NFAT4 Movement in Native Smooth Muscle

A ROLE FOR DIFFERENTIAL Ca\textsuperscript{2+} SIGNALING

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The transcription factor NFAT (nuclear factor of activated T-cells) plays a central role in mediating Ca\textsuperscript{2+}-dependent gene transcription in a variety of cell types. Sustained increases in intracellular calcium concentration ([Ca\textsuperscript{2+}]) are presumed to be required for NFAT dephosphorylation by the Ca\textsuperscript{2+}/calmodulin-dependent protein calcineurin and its subsequent nuclear translocation. Here, we provide the first identification and characterization of NFAT in native smooth muscle, showing that NFAT4 is the predominant isoform detected by reverse transcriptase-polymerase chain reaction and Western blot analysis. PDGF induces NFAT4 translocation in smooth muscle, leading to an increase in NFAT transcriptional activity. NFAT4 activation by PDGF depends on Ca\textsuperscript{2+} entry through voltage-dependent Ca\textsuperscript{2+} channels, because its nuclear accumulation is prevented by the Ca\textsuperscript{2+} channel blocker nisoldipine and the K\textsuperscript{+} channel opener pinacidil. Interestingly, elevation of [Ca\textsuperscript{2+}]\textsubscript{i} by membrane depolarization or ionomycin treatment are not effective stimuli for NFAT4 nuclear accumulation, indicating that Ca\textsuperscript{2+} influx is necessary but not sufficient for NFAT4 activation. In contrast, membrane depolarization readily activates the Ca\textsuperscript{2+}-dependent transcription factor CREB (cAMP-responsive element-binding protein). The calcineurin blockers CsA and FK506 also prevented the PDGF-induced NFAT4 nuclear localization. These results indicate that both the nature of the calcium signal and PDGF-induced modulation of nuclear import-export of NFAT are critical for NFAT4 activation in this tissue.

Calcium ions (Ca\textsuperscript{2+}) play a central role in the physiology of all cells. In arterial smooth muscle, for example, global changes in intracellular calcium concentration ([Ca\textsuperscript{2+}],\textsuperscript{[3]} control tonic contractions that lead to changes in arterial diameter (1, 2). In the gut, the phasic behavior of ileal smooth muscle cells that is essential for the normal function of this tissue is determined by recurrent action potentials that are mediated by voltage-dependent Ca\textsuperscript{2+} channels (VDCC). These action potentials deliver surges of Ca\textsuperscript{2+} to the interior of the cell in the form of repetitive Ca\textsuperscript{2+} spikes (3). Ca\textsuperscript{2+} signal modulation in smooth muscle takes on additional forms, including localized transient releases of Ca\textsuperscript{2+} through ryanodine receptors in the sarcoplasmic reticulum, known as Ca\textsuperscript{2+} sparks, as well as propagating Ca\textsuperscript{2+} waves that traverse the length of the cell and display distinctive frequency and amplitude properties (2, 4, 5).

It is becoming increasingly clear that [Ca\textsuperscript{2+}]\textsubscript{i}, also serves an important second messenger role in the regulation of gene expression in smooth muscle. We have recently demonstrated that activation of the cAMP-responsive element-binding protein (CREB) and subsequent c-fos expression can be induced by depolarization in native arterial smooth muscle, a process that is mediated by calmodulin-dependent kinase (CaMK) and is dependent on Ca\textsuperscript{2+} influx through VDCC (6). The Ca\textsuperscript{2+}-sensitive transcription factor, NFAT (nuclear factor of activated T-cells) may play a role in smooth muscle, as suggested by results from the A7r5 aortic smooth muscle cell line (7), but this possibility has remained unexplored in native tissue.

NFAT represents a family of Ca\textsuperscript{2+}-dependent transcription factors comprising four well-characterized members, designated NFAT1 (NFATc2/p), NFAT2 (NFATc1/c), NFAT3 (NFATc4), and NFAT4 (NFATc3/x) (8). Although originally thought to be largely restricted to cells of the immune system, NFAT has since been shown to play a role in other cell types, including cardiac and skeletal myocytes and neurons. In non-immune cells, NFAT has been shown to regulate heart valve development, control the differentiation of skeletal myocytes into slow- or fast-twitch fiber types, and contribute to the development of hypertrophy in cardiac and skeletal myocytes (9–13). It has also been suggested that NFAT plays a role in long term memory in neurons (14).

Most NFAT isoforms are constitutively expressed and exist as transcriptionally inactive, cytosolic phosphoproteins. Stimuli that activate NFAT do so, in part, by increasing [Ca\textsuperscript{2+}], and NFAT activation appears to be strictly dependent on this increase. Upon elevation of [Ca\textsuperscript{2+}], the Ca\textsuperscript{2+}/calmodulin-dependent phosphatase calcineurin dephosphorylates NFAT, leading to the unmasking of nuclear localization signals and the transcription of NFAT to the nucleus (15–17). Calcineurin-dependent dephosphorylation of NFAT has been shown to be inhibited by the immunosuppressant drugs cyclosporin A (CsA) and FK506 (16). The subcellular localization of NFAT is dynamically dependent on a balance between the cytosolic phosphatase activity of calcineurin and the activity of incompletely characterized nuclear kinases, which promote export of NFAT.

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NFAT4 and CREB Discriminate between Different Ca²⁺ Signals

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from the nucleus. Both import and export processes can be regulated in an isofrom-selective manner (18–22).

Temporal and spatial features of the Ca²⁺ signal are important determinants of NFAT activation. Stimuli that provoke a sustained moderate elevation in global [Ca²⁺], have consistently been shown to effectively promote NFAT nuclear translocation in non-excitable cells. In these cell types, the Ca²⁺ requirement may be provided by release from intracellular stores coupled with influx of extracellular Ca²⁺ through capacitative Ca²⁺ entry pathways (23). In hippocampal neurons, NFAT activation is mediated by Ca²⁺ influx through L-type Ca²⁺ channels, but not NMDA receptors, suggesting the possibility of privileged communication between specific Ca²⁺ entry pathways and NFAT activation (14). NFAT activity can also be induced in neurons by a brief depolarizing stimulus, indicating that the Ca²⁺ signaling component of NFAT activation may display cell-type specific requirements. Ca²⁺ oscillations, which can occur in both excitatory and non-excitable cells, can also increase the efficiency and specificity of gene expression by selectively activating certain transcription factors, including NFAT (24, 25).

Platelet-derived growth factor (PDGF) plays an important role in a number of pathophysiological processes. It has been shown to contribute to the healing of gastrointestinal ulcers, which can occur in both excitatory and non-excitable cells, can also increase the efficiency and specificity of gene expression by selectively activating certain transcription factors, including NFAT (24, 25).

EXPERIMENTAL PROCEDURES

Tissue Preparation—Adult female CD-1 mice (20–25 g, Charles River Laboratories, Canada) were euthanized by peritoneal injection of pentobarbital solution (200 mg/kg). For isolation of ileal strips, a segment of ileum was detached from mesenterium and the outer longitudinal and several layers of the inner circular smooth muscle were removed. Ileal smooth muscle sheets were cut into sections for use in immunofluorescence experiments. For RT-PCR analysis of NFAT expression in isolated cells, smooth muscle cells were enzymatically dispersed as described by Gomez and Sward (29). Briefly, the sheets were cut into pieces and incubated for 10 min at 35 °C in 2 ml of the dispersion medium (in mM: 110 NaCl, 5 KCl, 0.16 CaCl₂, 2 MgCl₂, 10 HEPES, 10 NaHCO₃, 0.5 KH₂PO₄, 0.5 NaH₂PO₄, 10 glucose, 0.49 EDTA, 10 taurine, pH 7.0) containing 1.5 mg ml⁻¹ collagenase (FLUKA, Switzerland), 1 mg ml⁻¹ papain ( Worthington Biochemical Corporation, Lakewood, NJ), 4.5 mg ml⁻¹ bovine serum albumin (Sigma) and 1 mg ml⁻¹ dithioerythritol. After isolation, cells were allowed to settle in a chamber mounted on an inverted microscope and collected using a micropipette (5–10 μm diameter). The cells were transferred to nuclease-free microcentrifuge tubes, flash-frozen in liquid nitrogen, and stored at −80 °C for future use in RT-PCR.

Immunofluorescence—Ileal sheets (1–3 mm wide, 5–8 mm long) were treated at room temperature with various agents and times as specified in the text and then mounted onto glass slides. After air-drying for 5 min, sheets were fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS, pH 7.4) for 15 min, permeabilized with 0.1% Triton X-100 in PBS for 10 min and blocked for 1 h with 2% bovine serum albumin in PBS. Primary antibody dilutions in 2% bovine serum albumin/PBS, rabbit anti-NFAT4/c3 (Santa Cruz Biotechnologies) (1:250 dilution) and rabbit anti-P-CREB (1:250 dilution), were applied overnight at 4 °C. Secondary antibody, Cy3-anti-rabbit IgG or Cy5-anti-rabbit IgG (Jackson ImmunoResearch Laboratories) (1:500 dilution) was applied for 1 h at 25 °C. The fluorescent nucleic acid dye YOYO-1 (1:10 000 dilution) (Molecular Probes) was applied for 10 min. After washing, nuclei were mounted (Aquapharm Mounting medium, Polysciences) and examined at ×40 magnification using a Bio-Rad 1000 laser scanning confocal microscope. Red fluorescence of NFAT4/P-CREB was measured at an excitation wavelength of 550 and/or 650 nm, and emission was measured at 570 and/or 670 nm, for Cy3 and Cy5 respectively. Specificity of immune staining was confirmed by the absence of fluorescence signals when incubated with primary or secondary antibodies alone. For scoring of NFAT4- and P-CREB-positive nuclei, multi-field for each smooth muscle strip were imaged and counted by two independent observers under double-blind conditions. For quantification, a cell was considered positive if co-localization (yellow) was observed in the nucleus, whereas a cell was considered negative if no co-localization (green) was visualized.

Western Blot Analysis—Tissue samples were homogenized in cell lysis buffer (in mM: 50 Tris, 150 NaCl, 1 EDTA, 1% Nonidet P-40, pH 8.0) containing phenylmethylsulfonyl fluoride (1 μl), pepstatin (20 μg/ml), leupeptin (20 μg/ml), and aprotinin (0.5 μg/ml). Protein concentrations were determined by the Bradford dye-binding assay using bovine serum albumin as a standard. Aliquots of tissue extracts containing equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis on 8% gels using the Laemmli buffering system. Proteins were transferred into Immuno-Blot polyvinylidene difluoride membranes (Bio-Rad) and blocked by rocking for 1 h at room temperature. Membranes were incubated with a mouse monoclonal antibody against NFAT4, Santa Cruz Biotechnology) and secondary antibodies were applied as a standard. Optical density measurements were normalized to starting protein concentration determined using a protein assay reagent (Bio-Rad) and expressed as relative luciferase units.

Statistics—Results are expressed as means ± S.E. where applicable. All statistical analysis was performed using GraphPad software (Prism 3.0). Statistical significance was determined using the two-tailed unequal paired Student’s t test.
**RESULTS**

**NFAT4 Is the Predominant Isoform Expressed in Native Smooth Muscle**—We have used RT-PCR analysis and immunoblotting to identify NFAT isoforms expressed in native ileal smooth muscle. Previous results from experiments employing the rat A7r5 aortic smooth muscle cell line suggested that the NFAT1 and NFAT2 isoforms are expressed in smooth muscle (7), although expression of additional isoforms could not be ruled out in this study. Using RT-PCR analysis, we find no evidence for NFAT1 or NFAT2 expression in ileal smooth muscle sheets (Fig. 1B) using primer pairs that efficiently amplify NFAT1 from spleen and NFAT2 from thymus (Fig. 1A). Instead, we find that the NFAT4 and, perhaps to a lesser extent, the NFAT3 isoforms are constitutively expressed, as shown in Fig. 1B. We have found a similar pattern of NFAT isoform expression in native arterial smooth muscle of the aorta and the cerebral vasculature (30, 31). NFAT4 appears to be the predominant isoform expressed, based on results obtained from an RT-PCR analysis of isolated smooth muscle cells (Fig. 1C). NFAT3 identified in RT-PCR analysis of intact ileal strips (Fig. 1B) may represent expression in co-isolated neurons of the nerve plexus between longitudinal and circular smooth muscle layers, which is consistent with previous observations in neurons (14).

At the protein level, NFAT4 isolated from ileal smooth muscle strips runs as multiple high molecular mass bands on SDS gels with a single band at ~160 kDa, which is similar to that previously reported in Jurkat cells (32) and as additional, apparently nonspecific bands at 75–100 kDa (Fig. 1D). Differences in the mobility of the high molecular mass band between smooth muscle and thymus (which expresses NFAT4 at high levels) may reflect tissue differences in the expression of known NFAT4 splice variants (33).

**NFAT4 Nuclear Translocation Is Induced in Ileal Smooth Muscle by Treatment with PDGF**—The potent smooth muscle cell mitogen PDGF has been previously shown to induce NFAT activation in the rat A7r5 aortic smooth muscle cell line (7). To explore NFAT activation in native smooth muscle cells, we treated ileal smooth muscle strips with PDGF (10 ng/ml) for 30 min and analyzed for NFAT nuclear accumulation by immunostaining with an antibody specific for NFAT4. Sections were also stained with the DNA binding dye YOYO-1 to identify nuclei. PDGF induces nuclear translocation of NFAT4 in ileal smooth muscle (Fig. 2), with 47.4 ± 6.7% of the cells in PDGF-treated strips exhibiting nuclear localization of NFAT4, compared with 5.8 ± 1.2% of the cells in untreated control strips. PDGF-induced nuclear accumulation is completely blocked in the presence of the calcineurin inhibitors CsA or FK506 (Fig. 2), which reduce the percentage of NFAT-positive nuclei to 5.7 ± 5.7 and 4.3 ± 2.0, respectively. PDGF-induced NFAT4 nuclear translocation appears to reflect a direct action on smooth muscle rather than an indirect action through associated neurons in the plexus layer since stimulation with PDGF in the presence of a mixture of neurotransmitter inhibitors that includes atropine, tetrodotoxin, phentolamine, and propanolol (1 μM each), does not prevent NFAT4 nuclear accumulation (60.9 ± 12.8% NFAT4-positive nuclei, Fig. 2). The percentage of NFAT4-positive nuclei (5.4 ± 1.9%) in ileal strips treated with the neurotransmitter inhibitory mixture alone does not significantly differ from controls (Fig. 2).

Nuclear translocation of NFAT4 is first evident at 10–15 min after exposure to PDGF, with a clustering of NFAT4 staining in the vicinity of the nuclear envelope (Fig. 3). After 30 min of PDGF treatment, redistribution to the nucleus is observed. The clustering of NFAT4 around the nucleus prior to translocation suggests the possibility that a distinct docking step is involved in the nuclear translocation process.

**PDGF Induces Transcriptional Activity in Ileal Smooth Muscle**—To determine whether the observed NFAT4 nuclear accumulation is associated with increased transcriptional activity, ileal smooth muscle strips from a transgenic mouse that uniformly expresses an NFAT luciferase reporter-promoter construct (34) were treated with PDGF (10 ng/ml) and harvested after 6 or 24 h in serum-free medium (37 °C). Samples were homogenized as described under “Experimental Procedures” and assayed for protein-normalized luciferase activity. We found that the PDGF-induced NFAT4 accumulation is accompanied by an increase in NFAT-dependent transcriptional activity (Fig. 4). This transcriptional activity is transient, being evident at 6-h post-PDGF treatment, but not after 24 h, and is effectively induced by a brief (30 min) exposure to PDGF.

**PDGF-induced NFAT4 Nuclear Translocation Is Dependent on Voltage-dependent Ca2+ Channels**—[Ca2+]i, regulates nuclear accumulation of NFAT, and both the source of Ca2+ and
the nature of the Ca\(^{2+}\) signal may be important in determining the nature of the response (24, 25). In ileal strips, treatment with the dihydropyridine inhibitor of voltage-dependent L-type Ca\(^{2+}\) channels, nisoldipine (100 nM), results in a complete abrogation of PDGF-induced NFAT4 nuclear accumulation (Fig. 5C; 6.7 ± 5.6% NFAT4-positive nuclei). Nisoldipine is equally effective when added before (Fig. 5C) or concurrent with PDGF exposure (data not shown). Thus, PDGF-induced mobilization of NFAT4 is critically dependent on Ca\(^{2+}\) influx through VDCC. To provide additional evidence for VDCC involvement, we examined the effects of hyperpolarizing the cell membrane, which would decrease Ca\(^{2+}\) entry by closing VDCC. Pinacidil (1 mM), which induces membrane hyperpolarization by opening ATP-sensitive K\(^{+}\) channels (35), completely blocked the PDGF-induced nuclear translocation of NFAT4 (Fig. 5D; 6.74 ± 6.17% NFAT4-positive nuclei). These results indicate that functional VDCC are required for PDGF-induced nuclear accumulation of NFAT. To determine whether direct activation of VDCC is sufficient to induce NFAT4 nuclear accumulation, we treated ileal smooth muscle with 60 mM K\(^{+}\) for up to 30 min, a treatment that has been previously shown to cause a graded and sustained increase in [Ca\(^{2+}\)]\(_i\) in this preparation as a result of VDCC activation (29, 36). Surprisingly, depolarization was incapable of inducing the nuclear accumulation of NFAT4 (Fig. 5 and 6, 10.5 ± 3.5% NFAT4-positive nuclei). Similar results were obtained with the Ca\(^{2+}\) ionophore ionomycin (1 mM, 30 min, 13.1 ± 2.3% NFAT4-positive nuclei, n = 4, total cells = 930). These data indicate that although Ca\(^{2+}\) influx is necessary for PDGF-mediated NFAT4 nuclear accumulation, it is not a sufficient stimulus alone.

Further, we tested whether PDGF was still capable of inducing NFAT4 nuclear accumulation in the presence of a depolarizing stimulus. The PDGF-stimulated increase in NFAT4 nuclear localization was completely blocked in ileal strips treated for 30 min with PDGF (10 ng/ml) and 60 mM K\(^{+}\) (Fig. 5, 8.6 ± 3.8% positive nuclei). These results suggest that NFAT4 nuclear accumulation may require the repetitive Ca\(^{2+}\)-spiking characteristic of ileal smooth muscle (3), which might be modified by PDGF, but is completely abolished by high K\(^{+}\) (29, 36). In time course experiments, neither continuous exposure (30 min) nor brief pulses of 60 mM K\(^{+}\) (5, 10, 15 min) were able to induce NFAT4 nuclear accumulation (Fig. 6). Even when time course experiments were performed in the presence of the L-type Ca\(^{2+}\) channel agonist Bay K 8644 to increase Ca\(^{2+}\) influx...
nuclear accumulation (data not shown).

Ca\textsuperscript{2+}-elevating Stimuli Have Differential Effects on NFAT4 and CREB—We have previously reported that depolarizing stimuli activate the Ca\textsuperscript{2+}-sensitive transcription factor CREB in cerebral artery smooth muscle (6), increasing the fraction of nuclei staining for P-CREB. This response is dependent on the influx of extracellular Ca\textsuperscript{2+} through VDCC and appears to be mediated by CaMK. In ileal smooth muscle, depolarization with 60 mM K\textsuperscript{+} also results in a rapid and robust increase in P-CREB (Fig. 7B). Like NFAT4, CREB is also activated by PDGF in ileal smooth muscle, as evidenced by the prominent nuclear P-CREB staining observed after 30 min of PDGF stimulation (Fig. 7C). Thus, CREB is activated by both PDGF and depolarizing stimuli, whereas depolarization-induced Ca\textsuperscript{2+} influx through VDCC alone is insufficient to stimulate NFAT4 nuclear translocation. These results clearly indicate that these two Ca\textsuperscript{2+}-sensitive transcription factors respond differentially to a given Ca\textsuperscript{2+}-elevating stimulus and provide evidence for Ca\textsuperscript{2+} signal discrimination at the transcription factor level.

**DISCUSSION**

We have found that NFAT4 is expressed in native smooth muscle and can be activated by stimulation with the smooth muscle mitogen PDGF. This activation is dependent on Ca\textsuperscript{2+} influx through VDCCs and can be blocked by calcineurin inhibitors CsA and FK506. Surprisingly, sustained increases in global [Ca\textsuperscript{2+}], induced by treatment with ionomycin or membrane depolarization with K\textsuperscript{+} failed to activate NFAT4 in this tissue. The predominant isoform of NFAT expressed in native ileal smooth muscle is NFAT4, which we have also found abundantly expressed in aortic and cerebral artery smooth muscle tissue (30, 31). NFAT1 and NFAT2 isoforms, which are ubiquitously expressed in cells of the immune system and in other cell types, do not appear to be constitutively expressed at significant levels in these tissues. This is in sharp contrast to results reported for the A7r5 smooth muscle cell line, which expresses both NFAT1 and NFAT2 isoforms (7), suggesting that significant changes in the regulation of NFAT expression take place during the process of cell line establishment. Although it is known that NFAT4 is highly expressed in thymus cells (37) and is also expressed in skeletal muscle (33), these data provide the first evidence that this NFAT isoform is highly expressed in smooth muscle.

It has become axiomatic that stimuli that induce sustained increases in [Ca\textsuperscript{2+}], are sufficient and necessary for NFAT nuclear accumulation. The failure of depolarization-induced elevation in [Ca\textsuperscript{2+}], to induce NFAT4 nuclear accumulation in native ileal smooth muscle was therefore unexpected. Ca\textsuperscript{2+} influx is clearly necessary for PDGF-induced NFAT4 nuclear translocation in native ileal smooth muscle, as evidenced by the ability of VDCC inhibitors and membrane hyperpolarizing agents to completely prevent this translocation. It is possible that modulation of the Ca\textsuperscript{2+} signal may play an important facilitating role in PDGF-induced NFAT4 nuclear accumulation, such that the tonic increase in [Ca\textsuperscript{2+}], induced by depolarization with K\textsuperscript{+} may lack information that is critical to the response of NFAT in this tissue. In ileal smooth muscle, the presence of spontaneous action potentials generates repetitive [Ca\textsuperscript{2+}], spiking that may constitute an appropriately modulated Ca\textsuperscript{2+} signal. PDGF has also been shown to give rise directly to Ca\textsuperscript{2+} waves or oscillations (38, 39). The spontaneous

![Fig. 4. PDGF exposure induces a transient increase in NFAT4 transcriptional activity. Ileal smooth muscle strips from NFAT-luciferase transgenic mice were pooled and divided into five treatment groups. Strips were collected 6 or 24 h after exposure to 10 ng/ml PDGF for 30 min (PDGF 0.5h) or continuously for the duration of the experiment (PDGF 6h, PDGF 24h). Data are expressed as optical density normalized to protein concentration (n = 3, * p < 0.05).](image)

**Fig. 5. Inhibition of VDCC prevents PDGF-induced NFAT4 nuclear accumulation.** Representative images of ileal smooth muscle stained with anti-NFAT4 antibody (red) and with the DNA-binding dye YOYO-1 (green). Yellow nuclei reflect co-localization of NFAT4 and YOYO-1. Strips were treated for 30 min at room temperature as indicated below each field. Lower panel summarizes the percentage of NFAT4 nuclear localization observed in ileal strips in control conditions and after a 30-min treatment with PDGF (10 ng/ml), nisoldipine (100 nM), pinacidil (1 μM), and high K\textsuperscript{+} buffer (60 mM). **PDGF** compared with control, PDGF + nisoldipine, PDGF + pinacidil, high K\textsuperscript{+} buffer, and PDGF + high K\textsuperscript{+} buffer (n, number of animals; i, analyzed images; and c, total number of cells counted).
Phasic contractile behavior and repetitive Ca\(^{2+}\) spiking is blocked in the presence of 60 mM K\(^+\), and it is likely that any complex or modulated Ca\(^{2+}\) signal generated by PDGF stimulation would be predicted to promote the constitutive nuclear localization of NFAT. Thus, ileal smooth muscle may be unique with respect to NFAT activation in that a sufficient Ca\(^{2+}\) signal may exist under basal conditions, and the regulation of nuclear export dynamics may be the critical factor that determines the cellular localization of NFAT.

Accordingly, PDGF may induce NFAT4 nuclear accumulation primarily by decreasing the basal rate of nuclear export. Nuclear export of various NFAT isoforms is promoted by the activity of glycogen synthase kinase-3 (GSK-3), which phosphorylates conserved serines in the N terminus that are required for nuclear export. There is evidence that PDGF inhibits GSK-3 activity in fibroblasts, suggesting a possible mechanism by which PDGF might promote NFAT nuclear accumulation (40). NFAT4 is unique among NFAT isoforms in that its nuclear export can be regulated by the activity of c-Jun N-terminal kinase (JNK; Ref. 21) and it is conceivable that the effects of PDGF on NFAT4 may be caused by inhibition of JNK activity.

NFAT nuclear import can also be inhibited by a number of mechanisms and PDGF might act to disinhibit these processes or directly promote NFAT4 translocation by other means. Although inhibition of nuclear export or potentiation of import represent attractive mechanisms to account for these results, the Ca\(^{2+}\)-independent mechanisms by which PDGF may regulate NFAT4 localization remain to be elucidated.

Unlike NFAT4, CREB is activated by membrane depolarization with elevated external K\(^+\) in ileal smooth muscle, suggesting that CREB is capable of responding to global increase in [Ca\(^{2+}\)]\(_i\), alone, whereas NFAT4 is not. These results clearly indicate that Ca\(^{2+}\)-sensitive transcription factors are capable of discriminating between different Ca\(^{2+}\) signals, resulting in their differential activation. The physiological or pathological implication of differential transcription factor activation mechanisms has not been explored in native smooth muscle, which exhibits a rich diversity of Ca\(^{2+}\) signaling modalities and a phenotypic plasticity that clearly reflects the activity of underlying genetic regulatory mechanisms.

The targets of Ca\(^{2+}\)-sensitive transcription factors that may play a role in maintaining the contractile smooth muscle phenotype, or contribute to adaptive or pathological changes in smooth muscle structural or functional properties, are un-
known. Smooth muscle cells have been found to undergo rapid and profound phenotypic changes during culture in vitro (41). In guinea pig ileal strips cultured in the presence of serum, it has been shown that force development in response to contractile stimuli is diminished and that expression of L-type Ca\textsuperscript{2+} channels is down-regulated. The down-regulation of VDCCs is prevented by pretreatment with the calcineurin inhibitor CsA (42), suggesting that the changes in VDCC expression are Ca\textsuperscript{2+}-dependent and that the Ca\textsuperscript{2+}-dependent modulation of VDCC expression may be regulated by NFAT. A number of ion channel genes and regulatory subunits, including those for L-type Ca\textsuperscript{2+} channels (e.g. a1C), ryanodine receptors (e.g. RYR2), IP3 receptors (e.g. IP3R1), voltage-dependent K\textsuperscript{+} channels (e.g. Kv1.5) and the \( \beta \) subunit of the large conductance Ca\textsuperscript{2+}-dependent K\textsuperscript{+} channel contain NFAT response elements within their promoter regions. These observations suggest the possibility that this Ca\textsuperscript{2+}-dependent transcription factor may play a role in regulating Ca\textsuperscript{2+} homeostasis by regulating the expression of ion channel gene assemblages. Ultimately, the identification of NFAT-regulated genes in native smooth muscle will be crucial for developing an understanding of the physiological role that this transcription factor plays in this genetically dynamic tissue.

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