Calcium, Calcineurin and the Control of Transcription

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Three Figures

Abstract
Calcineurin (PP2B), a serine threonine kinase controlled by cellular calcium, was originally identified by Klee and colleagues in extracts of mammalian brain. Within the past few years this phosphatase has been implicated in a wide variety of biological responses including lymphocyte activation, neuronal and muscle development, axonal pathfinding and morphogenesis of vertebrate heart valves. Progress in this area has been greatly accelerated by the drugs cyclosporin A and FK506, which use a unique mechanism of action to achieve near genome-specific nanomolar inhibition of calcineurin. This minireview will focus on the role of calcineurin in regulating transcription during development, primarily through its dephosphorylation of downstream targets, such as the cytoplasmic subunits of the NF-AT transcription complex.
Calcineurin and the regulation of nuclear import of the NF-ATc family members

The Ca\(^{2+}\), calcineurin/NF-AT signaling pathway was defined about 10 years ago (1-4) and was one of the first signaling pathways to bridge the cell membrane with the nucleus. Although the pathway is simple (Fig. 1), multiple levels of regulation impinge upon it, making it adaptable for many functions in a wide variety of cell types. The pathway was defined by a strategy of working backward from the nucleus to the cell membrane Ca\(^{2+}\) channels in T lymphocytes. The regulatory regions of the IL-2 gene were identified and found to bind a cyclosporin-sensitive transcription complex called NF-AT1 (1,5-7). This transcription complex was shown to be made up of cytoplasmic (NFATc) and nuclear (NFATn) components (2). The cytoplasmic component translocated into the nucleus with a calcium signal and the import was blocked by the drugs FK506 and cyclosporin A (2). Identification of calcineurin as the \textit{in vitro} and \textit{in vivo} target of FK506 and cyclosporin A (3,4), established that calcineurin was required for the nuclear import of NF-ATc (8). Finally, a screen for somatic cell mutations that prevented NF-AT transcriptional activation yielded many mutations that abolished the activity of the Capacitance Regulated Activation Channels (CRAC) and hence identified it as the source of Ca\(^{2+}\) that regulated NF-AT import (9-11).

Calcineurin functions in this pathway by directly dephosphorylating the cytoplasmic subunits of the NF-AT1 transcription complex (12-14), which are encoded by four genes (NF-ATc1-4) (15), and which undergo cytoplasmic-to-nuclear translocation as originally described(2). Calcineurin binds directly to NF-ATc family members through a conserved motif in the N-terminus of the protein which functions as an effective dominant negative (16-18). This unconventional direct interaction between the phosphatase and its substrate outside of the enzymatic site results in a surprisingly specific relationship between the phosphatase and its substrate. Calcineurin dephosphorylates serines within the SP-repeats (SP1 to SP3) and the Serine Rich Region (SRR) of NF-ATc family members (13,19). When phosphorylated, these residues appear
to obscure the two nuclear localization sequences required for nuclear import, perhaps by forming salt bridges between the basic NLS and the acidic phosphoserines of NF-ATc family members (13,20).

Once dephosphorylated by calcineurin, NF-ATc family members move into the nucleus where they are maintained by persistent elevation of intracellular Ca\(^{2+}\) and the continuous activity of calcineurin (11). Persistent activation of calcineurin is required because NFATc proteins are rapidly exported from the nucleus. Export of NF-ATc1 and c4 was found to be due to the phosphorylation of the same residues that are dephosphorylated by calcineurin to bring about nuclear import. Biochemical purification of the nuclear kinase that phosphorylated these residues identified GSK3 as a potential export kinase (19). The persistent activation of calcineurin necessary to maintain NF-ATc family members in the nucleus requires the CRAC channel which is known to provide a sustained high level of Ca\(^{2+}\) (21). Indeed overexpression of constitutively active calcineurin or a NF-ATc1 mutant that is constitutively nuclear suppresses mutations in the CRAC channel allowing the activation of immune response genes such as IL-2 with only a PKC signal (11).

**NFAT complexes as coincidence detectors and integrators of Ras and Ca\(^{2+}\) signaling.**

Early studies indicated that NF-AT integrated Ca\(^{2+}\) signals with signals transduced by MAP kinase and/or protein kinase C (1,22,23). Thus, the NF-AT1 transcription complex acts as a coincidence detector in that it requires that both Ras/PKC and Ca\(^{2+}/\)Calcineurin signaling be coincident to achieve activation (Fig 2). In fact, DNA binding by NF-ATc proteins is quite weak due to the unusual structural features of its rel-like DNA binding domain (24), and, as a consequence the protein requires a partner for tight association with DNA. Thus, Ca\(^{2+}\) signaling becomes dependent on coincident Ras/PKC signaling and vice versa. In nearly all cell types studied, activation of Rac, Ras or PKC must accompany a Ca\(^{2+}\) signal for activation of NF-AT-dependent transcription (Fig 2).
In addition to the effects of GSK3 on NF-ATc1, the movement of NF-ATc3 into the nucleus appears to be opposed by the actions of either JNK kinase (25) or perhaps the combination of MEKK1 and CK1(20). Although the evidence is conflicting, these two kinases have both been reported to provide opposition to the cytoplasmic dephosphorylation by calcineurin in cell lines. However, since the transcriptional activity of NF-AT complexes is enhanced, rather than blocked by agents that activate MEKK1 and JNK (1,23,26-28) the physiological roles of these kinases remain to be understood.

**Signaling through Ca\(^{2+}\), Calcineurin and NF-ATc family members in neurons.**

Calcineurin has been shown to have important roles in axonal guidance (29) as well as memory and learning (30,31). In general, these functions in neurons have been thought to be independent of transcription. However, recent evidence indicates that the NF-ATc family members may have critical roles in the development of synaptic connections. NF-ATc4 is expressed in hippocampal neurons and undergoes translocation to the nucleus with brief depolarization or even with physiological 5 hertz stimulation (32). The latter is significant in that it implies that normal synaptic activity is sufficient to send NF-ATc4 into the nucleus, i.e., when you think about it NF-ATc4 enters the nucleus. A potential downstream gene appears to be IP3R1, which encodes a Ca\(^{2+}\) channel and is activated shortly after birth in response to the neural activity of the newborn animal (33). The promoter region of this gene has a cluster of NF-ATc sites and appears to be dependent on the activity of the NFATc4 protein, which is expressed in neurons (32). In addition, the activation of this gene after birth or in cultured hippocampal neurons is blocked by FK506 or cyclosporin A (33). These studies suggest that signaling by Ca\(^{2+}\), calcineurin and NF-ATc4 regulate a positive feedback pathway that could reinforce synaptic connections (Fig. 1). However, additional studies with null mutations of NF-ATc4 and possibly other redundant family members will be necessary to confirm this hypothesis.

**The morphogenesis of heart valves and the calcium, calcineurin, NFAT pathway.**
Null mutations in the NF-ATc1 gene result in a failure of heart valve development and abnormalities of the cardiac septum that recall the common cardiac abnormalities that occur in nearly 1% of all live births (34,35). Although calcineurin is expressed ubiquitously, at embryonic days 8.5 to 13, NF-ATc1 is expressed only in the cells that are destined to contribute to heart valves. Remarkably, the protein is nuclear in the endocardial cells that are adjacent to the cardiac jelly, suggesting that a local signal results in its nuclear localization (Fig 1). Both cyclosporin or FK506 block this translocation and result in an arrest in valve development indicating that an as yet uncharacterized signal (presumably a cytokine or growth factor) activates calcineurin leading to nuclear localization of NFATc1 and activation of genes that orchestrate the formation of a cardiac valve (Fig. 1). Much needs to be learned about this extremely important pathway including the nature of the signal, the downstream genes and the role of this pathway in the cardiac valve abnormalities that effect nearly 50 million individuals world wide.

The role of signaling through Ca\(^{2+}\), calcineurin and NF-ATc4 in myocardial hypertrophy.

The heart responds to stress with the hypertrophy of cardiomyocytes. This response is probably normal during development but becomes damaging in hypertension or after multiple myocardial infarctions. The signals for the stress-induced hypertrophy of cardiac muscle have long been known to be dependent on calcium and recently it was discovered that severe cardiac hypertrophy could be induced by the over-expression of a truncated constitutively active calcineurin A (36). In addition, a similar pathology could be induced in animals in which a truncated NF-ATc4 was overexpressed (36). Furthermore, cyclosporin A prevents the development of cardiac hypertrophy in response to certain, but apparently not all, stimuli (37,38). Additional studies with mice bearing mutations in calcineurin genes or the NFATc4 gene will be necessary to confirm these pharmacologic and overexpression studies, but the present results indicate that
calcineurin and NF-ATc4 represent new targets for development of antihypertrophic agents.

**Calcineurin and the control of transcription in yeast.**

In yeast signals initiated by pheromone or other agents that increase intracellular Ca\(^{2+}\) lead to the activation of transcription of FKS2, PMR2, PMC1, PMR1 and other genes (39-41). This transcriptional response requires the Crz1/Tcn1 gene and plays an important role in controlling ion homeostasis (40). The mechanism of control of Crz1p/Tcn1p is remarkably similar to the control of NF-ATc proteins in mammalian cells (Fig. 3). Like NF-ATc proteins which translocate from cytoplasm to nucleus upon activation of calcineurin, crz1p/Tcn1p also translocates and furthermore is dephosphorylated at critical serines similar to NF-ATc family members (42). Despite these mechanistic similarities, there is relatively little sequence similarity in the two proteins other than a conserved group of serines in the N-terminal translocation domain and while Crz1/Tcn1 use a zinc finger for DNA binding the NFATc family use a rel domain.

**Calcineurin inhibitors.**

In recent years, five different classes of calcineurin inhibitors have been discovered (Table 1) raising questions regarding their various roles in Ca\(^{2+}\)/calcineurin signaling. Each of these inhibits calcineurin by binding to the protein and inhibiting its ability to dephosphorylate substrates, such as NFATc family members, thereby preventing their nuclear localization. Perhaps the most interesting of these is the DSCR1 gene and its relatives, DSCR2 and ZAK14 (43). DSRC1 is located on chromosome 21 in the so-called critical region, hence its name Down’s Syndrome Critical Region 1 gene. One possibility is that over expression of DSCR1 as a result of trisomy leads to inhibition of calcineurin and subsequent effects on the development of the brain, immune system, heart and the skeleton. However, the Down’s Syndrome Critical Region includes a number of genes, and it is possible that the syndrome could arise from overexpression of...
several of these. Two pieces of evidence indicate that overexpression of DSCR1 might underlie the pathogenesis of Down’s Syndrome. First, Estivill, de la Luna and co-workers have shown that that DSCR1 protein is actually overexpressed in the brains of Down’s Syndrome patients (43). Secondly, some of the symptoms of Down’s Syndrome appear in mice with mutations of the different calcineurin-dependent (NF-ATc) subunits of the transcription complex. Recently, yeast have been shown to have a related protein, pcn1p that binds and inactivates calcineurin (44,45) (Fig 3).

Two additional interesting classes of calcineurin inhibitors are cabin/cain (46,47) which are novel proteins and the CHP protein that has similarity to calcineurin B (48,49). The CHP proteins appear to compete with calcineurin B for binding to the A protein and thereby inhibit the Ca\(^{2+}\)-dependentactivation of calcineurin A. On the other hand, the DSCR1, 2 and 3 proteins act as competitive inhibitors of phosphatase activity with nanomolar binding constants. Cabin (also called Cain) is a non-competitive inhibitor of calcineurin phosphatase activity with a Ki of 440nM. A physiologic role of these proteins is still unclear but they do antagonize NF-ATc translocation.

A fourth class of calcineurin inhibitors are found in the genome of certain viruses, most notably African Swine Fever virus. Here the A238L protein encoded by the virus binds tightly to calcineurin and blocks NF-ATc translocation and function (50). A238L shares sequence similarity with NF-ATc family members throughout the calcineurin interaction domain, hence it is likely that A238L induces cyclosporin-like immunosuppression in the host, allowing the virus to invade the host.

Finally, the AKAP 79 protein (51) was the first calcineurin inhibitor to be found and is also a scaffolding protein. AKAP79 binds both calcineurin and PKA and may anchor calcineurin at specific sites that allow the protein to engage the proper substrates when activated.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Function</th>
<th>Kd nM or Ki nM</th>
<th>Reference</th>
</tr>
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<tr>
<td>AKAP79</td>
<td>Scaffolding protein, Inhibits NF-AT function</td>
<td>100-200</td>
<td>(51)</td>
</tr>
<tr>
<td>Cabin/ cabin</td>
<td>Implicated in T cell activation and exocytosis in</td>
<td>440</td>
<td>(46,47)</td>
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Crabtree, G.R. 8 Ca\(^{2+}\)/calcineurin signaling

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<tr>
<th></th>
<th>Neurons inhibit NF-AT function</th>
<th>Inhibits nuclear translocation of NF-ATc1</th>
<th>4000</th>
<th>(49)</th>
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<tbody>
<tr>
<td>CHP had Similar to CaB, Prevents nuclear translocation of NF-ATc1</td>
<td></td>
<td>4000</td>
<td>(49)</td>
<td></td>
</tr>
<tr>
<td>DSCR1 (MCIP1)</td>
<td>May be involved in Down’s Syndrome. Prevents nuclear translocation of NF-ATc proteins</td>
<td>70 nM**</td>
<td>(43,52,53)</td>
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<tr>
<td>DSCR2 (MCIP2)</td>
<td>N.D.</td>
<td>N.D.</td>
<td>(52,53)</td>
<td></td>
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<tr>
<td>ZAK4 (DSCR3)</td>
<td>Prevents nuclear translocation of NF-ATc1</td>
<td>N.D.</td>
<td>(43,54)</td>
<td></td>
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<tr>
<td>rcn1p/ CBP1</td>
<td>Related to DSCR1, mutants are cation sensitivity and defective for crzy/tcn function.</td>
<td>6,500* (peptide) 7,000 (protein**)</td>
<td>(44,45)</td>
<td></td>
</tr>
<tr>
<td>A23SL</td>
<td>Blocks NF-ATc translocation; viral encoded</td>
<td>N.D.</td>
<td>(50)</td>
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*This measurement was made with a synthetic peptide derived from rcn1p/CBP1 and the Ki for the full-length protein might be much lower. ** Kyle Cunningham, personal communication

**Calcineurin and the regulation of NF-kB and AP-1 transcriptional activity.**

NFkB and other rel proteins play critical roles in the development of the liver, skin, inflammatory responses and in some aspects of the recombinational immune response. Unlike NF-AT which absolutely requires a calcium stimulus, NFkB can be fully activated by PKC activators in the absence of calcium (55). However, suboptimal stimuli can be augmented with a calcium signal (7,56). This calcium facilitation of NFkB activity can be mimicked with overexpression of a constitutively active calcineurin and can be partially (about 40-70%) blocked with cyclosporin A or FK506, indicating that calcineurin is likely to be required for full induction of NFkB activity in certain circumstances (57). The reduction in NF-kB activity by CsA or FK506 appears to result from an inhibition of the transcriptional induction of the p50 subunit and the c-rel protein (58) as well as reduced degradation of IkB (57). However, the mechanism underlying the later effect is not known.

The AP-1 transcription complex consists of fos and jun proteins which are encoded by families of genes including cjun, junB, junD as well as c-fos, fra, and others. Transcription controlled by most AP-1 sites is not sensitive to inhibition by CsA or FK506 (7,59). However, a site in the IL-2 promoter which binds junD and perhaps c-jun requires the actions of calcineurin for full function (60,61). In addition, an apparent AP-1 site in the collagenase promoter is sensitive to FK506 (62) and hence likely to be calcineurin-dependent. The activity of Jun N-terminal Kinase, JNK is partially inhibited
Calcineurin and the Regulation of MEF2 Transcription factors

Muscle cells respond to Ca$^{2+}$ signals by differentiation and the conversion from fast fibers to slow fibers (63). Calcineurin appears to mediate the later transition by controlling the activity of NF-AT and Mef2 (Myocyte Enhancer binding Factor 2) transcription factors which in turn control genes essential for muscle differentiation. The Mef2 proteins are encoded by at least four genes, Mef2A-D, which are related in their DNA binding domain to MCM-1, Agamous, ARG80, deficiens, serum response factors (64) and hence are referred to as MAD box proteins. The mechanism of the control of Mef2 proteins by calcineurin appears to be due to the Ca$^{2+}$-dependent dissociation of histone deacetylase 4 (HDAC) (65). In addition, Ca$^{2+}$ activates CaMKinase which in turn phosphorylates and exports HDAC4 from the nucleus (Eric Olson, personal communication).

The calcium dependent control of Mef2 might have important roles in regulating programmed cell death. Greenberg and colleagues recently found that Ca$^{2+}$ stimuli that protected neurons from cell death activated MEF2-dependent transcription in a calcineurin-dependent fashion (66). Presumably, the genes that are activated by MEF2 in neurons protect them from cell death. In lymphocytes, the Ca$^{2+}$-dependent activation of MEF2 appears to lead to programmed cell death by activating the Nur77 transcription factor (67). Why neurons and lymphocytes would have opposite responses to the calcium/calcineurin-dependent activation of MEF2 is not clear, but probably relates to the cellular context in which the Ca$^{2+}$-signal is delivered (15).
FIG. LEGENDS

Fig. 1 Signaling Through Calcium, Calcineurin and NF-AT in Different Cell Types. The Ca\(^{2+}\) calcineurin/NFATc signaling cassette (light blue) is used in several different tissues for quite different outcomes. In lymphocytes NF-ATc1, c2, and c3 are expressed and the genes activated include those encoding cytokines and cell surface molecules involved in cell-cell communication and cell death. In cardiac endocardial cells, the same signaling pathway uses NF-ATc1 to regulate the expression of genes essential for heart valve morphogenesis. In hippocampal cells, L-type Ca\(^{2+}\) channels and NMDA receptors activate the NF-ATc4 protein and lead to activation of the IP3R1 gene, which may participate in a positive feedback loop reinforcing synaptic connections. The nomenclature used for the different cytoplasmic, calcium-sensitive subunits of the NF-AT is the Genome Database nomenclature as per the original definition of the cytoplasmic and nuclear subunits of the complex (2).

Fig. 2. Coincidence detection and signal integration by the NF-AT1 transcriptional complex. Ras or Rac or PKC signaling must be coincident with Ca\(^{2+}\) /calcineurin signaling to assemble the NF-AT1 transcription complex and to activate downstream genes. Two receptors are shown that independently activate Ras/Rac/PKC signaling and Ca\(^{2+}\) signaling. However, the T cell receptor as well as a number of other receptors activate both pathways. In some situations, NF-ATn may be a tissue-specific component of the NF-AT complex.

Fig. 3. Calcineurin regulation of transcription in yeast and mammalian cells.

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References

Signaling Through Calcium, Calineurin and NF-AT in Different Cell Types

**T Lymphocyte**
- **TCR**
- **Ca$$^+$$**
- **CRAC**
- **Calcineurin**
- **NF-ATc1,c2,c3**
- **IL-2,3,4, CD40L, GMCSF, FasL, others?**

**Cardiac Endothelial Cell**
- **Receptor.**
- **Ca$$^+$$**
- **Calcineurin**
- **NF-ATc1**
- **Genes required for Cardiac Valular Formation**

**Hippocampal Neuron**
- **L Type Ca$$^{2+}$$ Channel**
- **Calcineurin**
- **NF-ATc4**
- **IP3R1 Gene**

Crabtree et al. JBC 2000, Figure 1
Coincidence Detection and Signal Integration by the NF-AT1 Transcriptional Complex
Calcineurin Regulation of Transcription in Yeast and Mammalian Cells

Receptor

CsA/CpH
AKAP-79
DSCR1
Cain
CHP

Calcineurin
NF-ATc

Ca++

Cytokines, calcium channels,
cell-cell interaction molecules

Mammalian Cells

Pheromone Receptor

CsA/CpH

Calcineurin
NF-ATc

Ca++

Calcium Channels,
ion responsive genes

Saccharomyces cerevisiae

PO3