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Calcineurin regulation of cytoskeleton organization: a new paradigm to analyse the effects of calcineurin inhibitors on the kidney

Virginie Descazeaud [a, b], Elodie Mestre [a, b], Pierre Marquet [a, b], Marie Essig [a, b, c]

[a] *Inserm, UMR-S850, Limoges, France*
[b] *Limoges University, Limoges, France*
[c] *CHU de Limoges, Service de Nephrologie, Hemodialyse, Transplantations, Limoges, France*

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Abstract

Calcineurin is a serine/threonine phosphatase originally involved in the immune response but is also known for its role as a central mediator in various non-immunological intracellular signals. The nuclear factor of activated T cell (NFAT) proteins are the most widely described substrates of calcineurin, but ongoing work has uncovered other substrates among which are the cytoskeleton organizing proteins (*i.e.* cofilin, synaptopodin, WAVE-1). Control over cytoskeletal proteins is of outmost interest because the phenotypic properties of cells are dependent on cytoskeleton architecture integrity, while rearrangements of the cytoskeleton are implicated in both physiological and pathological processes. Previous works investigating the role of calcineurin on the cytoskeleton have focused on neurite elongation, myocyte hypertrophic response and recently in kidney cells structure. Nuclear factor of activated T cell activation is expectedly identified in the signalling pathways for calcineurin-induced cytoskeleton organization, however new NFAT-independent pathways have also been uncovered. The aim of this review is to summarize the current knowledge on the effects of calcineurin on cytoskeletal proteins and related intracellular pathways. These newly described properties of calcineurin on cytoskeletal proteins may explain some of the beneficial or deleterious effects observed in kidney cells associated with the use of the calcineurin inhibitors, cyclosporine and tacrolimus.

Keywords: calcineurin • cytoskeleton • kidney • nephrotoxicity

Introduction

Calcineurin (CaN) is a serine/threonine phosphatase widely distributed in mammalian tissues and unique among phosphatases for its ability to sense Ca^{2+} through its activation by calmodulin. This latter property makes it a powerful mediator of intracellular signals commonly involved in physiological and pathological processes. Calcineurin is composed of two subunits. A catalytic A subunit, which contains the phosphatase domain and mediates interaction with phosphorylated substrates, and a regulatory B subunit which binds Ca^{2+} and calmodulin and facilitates the conformational change needed for phosphatase activity [1]. First identified in the late 1970s as an inhibitor of the calmodulin-dependent cyclic nucleotide phosphodiesterase [2], CaN was then recognized as the target of the immunosuppressive drugs cyclosporine A (CsA) and tacrolimus (FK506) and was involved in the activation

Correspondence to: Marie ESSIG,
Inserm UMR-S850, Facult de Mdecine,
2 rue du Dr Marcland, 87025 Limoges Cedex, France.
Tel.: +33-555-43-58-95
Fax: +33-555-43-59-36
E-mail: marie.essig@inserm.fr

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of nuclear factor of activated T cell (NFAT) proteins [3, 4]. Activation of T cell receptor by extracellular stimuli leads to elevation of intracellular Ca^{2+} and activation of CaN, which dephosphorylates NFAT, allowing its translocation to the nucleus and the activation of gene expression. CaN was originally thought to have a narrow substrate specificity, mainly limited to NFAT [3], but numerous other substrates were thereafter discovered such as the transcription factors Elk-1 and MEF2 [5, 6], the heat-shock protein Hsp25 [7], the growth factor neuromodulin [8], the NMDA receptor [9] and various cytoskeleton organizing proteins (*e.g.* Tau factor, MAP-2, cofilin, synaptopodin, WAVE1) [10–13]. These later proteins are of particular interest due to their major role as cytoskeletal proteins, determining cell phenotype.

The cytoskeleton is a three-dimensional filamentous protein network providing structural scaffolding to the cell and thereby determining its size, shape and mechanical properties. The cytoskeleton is composed of three distinct but integrated cytoplasmic fibrous polymers: actin microfilaments, microtubules and intermediate filaments. Cytoskeleton is a key regulator of various cellular and molecular events, such as movement of chromosomes during cell division, vesicular trafficking, cellular adhesion and migration and acquisition of cellular polarity. It also acts as a signalling platform and modulates cellular pathways by controlling the activity and/or the subcellular localization of signalling proteins and their targets.

Several studies have provided evidence that CaN influences the cytoskeleton of neurons, myocytes and recently of kidney cells, potentially contributing to some of the non-immunological effects observed with the use of the CaN inhibitors. The aim of this review is to summarize the current knowledge on the effects of CaN on cytoskeletal proteins and the intracellular pathways involved. These newly described properties of CaN on cytoskeletal organization may explain some of the beneficial or deleterious effects observed in kidney cells with the use of the CaN inhibitors.

Calcineurin effects on neuronal cytoskeleton

Calcineurin was first implicated in the regulation of the structural integrity and the activity-dependent modelling of the neuronal cytoskeleton by Goto *et al.*, who highlighted its ability to control the assembly–disassembly cycle of microtubules [10]. Today, CaN is known to directly control microtubules assembly, through dephosphorylation of tubulin and microtubules associated proteins such as Tau and MAP-2 [10, 14–16], and neurofilaments organization in cultured-neuronal cells [8, 17, 18]. These effects were confirmed in mice lacking neuronal CaN Aα subunit, which displayed abnormal phosphorylation of microtubules, diminution of neurofilament content and abnormal cytoskeleton organization [19].

Calcineurin has also been involved in neuron elongation. This physiological process involves cytoskeletal rearrangements in response to extracellular signals that increase intracellular Ca^{2+} [20]. Growth cones at the distal ends of growing neurites determine the rate and the direction of neuron elongation. Their ability to reorganize rapidly in response to a variety of molecular signals allows the axon to advance, retract, turn or branch mainly through reorganization of actin and microtubule cytoskeleton. CsA and FK506 were shown to inhibit the polarized axon elongation of cerebellar macroneurons [14], shorten the length of neurites produced in culture of chicken dorsal root ganglia neurons on laminin [21] and suppress the neurite outgrowth imposed by Ca^{2+} waves in cultured *Xenopus* spinal neurons [22]. Transitory and local inactivation of CaN induced a retraction of the growth cone and a deviation of its progression from the inactivated site, suggesting that CaN controls filopodia motility [21]. Expression of CaN is also dramatically increased during neuron development and its localization varies during neuron elongation [14, 23].

Calcineurin effects on myocytes

Investigations into the possible role of CaN in myocyte hypertrophic response started when increase in intracellular Ca^{2+} provoked by hypertrophic agonist (*e.g.* Angiotensin II, Phenylephrine or myocyte stretch) was demonstrated to be a major event leading to cardiac hypertrophy [24–28].

Cardiac hypertrophy is characterized by a reorganization of the microtubule network [29–33] and a transcriptional up-regulation of α-tubulin, β1-tubulin and MAP-4 [34–36]. β-Actin is also increased in different models of cardiac hypertrophy [37]. Finally, the intermediate filament desmin is increased in models of cardiac hypertrophy in the guinea pig [38] and is both increased and disorderly rearranged in human end-stage dilated cardiomyopathy [39].

Calcineurin enzymatic activity and protein levels were found to be significantly up-regulated in hearts from numerous cardiac hypertrophy models [40–52] and in human failing or hypertrophied hearts [53–55].

Molketin *et al.* generated transgenic mice, expressing a constitutively active cardiac form of either CaN A subunit or NFAT3 protein, and showed a profound hypertrophic response in the transgenic mice compared to the control mice. Cardiomyocytes were highly disorganized and hypertrophic with dramatic karyomegaly and myofibre degeneration [56]. A similar hypertrophic response was observed in cultured cardiomyocytes, expressing a constitutively active form of mouse CaN Aα [57].

Cellular, morphological and molecular changes associated with cardiac hypertrophy in activated-CaN transgenic mice were prevented by administration of CsA and FK506 [56]. Both drugs also blocked the ability of cultured cardiomyocytes to undergo hypertrophy in response to Angiotensin II and phenylephrine [56]. Furthermore, Sussman *et al.* demonstrated that CaN inhibitors prevented the phenotypic manifestations of hypertrophic cardiomyopathy and the disorganization of myofibrils in transgenic

Table 1 Proteins involved in calcineurin's regulation of cytoskeleton

Calcineurin substrate	Cell's modifications	*References, animal models*
Cofilin	Induction of neurite development	*[82,83]*
	Dendritic spine loss	*[84]*
	Platelet activation	*[85]*
	Tracheal muscle cells activation	*[86]*
NFAT	*Neurite development*	
	Induction on neuronal elongation	*[74, 75] → NFATc3/c4$^{-/-}$ and c2/c3/c4$^{-/-}$ mice; VIVIT-treated zebrafish embryos*
	Inhibition of neuronal outgrowth	*[76] → NFAT3$^{-/-}$ mice*
	Induction of cardiac hypertrophy	
	NFAT3	*[56, 78] → NFAT3$^{+/+}$ mice ;*
		NFAT3$^{-/-}$ transfected cardiomyocytes
	NFAT4	*[77] → NFAT4$^{-/-}$ mice*
	Glomerulosclerosis, proteinuria	*[102] → NFATc1 conditional induction*
Synaptopodin	Podocytes actin cytoskeleton disorganization	*[12]*
WAVE-1	Neurite development in response to neurotransmitters	*[13]*

CaN: calcineurin; NFAT: nuclear factor of activated T cells; WAVE1: WASP-family verprolin homologous protein 1.

mice or pressure-overload hypertrophy rat model [50]. Numerous other studies using a similar pharmacological approach demonstrated that CaN is a key mediator in the hypertrophic response in pleiotropic rodent models [41, 44, 45, 48, 49, 58–69]. The role of CaN was confirmed in transgenic models expressing negative mutants of CaN or inhibitory domains of CaN-interacting proteins [40, 70, 71]. Transgenic mice lacking the CaN Aβ subunit or expressing a negative mutant of CaN displayed a reduced hypertrophic response to aortic banding or agonist stimulation [52, 72].

Intracellular pathway involved in calcineurin-induced effects on cytoskeletal organization (Table 1)

CaN/NFAT pathway was first implicated in cytoskeletal control; however, the discovery of new CaN substrates in the brain, known as cytoskeletal organizing proteins, highlights the fact that CaN may act directly on cytoskeletal organization, in parallel to the well-known NFAT-dependent transcriptional effects.

Calcineurin/NFAT pathway

Nuclear factor of activated T cell family is composed of five proteins (NFAT1/c2, NFAT2/c1, NFAT3/c4, NFAT4/c3 and NFAT5/TonEBP). All of them, except NFAT5, contain a Ca^{2+} sensor/translocation domain and are specifically activated by CaN-mediated dephosphorylation. Serine dephosphorylation within the amino-terminus domain triggers the cytoplasmic-to-nuclear translocation of NFAT and its binding to the promoter of target genes [73].

Calcineurin/NFAT in neuronal elongation

Graef *et al.* were the first to provide evidence for the role of NFAT in controlling the neurotrophin-dependent outgrowth of embryonic axons [74]. They demonstrated abnormal sensory axon projection and commissural axon growth in double (c3/c4) and triple (c2/c3/c4) NFAT mutant mice, while no defect was observed in single mutants. Abnormal axonal growth is specific to NFAT activation by CaN because similar defects were found in CaN B mutant mice and in embryos from wild-type pregnant mice treated with CsA [74]. More recently, CaN/NFAT pathway was also demonstrated to regulate morphological remodelling of axon terminals of olfactory sensory neurons in zebrafish [75]. Using growth-associate

protein-43-EGFP (GAP-43-EGFP) as *in vivo* visual marker for axon terminal maturation, Yoshida *et al.* showed that axon terminal remodelling was prevented by CsA and VIVIT, a specific NFAT inhibitor.

All these results support the idea that NFAT activation is required for proper neural development and functions but, conversely, a recent study reported an unexpected role of NFAT3 in decreasing GAP-43 gene expression during latter part of embryonic neurite development [76]. This work is the first to report a direct control of NFAT proteins on axon outgrowth-related genes in brain and provides an unexpected new role for NFAT3 in negative transcriptional regulation of the neuronal outgrowth program.

Calcineurin/NFAT in cardiac hypertrophy

Nuclear factor of activated T cell activity was found to be increased in primary rat cardiomyocytes subjected to angiotensin II or phenylephrine infusion, and completely abolished by CsA or FK506 [56]. However, the identification of the specific isoform involved remains complex because all of the four CaN-regulated NFAT proteins were identified in the heart and present a high degree of homology within the DNA-binding domain [77, 78].

NFAT3 pathway was first implicated as a pivotal transducer of the cardiac hypertrophy response by interacting with the cardiac transcription factor GATA4 and by activating expression of numerous cardiac genes stimulated during cardiac hypertrophy [56]. Involvement of NFAT3 in cardiac hypertrophy was confirmed in transgenic mice expressing a constitutively activated NFAT3 mutant in the heart and displaying pronounced cardiac hypertrophy with extensive fibrosis [56]. Conversely, cardiomyocytes transfected with a negative NFAT3 transcript displayed neither hypertrophic remodelling nor increase in atrial natriuretic peptide expression in response to hypertrophic stimuli [78]. Together, these results defined a signalling pathway coupling hypertrophic signals to pathological changes in cardiac morphology and gene expression though activation of NFAT3.

However, Wilkins *et al.* did not reproduce the previous results and supported the idea that NFAT3 function might be compensated by another heart-expressed NFAT protein [77]. Expressing a negative mutant of CaN Aα in wild-type and NFAT3 null mice, they observed that loss of NFAT3 did not diminish the magnitude in CaN-induced hypertrophy. Similar reports were made in NFAT3 null mice exposed to angiotensin II or aortic banding, which showed identical cardiac pathology and morphologic hypertrophy than wild-type mice exposed to the same stimuli [77]. On the other hand, NFAT4 null mice showed a significant and long-standing reduction in CaN-induced hypertrophy, or were compromised in their ability to mount an efficient hypertrophic response following aortic banding or angiotensin II infusion [77]. These results support the idea that NFAT4, which is the closest structural homologue to NFAT3, may also act as a downstream effector of CaN in heart.

The hypothesis that NFAT is a critical mediator of CaN signalling is also supported by studies in which cardiac hypertrophic growth was reduced by overexpression of glycogen synthase kinase 3β (GSK3β), known to directly phosphorylate NFAT2 proteins and, thus antagonizing the action of CaN [79, 80].

These results provide multiple lines of evidence for the role of NFAT factors as necessary mediators of CaN-regulated hypertrophic signalling, but further studies are needed to discriminate the proper role of each NFAT isoform.

Calcineurin/actin-associated proteins pathway

ADF and cofilin are major actin-organizing proteins, which regulate actin assembly/disassembly. ADF/cofilin activity is regulated by the state of phosphorylation at a unique site (ser3). Dephosphorylation/activation is achieved by CaN through slingshot phosphatase. Phosphorylation occurs by LIM kinase and inhibits the binding to actin monomers as well as the actin-depolymerizing activity [81]. Meberg *et al.* demonstrated that ADF/Cofilin are involved in the dynamic changes of actin filaments during neurite extension through Ca^{2+}-dependent dephosphorylation/activation by CaN [82]. Treatment of HT4 cells or primary cortical neurons with Ca^{2+} ionophore decreased phospho-ADF/Cofilin [p(ADF/cofilin)] level through CaN activation, and conversely, CsA increased p(ADF/Cofilin) level. Regulation of ADF/cofilin phosphorylation was also found to be an important determinant of growth cone motility and neurite outgrowth because decrease of p(ADF/Cofilin) level was associated with increased process extension, whereas agents increasing p(ADF/Cofilin) level inhibited process extension. Moreover, NGF-induced differentiation was accompanied by decrease of p(ADF/Cofilin) level and accumulation of non-phosphorylated proteins co-localized with actin at the tips of lamilipodia. The control of neuronal actin organization by CaN through cofilin dephosphorylation was confirmed by Homma *et al.*, who highlighted the role of CaN-dependent activation of cofilin in the formation of cofilin–actin rods during neurite outgrowth [83]. Pharmacological inhibition of CaN by cypermethrin or expression of an unphosphorylable variant of cofilin both inhibited the formation of cofilin rods and consequently neurite extension. Finally, CaN inhibitors were also shown to inhibit cofilin dephosphorylation and subsequent depolymerization of actin in a pilocarpine model of status epilepticus [84].

As suggested by a recent study, this effect of CaN on cofilin seems not to be restricted to the brain and neurite outgrowth. Indeed, an effect of CaN on cofilin has also been shown in platelets in response to thrombin and in tracheal smooth muscle cells in response to stimulation with acetylcholine [85, 86]. Interestingly, it has been recently demonstrated that cofilin modifies the length of the primary cilium, an organelle located on the apical surface of many cells, such as endothelial or epithelial renal cells, that is involved in mechanosensing of the cell. This effect is observed after cofilin dephosphorylation by the phosphatase PP-1 and is mediated by a reorganization the actin network [87].

WAVE1 protein was recently identified as a new substrate for CaN, able to modify actin organization [13]. WASP-family verprolin homologous protein 1 (WAVE1) is a WASP protein that stimulates the Arp 2/3 complex and nucleates the *de novo* synthesis and branching of actin filaments [88]. In brain under basal conditions, WAVE1 is inactive and phosphorylated by cyclin-dependent kinase

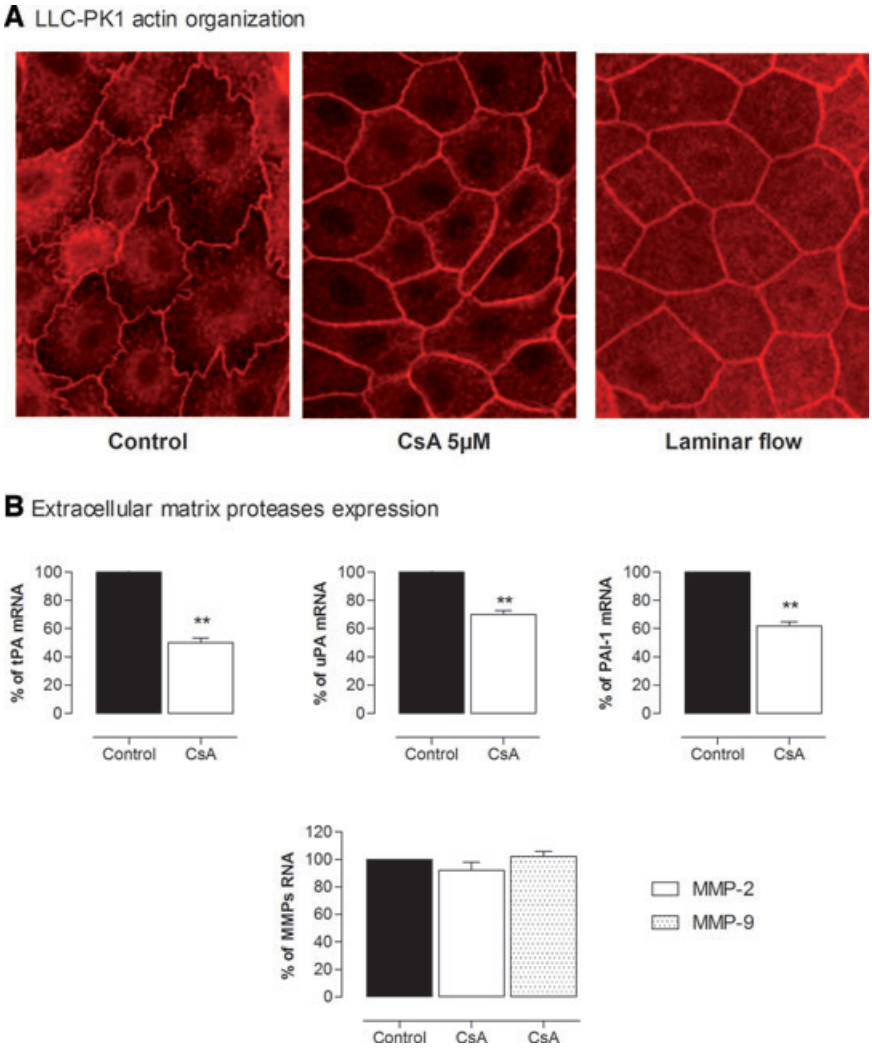

Fig. 1 CsA-induced reorganization of actin filaments and decrease in extracellular matrix proteases in proximal tubular cells. (**A**) LLC-PK1 cells were exposed to CsA 5 μM or laminar flow (1.65 mm/sec.) for 24 hrs then stained with fluorescein-labelled phalloidin and analysed by immunofluorescence microscopy (40×). (**B**) mRNA of tPA, urokinase, PAI-1 and metalloproteinases 2 and 9 (MMP2, MMP9) were quantified by qPCR. Pictures highlight modification in global cell's shape, stiffening of the lateral actin network, and a decrease in tPA, urokinase and PAI-1 expression under CsA conditions.

5 at multiple sites [89]. Ceglia *et al.* have recently revealed a complex pattern of regulation of WAVE1 in response to increase in intracellular Ca^{2+} and CaN activation [13]. Calcineurin was found to dephosphorylate different sites of WAVE1 depending on the neurotransmitter-mediated pathway involved. Activated WAVE1 induces actin polymerization, enabling neurite outgrowth and neuronal plasticity in response to the distinct neurotransmitters.

WAVE1 is predominantly present in the brain but other members of the WAVE family are more ubiquitous, playing a major role in the actin organization of numerous cells. For example, WAVE2 has been involved, through actin organization, in fibroblast or myogenic cell migration, vasculogenesis, cell–cell adhesion or formation of the immunological synapse during T cell activation [90–94]. Ceglia's results showing WAVE1 regulation by CaN of open thus a large field of investigation if a similar modulation by CaN of WAVE2 phosphorylation could be demonstrated.

Calcineurin effects on kidney cells cytoskeleton

The importance of CaN in the kidney is suggested by the frequently observed nephrotoxicity of CsA and FK506. The physiologic role of CaN in kidney cells remained unknown until the recent exhibition of abnormal kidney development in mice invalidated for the CaN Aα subunit. However, the authors do not forward a mechanistic explanation [95].

More recently, Faul *et al.* have opened a new field of investigation of the role of CaN in kidney by analysing the intracellular pathway involved in the anti-proteinuric effect of CsA in nephrotic syndrome. They identified synaptopodin as a new target of CaN in podocytes and a non-immunological effect of CsA on the podocyte cytoskeleton [12].

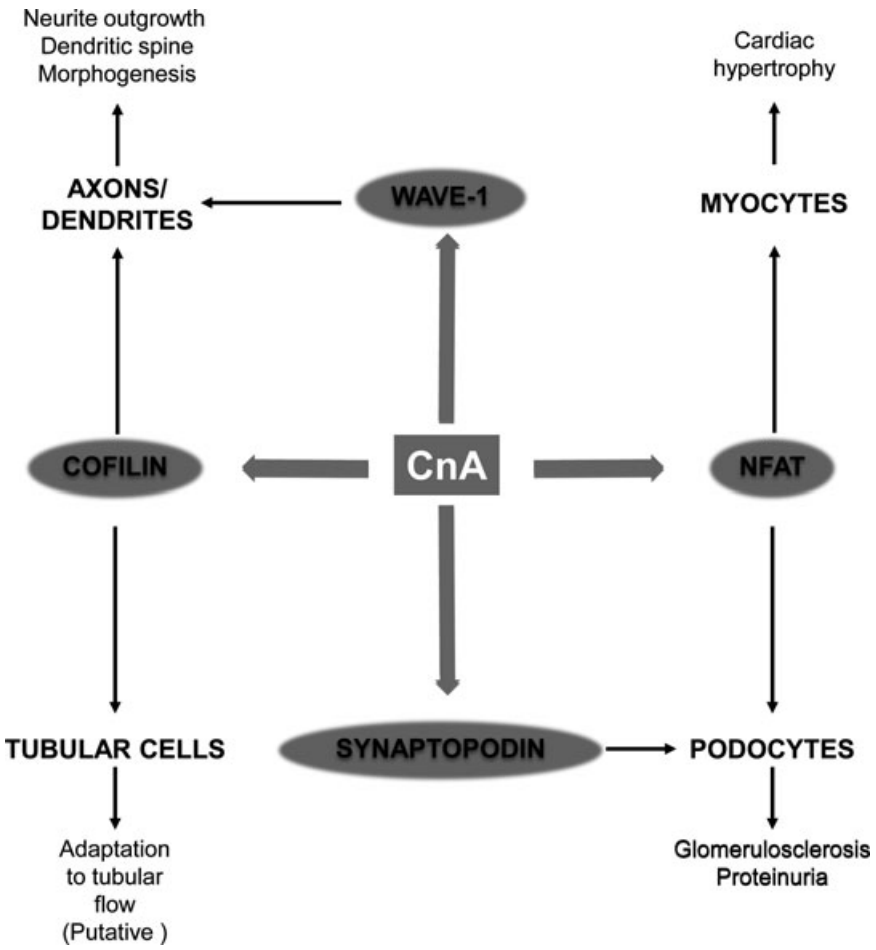

Fig. 2 Role of calcineurin-induced organization of cytoskeleton in brain, heart and kidney. CaN: calcineurin; NFAT: nuclear factor of activated T cells; WAVE1: WASP-family verprolin homologous protein 1.

Synaptopodin is an actin-associated protein specifically expressed in differentiated podocytes and in a subpopulation of telencephalic neurones, and involved in actin regulation, synaptic plasticity and organization of podocyte foot processes [96, 97]. Synaptopodin is selectively down-regulated by nephrotic plasma or after puromycin aminonucleoside treatment [98, 99]. Synaptopodin-deficient mice display impaired recovery from protamine sulfate-induced foot process effacement and lipopolysaccharide-induced nephrotic syndrome [100]. Gene silencing of synaptopodin in podocytes causes the loss of stress fibres, the formation of aberrant non-polarized filopodia and impairment of cell migration [101].

In their work, Faul *et al.* highlighted that CaN dephosphorylates synaptopodin, rendering it unable to bind to chaperone molecules of the 14-3-3 proteins family and thus, facilitating its degradation by cathepsin L [12]. CsA, by inhibiting the dephosphorylation of synaptopodin, blocks its degradation by cathepsin and allows a stabilization of the podocyte actin cytoskeleton. Faul *et al.* also demonstrated that transgenic mice expressing a constitutively active form of CaN developed significant proteinuria. Such an activation of the CaN pathway may be observed in focal and segmental glomerulosclerosis due to mutation of the Ca^{2+} channel TRPC6 [102]. However, the effect of CaN on the podocyte cytoskeleton is not solely mediated by synaptopodin dephosphorylation. In fact, Wang *et al.* recently provided *in vivo* evidence that NFAT activation, either *in utero* or post-developmentally, leads to proteinuria and glomerulosclerosis [103]. Ultrastructural studies revealed podocyte foot process effacement and deposition of extracellular matrix whereas NFAT activation did not initially affect expression of synaptopodin.

The results of Faul *et al.* lead us to hypothesize that the deleterious effects of CaN inhibitors on tubular structure observed in kidney transplantation could be due to a disorganization of tubular cytoskeleton and an incorrect adaptation to the increase in tubular flow. To investigate this, we analysed the effect of CsA on the proximal tubule cytoskeleton and demonstrated that CsA induced a strong reorganization of actin filaments [104]. The stiffening of the actin network impacts the phenotype of proximal tubular cells because it is associated with an inhibition of extracellular matrix protease expression with a decrease in tissue-type Plasminogen

Activator (tPA) and urokinase and their inhibitor PAI-1 (Fig. 1). These modifications are similar to what is observed when mechanical strains induced by tubular flow on tubular cells are increased such as after subtotal nephrectomy [105]. As regards to the intracellular pathway involved, we demonstrated that CsA-induced actin reorganization was independent of NFAT inhibition because a specific inhibitor of NFAT dephosphorylation did not reproduce the effect of CsA on the cytoskeleton.

The effect of CaN on renal cells seems thus to be beneficial or detrimental according to the structure triggered. CaN and NFAT activation in podocytes seems to play an important role in the development of podocytes damaged within states of nephrotic syndrome and glomerulosclerosis. In glomerular cells, CsA inhibits CaN acting on actin filament, thus having a beneficial effect by restoring normal podocyte structure. On the contrary, in tubular structure, the stiffening of actin network induced by CsA may alter the response of proximal cells to modifications of tubular flow, thus exerting a detrimental effect on tubular structure.

Conclusion

Calcineurin was initially known for its immunological properties through the activation of NFAT proteins in immune cells. Further studies have underlined its role in the regulation of cytoskeleton organization in other cell types such as neurons, myocytes, and recently podocytes and proximal cells, highlighting some of its non-immunological properties (Fig. 2).

The control of cytoskeleton organization is mediated by both its NFAT-dependent transcriptional effects and by the direct control of filament organization through dephosphorylation of cytoskeletal organizing proteins such as cofilin, WAVE-1 and synaptopodin. The recent identification of these pathways in kidney cells opens new fields of investigation to explain some beneficial or detrimental effects of CaN inhibitors on kidney and the phenotypic changes observed after long-term exposure to these drugs.

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Conflict of interest

The authors confirm that there are no conflicts of interest.

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