletal muscle (Mishra et al., 1995), as recently shown for bone (Simon et al., 2002; Zhang et al., 2002). Our data suggest that by blocking the production of PGF₂α in skeletal muscle, muscle growth and repair may be impaired in patients taking nonsteroidal antiinflammatory drugs after muscle atrophy, disease, or injury.

Materials and methods

Cell culture

Primary myoblast cultures were prepared from tibialis anterior muscles of three adult wild-type and three NFATC2⁻/⁻ Balb/c mice (Horsley et al., 2001) and purified to >99% as described previously (Rando and Blau, 1994; Pavlath et al., 1998). Growth media consisted of Ham's F10, 20% FBS, 5 ng/ml bFGF, 100 U/ml penicillin G, and 100 μg/ml streptomycin. Differentiation was induced by plating cells on E-C-L (Upstate Biotechnology)-coated dishes and switching the media to a low serum DM in DME with either 2% horse serum or insulin-transferin-selenium-A supplement (GIBCO BRL).

Plasmid production, retroviral infection, and transient transfections

The GFP-VIVIT and NFATC2 retroviral constructs have been described previously (Friday et al., 2000; Horsley et al., 2001). An NFATC2-GFP construct was created by PCR amplification of NFATC2 CDNA from the vector pREP4-NFATC2 to generate a 2.8-kb product (Ranger et al., 2000). The forward primer consisted of an EcoRI site followed by bases 224–241 of the human NFATC2 mRNA (GenBank/EMBL/DDBJ accession no. U43342), whereas the reverse primer contained a XmaI site and bases 9251–9253 of the human NFATC2 mRNA (GenBank/EMBL/DDBJ accession no. U43342), whereas the reverse primer contained a XmaI site and bases 9251–9253 of the human NFATC2 mRNA (GenBank/EMBL/DDBJ accession no. U43342). The NFATC2 vector contained NFATC2 cDNA linked to nine copies of a consensus NFAT binding site without an associated minimal MHC promoter linked to nine copies of a consensus NFAT binding site without an associated AP-1 site. Production of infectious retrovirus and infection of primary myoblasts were performed as described previously (Abbott et al., 1998). Cells were subject to two rounds of infection with an efficiency of gene transfer of >90% based on visualization of green fluorescent protein. Experiments us-
ing the NFATC2 retrovirus were begun 48 h after the final infection when maximum levels of gene expression were achieved. In contrast, experiments using VIVIT retrovirus were begun 24 h after the final infection, before maximum levels of gene expression in order for normal myotube formation to occur.

For transient transfections, cultures were plated in 6-well dishes at 2.5 × 10^5 cells/well. For each well, 4 μg DNA was complexed with 6 μl Lipofectamine 2000 (Promega) for 20 min at room temperature in a total volume of 500 μl in Ham's F10. The DNA–Lipofectamine mixture was added to wells containing 2 ml Ham's F10 and incubated for 4 h at 37°C after which the cells were re-fed with fresh growth media.

Drug treatment
Primary myotubes were plated at 2 × 10^5 cells per well of 6-well dishes. After 2 h, cells were placed in DM with either vehicle (0.095% ethanol); PGF_2α (Sigma-Aldrich), 17-phosphodiesterase (Cayman Chemical), or a protostroph (BIOMOL Research Laboratories, Inc.), and drugs were replenished at 24 h unless otherwise noted. Cells were treated with AL-8810 (Sigma-Aldrich) after 24 h in DM. Doses were chosen and used in the range where maximal effects were shown in a variety of assays using cardiac (Adams et al., 1996; Kunapuli et al., 1998; Yew et al., 1998) and smooth muscle cells (Griffin et al., 1998; Katsuyama et al., 2002).

Differentiation and fusion assays
After 24 or 48 h in DM, cells were fixed in either ice-cold methanol or 3.7% formaldehyde for 10 min and nonspecific binding was blocked with TMB buffer (DENL Life Science Products) for 1 h at room temperature. The cells were incubated with an antibody against EMyHC (F.I.632, neot hybride Ia [Denmark]; Developmental Studies Hybridoma Bank) for 1 h at room temperature. Cells were washed in PBS with 0.1% Tween, and then incubated in biotinylated goat anti–mouse IgG (1:200; Jackson Immunoresearch Laboratories). Antibody binding was detected using Vectastain Elite ABC reagent (Vector Laboratories) and diaminobenzidine.

To analyze differentiation, the number of nuclei in EMyHC-positive cells was counted and expressed as a percentage of the total number of nuclei analyzed (250). The fusion index was determined by dividing the number of nuclei within myotubes (two or more nuclei) by the total number of nuclei analyzed (100–250). Fusion was also analyzed by performing nuclear number assays. The number of nuclei within individual myotubes was counted for 50–100 myotubes. Myotubes were grouped into two categories: those with two to four nuclei and those with five or more nuclei. The percentage of myotubes in each category was calculated.

DNA quantification
The DNA content of whole cell lysates was quantified after 48 h in DM for vehicle and PGF_2α-treated cells. Cells were washed twice in ice-cold PBS, scraped off of the dish, and collected by centrifugation at 10,000 g at 4°C. Cell lysates were resuspended in 250 μl of saline phosphate buffer (0.05 M NaPO_4, and 2 M NaCl, pH 7.4) and frozen. Samples were thawed and sonicated for 15 s, and a 50-μl aliquot of each sample was added to the buffer containing 0.5 μg/ml Hoechst 33258 (Molecular Probes). DNA concentration was determined by measuring the emission at 465 nm after excitation at 365 nm (Labarca and Paigen, 1980) using an Amicon-Bowman luminescence spectrophotometer (Spectronic Instruments). Calf thymus DNA was used to construct the standard curve.

FP receptor RT-PCR
RNA was isolated from wild-type and NFATC2−/− muscle cells after 24 h in DM using TRizol reagent (Life Technologies). RT-PCR was performed for each sample using specific primers for the murine FP receptor (GenBank/EMBL/DDBJ accession D17431): sense, 5′-CACAACCTGGCCAGACGAGAAC-3′; antisense, 5′-ATGGCGGACACAGCGCGACGAC-3′; 452 bp pair. 18S RNA was used as an internal control in each sample using QuantumRNA 18S primers (Ambion). The amplification cycle for the FP receptor consisted of 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 61.5°C for 30 s, 72°C for 45 s, and termination at 72°C for 5 min. The amplification cycle for the 18S RNA consisted of 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 57°C for 30 s, 72°C for 30 s, and termination at 72°C for 5 min. The products were resolved by electrophoresis on a 1.5% agarose gel and visualized by ethidium bromide staining.

NFATC2 nuclear translocation
24 h after transient transfection, cells were plated at 2 × 10^5 cells per well of ECL-coated 6-well plates, and switched to DM after 2 h. After 24 h in DM, cells were placed in DME for 3 h, treated with 10−6 M PGF_2α alone or in combination with 10−6 M CsA or 10−6 M AL-8810 for 30 min, and fixed with 3.7% formaldehyde.

Reporter assays
24 h after transient transfection with an NFAT reporter construct, myoblasts were plated at 7.5 × 10^5 cells per well of ECL-coated 24-well plate. The medium was replaced with DME and the cells were allowed to differentiate for 24 h. Cells were stimulated with 10−6 M PGF_2α alone or in combination with 10−6 M CsA for 5 h and assayed for luciferase as described previously (Abbott et al., 1998).

Statistics
To determine significance between two groups, comparisons were made using t tests. Analyses of multiple groups were performed using one-way ANOVA with Bonferroni's post test using GraphPad Prism version 3.02 for Macintosh (GraphPad Software). For all statistical tests, the 0.05 level of confidence was accepted for statistical significance.

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