NFAT Regulation in Smooth Muscle

David C. Hill-Eubanks, Maria F. Gomez, Andra S. Stevenson, and Mark T. Nelson*

First identified in activated T cells, the calcium (Ca\(^{2+}\))-dependent transcription factor, nuclear factor of activated T cells (NFAT), has since been shown to play a role in nonimmune cells, including cells of the cardiovascular system. In arterial smooth muscle, the diverse array of calcineurin--ionizing modalities, the functional interplay between smooth muscle and endothelial cells, and the influence of intravascular pressure on calcium and other signaling pathways creates a calcium-regulatory environment that is arguably unique. This review focuses on mechanisms that control the initial Ca\(^{2+}\)/calcineurin-dependent events in NFAT activation, with a particular emphasis on NFAT regulation in native vascular smooth muscle. Also addressed is the role of additional mechanisms that act to modulate calcineurin-dependent NFAT nuclear import/export, mechanisms that may have particular relevance in this tissue. (Trends Cardiovasc Med 2003;13:56–62).

© 2003, Elsevier Science Inc.

David C. Hill-Eubanks and Mark T. Nelson are at the Department of Pharmacology, University of Vermont, Burlington, Vermont, USA. Maria F. Gomez is at the Department of Physiological Sciences, University of Lund, Lund, Sweden. Andra S. Stevenson is at the Department of Molecular Physiology & Biological Physics, University of Virginia, Charlottesville, Virginia, USA.

*Address correspondence to: Mark T. Nelson, Dept. of Pharmacology, University of Vermont, 89 Beaumont Avenue, Burlington, VT 05405, USA. Tel.: (+1) 802-656-2500; fax: (+1) 802-656-4523; e-mail: mark.nelson@uvm.edu.

© 2003, Elsevier Science Inc. All rights reserved. 1050-1738/03/$-see front matter

PII S1050-1738(02)00211-6

TCM
Calcium (Ca\(^{2+}\)) plays a central role in the regulation of diverse intracellular processes in all tissues (see Carafoli and Klee 1999, and references therein). Changes in intracellular Ca\(^{2+}\) ion concentrations ([Ca\(^{2+}\)]\(_i\))—often mediated by the ubiquitous Ca\(^{2+}\)-binding protein, calmodulin— regulate the activity of kinases and phosphatases, as well as other enzymes and proteins. Elevations in intracellular Ca\(^{2+}\) also mediate specialized cellular functions, including synaptic neurotransmitter release, hormone secretion by endocrine and exocrine cells, and contraction of cardiac, smooth, and skeletal muscle. Intracellular Ca\(^{2+}\) changes may be restricted to a selected region or cellular compartment to produce localized effects, or may be experienced by the cell as a whole. Similarly, the effects of Ca\(^{2+}\) signals may be rapid and transient—as is the case with the regulation of some ion channels (Nelson et al. 1995)—or sustained, producing long-term changes such as those associated with synaptic plasticity and tissue remodeling. The mechanisms that enable this simple divalent cation to provide simultaneous regulation of diverse cellular processes on multiple spatial and temporal levels are incompletely understood, but a growing body of evidence reveals a signaling landscape that is more subtle and complex than early investigators might have anticipated.

An important aspect of calcium's function that has received considerable attention in recent years is its role in the regulation of gene expression. Four basic mechanisms by which Ca\(^{2+}\) signals can be translated into a change in gene activity have been described: (a) direct activation of Ca\(^{2+}\)-binding, EF-hand domain-containing transcription factors by nuclear Ca\(^{2+}\) (Carrion et al. 1999), (b) negative regulation of basic helix–loop–helix transcription factor DNA binding by Ca\(^{2+}\)/calmodulin-S100 protein complexes (Hermann et al. 1998), (c) activation of Ca\(^{2+}\)/calmodulin-dependent protein kinases with subsequent phosphorylation/activation of DNA-binding transcription factors (Hook and Means 2001), and (d) dephosphorylation-dependent transcription factor activation by Ca\(^{2+}\)/calmodulin-dependent phosphatases (e.g., calcineurin). A prototype of this last mechanism is the calcineurin-mediated activation of nuclear factor of activated T cells (NFAT).

NFAT has been described as a signal integrator and coincidence detector (Crabtree and Olson 2002, Graef et al. 2001b), interlinking Ca\(^{2+}\) signaling and other signaling pathways with the induction of specific genetic programs. In T cells, NFAT regulates the coordinate expression of genes such as those encoding cytokines and their receptors, which are important in defining the activated T-cell phenotype (Rao et al. 1997). NFAT is involved in regulating the properties of skeletal muscle, playing a role in development (Delling et al. 2000); growth (Horsley et al. 2001); fast-twitch, slow-twitch isotype switching (Chin et al. 1998); and hypertrophy (Musaro et al. 1999). NFAT is also important in the cardiovascular system, where it contributes to the pathogenesis of cardiac hypertrophy (Molkentin et al. 1998), and regulates heart valve formation (Ranger et al. 1998) and vascular development (Graef et al. 2001a). The role of NFAT in cardiac hypertrophy is an important issue in cardiac biology that has been extensively reviewed by others (see Bueno et al. 2002, McKinsey and Olson 1999, and references therein), and is not a focus of this review. The reader is also directed to several excellent recent reviews on NFAT structure, function, and regulation (Crabtree and Olson 2002, Horsley and Pavlath 2002, Macian et al. 2001, Rao et al. 1997, Zhu and McKeon 2000).

### The NFAT Family

The NFAT transcription factor family is comprised of four well-characterized members, designated NFAT1 (NFAT2/c), NFATc2 (NFAT1/p), NFATc3 (NFAT4/x), and NFATc4 (NFAT3) (reviewed in Rao et al. 1997). A signature of this family is a shared NFAT homology region in the N-terminal portion of the protein, which mediates regulatory functions of the molecule, including calcineurin binding, necessary for regulated nuclear translocation. This region also contains nuclear localization and nuclear export sequences, and phosphorylation sites for a number of serine/threonine kinases, many of which are localized to a serine-rich region and three SP (serine-proline) repeats (Figure 1). The C-terminal region of NFAT proteins contains a DNA-binding domain that shows moderate sequence homology to the DNA-binding domains of Rel-family proteins. A fifth putative member of the family (NFAT5) is a constitutively nuclear phosphoprotein that is insensitive to calcineurin activity and has only limited sequence homology to other members of the NFAT family, primarily within the Rel domain (Lopez-Rodriguez et al. 1999).

### General Features of NFAT Activation

NFAT activation is regulated primarily through control of its subcellular localization (Zhu and McKeon 2000). In unstimulated cells, NFAT is a hyperphosphorylated cytosolic protein. An elevation in intracellular Ca\(^{2+}\), induced by a variety of mechanisms, increases the activity of the Ca\(^{2+}\)/calmodulin-dependent phosphatase, calcineurin (reviewed in Aramburu et al. 2000). Activated calcineurin dephosphorylates multiple serine residues within the regulatory region of the NFAT molecule (13 in NFATc2; Okamura et al. 2000), inducing a conformational change in NFAT that exposes nuclear localization signals and allows import of NFAT into the nucleus (Okamura et al. 2000, Shibasaki et al. 1996, Zhu et al. 1998). The molecular details of NFAT nuclear translocation are incompletely understood, but it appears that NFAT is translocated as a complex with calcineurin through nuclear pores by a mechanism that depends on Ran and specific nuclear importins (Kehlenbach et al. 1998, Shibasaki et al. 1996). Calcineurin also plays a role in promoting nuclear retention of NFAT by masking nuclear export signals recognized by the exportin protein, Crm-1, and by maintaining NFAT in a dephosphorylated state (Shibasaki et al. 1996, Zhu and McKeon 1999).

Sustained NFAT nuclear accumulation is the outcome of a dynamic process subject to regulation on multiple levels. Calcium provides the initial required signal for NFAT nuclear translocation through Ca\(^{2+}\)/calmodulin-dependent activation of calcineurin. At the next level, vigorous nuclear kinases act to rephosphorylate NFAT and hasten its export from the nucleus (Beals et al. 1997, Zhu and McKeon 2000), thus opposing the activity of calcineurin. Phosphorylation by cytosolic kinases may also promote cytosolic retention by disrupting NFAT-calcineurin interactions (Chow and Davis 2000) or masking of nuclear localization signals (Zhu et al. 1998). Some of these regulatory mechanisms rely on NFAT target sites that are conserved in all
Figure 1. (Top panel) Domain structure of nuclear factor of activated T cell (NFAT) family proteins. Common features include an NFAT homology region (NHR) that serves regulatory functions, a Rel homology domain (RHD) that mediates DNA binding, an N-terminal (N-term) transactivation domain (TAD) rich in prolines and acidic residues, and a variable C-terminal (C-term) region. Expanded view of regulatory region shows arrangement of calcineurin (Cn) binding motif (PxIxIT), serine-rich region (SRR), SP (serine-proline) motif (SPxxSPxxSPxxxxxx D/E D/E) repeats (SP1–3), and nuclear localization sequence-1 (NLS-1). (Bottom panel) Sequence alignment of the regulatory domain of human NFAT isoforms, with positions at which human and mouse sequences differ shown in blue; phosphorylation sites (in red) are targets of the indicated kinases. Bold upward arrows indicate experimental evidence for phosphorylation, positions marked with black dots indicate possible phosphorylation sites that could not be individually resolved by the experimental methods employed, “p” indicates predicted phosphorylation sites based on consensus sequence, and asterisks above sequence alignment show serines in cytosolically localized mouse NFATc2 that are dephosphorylated by calcineurin in association with nuclear translocation. aBeals et al. 1997, bMarin et al. 2002, cPorter et al. 2000, dChow et al. 2000, eChow et al. 1997, fAvots et al. 1999, gYang et al. 2002, hChow and Davis 2000. GSK, glycogen synthase kinase; CK, casein kinase; JNK, c-Jun terminal kinase; ERK, extracellular signal-regulated kinase; PKA, protein kinase A.

Figure 2. Simplified schema depicting regulatory pathways implicated in the control of nuclear factor of activated T cell (NFAT) nuclear accumulation in native arterial smooth muscle. Positive effects, negative effects, no effects, and undefined effects on NFAT nuclear accumulation associated with a particular pathway or stimulus are indicated by circled “+”, “−”, “x”, and “?”, respectively. ER, endoplasmic reticulum; NOS, nitric oxide synthase; NO, nitric oxide; PKG, protein kinase G; JNK, c-Jun terminal kinase; NK, nuclear kinase; UTP, uridine triphosphate; IP3R, inositol trisphosphate receptor; SR, sarcoplasmic reticulum; RyR, ryanodine receptor.
NFAT isoforms and are likely to apply universally (Beals et al. 1997, Chow and Davis 2000), whereas other kinases target some NFAT isoforms, but not others (Avots et al. 1999, Chow et al. 1997, Gomez del Arco et al. 2000). An example of apparent subtype-selective regulation is c-Jun N-terminal kinase 2 (JNK2), which promotes nuclear export of NFATc3, but not other NFAT isoforms (Chow et al. 1997). Additional kinases, including JNK1 (Chow et al. 2000), JNK3 (Porter et al. 2000), extracellular signal-regulated kinases ERK1 (Porter et al. 2000) and ERK2 (Avots et al. 1999), and casein kinases I (Marin et al. 2002, Zhu et al. 1998) and II (Porter et al. 2000), have also been proposed to play a role in regulating NFAT nuclear accumulation (see Figure 1; reviewed in Crabtree 1999, Crabtree and Olson 2002).

In the nucleus, NFAT must associate with a transcriptional coactivator to exhibit significant transcriptional activity. Under most circumstances, the expression of required cofactors, which may include AP-1 (Jain et al. 1992), MEF2 (Chin et al. 1998), and members of the GATA family (Molkentin et al. 1998, Musaro et al. 1999, Wada et al. 2002), is also subject to regulation and may be tissue dependent. In addition to providing a final level of control over NFAT activation, this cofactor requirement serves to integrate Ca\(^{2+}\)/calcineurin signaling with other signaling pathways.

**Calcium Signal Requirements**

Early studies (Crabtree 1999, Dolmetsch et al. 1997, Timmerman et al. 1996) using cells of the immune system suggested that a sustained, graded increase in global intracellular Ca\(^{2+}\) was both necessary and sufficient to induce NFAT nuclear accumulation, whereas transient Ca\(^{2+}\) pulses were not. Subsequent work by Dolmetsch and others (Dolmetsch et al. 1998, Li et al. 1998) showed that oscillatory elevations in intracellular Ca\(^{2+}\) increased the efficiency of NFAT nuclear translocation in T and B cells. This work also suggested a mechanism whereby modulation of the frequency and amplitude of oscillating Ca\(^{2+}\) signals might encode information that could be parsed by Ca\(^{2+}\)-sensitive transcription factors, so that a given Ca\(^{2+}\) signal might activate certain transcription factors and not others. Such oscillating Ca\(^{2+}\) signals, integrated over time, increase average Ca\(^{2+}\) throughout the cell; thus, these observations represent a refinement to, rather than a refutation of, the established paradigm. Recent work in hippocampal neurons, however, challenges the universality of the presumed requirement for a sustained Ca\(^{2+}\) signal. In these cells, a brief (3 min) depolarizing stimulus with 90 mM K\(^{+}\) induced a transient increase in intracellular Ca\(^{2+}\), but promoted a sustained increase in NFAT nuclear localization and transcriptional activity (Graef et al. 1999). The molecular basis for this apparent difference in activation requirements is not clear, but it may be important in synaptic plasticity, which involves translating synaptic activity into long-term memory formation. At the other extreme, we have found that a chronic increase in intracellular Ca\(^{2+}\) induced by sustained depolarization with elevated K\(^{+}\) fails to induce a measurable increase in NFAT nuclear accumulation in native smooth muscle (Gomez et al. 2002, Stevenson et al. 2001). This lack of response to simple Ca\(^{2+}\)-elevating stimuli is not unique to K\(^{+}\), because the Ca\(^{2+}\) ionophore, ionomycin, also fails to induce NFATc3 nuclear accumulation in this tissue.

**NFAT in the Cardiovasculature**

In the heart, the NFATc1 isoform plays a role in cardiac morphogenesis, acting in cells of endothelial origin to regulate the formation of aortic and pulmonary valves (Ranger et al. 1998). Mice containing a targeted deletion of this isoform die in utero due to failure of normal valves (Ranger et al. 1998). Mice containing homozygous deletions of these two closely related NFAT family members die in utero due to failure of normal vascular patterning. Individual knockouts do not display this lethal phenotype, suggesting that either isoform is sufficient, or loss of one is compensated for by developmental upregulation of the other.

**NFAT Activation in Smooth Muscle**

We have used immunofluorescence confocal microscopy to study NFATc3 nuclear localization in native smooth muscle cells of both vascular (Gomez et al. 2002) and nonvascular (Stevenson et al. 2001) origin. In cerebral artery smooth muscle, we have found that a number of G\(_{q/11}\)-coupled vasoconstrictor agonists, including the physiologically relevant vasoactive compounds uridine triphosphate (UTP) and endothelin-1, are potent inducers of NFATc3 nuclear accumulation (Gomez et al. 2002). A smaller increase in nuclear NFATc3 is also associated with treatment with angiotensin II; prostaglandin F2\(\alpha\); and the receptor tyrosine kinase ligand, epidermal growth factor. Platelet-derived growth factor (PDGF) is ineffective in native cerebral artery smooth muscle, but provides a potent stimulus for NFATc3 nuclear accumulation in ileal smooth muscle (Stevenson et al. 2001)—a difference that may reflect distinct patterns of PDGF receptor expression in these tissues. In ileal smooth muscle obtained from transgenic mice that uniformly express an NFAT-responsive promoter-luciferase reporter construct, PDGF-induced NFAT nuclear accumulation is associated with an increase in luciferase activity, suggesting that this nuclear accumulation is functionally relevant.

Additional evidence for NFAT activation in smooth muscle-derived cells comes from experiments using rat aortic A7r5 cells expressing a retrovirally introduced NFAT-responsive promoter-reporter construct. In these cells, NFAT transcriptional activity was consistently induced by vasoconstrictor agonists, including
UTP and angiotensin, and by PDGF (Boss et al. 1998). Potential NFAT targets that have been identified in the A7r5 smooth muscle cell line include IL-6, but not other previously identified cytokine targets of NFAT (Abbott et al. 2000), and the NFIL3/E4BP4 transcriptional repressor of the bZIP family (Nishimura and Tanaka 2001). In cultures of human primary aortic smooth muscle cells, the NFATc1 isoform has been shown to couple to the cofactor, GATA6, to drive transcription of an NFAT-responsive promoter-reporter construct (Wada et al. 2002).

**Sources of Calcium**

In nonexcitable cells, the sustained increase in intracellular Ca\(^{2+}\) required to effectively translocate NFAT to the nucleus is provided by a capacitative mechanism by which depletion of intracellular Ca\(^{2+}\) stores is coupled to extra-cellular Ca\(^{2+}\) influx (Serafini et al. 1995). In smooth muscle, where the contribution from capacitative Ca\(^{2+}\) entry pathways remains unclear, the principal mediator of extracellular Ca\(^{2+}\) influx is the L-type voltage-dependent Ca\(^{2+}\) channel (VDCC). In both ileal and cerebral artery smooth muscle, NFAT nuclear accumulation may reflect a capacitative mechanism of extracellular Ca\(^{2+}\) influx (Nishimura and Tanaka 2001). In cultures of human primary aortic smooth muscle cells, the NFATc1 isoform has been shown to couple to the cofactor, GATA6, to drive transcription of an NFAT-responsive promoter-reporter construct (Wada et al. 2002).

**Regulatory Features Revealed**

The failure of high K\(^+\) to induce a detectable increase in NFAT nuclear localization in smooth muscle demonstrates that, at least in certain tissues, elevation of intracellular Ca\(^{2+}\) alone is not sufficient to promote a net accumulation of NFAT in the nucleus. Thus, although elevation of Ca\(^{2+}\) presumably increases the rate of NFAT nuclear import, other mechanisms can apparently come into play to override the normal sufficiency of the Ca\(^{2+}\) signal. Recent studies from our laboratory employing JNK knockout animals suggest such a role for JNK-dependent mechanisms (M. Gomez, A. Stevenson, unpublished observations). Quite unexpectedly, we have found that depolarization of cerebral arteries from JNK2-deficient mice induces a robust accumulation of NFATc3 that is comparable in magnitude to that induced by UTP. We have further found that the rate of NFATc3 nuclear export is slowed in UTP-treated cerebral arteries from JNK2 knockout animals compared with wild-type animals, suggesting that JNK2 negatively regulates NFATc3 nuclear accumulation in smooth muscle by promoting export. Thus, downregulation of the JNK2-dependent nuclear export pathway is apparently sufficient to allow for depolarization-
induced increases in \([\text{Ca}^{2+}]\), to effectively promote NFATc3 nuclear accumulation.

The provisional quality of K’ as an NFAT-activating stimulus has also provided provocative evidence for endothelial regulation of smooth muscle NFAT (L. Gonzalez-Bosc, K. Wilkerson, unpublished observations). Ongoing experiments suggest that K’ effectively induces NFATc3 nuclear accumulation in intact cerebral arteries in the presence of an exogenously added nitric oxide donor; implicating endothelial nitric oxide as a potential additional regulator of NFATc3 accumulation. The salient features of NFAT regulation in arterial smooth muscle identified to date are shown in Figure 2.

• Future Directions

Multiple NFAT isoforms are expressed in smooth muscle, and NFAT transcriptional activity can clearly be induced in these cells. Some important features of NFAT regulation in native smooth muscle are also beginning to emerge, and studies in progress should help to define the interrelationships among the regulatory pathways that have been identified to date. However, the functions that NFAT might serve in this tissue are largely unknown. Given the recurrent theme of NFAT regulation of hypertrophic tissue responses in the cardiac and skeletal muscle literature, it is tempting to speculate about a similar role for NFAT in smooth muscle hypertrophy as well as an additional role for isoform-specific import/export targeting of the protein phosphatase calcineurin to effectively modulate smooth muscle NFAT activity.

The identification of specific NFAT target genes in smooth muscle will go a long way toward establishing a possible physiological role for NFAT in this tissue. Additional experimental tools, currently under development, will allow NFAT regulation and activity to be more fully explored in native smooth muscle, and should help to define the expanding functional repertoire of this important transcriptional mediator of Ca\(^{2+}\) signals.

• Acknowledgments

The authors would like to thank Dr. Mercedes Rincon for helpful discussions, and Drs. Laura Gonzalez Bosc and Keith Wilkerson for unpublished observations and for reviewing the manuscript. This work was supported by grants HL63722, DDK53832, HL44455, and HL07647-12 from the National Institutes of Health; grants from the Swedish Medical Research Council, K. & A. Wallenberg, and Dr. P. Häkansson Foundation; and by a grant from the Tomton Trust for Medical Research.

References


Dolmetsch RE, Lewis RS, Goodnow CC, Healy JI: 1997. Differential activation of transcription factors induced by Ca\(^{2+}\) response amplitude and duration [published erratum...


