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Review

Molecular Diagnostics of Calcineurin-Related Pathologies

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BACKGROUND: The Ca^{2+}-dependent protein phosphatase enzyme calcineurin (Cn) (protein phosphatase 3) is best known for its role as director of the adaptive immune response. One of its principal substrates is the nuclear factor of activated T cells (NFAT), which translocates to the nucleus after dephosphorylation to mediate gene transcription. Drugs targeting Cn (the Cn inhibitors tacrolimus and cyclosporin A) have revolutionized posttransplantation therapy in allograft recipients by considerably reducing rejection rates.

CONTENT: Owing primarily to intensive study of the side effects of the Cn inhibitors, the unique importance of Cn and Cn/NFAT signaling in the normal physiological processes of many other cell and tissue types is becoming more evident. During the last decade, it has become clear that an extensive and diverse array of clinical conditions can be traced back, at least in part, to a disturbed Cn-signaling axis. Hence, both diagnostics and therapeutic monitoring could benefit from a technique that conveniently reads out Cn/NFAT operative status.

SUMMARY: This review outlines the current knowledge on the pathologic conditions that have calcineurin as a common denominator and reports on the progress that has been made toward successfully applying Cn and Cn/NFAT activity markers in molecular diagnostics.

Calcineurin (Cn)[3], a heterodimeric serine/threonine phosphatase enzyme, is the only protein phosphatase dependent on Ca^{2+}. The enzyme is composed of an A subunit and a B subunit. Its active site, which contains both a Fe^{2+} ion and a Zn^{2+} ion, resides in the 59-kDa A subunit, whereas the Ca^{2+}-binding 19-kDa B subunit serves a regulatory purpose. The A subunit features several well-defined domains that are responsible for interacting with the B subunit and the Ca^{2+} messenger protein calmodulin and for blocking the active site when the intracellular Ca^{2+} concentration is low [reviewed in *(1)*]. Three CnA isoforms (α, β, γ) with divergent expression profiles have been described. Expression of the γ isoform is restricted to the testes and specific parts of the brain. The other isoforms appear in all tissues, albeit in varying ratios *(2)*. These isoforms differ quite extensively in their enzyme kinetics and physiological functions *(3, 4)*. For instance, lack of CnAβ, the predominant isoform in lymphocytes, leads to inadequate T-cell development and a compromised immune response in mice *(5)*. In addition, 2 isoforms of the B subunit have been identified. CnB1 is produced ubiquitously, whereas CnB2 has been detected only in testes *(2)*.

Many Cn substrates have been identified and are discussed in detail in the next section. The primary substrate of Cn, however, is the transcription factor NFAT (nuclear factor of activated T cells). The NFAT (formally NFATc) family consists of several members: NFAT1 (NFATp, NFATc2), NFAT2 (NFATc, NFATc1), NFAT3 (NFATc4), NFAT4 (NFATx, NFATc3), and NFAT5. NFAT members 1–4 are under control of Cn *(6)*. After dephosphorylation by Cn, NFATc translocates to the nucleus to mediate the transcription of specific genes (see Fig. 1). NFATc can partner with other, often tissue-specific, nuclear transcription factors (often referred to as "NFATn") to activate genes crucial for the respective tissue or cell type. This feature explains how NFAT can play a role in many unrelated organ systems *(7)*.

In lymphocytes, NFAT activation leads to the production of such cytokines as interleukin-2 (IL-2), IL-4, and interferon γ *(8)*. The therapeutic effectiveness of the Cn inhibitors (CnIs)—principally cyclosporin A (CsA) and tacrolimus—widely in use for the prevention of allograft rejection in transplantation patients *(9)* and for the treatment of inflammatory skin conditions *(10, 11)* is based on their ability to suppress the immune system by inhibiting the synthesis of these proinflammatory and T cell–recruiting cytokines *(12)*. Complexes of CsA and tacrolimus with carrier proteins known as "immunophilins" bind to Cn in such a man-

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[3] Nonstandard abbreviations: Cn, calcineurin; NFAT, nuclear factor of activated T cells; IL-2, interleukin 2; CnI, Cn inhibitor; CsA, cyclosporin A; RCAN1, regulator of calcineurin 1; DYRK1A, dual-specificity tyrosine phosphorylation–regulated kinase 1A; AD, Alzheimer disease; TGF-β, transforming growth factor β; TRPC, transient receptor potential channel; COX-2, cyclooxygenase 2; NKCC2, Na^+-K^+-$2Cl^-$ cotransporter 2; CREBc, AMP-response element–binding protein; NGAL, neutrophil gelatinase–associated lipocalin; KIM-1, kidney injury molecule 1; CTGF, connective tissue growth factor; BMP-7, bone morphogenetic protein 7.

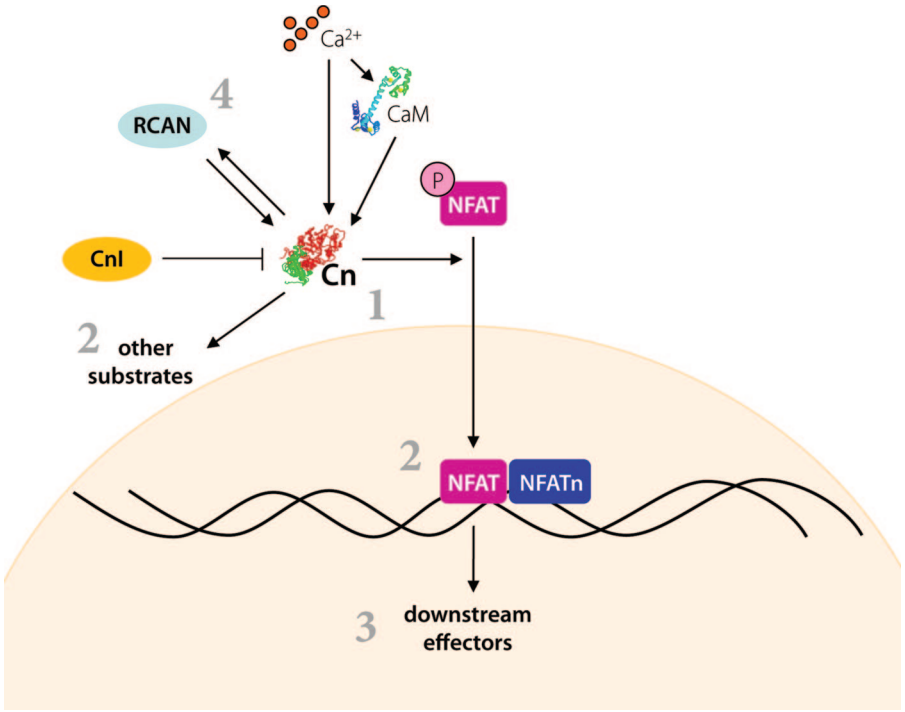

Fig. 1. General overview of Cn signaling showing the main actors and the levels at which the activity of the cascade can be probed.

Gray numbers correspond to individual paragraphs in section II, as follows: 1, Cn activity level; 2, substrate level (e.g., NFAT/nuclear factor κB presence in the nucleus or τ phosphorylation state); 3, downstream effector level (e.g., IL-2, IL-4, IL-10, granulocyte-macrophage colony-stimulating factor, interferon γ); 4, RCAN feedback inhibition level. CnIs comprise CsA and tacrolimus, among others. CaM, calmodulin; NFATn, nuclear proteins partnering with NFAT.

ner that docking and dephosphorylation of NFAT is severely hampered *(1)*.

In the laboratory, the CnIs have evolved to become valuable tools for studying the regulation of Cn activity. In addition, now that the full extent of their side effects—both acute and long-term—is becoming known, the discovery of novel functions of Cn steadily continues. Cn has been found to be essential for a plethora of cellular processes in a multitude of organ systems. A wide array of pathologies have been associated with defects in or deviations from normal Cn signaling. These properties are discussed in the first section of this review. The focus of attention is on the components of the Cn-signaling cascade that qualify as potential diagnostic biomarkers of these pathologies. The second section closely evaluates the technical aspects, preanalytical requirements, and analytical value of these proposed markers. After many in vitro and animal studies, the early case reports now emerging show promising results and encouraging potential for some of these markers in human samples in a typical clinical setting. Establishing the analytical and clinical validity of these markers in practice now tops the agenda toward their ultimate implementation into the clinical routine.

I. Calcineurin Signaling

In many organ systems, Cn translates Ca^{2+} messaging into gene expression. By now, often owing to side effects of the CnIs, several of these organs—brain, pancreas, skin, heart, and kidney—stand out on grounds of interest in Cn signaling and its potential or apparent clinical and diagnostic merit. We cover these systems in more detail below. Finally, we discuss selected examples of Cn functioning in other organ systems.

BRAIN

The name "calcineurin" was derived from its calcium dependence and its being one of the most prominent proteins in brain tissue. Among its substrates in nerve tissue are the vesicle protein synapsin 1 *(13)*, neuromodulin, MARCKS (myristoylated alanine-rich C-kinase substrate), and neurogranin. These proteins control various

aspects of neurotransmitter release and neuronal structure and development. Cn counteracts the activation of these proteins after their phosphorylation by protein kinase C *(14)*. In addition, Cn/NFAT signaling has been shown to be required for neuronal circuit development and refinement, synaptic plasticity, and neuronal excitability *(15)*: Binding of Cn by the A kinase anchoring protein AKAP79/150 appears to regulate the translation of neuronal Ca^{2+} channel activity to nuclear signaling *(16)*. In addition, the differentiation of Schwann cells, the myelin-forming support cells of neurons, has been found to rely on Cn/NFATc4 *(17)*. In contrast to most other tissues, parts of the brain produce the CnAγ isoform. The gene encoding this isoform has been named a candidate susceptibility gene for schizophrenia, because single-nucleotide polymorphisms within and near this gene have been associated with schizophrenia in different ethnic groups *(18, 19)*.

Cn is thought to be a major factor linking Ca^{2+} homeostasis to brain function. For instance, Cn activation due to Ca^{2+} dysregulation has been found to correlate with age-related changes in neural function, such as cognitive decline and memory impairment *(20)*. Overactivation of Cn, such as that occurring after prolonged Ca^{2+} overload following glutamate accumulation, could produce symptoms of excitotoxic neurodegeneration *(21)*. In view of its extreme sensitivity to deactivation by reactive oxygen species, especially in a sustained active state, Cn closely interacts with superoxide dismutase, which protects Cn against oxidation and the concomitant loss of metal ions at the active site *(22)*. On the other hand, chronic blockage of Cn by such CnIs as tacrolimus and CsA could be responsible for the extensive portfolio of these drugs' adverse effects, including mood disorders, headaches, seizures, hallucinations, and ataxia, that have been observed in transplantation patients—observations that illustrate the importance of Cn as a brain enzyme *(23, 24)*. Of note, however, is that the susceptibility of the brain to CnIs seems to be lower than for other organs. The blood–brain barrier may prevent CnI accumulation in the brain, and the immunophilin/Cn ratio is also lower in the brain *(25)*.

Disturbed Cn signaling has also been implicated in the pathogenesis of Down syndrome. Chromosome 21 contains the genes for regulator of calcineurin 1 (RCAN1) and dual-specificity tyrosine phosphorylation–regulated kinase 1A (DYRK1A); RCAN1 is an endogenous feedback regulator of Cn activity that facilitates activity at low levels but restrains Cn at higher levels to mitigate the effects of oxidative and calcium stress *(26)*. DYRK1A phosphorylates NFAT, thereby mediating its nuclear export *(27)*. The increase in the production of these 2 proteins has been suggested to lead to NFAT dysregulation and could explain many of the features of Down syndrome *(28)*. The concomitant suppression of proangiogenic signaling by Cn via vascular endothelial growth factor could explain the reduced incidence of cancers in Down syndrome patients *(29)*.

The presence of neurofibrillary tangles, which are composed of aggregates of hyperphosphorylated τ protein, is one of the key neuropathologic hallmarks of Alzheimer disease (AD). Together with protein phosphatase 2A, Cn is normally responsible for the dephosphorylation of τ protein. This feature is illustrated by observations that tacrolimus induces τ hyperphosphorylation in mouse brain *(30)*, whereas phosphorylation balance is maintained by counteracting kinases. Specific parts of the postmortem cerebral cortex of AD patients, however, show reductions in Cn activity that correlate with tangle formation *(31)*. Given that Down syndrome patients almost universally develop an AD-like neuropathology, the decreased Cn activity could be caused by increased RCAN production. According to a recent publication, increased RCAN concentrations could be responsible for neuronal degradation by facilitating neuronal apoptosis *(32)*. On the other hand, the co-occurrence on chromosome 21 of the gene encoding the amyloid β precursor protein, another important predisposing factor for AD, inherently increases the risk of forming amyloid β deposits *(33)*.

Conversely, there is evidence that inhibition of Cn might have advantageous effects in patients with Huntington disease or Parkinson disease. Huntingtin protein, the primary malefactor in Huntington disease, is a positive regulator of vesicular trafficking for neurotrophins. This function is compromised in Huntington disease patients, owing to the typical poly(Q) expansion mutation present in their huntingtin protein, but it can be restored by phosphorylation of huntingtin at Ser421. Cn activity in Huntington disease brain appears to be conspicuously increased, possibly because of lower RCAN1 concentrations *(34)*, and continuous dephosphorylation of huntingtin by Cn appears to ultimately lead to neuron death. In mice, suppression of Cn activity with either tacrolimus or small interfering RNA has been shown to reinstate vesicular transport and thereby relieve toxicity symptoms *(35)*. CnIs and their analogs also display neuroprotective effects in Parkinson disease, although it is not entirely clear whether their beneficial actions proceed through Cn or via the suppression of the rotamase activity of their immunophilin ligands, given that the latter have been found to accelerate the aggregation of α-synuclein, the fundamental causative event of Parkinson disease *(36)*.

PANCREATIC β CELLS

CnIs, particularly tacrolimus, decrease the insulin content of β cells and preproinsulin mRNA production

and dampen glucose activation of the insulin promoter *(37)*, thereby giving rise to diabetes mellitus–like symptoms, such as insulin resistance, hyperglycemia, and islet cell antibody formation [reviewed in *(38)*]. These findings set off a wave of research into the relationship between Cn/NFAT signaling and glucose and insulin homeostasis. High plasma glucose concentrations stimulate β cells by increasing cytosolic Ca^{2+} concentrations after membrane depolarization. A binding site for NFAT was discovered in the rat insulin promoter region, and promoter activity was found to respond to intracellular Ca^{2+}, a finding that verifies Cn involvement and adds NFAT to the many transcription factors already known to influence insulin production *(39)*. Heit et al. established that Cn/NFAT signaling is uniquely essential in facilitating pancreatic β-cell adaptive growth and function by demonstrating that mice with a β cell–specific deletion of CnB1 develop age-dependent diabetes and reduced production of regulators of β-cell proliferation. On the other hand, NFAT activation alone was found to be sufficient to increase β-cell proliferation and mass and to induce hyperinsulinemia *(40)*. Among the implications of this study are both a potential role for Cn/NFAT signaling in insulinoma *(40)* and the prospective value of Cn-inhibition therapy for disorders of β-cell overgrowth *(41)*. Continuous Cn activation (in cases of chronic depolarization, for instance), on the other hand, would produce the classic signs of Ca^{2+} "overload" known for other cell types, namely decreased proliferation and enhanced apoptosis. Such activation would lead to glucose intolerance and hyperglycemia *(42)*. Interestingly, Yang et al. found that mice deficient in selected isoforms of NFAT (NFATc2 and NFATc4) exhibit not only reduced insulin concentrations in the fasting state but also hypersensitivity to insulin. Moreover, these mice do not accumulate fat. These findings could arise from changes in adipokine signaling, because NFAT also seems responsible for the production of resistin, a protein that confers insulin resistance *(43)*. During the last few years, several genes predisposing to posttransplantation diabetes mellitus have been discovered *(44)*. Such genetic studies may uncover not only potential diagnostic markers but also new molecular pathways of glycemic regulation under the control of Cn.

The sensitivity of Cn to oxidation suggests that the pathophysiology of conditions featuring high amounts of oxidative stress may reflect disturbances in cellular processes that are governed by Cn signaling. Serum measurements in patients with type II diabetes have revealed not only a generally lowered Cn activity and increased oxidative stress but also a reciprocal correlation between Cn activity and diabetic markers (fasting blood sugar, glycohemoglobin). Oxidative stress, which is exacerbated in diabetic patients, could therefore influence glycemic control via Cn *(45)*.

SKIN

CnIs are used topically as an alternative to ultraviolet phototherapy for the treatment of patients with a variety of inflammatory skin diseases, including psoriasis, lupus erythematosis, atopic eczema, and hypersensitivity reactions *(11)*. The alleviation of inflammatory symptoms by CnIs is generally assumed to proceed through the manipulation of T cells infiltrating from the lower dermal layers; however, a great deal of evidence points to direct effects of CnIs on skin cells, although these effects are at times poorly understood, as illustrated by the common occurrence of gingival hyperplasia in patients who receive immunosuppressants *(46)* and the antiproliferative effects of these drugs on cultured keratinocytes *(47)*. A particularly disturbing side effect accompanying systemic CnI therapy in transplantation patients is the increased incidence of skin cancer, especially the nonmelanoma skin cancers *(48)*. Although many of the toxic effects of CnI seem to be mediated via transforming growth factor β (TGF-β) upregulation *(49)*, more attention is also being paid to the role of Cn/NFAT signaling in skin cells. Cn activity, NFAT nuclear translocation, and the inhibition of both by CnIs have been established in keratinocytes, melanocytes, and fibroblasts *(50–52)*. Furthermore, Cn signaling in skin, skin cells, and lymphocytes has been found to be strongly suppressed by high-dose ultraviolet A radiation, presumably through oxidative damage to the enzyme. Lower levels of oxidative stress, on the other hand, could increase the activity of the Cn/NFAT pathway *(53)*. In keratinocytes, NFAT members have been implicated in the control of p21 production, thus regulating the switch between proliferation and differentiation *(54, 55)*. This finding may account for the effectiveness of CnIs in treating skin disorders such as psoriasis *(51)*. Knock-down of Cn has been found to reduce repair of DNA lesions in keratinocytes *(56)*. In addition, Cn seems to mediate calcium-dependent apoptosis by dephosphorylating the proapoptotic protein Bad *(57)*. By sequestering Cn, the antiapoptotic protein Bcl-2 can suppress apoptosis *(58)*. With these results, taken together with the recent finding that intact Cn/NFAT signaling is essential for p53 and senescence-associated mechanisms that protect against skin cancer *(59)*, the relationship between Cn suppression and skin tumor development is starting to take shape.

HEART

Cn signaling has been receiving much attention with respect to its role in striated muscle. In both skeletal muscle and heart tissue, Ca^{2+} is used as a second messenger in the individual's response and adaptation to

environmental stimuli. Activation of Cn in skeletal muscle promotes fiber-type switching through altered myosin heavy chain production, whereas in heart muscle, Cn regulates the production of proteins responsible for cardiomyocyte maturation and remodeling [reviewed in *(60)*]. The importance of Cn/NFAT signaling may begin during the embryonic phase, because mice containing a deletion in the gene encoding NFATc1 show features of impaired cardiac morphogenesis, such as deformed heart valves *(61)*. Furthermore, studies have recently pointed out that Cn may be directly linked to the proper control of basic heart functions, such as rhythm and contractility *(62, 63)*. The most recognized and established role of Cn in heart muscle, however, is its function in the development of cardiac hypertrophy. More than a decade ago, Molkentin et al. found in a number of rodent models that overproduction of CnA produced a profound hypertrophic response and, ultimately, heart failure *(64)*, whereas genetic inhibition of Cn (specifically of the CnAβ isoform) led to a reduced basal heart size and even loss of viable myocardium *(65)*. In addition, a strong link between NFATc2 and cardiac enlargement was found after pathological but not physiological (voluntary exercise training) stress *(66, 67)*. The endogenous regulatory protein RCAN1 (also known as MCIP1), which was mentioned earlier for its role in the regulation of Cn in brain, is envisaged to be important in the heart, because it not only enables the onset of but also bridles the hypertrophic response, depending on the nature of the hypertrophic stimulus *(68, 69)*. Another crucial role seems to be played by transient receptor potential channels (TRPCs), the expression and activity of which are upregulated during pathologic hypertrophy. Because TRPCs activate Ca^{2+}/Cn signaling and the promoters of several TRPC members contain NFAT consensus sites, a positive-feedback circuit is created, maintaining a hypertrophy-producing state *(70)*. Assessment by Ritter et al. of Cn activity and the NFAT phosphorylation state in tissue acquired from patients with hypertrophic cardiomyopathy revealed an increase in Cn capacity, as well as an increased electrophoretic mobility of NFATc1. Their results also suggest that Cn may undergo calpain-mediated truncation of its autoinhibiting carboxyl end after persistent activation, as is the case in the etiology of cardiac hypertrophy *(71, 72)*.

KIDNEY

It is fairly ironic that CnIs are particularly harmful to the very same organ they often ought to protect: the kidney. The nephrotoxic side effects of the CnIs have been acknowledged since the 1980s and continue to hamper their therapeutic use *(73)*. Long-term exposure to CsA is associated with histologic damage to all compartments of the kidney [reviewed in *(74)*]. TGF-β–induced tubulointerstitial fibrosis is thought to be the primary mechanism driving progression of CsA nephropathy, which is characterized by loss of tubular epithelial cells and deposition of extracellular matrix in the tubulointerstitium *(75)*. Another pathologic hallmark is arteriolar vasoconstriction, which leads to glomerular ischemia *(76)*. Both angiotensin II and aldosterone may independently aggravate these pathologic effects because of their stimulating effects on TGF-β production *(75, 77, 78)*. Tacrolimus has a similar nephrotoxic profile, although it is seemingly somewhat less severe *(79)*.

Several important functions of Cn signaling in the kidney have been recognized. Cn is indispensable for renal growth, development, and maturation at its earliest stages *(80, 81)* and functions in close association with COX-2 (cyclooxygenase 2) *(82)*. Cn has been shown to relay Ca^{2+} signals generated by such factors as Wnt, TGF-β, insulin-like growth factor I, angiotensin II, and even polycystin 1, to activate NFAT *(4, 80, 83)*. Furthermore, both NFAT5 and Ca^{2+}/Cn/NFAT signaling seem to be key mediators of tonicity-response gene expression, ensuring an adequate reaction to osmotic stress *(84, 85)*. NFAT is also implicated in salt and water homeostasis by decreasing sodium reabsorption, owing to its suppression of the activity of Na^+-K^+-$2Cl^-$ cotransporter 2 (NKCC2), a finding that could explain the fact that CsA therapy is often accompanied by hypertension *(86, 87)*. Grossmann and coworkers recently showed an intriguing link between activation of the mineralocorticoid receptor and inhibition of the cAMP-response element–binding protein (CREB) due to dephosphorylation by Cn. As the Na^+-K^+ ATPase promoter contains a cAMP-response element, these findings could implicate Cn in defining the mineralocorticoid responsiveness of the Na^+-K^+ pump *(88)*.

Interestingly, whereas the CnAα isoform is indispensable for kidney growth and function and the effects of its knock-down closely resemble the nephrotoxicity profile of the CnIs, the CnAβ isoform, although also produced throughout the kidney, seems to be unnecessary for proper kidney structure and function. Young mice lacking CnAα showed renal dysfunction, increased TGF-β concentrations in the urine and kidneys, increased serum creatinine concentrations, matrix accumulation, fibrosis, and increased cellular death. Given that p27 (a cyclin-dependent kinase inhibitor) concentrations were increased dramatically, disruption of normal cell cycle patterns could be a major component of CnI toxicity *(4, 89, 90)*. Mice lacking CnAβ, however, show a complete absence of NFATc translocation after ionomycin treatment, which suggests distinct roles for the 2 Cn isoforms *(90)*. Because

CsA has secondary targets that may contribute to its nephrotoxicity *(91)*, these knock-down experiments show that Cn inhibition itself is a major causative factor for kidney damage. Cn overproduction, on the other hand, may also have undesirable effects: Accumulation of extracellular matrix accumulation is also seen during diabetic nephropathy, along with renal cell hypertrophy. In the diabetic kidney, however, both TGF-β and Cn/NFATc1 signaling are clearly upregulated *(4, 92)*.

There is an urgent need for diagnostic tools to detect CnI-induced kidney damage before patients become symptomatic, so that therapy can be adjusted in time. There are a number of sensitive histologic markers, such as afferent arteriolar hyalinosis; but they are generally very nonspecific; and invasive biopsy should be avoided as much as possible. Unfortunately, standard kidney function tests also prove mostly inadequate for this purpose. Owing to internal compensation in the kidney, the glomerular filtration rate does not decline until structural damage has already reached an advanced and irreversible state. By that time, serum creatinine and blood urea nitrogen tests may still yield normal values. Microproteinuria (particularly β_2- and α_1-microglobulins), however, was recently found to be present far in advance of rising serum creatinine concentrations in a group of patients who had undergone liver transplantation *(93, 94)*. Markers of structural damage, such as neutrophil gelatinase–associated lipocalin (NGAL) and kidney injury molecule 1 (KIM-1) *(95, 96)*, respond early but may also signal other sources of kidney injury. Research efforts to discover new biomarkers that reveal very early CnI nephrotoxicity more specifically have pointed to several candidates in close proximity to the disturbed Cn-signaling pathway, such as TGF-β and its downstream modulators CTGF (connective tissue growth factor) and BMP-7 (bone morphogenetic protein 7), known mediators of tubulointerstitial fibrosis *(97)*. Alternatively, toxicogenomic studies have identified molecular mechanisms that may specifically reveal CsA toxicity, such as endoplasmic reticulum stress and the epithelial-to-mesenchymal transition. Diagnostic markers of these processes, such as the chaperone protein BiP and the epithelial marker E-cadherin, are currently being evaluated as potential biomarkers of early kidney damage in CsA-treated renal allograft recipients *(98)*. Urine screening in mice receiving CsA revealed several proteins that may also qualify as biomarkers of early kidney damage, including vinculin, podocin, uromodulin, and, again, E-cadherin *(97)*.

OTHER ORGAN SYSTEMS

CnIs have proved very useful in alleviating the symptoms of rheumatoid arthritis *(99)*. Patients treated with CsA, however, often develop osteoporosis *(100)*. A large number of genes in endothelial cells, osteoblasts, chondrocytes, and osteoclasts have been linked to NFAT transcriptional control, suggesting a multifaceted role for the Cn/NFAT axis in bone remodeling and control of joint architecture *(101, 102)*. Topical CnIs, including the new agent voclosporin, show good results in treating several types of inflammatory eye disease *(103, 104)*. Cn is produced in various eye tissues, with the highest concentrations occurring in the retina, cornea, and optic nerve. Its functions in ocular structure and possible involvement in the processing of visual information have recently been reviewed *(105)*. Increased ocular pressure has been shown to lead to apoptosis of retinal ganglion cells in glaucoma via a Cn-mediated mechanism *(106)*.

II. Calcineurin Assays

Probing the real-time activity of Cn and the signaling pathways under its control presents several experimental challenges, and therefore its application for clinical purposes is still in its infancy. As has become clear from section I, Cn is a ubiquitous enzyme in the human body, and administration of CnIs causes systemic suppression of Cn activity. In allograft recipients receiving treatment with CnIs, Cn activity measured in peripheral blood mononuclear cells is being applied as an index of T-cell activation and a marker of graft-vs-host disease *(107, 108)*. It is still unclear, however, whether Cn activity as measured in the blood is a reliable measure of systemic Cn activity and whether it permits conclusions on the operational status of Cn signaling in other organs, given that basal Cn activity tends to vary greatly from one cell type to another *(52)*, as well as between individuals *(109)*. For example, it would be helpful to know how the Cn measurements in serum samples from patients with type II diabetes reported by Sankaranarayanan et al. *(45)* translate into Cn activity levels in the pancreas. Alternatively, Cn activity could be assessed in biopsy samples of specific tissues, such as skin, for diagnostic purposes or for monitoring of treatment efficacy, although the invasive aspect of tissue biopsy makes this option less of a routine intervention.

Another facet concerns the choice of readout. Cn signaling can be probed at different levels in the cascade (see Fig. 1). Each level, however, has its own experimental pros and cons. Directly measuring Cn activity toward an artificial substrate represents the first, most obvious approach to probing Cn signaling. Early mechanistic experiments that assessed Cn phosphatase activity involved the detection of free phosphate released from Cn-catalyzed hydrolysis of *p*-nitrophenylphosphate *(110)*. When it became ap-

preciated that the kinetic behavior of Cn toward small organic phosphates differs markedly from its behavior toward phosphorylated peptides and proteins *(111)*, a method based on the radioactive detection of phosphate released from ^{32}P-labeled RII peptide, a fragment derived from the regulatory subunit of protein kinase A, came into use *(112, 113)*. A spectrophotometric assay of Cn activity based on the colorimetric detection of phosphate is currently available *(114)*. Originally designed for pharmacodynamic monitoring purposes, this assay circumvents the safety issues involved with the use of ^{32}P and has already proved its value, not only with blood samples and blood cell subsets, but also with skin and primary skin cells *(52, 114, 115)*. Alternatively, an HPLC-based method has been validated to quantify Cn activity in lymphocytes *(116)*. This method monitors the amount RII peptide dephosphorylated by Cn. Although both approaches are, in principle, very flexible and versatile, some caveats exist. Because Cn is readily oxidized and sensitive to degradation, all buffers require a supplement of reducing agents and protease inhibitors *(114)*, and lengthy workup procedures should be avoided. In addition, some cross-reactivity for RII exists for other protein phosphatases, which should be inhibited completely to improve the assay's background signal and its specificity. Even then, the relevance of information obtained with these Cn assays requires careful interpretation, because Cn activity can be highly variable in time, depending on the oscillations in the intracellular Ca^{2+} concentration and the production of endogenous regulatory proteins *(69)*. In fact, by measuring Cn activity at one specific moment and under optimal, nonlimiting conditions (excess Ca^{2+}, calmodulin, and substrate), most assays actually measure Cn capacity rather than Cn activity *(109)*. Although this approach smoothes out the effect of Ca^{2+} oscillations over time, the prediction of biological effects becomes less trivial. A recently developed Cn activity sensor based on Förster resonance energy transfer allows real-time visualization of Cn activity in living cells (e.g., heart muscle) in response to Ca^{2+} dynamics *(117)*. Data obtained with this type of sensor may provide more insight into Cn behavior over time, with respect to its reaction to common stimuli, or in a particular cellular context—all of which could be of benefit in constructing a basal activity profile.

Second, Cn signaling can be probed indirectly at the substrate or transcriptional level. NFAT activation could be viewed as a more balanced representation of Cn activity over a selected period of time and, as such, could be roughly designated as the integral of Cn activity *(118)*. Therefore, measurements of NFAT transcriptional activity by means of a reporter construct could provide a more reliable and objective means of quantifying Cn activity, although one should keep in mind that the existence of counteracting kinases that export NFAT from the nucleus may affect the results *(119)*. At this stage, unfortunately, this technique is typically more suitable for experimental purposes and is unlikely to make its way into a clinical routine setting, although direct quantification of the presence of NFAT in the cell nuclei of biopsy samples should be achievable with immunohistochemistry techniques *(50)*, electrophoretic mobility shift assays *(120)*, or an immunoassay *(115)*. The τ protein, another direct substrate of Cn and a useful biomarker of AD, can be detected in cerebrospinal fluid *(121)*. Given that the presence of single-nucleotide polymorphisms in the genes encoding the subunits of Cn is associated with altered τ and mRNA concentrations in cerebrospinal fluid *(122)* and that the infusion of CnIs into mouse brain induces hyperphosphorylated τ *(30)*, τ status may reflect Cn signaling. Nuclear factor κB is another transcription factor that can be activated by Cn *(123)*. In contrast to NFAT, however, many stress factors can lead to nuclear factor κB activation in a Cn-independent manner. Its suitability as a marker of Cn signaling is therefore debatable, although CnIs may be used as controls to determine Cn specificity.

Third, Cn activity could be examined even further downstream, at the level of effector molecules, such as cytokines and surface activation markers *(109)*. The production and/or excretion of these molecules can be measured both at the mRNA level (PCR) and at the protein level (ELISA, flow cytometry, immunoaffinity capillary electrophoresis). Assays for measuring cytokines in blood, blood fractions, and other body fluids are common and well documented *(124–126)*. Several reports have mentioned strong effects of disturbed Cn activity in lymphocytes on cytokine concentrations (e.g., IL-2, interferon γ) *(115, 124)*. One should keep in mind, however, that the choice of cell type determines which molecules are eligible downstream markers. In addition, many of these molecules are not exclusively dependent on Cn, and information on base values and reference intervals is still very limited. In T cells, however, activation accompanied by CD28 costimulation produces a selective and reproducible response of NFAT-dependent genes *(109, 127)*.

Fourth and lastly, RCAN1, one of the endogenous regulators of Cn, is currently receiving a great deal of interest for its supposed role in degenerative brain disease. Because of its close feedback relationship with Cn, RCAN1 could be a reliable marker of Cn signaling *(68)*.

Although downstream markers generally offer a better representation of the expected biological effects, they do not always correlate one to one with Cn activ-

ity. Therefore, the primary considerations underlying the choice of readout are the intended application and the cell type of choice. For instance, one may argue that mechanistic studies into the effects of inhibitors might best be performed at the Cn activity level, whereas concrete physiological changes might best be reflected by measuring downstream markers. The search for the most workable and reliable readout in the field of CsA dose optimization in individual allograft recipients is nicely illustrated by the efforts of Van Rossum, Press, and coworkers and by the Giese/Sommerer group *(109, 128, 129)*.

Dose adjustment is crucial to guarding the balance between preventing rejection on one side and protecting against infections and malignancy on the other. At the moment, pharmacokinetic measurements (trough and/or peak concentration) are the gold standard for CnI monitoring. Advanced techniques such as Bayesian estimation of the area under the curve are fairly capable of compensating for interindividual differences in the pharmacokinetic profile and therefore provide a better measure of total drug exposure *(130, 131)*. Nonetheless, because the CnIs display both a very intricate mechanism of action *(1)* and a complex cellular and subcellular distribution profile *(132)*, the extent of their biological consequences is very difficult to deduce from a blood concentration. Pharmacodynamic (drug effect) monitoring represents the next logical step, but its success depends on the presence of a quick, quantitative, and reliable marker of the level of inhibition of Cn signaling. In light of the obvious advantage of measuring a drug effect directly at its target for determining individual susceptibility, Van Rossum et al., building on earlier studies [reviewed in *(133)*], have recently investigated the relationship between Cn activity and T-cell cytokine secretion at therapeutic CsA concentrations *(109)*. Their results demonstrate the possible value of the Cn activity marker in pharmacodynamic monitoring of the CnI; however, a thorough validation that addresses such fundamental issues as the effect of sample composition and the amount of intra- and interindividual variation has not yet been completed. For now, it seems that the origin of interindividual variation and the response to CnIs in general are determined by a complex set of factors, among which genetic variation—both in drug metabolism and in Cn isoforms—has received particular interest in view of future improvements to CnI therapy *(128, 134)*. In addition, attempts are being made to overcome the current Achilles' heel of this monitoring method, which seems to be the critical sample-storage conditions required to maintain Cn stability in solution. This unresolved issue is responsible, at least in part, to the high intraindividual variation in observed Cn activity *(109, 128)*.

Giese et al. have described quantitative measurements of mRNA transcription for a number of NFAT-regulated genes, among which are those encoding IL-2 and interferon γ in stimulated peripheral blood from both healthy volunteers and transplant recipients on a CsA regimen. Gene expression was chosen over protein production as the choice of readout, because the former was found to be more dynamic. Gene expression levels decreased dramatically 2 h after oral CsA administration, returning to normal 6–10 h after uptake. Most patients showed a comparable expression profile over time *(135)*. Intraindividual variation over a period of several months was found to be $<$10% *(136)*. A follow-up study monitored CsA dose tapering with the same readout technique. The ratio of residual NFAT-regulated gene expression at the CsA peak (C2) level to that at the CsA trough (C0) level was shown to increase up to 20% in the case of a typical safe reduction in CsA dose *(137)*. Meanwhile, a parallel study once again underlined the importance of preventing CsA overdosing. Elderly long-term renal transplant recipients who developed nonmelanoma skin cancers exhibited substantially lower expression of NFAT-regulated genes than the group that did not develop malignancies *(138)*. Interestingly, CsA dosing and C0 and C2 gene expression levels of the 2 groups were comparable, implying that the observed correlation between NFAT-regulated gene expression and the incidence of nonmelanoma skin cancers would not have been noticed by pharmacokinetic monitoring alone. A subsequent case report showed that the information derived from newly developed methods may even have evolved beyond prediction alone. In a patient experiencing recurring skin malignancies, adjustment of immunosuppressive therapy based on pharmacodynamic monitoring brought the development of new lesions to a complete halt *(139)*. Given the amount of evidence for their value in predicting clinical outcome, as well as such criteria as ease of use and clinical pertinence, these pharmacodynamic monitoring methods do not yet measure up to current mainstream methods that are based on pharmacokinetics *(140)*. Nevertheless, it appears that their clinical benefit has been demonstrated and that a promising role as a supportive tool seems to lie ahead for them.

The first section of this review illustrates that specific isoforms of both Cn and NFAT feature different activity kinetics in different organs and hence may fulfill divergent roles and cellular functions, depending on the place and time. Future improvements and refinements to both therapeutic and diagnostic strategies involving Cn/NFAT signaling will undoubtedly have to confront this issue and, through the ability to specifically target one isoform, may even exploit it.

Conclusion

During the last 20 years, Cn has proved itself a key player in the normal operation and maintenance of many cells and tissues in the human body. Defective Cn signaling lies at the root of a growing number of clinical conditions and has conspicuously been linked to 3 epidemics of the 21st century: cancer, metabolic aberrations, and degenerative brain diseases. In addition, treating transplant recipients with CnIs to prevent rejection has to be carefully controlled because of the systemic effects of CnIs. Meaningful and effective measures of Cn activity are obviously very important for further clarifying the role of Cn in the aforementioned conditions. In some cases, they already have prognostic and/or diagnostic value. Furthermore, such markers will be very convenient for therapeutic monitoring purposes, especially if they enable early detection of the adverse effects of CnIs. This review has highlighted the involvement of Cn in a diverse array of pathologies and has summarized the state of affairs regarding the application and applicability of Cn and Cn/NFAT activity markers. The first signs of their clinical value and viability are emerging, and we hope that they will inspire future research endeavors on this subject.

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