Cover Page

The handle http://hdl.handle.net/1887/20134 holds various files of this Leiden University dissertation.

Author: Musson, Ruben Eduardus Antonius
Title: Calcineurin in skin : rising star or fallen angel ?
Date: 2012-11-15

CHAPTER 2

regulatory mechanisms of calcineurin phosphatase activity

R.E.A. Musson and N.P.M. Smit

Current Medicinal Chemistry 2011; 18: 301-315

ABSTRACT

Calcineurin (protein phosphatase 3, Cn) is best known for its central position in Ca^{2+}-dependent T cell signaling. Interest in calcineurin has, however, conserved its momentum as new Ca^{2+}-dependent pathways have been steadily surfacing in several other cell types, such as brain, heart, skin cells, and beta pancreatic cells, and Cn appears to serve as a central controller of stress, immune response, and cellular proliferation and differentiation.

Calcineurin is the principal target of the immunosuppressive drugs cyclosporin A (CsA) and tacrolimus (TRL). Therapy based on these immunosuppressants has markedly reduced the incidence of transplant rejection in allograft recipients. In addition, these drugs have proven very useful for patients suffering from chronic inflammatory skin conditions. Unfortunately, their application is somewhat limited by a broad spectrum of toxic side-effects, affecting several organ systems. This calls for enhancements in the design of this class of immunosuppressants.

An intricate constellation of regulatory systems allows for precise modulation and adaptation of calcineurin activity *in vivo*. The last few years have been very fruitful in elucidating several long-standing issues regarding the binding patterns of substrates and inhibitors to Cn. This new knowledge may enable more precise manipulation of the Ca^{2+}-calcineurin pathway in the near future, preferably targeted towards one specific substrate or cell system. In this review, we will discuss the factors and mechanisms underlying calcineurin activity regulation and their exploitation in recent approaches towards better immunosuppressants.

INTRODUCTION

Calcineurin, or protein phosphatase 3 (Cn, PP3, formerly PP2B), is a heterodimeric, Ca^{2+}-dependent serine/threonine phosphatase enzyme that fulfills important mediating functions in the T cell activation programme (*1*), muscle growth and development (*2*), apoptosis (*3*), DNA repair (*4, 5*), and cardiac and neural health (*6, 7*) (reviewed in (*8*)). Our understanding of additional roles of Cn in other cell systems, such as pancreatic beta-cells (*9, 10*) and skin (*11-13*), is gradually taking shape. Various muscle and neuronal proteins and enzymes are directly dephosphorylated by Cn; however, through the transcription factors NFAT, NF-κB, and Elk-1 (*14-19*), the realm of Cn extends to the level of regulation of gene expression, as depicted in figure 1.

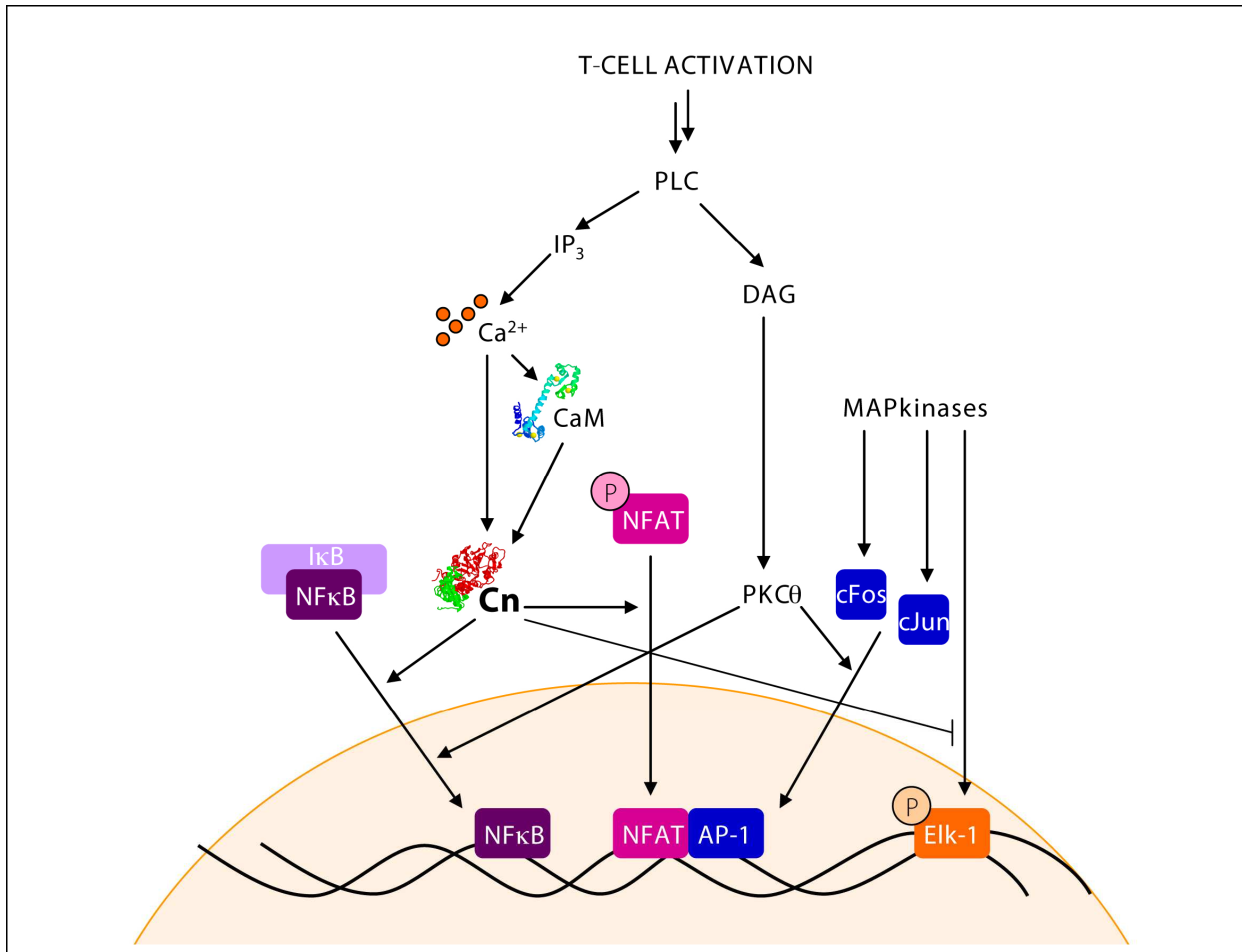

FIGURE 1 Simplified overview of the position of Cn in T cell signaling. Activation of the T cell receptor leads to hydrolysis of PIP2 by phospholipase C (PLC) to diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). IP_3 stimulates Ca^{2+} release from the endoplasmic reticulum and, hence, activates calcineurin. DAG stimulates protein kinase C theta (PKCθ). Together with the MAPkinases, these enzymes modulate the activity of an important set of transcription factors. PIP_2 = phosphatidylinositol 4,5-bisphosphate; NFAT = Nuclear Factor of Activated T cells; NF-κB = Nuclear Factor κB; IκB = Inhibitor of κB; AP-1 = Activator Protein 1; Elk-1 = Ets-like protein 1.

Beside representing useful means to acquire further insight into cellular signaling pathways, charting the conspicuously versatile mechanisms of calcineurin activity regulation has an apparent therapeutical concern: Cn inhibition is one of the cornerstones of contemporary immunosuppressive protocols, which generally revolve around cyclosporin and tacrolimus as part of a multidrug regimen (20). Since both drugs feature a daunting repertoire of toxic side-effects and their all-round suppression of Cn not only abolishes the adaptive immune response but also has the potential to affect other signaling routes and components of the cellular machinery, much effort is put in the optimization of pharmacodynamic monitoring strategies (21) and in the pursuit of less toxic and more specific alternatives (reviewed in (22)). The design of new modulators of

Cn activity, however, heavily relies on thorough knowledge of the many facets and features of Cn regulation (see table 1). Here, we will catalogue this knowledge, covering the different domains of Cn, the mechanisms of substrate binding and dephosphorylation, the structural requirements of Cn inhibitors, and the cellular actors that may limit or control Cn activity.

TABLE 1 The regulatory landscape of calcineurin signaling. Numbers between parentheses refer to the respective sections.

	ENDOGENOUS		EXOGENOUS
Activating	CnB, calmodulin (1) Chaperone-like activators (3) Ca^{2+} (5)		
	RCAN family (3) Anchoring proteins (3)	ROS (4) Metal ions (5)	Miscellaneous compounds (6)
Inhibiting	Sequestrating proteins (3) Substrate binding inhibitors (3)		CsA/TRL and analogues (2)

1. REGULATORY DOMAINS

Well before mainstream interest in Cn was aroused, Klee and coworkers had already taken the lead in elucidating the structure of calcineurin and performed pioneering work in investigating the nature of its protein-protein interactions with calmodulin (CaM). Like all other forms of calcineurin that have been isolated to date, human Cn is composed of two subunits: the A subunit (CnA) is the seat to the enzyme's catalytic center, while the B subunit (CnB) is a Ca^{2+}-binding regulatory subunit. Several isoforms of both subunits have been identified (*23-25*), displaying different kinetic parameters towards a variety of substrates and divergent expression ratios within and between tissues (*26-28*). The B subunit is tightly associated with the A subunit and can only be dissociated under denaturing conditions or at extremely low Ca^{2+} levels (*29*). Within the A subunit, regions with specific assignments can be distinguished, as verified by limited proteolysis (*30*) and structure determination studies (*31*) (see figure 2). The CnA chain comprises a catalytic domain (human α isoform ≈ aa 20-340), a CnB binding helix (BBH, ≈ aa 349-372), a calmodulin docking segment (≈ aa 390-414) and a C-terminal autoinhibitory (AI) helix (≈ aa 469-486) occupying the active site access channel (*32*). Additional autoinhibitory elements are believed to exist throughout the entire CaM binding region (*33-35*).

Proteolysis with clostripain strips the A subunit from its calmodulin docking region and its AI helix. Additionally, a 2 kDa *N*-terminal sequence is removed from the enzyme. X-ray diffraction data on the α isoform showed part of this sequence to contribute to the binding interface with CnB (*31*). In the β isoform, however, this region is extremely proline-rich (*24*), the purpose of which is as of yet unclear. The proteolytically resistant enzyme core, which closely resembles the core of Cn's protein phosphatase siblings 1 and 2A (*36*), encompasses the CnB binding region as well as the catalytic domain; it was originally reported to have a tenfold higher activity than the integral enzyme and, surprisingly, even 40-fold higher activity in presence of Ca^{2+} (*30*). More recently, though, it became clear that the purification protocol used at that time does not take the extraordinary sensitivity of Cn to oxidation into account (*37*). In fact, after cautious work-up, the fully intact, Ca^{2+}-stimulated enzyme may even surpass the catalytic core in terms of activity, depending on the substrate (*38*).

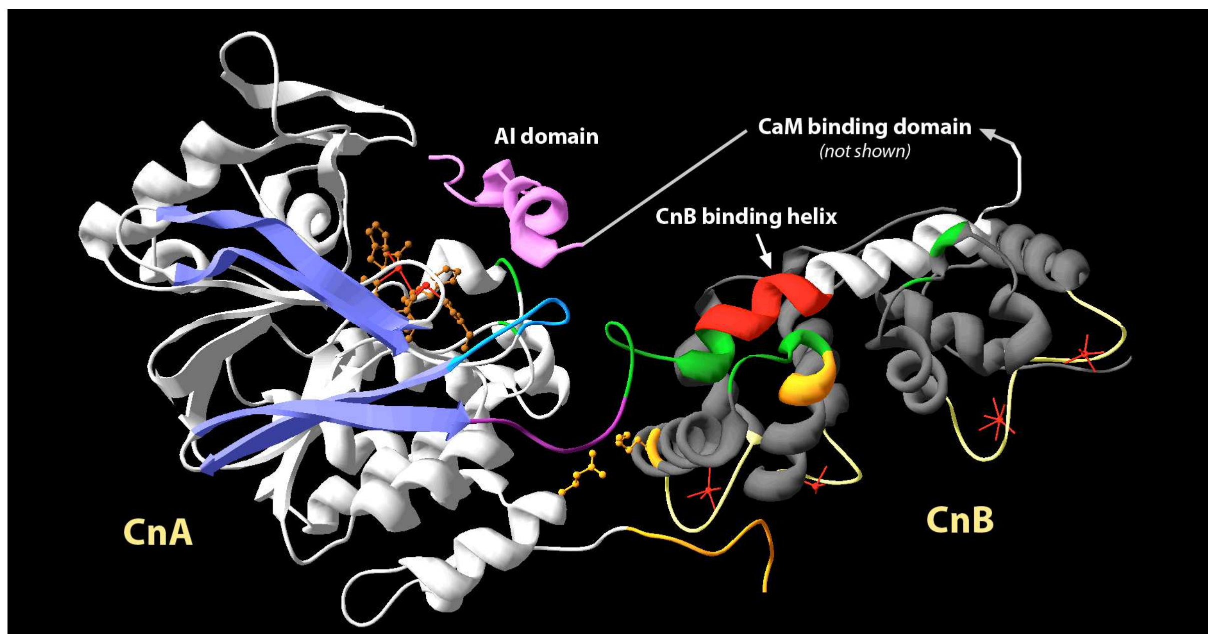

FIGURE 2 Overview of the calcineurin heterodimeric enzyme. The CaM binding aa 374-468 region is not present in the x-ray structure due to its disordered nature. The A subunit is displayed in light grey, the B subunit in dark grey. Metal ions are depicted in red. Distinctive regions and residues, as discussed throughout the text, are highlighted in color and specified in table 2. Image adapted from PDB entry 1AUI (*31*) and modeled using Swiss PDB viewer.

The CnA catalytic domain contains the active site, which features two intrinsic metal ions, iron and zinc (*39-41*), located at the bottom of a polypeptide substrate binding groove (*31*, *42*). An identical active site configuration was found in protein phosphatase 1 (*43*). Under oxidative conditions, exogenic transition metals can replace the intrinsic metals (*37*). The allocation of catalysis-related roles is not entirely clear and the need for both metals has

even been questioned (*44*), although it seems plausible that one of the metals activates water to a more nucleophilic species, while the other performs supporting and stabilizing functions (see section 5) (*45*). Both zinc and several adjacent Arg residues were proposed to aid in stabilizing the developing negative charge during the transition state (*36*, *46*).

TABLE 2 Summary of highlighted regions and residues in the Cn protein structure (figure 2).

RESIDUES	COLOR	FUNCTION
CnA		
14-23, 53	orange	CnB interaction
311-318	aqua	β12 - β13 loop (loop 7, CnI binding and activity regulation)
287-334	lavender	β11 - β14 strands (PxIxIT recognition moiety)
335-347	violet	hinge region/linker (required for PxIxIT interaction)
341-356, 122, 159-160	green	CnI interaction
352-356	red	LxVP binding motif
349-373		CnB docking helix (BBH)
469-486	pink	autoinhibitory helix (AI)
90, 92, 118, 150, 151, 199, 281	brown	active site metal coordinating ligands
CnB		
115-116, 134	orange	CnA interaction
118-123, 47, 50	green	CnI interaction
30-38, 62-70, 99-107, 140-148	yellow	Ca^{2+} binding

The AI domain and the B subunit

If intracellular [Ca^{2+}] is sufficiently high, calmodulin docks to the CaM-binding domain of Cn; in a similar fashion as in other CaM-activated enzymes, this results in displacement of the autoinhibitory fragment from the entrance to the catalytic center (*47*). Removal of this intrasteric inhibition constitutes Cn's primary on-switch (*48*, *49*). Interestingly, Burkard *et al.* found evidence of targeted proteolytic cleavage of CnA at residue 424 by calpain, sustaining Cn activity and causing persistent transcription of Cn-controlled genes, occuring *in vivo* in cardiomyocytes (50). Wu *et al.* showed three *in vivo* cleavage sites for calpain, including residue 424, in neuronal cells. These findings imply that, under specific circumstances – such as prolonged Ca^{2+} overload –, a complete severing of the AI domain rather than a mere retraction may occur. Here, the unrestrained Cn would

trigger caspase activity, thus presenting the classic image of excitotoxic neurodegeneration (*51*).

CnB features 4 EF-hand motifs, each able to accomodate a Ca^{2+} ion; contrary to CaM, however, CnB does not release its calcium ions at low Ca^{2+} levels (*52*). The Ca^{2+} binding sites in the *N*-terminal half of the B subunit proved crucial for activation of the A subunit. The carboxyl half was designated a mainly structural role, although one of its calcium sites interfaces with the CnA amino terminus (aa 14-23, see figure 2) to complete the activation signal (*31, 53*). A salt bridge between Glu-53 of CnA and Lys-134 of CnB was found to be essential for the correct orientation of the CnA *N*-terminus and an important handle to CnB to obtain accurate rapprochement to the A subunit (*54*).

The CnB subunit seems to act in synergy with calmodulin in stimulating Cn activity (*55*). CnB and CaM are structurally similar, but cannot substitute for one another (*29*). Both increase V_{max}, whereas the B subunit additionally lowers K_m (*56*). The event of calcium binding to the B subunit is believed to be accountable for this phenomenon (*33, 55*), causing a conformational change and exposure of a hydrophobic surface (*57*), which is subsequently being relayed to CnA by two residues with high affinity for the A subunit – Val-115 and Leu-116 (*58*). Conformational adjustments in the CnA regulatory domain are required for sufficient exposure of the calmodulin binding domain (*59*). The current opinion is that CnB enables CaM binding, whereas CaM subsequently accomplishes the conformational change needed for enzyme activity (*60*). This intricate allosteric interplay ensures that Cn phosphatase activity can be regulated both positively and negatively, depending on intracellular Ca^{2+} levels (*53*).

2. IMMUNOPHILIN COMPLEXES

Present-day transplantation medicine extensively uses two pharmaceutical agents based on their ability to inhibit Cn activity: the cyclic undecapeptide cyclosporin A (CsA) and the macrolide tacrolimus (FK506, TRL). Since the 1980s, a plethora of clinical studies have illustrated their value in a wide array of immune-related conditions, in particular prevention of rejection in allograft recipients (*61-63*). It did not take long before the interfering action of CsA with several cytokines involved in the immune response was discovered (*64*). In 1989, efforts from the Crabtree group made it possible to trace these cytokines back to specific transcription factors, principally the Nuclear Factor of Activated T cells (NFAT), whether or not in consort with AP-1 (*65*), but also NF-κB (*66, 67*) (see figure 1). However, by that time it was still unclear by what mechanism CsA and TRL manifested their immunosuppressive effects. This soon changed with the breakthrough discoveries of calcineurin as a common target for both CsA and TRL (*68*) and the blockage of NFAT-mediated transcription in T cells by CsA and TRL (*69*), which led

Clipstone *et al.* to speculate Cn may be responsible for the nuclear translocation of NFAT (*1*). The identification of NFAT as a substrate of Cn shortly afterwards (*70, 71*) eventually reconciled the IP_3-induced Ca^{2+} flux at the plasma membrane following T cell receptor activation with the earlier observed transcriptional events (*72*).

An unprecedented mechanistic hallmark is the fact that cyclosporin and tacrolimus need endogenous carrier proteins, named cyclophilin and FK-binding protein-12 (FKBP12), respectively, to exert their immunosuppressive actions. Both immunophilins exhibit peptidylprolyl rotamase activity, which is abolished upon binding of the immunosuppressant molecules (*73-75*). X-ray and NMR data on the TRL-FKBP complex revealed a marked difference in TRL structure before and after binding to FKBP, whereas the protein conformation did not change considerably on complexation (*76, 77*). Similar findings were reported concerning the binding characteristics of cyclosporin to cyclophilin, featuring *cis* to *trans* conversion of an amide bond in the drug molecule (*78, 79*). This may explain why both immunosuppressants in their unbound conformation are devoid of any Cn suppressing activity (*68*). In fact, the observed variations in efficiency of the calcineurin inhibitors across cell types correlate with deviations in the ratio of Cn to immunophilin expression levels (*80, 81*), which suggests that the immunophilin concentration is a limiting factor for Cn inhibition.

Binding to calcineurin

Para-nitrophenyl phosphate (*p*NPP) is a small substrate to Cn. Ever since it was described by Pallen and Wang as a rapid way of quantifying Cn functioning and probing reaction kinetics (*82*), it has been applied in a large number of mechanistic studies (see section 5). Paradoxically, in presence of TRL-FKBP12 and CsA-Cyp, which inhibit the activity of Cn towards its physiological targets, *p*NPP dephosphorylation is increased (*83, 84*). Although this finding conveys interesting information on the mode of inhibition by the drug-immunophilin complexes, it also entails that Cn assays cannot solely rely on *p*NPP as a substrate. Certain proteins and peptides – mainly RII, a peptide from the regulatory subunit of cAMP-dependent protein kinase – proved to be better alternatives (*83, 85*). The behavior of Cn towards different classes of substrates is a dynamic area that still receives attention (see section 3) (*86*).

The elucidation of the crystal structures of the TRL-FKBP-Cn complex and CsA-Cyp-Cn complex provided a substantial amount of new knowledge on Cn structure and regulation (*31, 32, 42, 87*). TRL-FKBP12 and CsA-Cyp both bind to a composite surface at the interface of the catalytic and regulatory subunits of Cn. Calmodulin increases the Cn affinity of both the TRL-FKBP12 and CsA-Cyp complexes (*88*), which could mean that the AI-domain poses a sterical hindrance and needs to withdraw before the drug-immunophilin complexes can start interacting with Cn. Subsequently, Cn undergoes subtle structural changes that are limited to the BBH (CnB binding helix) and the flexion of its hinge angle with the catalytic domain (*32*). Both drugs primarily engage in

interactions with predominantly hydrophobic residues of the BBH (aa 343-356, see figure 2) and CnB (aa 118-123); the TRL allyl function, for instance, is deeply embedded in a hydrophobic patch (*32, 89*). Important to mention is the suggestion put forward by Watanabe *et al.*, that the interaction of immunophilin complexes with the hydrophobic CnA binding region of CnB might weaken its stimulating effects on the A subunit (*58*). This viewpoint concurs with a mechanism proposed a few years earlier by Milan *et al.*, who had pointed out that mutations in the latch region of CnB not only impart resistance to CsA and TRL but also impair the stimulation of CnA (*90*). Since the presence of CnB has proven indispensable for binding of CsA (*91*), this may likely be the fundamental mechanism by which immunophilin complexes operate on Cn.

Although the binding repertoires of the CnI (calcineurin inhibitor) complexes CsA-Cyp and TRL-FKBP12 are largely overlapping, there are noteworthy differences in hydrogen bonding patterns that take care of secondary interactions (*46*). For example, coordination to CnA Tyr-341 is both unique and of paramount importance to CsA binding (*87, 92*). Speculation arose that these discrepancies may also reflect slightly different modes of inhibition. TRL-FKBP12 has been hypothesized to occlude the approach to the active site entrance, thus inhibiting substrate access (*42, 46*). CsA-Cyp, on the other hand, seems to interact strongly with an active site residue, Arg-122, that may be of importance to phosphate coordination (*46*).

As the aforementioned composite surface is located adjacent to the catalytic center and their mutual orientation is reminiscent of a docking cleft, it has been suggested that this moiety may serve as a universal recognition site for Cn binding molecules (*46*). Very recently, it was confirmed that the docking moiety for CnI complexes is highly conserved and that the docking sequence displayed by the CnI complexes is shared with NFATc1 (this sequence is depicted in figure 2). Mutations in this area (W352, S353, F356 in CnA; M118-V119 in CnB) impair the interaction of Cn with its substrates but also with several regulatory molecules (cf. section 3) (*93*).

Several research groups pointed out that some immunophilins can interact with or even inhibit Cn in absence of their exogenous ligands (*94-96*). Li *et al.* identified a striking Ca^{2+}, calmodulin- and TRL-independent interaction between FKBP51 and Cn (*97*). Cardenas *et al.* argued the immunophilins play a role in the regulation of the CnA-CnB subunit association/dissociation process. Whether this interaction amounts to a mere regulation of assembly rate or whether their peptidyl prolyl isomerase activity is somehow required for Cn assembly and folding to an active state, is unknown, though (*98*). Lastly, in absence of TRL, FKBP12 has been reported to facilitate the association of Cn with the inositol 1,4,5-triphosphate (IP_3) receptor and the ryanodine receptor and regulate their phosphorylation status. Quintessentially, this mechanism comes down to a dynamic feedback loop, in which the IP_3-mediated Ca^{2+} flux remodulates itself *via* calcineurin (*99*) (see also figure 1).

Current developments

The cyclosporin family consists of a substantial number of members. Each displays a unique pharmacological profile; inhibitory effects not only on Cn activity, but also on P-glycoprotein (*100*) and the mitochondrial permeability transition (*101*) have been established. Cyclosporin A proved the most adequately suited for immune suppression. However, CsA features an extensive portfolio of adverse effects, particularly nephrotoxicity and malignancy, which limits its long-term use (*102, 103*). Many strategies for the development of cyclosporin therapy aim towards improving its therapeutic index by closing down on these side-effects or by designing ways to reduce drug and metabolite load. Unfortunately, lack of functional groups on the CsA molecule fairly restricts the degree of freedom to apply (semi)synthetic modifications (*104*). Among the derivatives that have been created, almost none achieves high marks in both the potency and specificity criteria (reviewed in (*105*)). Therefore, naturally occurring cyclosporin analogues, such as FR901459 (*106*), still receive considerable interest. However, preclinical observations on ISA247, a CsA derivative developed by Isotechnika, shows this drug to be a possibly viable alternative. ISA247 has a modified functional group on aa1, which both makes it bind more tightly to Cn and shifts its metabolism to other sites. Affinity for CypA is also improved, while its potency at lower concentrations than CsA may aid reducing toxicity (*107*). Clinical trials assessing its applicability in the treatment of psoriasis are currently in an advanced stage (*108*). Among newly generated compounds are a series of deuterated CsA analogues that are more resistant to biotransformation and clearance (Isotechnika, patent #WO9918120) and CsA esters that are swiftly inactivated to minimize systemic exposure (Enanta Pharm, patent #WO02069902). Alternatively, CsA conjugates have been synthesized to increase water solubility and, thus, bioavailability or to attain tissue-specific delivery (*109*).

The macrolide CnI group currently consists of tacrolimus and the ascomycin family. Their key difference is the presence of an allyl group in TRL where the ascomycins feature an ethyl group. The ascomycins have been subject to extensive clinical testing in the past decade (*110-112*). Notable members of this group are, beside the parent molecule ascomycin (FK520), pimecrolimus (SDZ ASM-981), ABT-281, and SDZ 281-240. As with cyclosporin, analogues with varying affinities for FKBP12 and Cn have been constructed and tested in order to ultimately find the optimal balance between potency and specificity (*105*). Macrolide CnI are more suited for topical use than cyclosporin; pimecrolimus, due to its higher lipophilicity, shows large efficacy towards atopic dermatitis, while maintaining minimal systemic immunosuppression (*113*).

3. OTHER ENDOGENOUS PROTEINS

Liu discerned four classes of calcineurin binding and regulatory proteins (*96*). Calmodulin, being an activator of Cn, belongs to the first group. By now, heat shock proteins 70 and 90, known to increase Cn V_{max} by direct chaperone-like interactions (*114, 115*), may have secured themselves a position in this group as well.

The second class harbors the RCAN family of proteins with stimulating as well as inhibitory actions. RCAN1 (*116*) is the product of the DSCR1 gene, which is closely associated with the pathogenesis of neural defects in Down syndrome and Alzheimer's disease (*117, 118*). At low concentrations, RCAN1 serves as a vital initiator of Cn activity (*119*), in a way reminiscent of a chaperone molecule (*120*). Higher levels, on the other hand, inhibit Cn, probably by means of competition of its central SP-repeat segment with substrate (*121*). In addition, RCAN1 induces the Cn-antagonizing kinase GSK-3β (*122*). Since Cn itself instigates RCAN1 transcription (*123*), this would indicate a dual feedback mechanism (*119*). On top of this, RCAN1 can be phosphorylated by GSK-3β and dephosphorylated by Cn; its phosphorylation status is believed to be determinative for its affinity for and effects on Cn (*120*). Ermak *et al.* reported that transient expression of RCAN1 serves as a safeguard system against calcium-mediated stress and oxidative damage. Prolonged overexpression of RCAN1, however, may have a hand in the precipitation of neuronal degradation (*118, 124, 125*) *via* Cn activity suppression and a consequent hyperphosphorylated state of tau protein. Lian *et al.* found diminished Cn activity in the cerebral cortex of Alzheimer patients, correlating with neurofibrillary tangle pathology. Cn expression levels, however, were unaffected (*126*).

Inhibitors Cabin1/cain, FKBP38, CHP, and the African swine fever viral protein A238L (*127*) make up the third group. Cabin1/cain was identified as a phosphoprotein that becomes extensively phosphorylated upon PKC activation. Its ability to inhibit Cn resides in its *C*-terminal domain and is dependent on both PKC and calcium signals (*128*). In addition, Cabin1/cain has been found to enclose a second Cn binding domain that interacts with the Cn *N*-terminal region (*129*). The expression of Cabin1/cain in brain resembles that of Cn, which points at a physiological relation between these two proteins (*130*). Following stimuli that elicit apoptosis through elevation of intracellular [Ca^{2+}], Cabin1/cain is cleaved at its C terminus by calpain, eliminating its inhibitory effects on Cn; this would enable Cn to mediate the course of apoptotic cell death (*131*).

Several FK binding proteins can interact with Cn in a ligand-independent fashion (see section 2), but FKBP38 takes up a unique position, as it is incapable of binding TRL at all (*132*), yet effectively blocks Cn activity. After the discovery that FKBP38 and Cn crosstalk with Bcl-2 in counteracting apoptosis (*95*), Weiwad *et al.* showed this blockage to be founded in altered subcellular distribution of Cn following sequestration (*133*).

CHP is the ubiquitously expressed EF-hand Ca^{2+}-binding Calcineurin Homologous Protein, which displays a striking degree of structural homology with both CnB and calmodulin but inhibits calcineurin. Its dealings with Cn are limited to the A subunit and seem to be based on antagonization of calmodulin (*134*). CHP expression is upregulated solely when Cn is inactive. In T cells, this mechanism may ensure that Cn is kept silent unless set to work by activation of the T cell receptor (*134*).

The fourth group consists of anchoring proteins that could serve as scaffolds in order to physically link Cn to other proteins or structures; notable members of this group are, in addition to FKBP12 (*99*), AKAP79 (*135*), calsarcins (*136*), superoxide dismutase (*137*) (cf. section 4), and Bcl-2 (*138*). AKAP79 is the 79 kDa A kinase associated protein, which leads Cn within reach of protein kinases A and C (*135*). The mode of interplay between Cn, AKAP and PKA is, however, largely unclear, although AKAP79 anchoring to Cn does appear to regulate the translation of neuronal Ca^{2+} channel activity to nuclear signaling (*139*).

Bcl-2 has been known to prevent most cases of calcium-dependent apoptosis, for which Cn appears to be a crucial mediator (*140*). As the intrinsic phosphatase activity of Bcl-2-bound Cn remains fully intact, it was theorized that, during stress or damaging conditions, Bcl-2 shuttles active Cn to specific microenvironments and compartments in the cell to interact with other substrates, and prevents Cn from accessing NFAT (*138*, *141*). Upon heterodimerization with pro-apoptotic protein Bax, Bcl-2 dissociates from Cn. Back in the cytosol, Cn starts dephosphorylating the apoptotic death promoter Bad (*3*) and restores NFAT signaling (*141*).

The proteins that have been discussed so far typically target the CnA subunit or the heterodimer enzyme as a whole. Intriguingly, several proteins that associate with CnB alone, tubulin and Hsp60, have been reported; it is, therefore, conceivable that CnB performs additional regulatory functions in the cell, independent of the CnA subunit (*142*).

Cn recruitment sequences

The binding mode of many endogenous regulators of Cn strongly resembles the conventional interactions of Cn with its most celebrated substrate NFAT. Early work by the Rao and Hogan groups describes the association of Cn with a regulatory domain in both NFAT1 and NFAT2, *N*-terminal to its DNA-binding domain (*143*). Domain analysis revealed two Cn recruitment sites in this region (*144*). The primary recognition site was reduced to a short, ≈ 12 aa, motif containing a shared PxIxIT sequence (*145*). In presence of a SPRIEIT peptide, NFAT dephosphorylation was inhibited, whereas catalytic activity towards substrates that do not rely on recognition of a PxIxIT motif, such as RII peptide and tau protein, was not impaired (*145*). A subsequent publication recounts the mapping of a synergistic interaction site that would account for the increase in binding strength of NFAT when Cn becomes activated and would explain why binding of NFAT to Cn is

obstructed by immunophilin complexes (*146*). It was hypothesized that Cn cooperatively binds to both regions, with the strength of each interaction fluctuating among individual NFAT isoforms (*147*). Sequence analysis of this secondary motif identified a conserved LxVP sequence present in all NFAT-members that are regulated by Cn. An LxVP peptide proved able not only to disturb Cn-NFAT binding, but also to disrupt Cn phosphatase activity (*148*).

An important development was the identification of a region in the Cn A subunit crucial for enzyme regulability. The Wei group appreciated the importance of loop 7 (between β12 and β13, aa 311-318; see figure 2) for mediation of Cn activity, as deletions in this region increased phosphatase activity, while some even rendered the A subunit literally insensitive to CnB (*149*). Moreover, mutations in this region confer resistance to CsA, but not to TRL (*32*). By affinity-driven selection among PxIxIT peptides, a 16-residue peptide – colloquially referred to as 'VIVIT' – was identified as the most potent inhibitor of NFAT processing (*150*). Its docking site on Cn was roughly described as the β11-β14 region (*151*), containing loop 7. However, Rodríguez *et al.* found an intact CnA aa 335-347 linker region to be essential for NFAT binding as well (*152*). NMR modeling of the Cn-VIVIT complex revealed the VIVIT peptide to form a parallel β-sheet with the β14 strand of CnA; when extrapolated to NFAT, the topology of this interaction would orient NFAT's phosphorylated residues towards the Cn active site (*153, 154*). On the other hand, the LxVP motif, preferably preceded by a flanking aromatic residue, seems to bind to Cn in a similar fashion as immunophilin complexes do, as deduced from observed competition for Cn binding. Molecular dynamic simulations show that in a CnB/Ca^{2+}/CaM-dependent manner – since in the inactivated enzyme, this area is masked by the AI domain (*146*) – , it aligns with the CnB binding helix of Cn and slips into a hydrophobic pocket delineated by – mostly aromatic – amino acids 352-356, at the CnA/CnB interface, which may serve as a proline-recognition domain (*93*) (displayed in figure 2). Although still mainly based on *in silico* docking studies and not yet supported by structural evidence, this could concretely implicate that while both "ends" of the NFAT molecule are firmly secured by two regions adjoining the Cn active site, the NFAT regulatory region with its multiple phosphorylated residues can be processed by the active site in an extremely efficient manner (*155*).

Several endogenous Cn regulators, notably the RCAN proteins, interact with the Cn A subunit via one or two distinct regions at their carboxyl end; for the RCAN family, the one crucial sequence is the highly conserved CIC (Cn inhibitor calcipressin) motif (*156*). This motif typically features an ELHA sequence (E-motif) and a valine-rich region (V-motif, PSVVVHV). Understandably, due to its strong resemblance to the PVIVIT motif, the EV-moiety also interacts with the N330-I331-R332 sequence in the β14 strand (*151, 157*). Although binding of a regulatory protein *per se* does not necessarily impede phosphatase activity, competition for this region severely hampers NFAT docking to the

enzyme, causing disturbed Cn signaling anyway. In addition, the extreme C-terminal region of RCAN proteins, acting in unison with the docking motif, is envisaged to directly interfere with active site operations (*157*). Because of the ubiquitous nature of the PxIxIT signature – for example, AKAP79 (*158*) and Cabin1 (*128*) also display such a sequence –, competition is to be expected not only between regulatory proteins and NFAT, but also among regulatory proteins themselves (*157*). Still, small inconsistencies in binding patterns exist: mutations in the aforementioned Cn linker region that is supposed to be required for complete PxIxIT interaction, although disastrous for NFAT docking, do not adversely affect AKAP79 or Cabin1 binding (*159*).

This knowledge creates advantageous opportunities for specific silencing of the NFAT pathway, while leaving overall phosphatase activity and, hence, other Cn-dependent signaling pathways undisturbed. The Rao and Hogan groups used displacement of the high affinity VIVIT peptide to identify selective inhibitors of NFAT-Cn association (INCA) among a large number of small organic compounds (*160*), while Mulero *et al.* screened a chemical library for Cn-inhibiting capabilities based on competition with a stripped RCAN1-peptide (*161*).

4. REACTIVE OXYGEN SPECIES AND THE CELLULAR REDOX STATE

Cn is known to be a Fe^{2+}/Zn^{2+} metalloenzyme (*37*), the ferrous ion being a critical factor for enzyme functioning (*37, 162*). Before its high sensitivity to oxidation became widely acknowledged, the state of the active site iron was subject to some controversy. When Griffith *et al.*, in 1995, managed to solve the X-ray structure of a ternary complex composed of a CnA fragment, CnB, and FKBP12-bound TRL, the active site metals were initially modeled as Zn^{2+} and Fe^{3+} (*42*). Meanwhile, the Rusnak lab reported metal ion analysis data supporting the presence of both iron and zinc in the enzyme as well as EPR data strongly advocating a high-spin Fe^{3+} center in Cn isolated from bovine brain (*41*). This would parallel the common active site make-up and iron redox status in the family of kidney bean purple acid phosphatases, to which Cn shows remarkable sequence resemblance (*163*). Furthermore, both phosphatase activity and EPR signals suggesting the presence of Fe^{3+} collapsed upon treatment of Cn with the one-electron reductant dithionite (*41*). While the active sites of kidney bean purple acid phosphatases are built around a Fe/Zn dinuclear center, mammalian purple acid phosphatases typically feature two Fe ions. Therefore, in an ensuing paper, further EPR experiments were undertaken to verify the Fe^{3+} state in iron-substituted (Fe^{3+}-Fe^{2+}) recombinant rat calcineurin in addition to native (Fe^{3+}-Zn^{2+}) bovine brain Cn. Both forms proved equally susceptible to dithionite. Upon titration with hydrogen peroxide, the mixed-valence diiron form suffered from inactivation. All changes in metal ion charge were monitored and confirmed using

EPR (*164*). Yu *et al.* argued that substitution of one of the irons by zinc might be a way to confer resistance against oxidation (*164*).

However, a critical evaluation of their work by other studies rendered these conclusions untenable. First complications arose when Huang and colleagues failed to identify any EPR signals pointing at Fe^{3+} in freshly isolated, highly active Cn. Oxidation and consequent inactivation of Cn using $Fe(CN)_6^{3-}$, however, produced the distinctive EPR signal that Yu *et al.* had ascribed to adventitious iron or iron in a Fe^{3+}-Zn^{2+} binuclear center (*41*). Subsequent reduction by ascorbate reinstated the active, EPR-silent enzyme (*40*). These facts are in favor of a ferrous state. Secondly, the analogy to the configuration of the metal cluster of purple acid phosphatases does not hold; their iron ligand sphere contains a tyrosine, which tends to stabilize Fe^{3+}. Cn, on the other hand, features a histidine residue, which favors Fe^{2+} (*37*). Thirdly, the conclusions of the Rusnak group were also contested by studies into the effects of superoxide on Cn activity. Wang *et al.*, having observed a time- and calcium-/calmodulin-dependent decrease of Cn activity *in situ*, identified superoxide dismutase as a stabilizing factor to Cn purified from brain extract, effectively protecting the phosphatase against spontaneous inactivation. They found that paraquat, a herbicide as well as a notorious superoxide anion generator, abolished Cn activity. Furthermore, Cn was shown to be protected by ascorbate and anaerobic incubation, whereas aerobic incubation resulted in loss of activity. Reactivation could be attained using a source of ferrous ion (*137*). While the presence of an iron/zinc cluster in the Cn active site was acknowledged, the authors believed their results would be more consistent with a ferrous state (*137*). In resemblance of [4Fe-4S]-cluster enzymes – such as hydrolyases – that readily lose iron upon reaction with superoxide (*165*), the ferrous ion of the Cn active site could become subject to oxidation by superoxide. Such a mechanism could very well be Nature's turn of coupling calcium signaling to the intracellular redox potential (*137*). Similar thoughts were expressed by Sommer *et al.*, who also noticed the high sensitivity of Cn – both the purified protein and in lysates – to superoxide as well as the countereffects of superoxide dismutase (*166*), and Ullrich and coworkers (*37, 167*). It was hypothesized that binding of CaM to Cn and concomitant displacement of the autoinhibitory domain would lead to exposure of the active site, allowing access of substrates as well as superoxide anion (*37, 40, 137, 167, 168*). Attack on the Cn binuclear center by superoxide could lead to a peroxo-like intermediate bridging the two metal sites. Release of peroxide then leaves an inactive Fe^{3+}-Zn^{2+} enzyme. Due to its lability, the ferric ion is swiftly exchanged for several bivalent cations, restoring enzymatic activity (*167*). Finally, the inhibitory actions of dithionite on Cn activity does not qualify as justification of the assumption that Cn contains a ferric ion: oxidation of dithionite involves formation of free radicals as well as sulfite, all known Cn suppressors. It seems that the assumption that Cn harbors a ferric ion was the consequence of a badly established condition of the enzyme after an isolation procedure that encourages unanticipated oxidation (*37*).

A few years later, concrete implications of Cn inhibition by superoxide for signaling were described when Namgaladze *et al.* established a concomitant reduction in NFAT nuclear translocation in vascular endothelial cells (*169*). Sankaranarayanan *et al.*, while investigating the applicability of serum Cn activity as a biomarker reflecting oxidative stress in type II diabetes mellitus, found decreased SOD and Cn activity in diabetic individuals. Since the regulation and maintenance of insulin gene expression and secretion are believed to proceed through Cn/NFAT (*9, 10*), illustrated by the fact that CnI have been found to interfere with glucose metabolism (*170*), Cn may link glycemic control to oxidative stress (*171*). Remarkably, Agbas *et al.* showed that the protective effects of SODs on calcineurin activity may trace back to partnering protein-protein interactions between Cn and SODs rather than to the disproportionation of the superoxide radical anion. This could link the pathophysiology of neurodegenerative diseases that are characterized by alterations in SOD to disturbed functioning of Cn (*172, 173*).

Hydrogen peroxide

Failure of the thiol reducing agents DTT and TCEP to protect against superoxide-induced loss of Cn activity suggests that superoxide primarily targets the binuclear center (*37*). However, redox control of Cn activity need not exclusively involve the intrinsic metal ions. Indeed, other possible sites prone to oxidative damage have been identified. Modification of –SH groups readily resulted in diminished enzyme activity and reduced affinity for nickel ion, a Cn activating species (*174*). Kang *et al.* found that even small modifications of Cys-266 can induce a rearrangement of the nearby NFAT docking site, presumably involving the β11-β12 loop (*160*). Bogumil *et al.* observed inactivation of Cn by H_2O_2 and believed this inactivation to be most likely due to the formation of a disulfide bond, as a free thiol group assay revealed oxidized Cys residues and EPR showed no changes at the binuclear metal site. Moreover, the effects of H_2O_2 could be reversed using DTT or thioredoxin (Trx) (*175*), which corroborated their hypothesis. An interesting approach by Carruthers *et al.* led to the discovery that H_2O_2 oxidizes a Met residue in the calmodulin binding domain of CnA, thereby reducing the affinity of CaM for Cn (*176*). Alternatively, Lee *et al.* suggested a mechanism, mediated by H_2O_2, by which the Cn catalytic domain is proteolytically cleaved, disabling the enzyme (*177*). This series of events involves binding to Cn of RCAN1 (see section 3), which regulates the duration and amplitude of its activity and could serve as an important linker between oxidative stress and Ca^{2+}-signaling: knockdown of RCAN1 led to inhibition of H_2O_2-induced enzyme cleavage, while its overexpression accelerated proteolysis of Cn (*178*).

Numerous other reports mention Cn inhibition, both in cell cultures and the recombinant protein, by H_2O_2 (*166, 179*). Downstream of Cn, NFAT DNA binding is suppressed following administration of hydrogen peroxide or thiol deprivation (*180, 181*). Matters are, however, complicated by the fact that low, physiologically more common doses of H_2O_2 basically stimulate Cn phosphatase activity indirectly *via* a course of events

involving an increase in intracellular Ca^{2+} levels (*182*). Although NFAT signaling seems to make the cell less susceptible to oxidative damage (*125*), this acute boost of Cn activity could have the cell commit to apoptosis instead (*140*). Indeed, Choi *et al.* reported Cn inhibitors to be successful in preventing H_2O_2-induced cell death mediated by Ca^{2+} (*183*). As mentioned in section 3, a short, transient intervention by RCAN1 could have a similar protective effect (*124*).

UV radiation

In the last few years, several groups have pointed out possible involvement of NFAT in UV-induced signaling, particularly stimulation of NFAT by low doses of UVA radiation (*184-186*). Therefore, increasing attention is being paid to elucidating the roles and regulation mechanisms of Cn in skin, in order to gain better understanding of dermal immunomodulation by UV radiation. Smit *et al.* report a dose-dependent fallback in Cn capacity and a decline in production of cytokines dependent on a functional Cn/NFAT pathway in skin, skin cells, PBMC, and the Jurkat T-lymphoma cell line after exposure to UVA radiation (*187*). Interestingly, protein phosphatase 2A is not affected. Supplemental effects of UVA on already lowered Cn activity levels in patients receiving systemic or topical calcineurin inhibition therapy may be related to the higher incidence of skin malignancies encountered in these patients, due to disruption of anti-carcinogenic pathways mediated by Cn, such as immunosurveillance, apoptosis, and DNA repair (*187*). If the effects of UVA on Cn are caused by ROS, the stimulating effects of low doses of UVA on Cn observed by some groups (*184-186*) may very well reflect those elicited by low concentrations of H_2O_2 (*182*).

In conclusion, the high sensitivity of Cn to a variety of reactive oxygen species makes this enzyme a sensible candidate for the role of central regulator of the cellular UV and redox response. High doses of ROS, however, display unwelcome inhibitory effects that seem to be long-lasting (*187*).

5. METAL IONS

As soon as the (protein) phosphatase activity of Cn was appreciated, mechanistic studies into the primary dependencies of Cn activity started to thrive. Unfortunately, they were complicated by the not-yet-acknowledged sensitivity of Cn's active site iron to oxidation. Stewart *et al.* determined Cn activity to be Ca^{2+}-dependent and notably boosted by calmodulin (*188*). Ca^{2+} binds to both the regulatory B subunit of calcineurin and to calmodulin (*189*). Merat *et al.* also observed Mn^{2+} to be crucial for CnA activity (*190*); intriguingly, although Stewart *et al.* had found that Mn^{2+} could substitute for Ca^{2+} (*188, 191*), Ca^{2+} could not fully replace Mn^{2+} (*190*). Screening surveys on the effects of bivalent

metal ions in general showed not only Mn^{2+} but also several other transition metals (Ni^{2+}, Co^{2+}) to dramatically enhance bovine Cn activity, even beyond levels typically obtained by Ca^{2+}-stimulation, whereas enzyme activity practically reduced to zero in case of Zn^{2+} (*192*). An ensuing study led King *et al.* to speculate that Cn, next to the tightly bound Fe and Zn ions in the active site and the Ca^{2+} ions bound to the B subunit, requires an additional bivalent metal ion (such as Mn^{2+} and Ni^{2+}) for structural stability and full activity (*39*). In an effort to identify the part of the enzyme responsible for Ni^{2+} binding, they managed to trace a single Cys residue on the catalytic subunit as being important in achieving the Ni^{2+}-activated conformation of Cn (*174*). Meanwhile, Pallen *et al.* independently conducted several more in-depth experiments into the kinetic interplay between the enzyme and several metal ions. This approach revealed that gradual conformational changes induced by Ni^{2+} and Mn^{2+} may interconvert the inactive and active forms of Cn (*193*), the distinction based on the accessibility of the active site. A later contribution specifies high affinity Ni^{2+} and Mn^{2+} binding sites located on the A subunit. Interestingly, preincubation with Zn^{2+} could indeed substantially block Ni^{2+} binding (*194*).

Nevertheless, ascribing the impact of exogenous metal ions on enzyme activity solely to allosteric activation, by dictating a general conformational change in either or both subunits, eventually proved untenable. Physiologically relevant concentrations of the metal ions described occur in the micromolar or nanomolar range while even [Mg^{2+}], the most abundant bivalent metal ion inside the cell, does not exceed 1 mM. As purified Cn only contains the two intrinsic metal ions, it became clear that these ions should not be left out of the discussion. Martin and Graves devised a two metal, direct hydrolytic mechanism for calcineurin catalysis in which one metal was envisaged to be responsible for coordinating and orienting substrate, polarizing the phosphate ester bond, stabilizing the leaving group, and ensuring an appropriate overall structure of the active site, while the other would activate water for nucleophilic attack on the phosphoester bond. This mechanism left room for extrinsic metal to functionally replace one of the intrinsic metals (*45*, *195*). Subsequently, it was reported that incubation of Cn with the small metal chelator dipicolinic acid activated the enzyme, even in absence of exogenous metal such as Mn^{2+}. This activation could be reversed by addition of Zn^{2+} or Fe^{3+}. Martin *et al.* concluded that one of the active site metal ions, presumably Zn^{2+}, would be dispensable for catalytic activity, or even repress it; it was suggested that dipicolinic acid masks this zinc ion and that exogenous metal replaces the Zn^{2+} in orienting the substrate (*44*). *A fortiori*, the Stemmer group discovered that, among a large group of antioxidants that enhance Cn activity, the compounds able to chelate zinc were particularly effective in stimulating the enzyme (*166*).

Facts fell into place when Namgaladze and associates found that transition metals such as Mn^{2+} can actually substitute for the active site iron, which is easily lost after oxidation to Fe^{3+} during Cn isolation and purification (*37*). This rationalized the early reports by the Cohen and Klee labs mentioning Cn becoming Mn^{2+}-dependent after

affinity chromatography (*188, 191*) and put the results and interpretation of many earlier studies into the metal ion preferences of Cn in a new perspective. However, this also implicates that Cn dependence on extrinsic metal cannot be unequivocally demonstrated.

Another confusing issue concerned the mode of Cn stimulation by Mg^{2+}. Wolff and Sved, who studied metal cation-induced conformational shifts by far UV circular dichroism spectrometry, confirmed Mn^{2+} and Ca^{2+} as strong activators of Cn, but failed to see any effects of Mg^{2+} on Mn^{2+} or Ca^{2+} dependency (*196*). Li *et al.* found that addition of Mg^{2+} increased the activity of the Ca^{2+}/CaM stimulated enzyme by almost a factor of 20, but noticed a discrepancy with the *modus operandi* of the transition metals (*197*), supposedly due to the significant difference in chemistry between alkaline earth and transition metals (*198*): Mg^{2+} cannot replace Ca^{2+} as allosteric activator and could therefore only be assigned as a cofactor for catalytic action (*197*). By monitoring the individual steps of the catalytic process, however, Martin and coworkers found a distinct disparity in the kinetic parameters and rate constants between Mn^{2+} and Mg^{2+} (*199*). Meanwhile, since a parallel study acknowledged the substitution-inert $Co(NH_3)_6{}^{3+}$ complex as a viable replacement for $Mg(H_2O)_6{}^{2+}$ in stimulating Cn activity, it would seem that Mg^{2+} does not directly bind to the phosphate ester in the catalytic center, but instead stabilizes the leaving group by proton donation from its aqua ligands or by outer sphere hydrogen bonding (*200*). Consecutive experiments endorsed the hypothesis that the potency of a specific metal ion to activate Cn may largely be dependent on the pK_a of its aqua complex and the ease of formation of substrate-metal interaction (*45*). In light of the distinct properties with regard to the course of the reaction appointed earlier to the different metal ions (*45, 195*), differences in interaction geometry with substrate and active site residues could explain the discrepancy in stimulatory effects between transition metals and Mg^{2+} (*201*). Because of its less favorable interaction with the active site, Mg^{2+} was suggested to interact with several regulatory regions on the A subunit as well. This would explain the higher extent of activation of the complete, intact enzyme achieved by Mg^{2+} than by transition metals (*38, 197, 198*).

Biological relevance

Metal ions have been very useful as experimental tools to gain understanding of Cn regulation. However, among metal ions existing at their basal intracellular concentrations only Ca^{2+} has been shown to be of influence on Cn activity *in vivo*; Ca^{2+} levels are transient and, hence, qualify as a functional on/off switch (*202*). Exposure to high levels of exogenous metal particles, on the other hand, can lead to pleiotropic stimulation of multiple signaling cascades and transcription factors (*203, 204*), which could include the Cn-NFAT pathway. Huang *et al.* showed that both soluble and insoluble nickel compounds ($NiCl_3$ and Ni_3S_2) are able to effect NFAT nuclear translocation (*205*). Its mechanism seems to be mediated by H_2O_2, but direct effects on Cn should not be discounted immediately. Furthermore, clinical evaluations report tacrolimus to alleviate

nickel contact hypersensitivity symptoms (*206*). It remains to be clarified whether this can be fully ascribed to suppression of the immune response or is partly due to prevention of overstimulation of Cn by Ni^{2+}. Proceeding on the findings of Takahashi *et al.*, who showed that Zn^{2+} effectively counteracts Cn stimulation by nickel ions *in vitro* (*207*), Tanaka and colleagues established that Zn^{2+} suppresses IL-2 production by T cells, presumably by direct inactivation of Cn; they allude to possible therapeutic application of Zn^{2+} as an immunosuppressive agent (*208*).

6. MISCELLANEOUS AGENTS

An interesting detail of the Cn structure not yet touched upon is the myristoyl function at the *N*-terminal glycine of CnB. For many proteins, such a modification facilitates their association to membranes. Indeed, phospholipids can rapidly attach to CnB in a reversible and calmodulin/Ca^{2+}-sensitive manner, evoking a slow conformational change and stimulation of phosphatase activity (*209*). Kennedy *et al.*, however, stated that both Cn-phospholipid interactions and Cn activity are myristoyl-independent. Instead, myristoylation is believed to provide structural stability and thermal stability to the CnA-CnB heterodimer (*210*, *211*). Still, the effects of long chain aliphates on Cn do not go unnoticed. Kessen *et al.* described a novel mode of activation of *Dictyostelium* Cn by the unsaturated fatty acids, arachidonic acid, linoleic acid, and oleic acid. Activation was found to proceed in a Ca^{2+}/calmodulin/CnB independent fashion, while arachidonic acid even inhibited calmodulin docking to its corresponding binding region. Saturated fatty acids and the methyl ester derivative of arachidonic acid were unsuccessful in stimulating enzyme activity (*212*). The Martin group recognized members of the tyrphostin family, topologically similar to tyrosine, as well as retinoic acid as Cn inhibitors (*213*, *214*); competition with CaM for the CaM binding site was shown for polyunsaturated eicosa fatty acids and leukotriene derivatives using bovine Cn (*215*). They concluded that the presence of a conjugated π-system, as opposed to isolated double bonds, appears to boost inhibition potency.

Screening studies of natural compounds have produced a handful of putative non-protein direct activators and inhibitors of Cn, several among which have warranted further investigation (*105*). The mechanisms behind activity regulation by these small molecules are, however, multitudinous and often ambiguous. Binding of chlorogenic acid, a major phenolic species in coffee, to Cn resulted in reorientation of aromatic residues and reduced α-helical content (*216*); this chain of events eventually enhanced Cn activity. A negative effect on Cn signaling was found for the bioflavonoids kaempferol and quercetin (*217*, *218*). Studies into the mode of binding of kaempferol to Cn revealed a docking site located in the non-active site area of the catalytic domain (*217*, *219*). On

another note, disruption of calmodulin binding is believed to be responsible for some of the inhibitory effects of the polyphenolic aldehyde gossypol on Cn activity (*88*).

The quest for new Cn inhibitors is not in the least a synthetic one. Due to the high degree of structural conservation of their catalytic sites, compounds targeting the active center of one protein phosphatase typically end up exhibiting cross-reactivity towards others, the paragon example being the marine-sponge toxin okadaic acid. Some of these compounds, such as endothal and cantharidin, have successfully been used as a lead for a synthetic series of more specific and potent Cn inhibitors (*220, 221*).

Just now, screening of a series of diaryl substituted pyrimidine compounds for Cn inhibition has produced compound CN585 as a potent as well as non-cytotoxic inhibitor of NFAT translocation. Cn activity towards *p*NPP, however, remained unaffected. Its binding site was found to be located remote from the active site, possibly overlapping with the CsA-Cyp docking moiety (*222*).

CONCLUSION

In this review, we have laid down a wide gamut of regulatory mechanisms for Cn activity. Current research faces the task of translating the wealth of structural and mechanistic data presently at hand to specific, clinically relevant trends in calcineurin signaling. Ideally, exploitation of the extensive knowledge on Cn regulation mechanisms would permit precise tweaking and manipulation of the Ca^{2+}-calcineurin pathway, even selectively targeted towards one specific substrate or even one specific tissue or cell type, which would create new avenues towards tailored therapeutical approaches for a multitude of immune- and proliferation-related conditions.

REFERENCES

1. Clipstone NA, Crabtree GR. Identification of calcineurin as a key signalling enzyme in T-lymphocyte activation. Nature 1992;357:695-7.
2. Dunn SE, Burns JL, Michel RN. Calcineurin is required for skeletal muscle hypertrophy. J Biol Chem 1999;274:21908-12.
3. Wang HG, Pathan N, Ethell IM, Krajewski S, Yamaguchi Y, et al. Ca2+-induced apoptosis through calcineurin dephosphorylation of BAD. Science 1999;284:339.
4. Herman M, Ori Y, Chagnac A, Weinstein T, Korzets A, et al. DNA repair in mononuclear cells: role of serine/threonine phosphatases. J Lab Clin Med 2002;140:255-62.
5. Yarosh DB, Pena AV, Nay SL, Canning MT, Brown DA. Calcineurin inhibitors decrease DNA repair and apoptosis in human keratinocytes following ultraviolet B irradiation. J Invest Dermatol 2005;125:1020-5.

6. Molkentin JD, Lu JR, Antos CL, Markham B, Richardson J, et al. A calcineurin-dependent transcriptional pathway for cardiac hypertrophy. Cell 1998;93:215-28.

7. Graef IA, Crabtree GR, Wang F. Calcineurin/NFAT Signaling in Development and Function of the Nervous System. In: Transcription Factors in the Nervous System. Wiley-VCH Verlag; 2006. p. 353-78.

8. Rusnak F, Mertz P. Calcineurin: form and function. Physiol Rev 2000;80:1483-521.

9. Lawrence MC, Bhatt HS, Watterson JM, Easom RA. Regulation of insulin gene transcription by a Ca(2+)-responsive pathway involving calcineurin and nuclear factor of activated T cells. Mol Endocrinol 2001;15:1758-67.

10. Heit JJ, Apelqvist AA, Gu X, Winslow MM, Neilson JR, Crabtree GR, Kim SK. Calcineurin/NFAT signalling regulates pancreatic beta-cell growth and function. Nature 2006;443:345-9.

11. Al-Daraji WI, Grant KR, Ryan K, Saxton A, Reynolds NJ. Localization of calcineurin/NFAT in human skin and psoriasis and inhibition of calcineurin/NFAT activation in human keratinocytes by cyclosporin A. J Invest Dermatol 2002;118:779-88.

12. Santini MP, Talora C, Seki T, Bolgan L, Dotto GP. Cross talk among calcineurin, Sp1/Sp3, and NFAT in control of p21(WAF1/CIP1) expression in keratinocyte differentiation. Proc Natl Acad Sci U S A 2001;98:9575-80.

13. Pena JA, Losi-Sasaki JL, Gooch JL. Loss of calcineurin Aalpha alters keratinocyte survival and differentiation. J Invest Dermatol 2010;130:135-40.

14. Sugimoto T, Stewart S, Guan KL. The calcium/calmodulin-dependent protein phosphatase calcineurin is the major Elk-1 phosphatase. J Biol Chem 1997;272:29415-8.

15. Tian J, Karin M. Stimulation of Elk1 transcriptional activity by mitogen-activated protein kinases is negatively regulated by protein phosphatase 2B (calcineurin). J Biol Chem 1999;274:15173-80.

16. Crabtree GR, Olson EN. NFAT signaling: choreographing the social lives of cells. Cell 2002;109 Suppl:S67-79.

17. Biswas G, Anandatheerthavarada HK, Zaidi M, Avadhani NG. Mitochondria to nucleus stress signaling: a distinctive mechanism of NFkappaB/Rel activation through calcineurin-mediated inactivation of IkappaBbeta. J Cell Biol 2003;161:507-19.

18. Feske S, Okamura H, Hogan PG, Rao A. Ca2+/calcineurin signalling in cells of the immune system. Biochem Biophys Res Commun 2003;311:1117-32.

19. Guo L, Urban JF, Zhu J, Paul WE. Elevating calcium in Th2 cells activates multiple pathways to induce IL-4 transcription and mRNA stabilization. J Immunol 2008;181:3984-93.

20. Halloran PF. Immunosuppressive drugs for kidney transplantation. N Engl J Med 2004;351:2715-29.

21. Van Rossum HH. Pharmacodynamic monitoring of calcineurin inhibition therapy. PhD thesis. Leiden University; 2010.

22. Martinez-Martinez S, Redondo JM. Inhibitors of the calcineurin/NFAT pathway. Curr Med Chem 2004;11:997-1007.

23. Kincaid RL, Giri PR, Higuchi S, Tamura J, Dixon SC, et al. Cloning and characterization of molecular isoforms of the catalytic subunit of calcineurin using nonisotopic methods. J Biol Chem 1990;265:11312-9.

24. Guerini D, Klee CB. Cloning of human calcineurin A: evidence for two isozymes and identification of a polyproline structural domain. Proc Natl Acad Sci U S A 1989;86:9183-7.

25. Ueki K, Muramatsu T, Kincaid RL. Structure and expression of two isoforms of the murine calmodulin-dependent protein phosphatase regulatory subunit (calcineurin B). Biochem Biophys Res Commun 1992;187:537-43.

26. Perrino BA, Wilson AJ, Ellison P, Clapp LH. Substrate selectivity and sensitivity to inhibition by FK506 and cyclosporin A of calcineurin heterodimers composed of the alpha or beta catalytic subunit. Eur J Biochem 2002;269:3540-8.

27. Liu H, Ye W, Guan G, Dong Z, Jia Z, Yang T. Developmental regulation of calcineurin isoforms in the rodent kidney: association with COX-2. Am J Physiol Renal Physiol 2007;293:F1898-904.

28. Kilka S, Erdmann F, Migdoll A, Fischer G, Weiwad M. The proline-rich N-terminal sequence of calcineurin Abeta determines substrate binding. Biochemistry 2009;48:1900-10.

29. Stemmer PM, Klee CB. Dual calcium ion regulation of calcineurin by calmodulin and calcineurin B. Biochemistry 1994;33:6859-66.

30. Hubbard MJ, Klee CB. Functional domain structure of calcineurin A: mapping by limited proteolysis. Biochemistry 1989;28:1868-74.

31. Kissinger CR, Parge HE, Knighton DR, Lewis CT, Pelletier LA, et al. Crystal structures of human calcineurin and the human FKBP12-FK506-calcineurin complex. Nature 1995;378:641-4.

32. Jin L, Harrison SC. Crystal structure of human calcineurin complexed with cyclosporin A and human cyclophilin. Proc Natl Acad Sci U S A 2002;99:13522-6.

33. Perrino BA, Ng LY, Soderling TR. Calcium regulation of calcineurin phosphatase activity by its B subunit and calmodulin. Role of the autoinhibitory domain. J Biol Chem 1995;270:340-6.

34. Perrino BA. Regulation of calcineurin phosphatase activity by its autoinhibitory domain. Arch Biochem Biophys 1999;372:159-65.

35. Wang H, Du Y, Xiang B, Lin W, Li X, Wei Q. A renewed model of CNA regulation involving its C-terminal regulatory domain and CaM. Biochemistry 2008;47:4461-8.

36. Mondragon A, Griffith EC, Sun L, Xiong F, Armstrong C, Liu JO. Overexpression and purification of human calcineurin alpha from Escherichia coli and assessment of catalytic functions of residues surrounding the binuclear metal center. Biochemistry 1997;36:4934-42.

37. Namgaladze D, Hofer HW, Ullrich V. Redox control of calcineurin by targeting the binuclear Fe(2+)-Zn(2+) center at the enzyme active site. J Biol Chem 2002;277:5962-9.

38. Liu P, Huang C, Jia Z, Yi F, Yu DY, Wei Q. Non-catalytic domains of subunit A negatively regulate the activity of calcineurin. Biochimie 2005;87:215-21.

39. King MM, Huang CY. The calmodulin-dependent activation and deactivation of the phosphoprotein phosphatase, calcineurin, and the effect of nucleotides, pyrophosphate, and divalent metal ions. Identification of calcineurin as a Zn and Fe metalloenzyme. J Biol Chem 1984;259:8847-56.

40. Qin K-F, Khangulov S, Liu C, Huang CY. Role of Fe ion in calcineurin studied by EPR and activity measurements. FASEB J 1995;9:A1347.

41. Yu L, Haddy A, Rusnak F. Evidence that calcineurin accomodates an active site binuclear metal center. J Am Chem Soc 1995;117:10147-8.

42. Griffith JP, Kim JL, Kim EE, Sintchak MD, Thomson JA, et al. X-ray structure of calcineurin inhibited by the immunophilin-immunosuppressant FKBP12-FK506 complex. Cell 1995;82:507-22.

43. Goldberg J, Huang HB, Kwon YG, Greengard P, Nairn AC, Kuriyan J. Three-dimensional structure of the catalytic subunit of protein serine/threonine phosphatase-1. Nature 1995;376:745-53.

44. Martin BL. Selective activation of calcineurin by dipicolinic acid. Arch Biochem Biophys 1997;345:332-8.

45. Martin BL. Evidence for a Structure-Function Relationship in the Activation of Calcineurin by Metal Ions. Bioorg Chem 2000;28:17-27.

46. Ke H, Huai Q. Structures of calcineurin and its complexes with immunophilins--immunosuppressants. Biochem Biophys Res Commun 2003;311:1095-102.

47. Crivici A, Ikura M. Molecular and structural basis of target recognition by calmodulin. Annu Rev Biophys Biomol Struct 1995;24:85-116.

48. Hubbard MJ, Klee CB. Calmodulin binding by calcineurin. Ligand-induced renaturation of protein immobilized on nitrocellulose. J Biol Chem 1987;262:15062-70.

49. Wang JH, Sharma RK, Huang CY, Chau V, Chock PB. On the mechanism of activation of cyclic mucleotide phosphodiesterase by calmodulin. Ann N Y Acad Sci 1980;356:190-204.

50. Burkard N, Becher J, Heindl C, Neyses L, Schuh K, Ritter O. Targeted proteolysis sustains calcineurin activation. Circulation 2005;111:1045-53.

51. Wu HY, Tomizawa K, Oda Y, Wei FY, Lu YF, et al. Critical role of calpain-mediated cleavage of calcineurin in excitotoxic neurodegeneration. J Biol Chem 2004;279:4929-40.

52. Kakalis LT, Kennedy M, Sikkink R, Rusnak F, Armitage IM. Characterization of the calcium-binding sites of calcineurin B. FEBS Lett 1995;362:55-8.

53. Feng B, Stemmer PM. Ca2+ binding site 2 in calcineurin-B modulates calmodulin-dependent calcineurin phosphatase activity. Biochemistry 2001;40:8808-14.

54. Hou Q, Yi X, Jiang G, Wei Q. The salt bridge of calcineurin is important for transferring the effect of CNB binding to CNA. FEBS Lett 2004;577:294-8.

55. Perrino BA, Fong YL, Brickey DA, Saitoh Y, Ushio Y, et al. Characterization of the phosphatase activity of a baculovirus-expressed calcineurin A isoform. J Biol Chem 1992;267:15965-9.

56. Hemenway CS, Heitman J. Calcineurin. Structure, function, and inhibition. Cell Biochem Biophys 1999;30:115-51.

57. Jiang G, Wei Q. Function and structure of N-terminal and C-terminal domains of calcineurin B subunit. Biol Chem 2003;384:1299-303.

58. Watanabe Y, Perrino BA, Soderling TR. Activation of calcineurin A subunit phosphatase activity by its calcium-binding B subunit. Biochemistry 1996;35:562-6.

59. Yang SA, Klee CB. Low affinity Ca2+-binding sites of calcineurin B mediate conformational changes in calcineurin A. Biochemistry 2000;39:16147-54.

60. Shen X, Li H, Ou Y, Tao W, Dong A, Kong J, Ji C, Yu S. The secondary structure of calcineurin regulatory region and conformational change induced by calcium/calmodulin binding. J Biol Chem 2008;283:11407-13.

61. Iwatsuki S, Starzl TE, Shaw BW, Yang SL, Zitelli BJ, Gartner JC, Malatack JJ. Long-term use of cyclosporine in liver recipients. Reduction of dosages in the first year to avoid nephrotoxicity. Transplantation 1983;36:641-3.

62. Merion RM, White DJ, Calne RY. Early renal allograft rejection episodes are less aggressive with cyclosporin-A immunosuppression. Transplant Proc 1983;15:2172-7.

63. Starzl TE, Todo S, Fung J, Demetris AJ, Venkataramman R, Jain A. FK 506 for liver, kidney, and pancreas transplantation. Lancet 1989;2:1000-4.

64. Elliott JF, Lin Y, Mizel SB, Bleackley RC, Harnish DG, Paetkau V. Induction of interleukin 2 messenger RNA inhibited by cyclosporin A. Science 1984;226:1439-41.

65. Macian F, Garcia-Cozar F, Im SH, Horton HF, Byrne MC, Rao A. Transcriptional mechanisms underlying lymphocyte tolerance. Cell 2002;109:719-31.

66. Emmel EA, Verweij CL, Durand DB, Higgins KM, Lacy E, Crabtree GR. Cyclosporin A specifically inhibits function of nuclear proteins involved in T cell activation. Science 1989;246:1617-20.

67. Mattila PS, Ullman KS, Fiering S, Emmel EA, McCutcheon M, Crabtree GR, Herzenberg LA. The actions of cyclosporin A and FK506 suggest a novel step in the activation of T lymphocytes. EMBO J 1990;9:4425-33.

68. Liu J, Farmer JD, Lane WS, Friedman J, Weissman I, Schreiber SL. Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. Cell 1991;66:807-15.

69. Flanagan WM, Corthesy B, Bram RJ, Crabtree GR. Nuclear association of a T-cell transcription factor blocked by FK-506 and cyclosporin A. Nature 1991;352:803-7.

70. McCaffrey PG, Luo C, Kerppola TK, Jain J, Badalian TM, et al. Isolation of the cyclosporin-sensitive T cell transcription factor NFATp. Science 1993;262:750-4.

71. Jain J, McCaffrey PG, Miner Z, Kerppola TK, Lambert JN, et al. The T-cell transcription factor NFATp is a substrate for calcineurin and interacts with Fos and Jun. Nature 1993;365:352-5.

72. Crabtree GR, Clipstone NA. Signal transmission between the plasma membrane and nucleus of T lymphocytes. Annu Rev Biochem 1994;63:1045-83.

73. Fischer G, Wittmann-Liebold B, Lang K, Kiefhaber T, Schmid FX. Cyclophilin and peptidyl-prolyl cis-trans isomerase are probably identical proteins. Nature 1989;337:476-8.

74. Harding MW, Galat A, Uehling DE, Schreiber SL. A receptor for the immunosuppressant FK506 is a cis-trans peptidyl-prolyl isomerase. Nature 1989;341:758-60.

75. Snyder SH, Sabatini DM. Immunophilins and the nervous system. Nat Med 1995;1:32-7.

76. Van Duyne GD, Standaert RF, Karplus PA, Schreiber SL, Clardy J. Atomic structure of FKBP-FK506, an immunophilin-immunosuppressant complex. Science 1991;252:839-42.

77. Tanaka H, Kuroda A, Marusawa H, Hatanaka H, Kino T, et al. Structure of FK506, a novel immunosuppressant isolated from Streptomyces. J Am Chem Soc 1987;109:5031-3.

78. Fesik SW, Gampe RT, Holzman TF, Egan DA, Edalji R, et al. Isotope-edited NMR of cyclosporin A bound to cyclophilin: evidence for a trans 9,10 amide bond. Science 1990;250:1406-9.

79. Mikol V, Kallen J, Pflugl G, Walkinshaw MD. X-ray structure of a monomeric cyclophilin A-cyclosporin A crystal complex at 2.1 A resolution. J Mol Biol 1993;234:1119-30.

80. Fruman DA, Klee CB, Bierer BE, Burakoff SJ. Calcineurin phosphatase activity in T lymphocytes is inhibited by FK 506 and cyclosporin A. Proc Natl Acad Sci U S A 1992;89:3686-90.

81. Van Rossum HH, Romijn FP, Smit NP, de Fijter JW, van Pelt J. Everolimus and sirolimus antagonize tacrolimus based calcineurin inhibition via competition for FK-binding protein 12. Biochem Pharmacol 2009;77:1206-12.

82. Pallen CJ, Wang JH. Calmodulin-stimulated dephosphorylation of p-nitrophenyl phosphate and free phosphotyrosine by calcineurin. J Biol Chem 1983;258:8550-3.

83. Swanson SK, Born T, Zydowsky LD, Cho H, Chang HY, Walsh CT, Rusnak F. Cyclosporin-mediated inhibition of bovine calcineurin by cyclophilins A and B. Proc Natl Acad Sci U S A 1992;89:3741-5.

84. Yin M, Ochs RS. Mechanism for the paradoxical inhibition and stimulation of calcineurin by the immunosuppressive drug tacrolimus (FK506). Arch Biochem Biophys 2003;419:207-13.

85. Blumenthal DK, Takio K, Hansen RS, Krebs EG. Dephosphorylation of cAMP-dependent protein kinase regulatory subunit (type II) by calmodulin-dependent protein phosphatase. Determinants of substrate specificity. J Biol Chem 1986;261:8140-5.

86. Yu DY, Qiao N, Liu P, Wei Q. Different regulation modes of calcineurin regulatory subunit on its catalytic subunit with RII peptide and tau as substrates. Protein Pept Lett 2009;16:168-72.

87. Huai Q, Kim HY, Liu Y, Zhao Y, Mondragon A, Liu JO, Ke H. Crystal structure of calcineurin-cyclophilin-cyclosporin shows common but distinct recognition of immunophilin-drug complexes. Proc Natl Acad Sci U S A 2002;99:12037-42.

88. Carruthers NJ, Dowd MK, Stemmer PM. Gossypol inhibits calcineurin phosphatase activity at multiple sites. Eur J Pharmacol 2007;555:106-14.

89. Husi H, Luyten MA, Zurini MG. Mapping of the immunophilin-immunosuppressant site of interaction on calcineurin. J Biol Chem 1994;269:14199-204.

90. Milan D, Griffith J, Su M, Price ER, McKeon F. The latch region of calcineurin B is involved in both immunosuppressant-immunophilin complex docking and phosphatase activation. Cell 1994;79:437-47.

91. Haddy A, Swanson SK, Born TL, Rusnak F. Inhibition of calcineurin by cyclosporin A-cyclophilin requires calcineurin B. FEBS Lett 1992;314:37-40.

92. Zhu D, Cardenas ME, Heitman J. Calcineurin mutants render T lymphocytes resistant to cyclosporin A. Mol Pharmacol 1996;50:506-11.

93. Rodriguez A, Roy J, Martinez-Martinez S, Lopez-Maderuelo MD, Nino-Moreno P, et al. A conserved docking surface on calcineurin mediates interaction with substrates and immunosuppressants. Mol Cell 2009;33:616-26.

94. Kumar R, Adams B, Musiyenko A, Shulyayeva O, Barik S. The FK506-binding protein of the malaria parasite, Plasmodium falciparum, is a FK506-sensitive chaperone with FK506-independent calcineurin-inhibitory activity. Mol Biochem Parasitol 2005;141:163-73.

95. Shirane M, Nakayama KI. Inherent calcineurin inhibitor FKBP38 targets Bcl-2 to mitochondria and inhibits apoptosis. Nat Cell Biol 2003;5:28-37.

96. Liu JO. Endogenous protein inhibitors of calcineurin. Biochem Biophys Res Commun 2003;311:1103-9.

97. Li TK, Baksh S, Cristillo AD, Bierer BE. Calcium- and FK506-independent interaction between the immunophilin FKBP51 and calcineurin. J Cell Biochem 2002;84:460-71.

98. Cardenas ME, Hemenway C, Muir RS, Ye R, Fiorentino D, Heitman J. Immunophilins interact with calcineurin in the absence of exogenous immunosuppressive ligands. EMBO J 1994;13:5944-57.

99. Cameron AM, Steiner JP, Roskams AJ, Ali SM, Ronnett GV, Snyder SH. Calcineurin associated with the inositol 1,4,5-trisphosphate receptor-FKBP12 complex modulates Ca2+ flux. Cell 1995;83:463-72.

100. Saito T, Zhang ZJ, Tokuriki M, Ohtsubo T, Shibamori Y, Yamamoto T, Saito H. Cyclosporin A inhibits the extrusion pump function of p-glycoprotein in the inner ear of mice treated with vinblastine and doxorubicin. Brain Res 2001;901:265-70.

101. Uchino H, Elmer E, Uchino K, Li PA, He QP, Smith ML, Siesjo BK. Amelioration by cyclosporin A of brain damage in transient forebrain ischemia in the rat. Brain Res 1998;812:216-26.

102. Paul CF, Ho VC, McGeown C, Christophers E, Schmidtmann B, et al. Risk of malignancies in psoriasis patients treated with cyclosporine: a 5 y cohort study. J Invest Dermatol 2003;120:211-6.

103. Humes HD, Jackson NM, O'Connor RP, Hunt DA, White MD. Pathogenetic mechanisms of nephrotoxicity: insights into cyclosporine nephrotoxicity. Transplant Proc 1985;17:51-62.

104. Lazarova T, Weng Z. Cyclosporin A analogues: recent advances. Expert Opin Ther Patents 2003;13:1327-32.

105. Sieber M, Baumgrass R. Novel inhibitors of the calcineurin/NFATc hub - alternatives to CsA and FK506? Cell Commun Signal 2009;7:25.

106. Muramatsu Y, Furuichi Y, Tojo N, Moriguchi A, Maemoto T, et al. Neuroprotective efficacy of FR901459, a novel derivative of cyclosporin A, in in vitro mitochondrial damage and in vivo transient cerebral ischemia models. Brain Res 2007;1149:181-90.

107. Birsan T, Dambrin C, Freitag DG, Yatscoff RW, Morris RE. The novel calcineurin inhibitor ISA247: a more potent immunosuppressant than cyclosporine in vitro. Transpl Int 2005;17:767-71.

108. Papp K, Bissonnette R, Rosoph L, Wasel N, Lynde CW, et al. Efficacy of ISA247 in plaque psoriasis: a randomised, multicentre, double-blind, placebo-controlled phase III study. Lancet 2008;371:1337-42.

109. Lu ZR, Gao SQ, Kopeckova P, Kopecek J. Synthesis of bioadhesive lectin-HPMA copolymer-cyclosporin conjugates. Bioconjug Chem 2000;11:3-7.

110. Rappersberger K, Meingassner JG, Fialla R, Fųdinger D, Sterniczky B, et al. Clearing of psoriasis by a novel immunosuppressive macrolide. J Invest Dermatol 1996;106:701-10.

111. Mollison KW, Fey TA, Gauvin DM, Kolano RM, Sheets MP, et al. A macrolactam inhibitor of T helper type 1 and T helper type 2 cytokine biosynthesis for topical treatment of inflammatory skin diseases. J Invest Dermatol 1999;112:729-38.

112. Gupta AK, Chow M. Pimecrolimus: a review. J Eur Acad Dermatol Venereol 2003;17:493-503.

113. Paul C, Graeber M, Stuetz A. Ascomycins: promising agents for the treatment of inflammatory skin diseases. Expert Opin Investig Drugs 2000;9:69-77.

114. Someren JS, Faber LE, Klein JD, Tumlin JA. Heat shock proteins 70 and 90 increase calcineurin activity in vitro through calmodulin-dependent and independent mechanisms. Biochem Biophys Res Commun 1999;260:619-25.

115. Imai J, Yahara I. Role of HSP90 in salt stress tolerance via stabilization and regulation of calcineurin. Mol Cell Biol 2000;20:9262-70.

116. Davies KJ, Ermak G, Rothermel BA, Pritchard M, Heitman J, et al. Renaming the DSCR1/Adapt78 gene family as RCAN: regulators of calcineurin. FASEB J 2007;21:3023-8.

117. Park J, Oh Y, Chung KC. Two key genes closely implicated with the neuropathological characteristics in Down syndrome: DYRK1A and RCAN1. BMB Rep 2009;42:6-15.

118. Harris CD, Ermak G, Davies KJ. Multiple roles of the DSCR1 (Adapt78 or RCAN1) gene and its protein product calcipressin 1 (or RCAN1) in disease. Cell Mol Life Sci 2005;62:2477-86.

119. Vega RB, Rothermel BA, Weinheimer CJ, Kovacs A, Naseem RH, et al. Dual roles of modulatory calcineurin-interacting protein 1 in cardiac hypertrophy. Proc Natl Acad Sci U S A 2003;100:669-74.

120. Hilioti Z, Gallagher DA, Low-Nam ST, Ramaswamy P, Gajer P, et al. GSK-3 kinases enhance calcineurin signaling by phosphorylation of RCNs. Genes Dev 2004;18:35-47.

121. Kingsbury TJ, Cunningham KW. A conserved family of calcineurin regulators. Genes Dev 2000;14:1595-604.

122. Ermak G, Harris CD, Battocchio D, Davies KJ. RCAN1 (DSCR1 or Adapt78) stimulates expression of GSK-3beta. FEBS J 2006;273:2100-9.

123. Rothermel BA, Vega RB, Williams RS. The role of modulatory calcineurin-interacting proteins in calcineurin signaling. Trends Cardiovasc Med 2003;13:15-21.

124. Ermak G, Harris CD, Davies KJ. The DSCR1 (Adapt78) isoform 1 protein calcipressin 1 inhibits calcineurin and protects against acute calcium-mediated stress damage, including transient oxidative stress. FASEB J 2002;16:814-24.

125. Porta S, Serra SA, Huch M, Valverde MA, Llorens F, et al. RCAN1 (DSCR1) increases neuronal susceptibility to oxidative stress: a potential pathogenic process in neurodegeneration. Hum Mol Genet 2007;16:1039-50.

126. Lian Q, Ladner CJ, Magnuson D, Lee JM. Selective changes of calcineurin (protein phosphatase 2B) activity in Alzheimer's disease cerebral cortex. Exp Neurol 2001;167:158-65.

127. Miskin JE, Abrams CC, Goatley LC, Dixon LK. A viral mechanism for inhibition of the cellular phosphatase calcineurin. Science 1998;281:562.

128. Sun L, Youn HD, Loh C, Stolow M, He W, Liu JO. Cabin 1, a negative regulator for calcineurin signaling in T lymphocytes. Immunity 1998;8:703-11.

129. Jang H, Cho EJ, Youn HD. A new calcineurin inhibition domain in Cabin1. Biochem Biophys Res Commun 2007;359:129-35.

130. Lai MM, Burnett PE, Wolosker H, Blackshaw S, Snyder SH. Cain, a novel physiologic protein inhibitor of calcineurin. J Biol Chem 1998;273:18325-31.

131. Kim MJ, Jo DG, Hong GS, Kim BJ, Lai M, et al. Calpain-dependent cleavage of cain/cabin1 activates calcineurin to mediate calcium-triggered cell death. Proc Natl Acad Sci U S A 2002;99:9870.

132. Lam E, Martin MM, Timerman AP, Sabers C, Fleischer S, et al. A novel FK506 binding protein can mediate the immunosuppressive effects of FK506 and is associated with the cardiac ryanodine receptor. J Biol Chem 1995;270:26511-22.

133. Weiwad M, Edlich F, Erdmann F, Jarczowski F, Kilka S, et al. A reassessment of the inhibitory capacity of human FKBP38 on calcineurin. FEBS Lett 2005;579:1591-6.

134. Lin X, Sikkink RA, Rusnak F, Barber DL. Inhibition of calcineurin phosphatase activity by a calcineurin B homologous protein. J Biol Chem 1999;274:36125-31.

135. Coghlan VM, Perrino BA, Howard M, Langeberg LK, Hicks JB, Gallatin WM, Scott JD. Association of protein kinase A and protein phosphatase 2B with a common anchoring protein. Science 1995;267:108-11.

136. Frey N, Barrientos T, Shelton JM, Frank D, Rutten H, et al. Mice lacking calsarcin-1 are sensitized to calcineurin signaling and show accelerated cardiomyopathy in response to pathological biomechanical stress. Nat Med 2004;10:1336-43.

137. Wang X, Culotta VC, Klee CB. Superoxide dismutase protects calcineurin from inactivation. Nature 1996;383:434-7.

138. Erin N, Bronson SK, Billingsley ML. Calcium-dependent interaction of calcineurin with Bcl-2 in neuronal tissue. Neuroscience 2003;117:541-55.

139. Oliveria SF, Dell'Acqua ML, Sather WA. AKAP79/150 anchoring of calcineurin controls neuronal L-type Ca2+ channel activity and nuclear signaling. Neuron 2007;55:261-75.

140. Shibasaki F, McKeon F. Calcineurin functions in Ca(2+)-activated cell death in mammalian cells. J Cell Biol 1995;131:735-43.

141. Shibasaki F, Kondo E, Akagi T, McKeon F. Suppression of signalling through transcription factor NF-AT by interactions between calcineurin and Bcl-2. Nature 1997;386:728-31.

142. Li W, Handschumacher RE. Identification of two calcineurin B-binding proteins: tubulin and heat shock protein 60. BBA-Proteins Proteom 2002;1599:72-81.

143. Luo C, Shaw KT, Raghavan A, Aramburu J, Garcia-Cozar F, et al. Interaction of calcineurin with a domain of the transcription factor NFAT1 that controls nuclear import. Proc Natl Acad Sci U S A 1996;93:8907-12.

144. Liu J, Masuda ES, Tsuruta L, Arai N, Arai K. Two independent calcineurin-binding regions in the N-terminal domain of murine NF-ATx1 recruit calcineurin to murine NF-ATx1. J Immunol 1999;162:4755-61.

145. Aramburu J, Garcia-Cozar F, Raghavan A, Okamura H, Rao A, Hogan PG. Selective inhibition of NFAT activation by a peptide spanning the calcineurin targeting site of NFAT. Mol Cell 1998;1:627-37.

146. Garcia-Cozar FJ, Okamura H, Aramburu JF, Shaw KT, Pelletier L, et al. Two-site interaction of nuclear factor of activated T cells with activated calcineurin. J Biol Chem 1998;273:23877-83.

147. Park S, Uesugi M, Verdine GL. A second calcineurin binding site on the NFAT regulatory domain. Proc Natl Acad Sci U S A 2000;97:7130-5.

148. Martinez-Martinez S, Rodriguez A, Lopez-Maderuelo MD, Ortega-Perez I, Vazquez J, Redondo JM. Blockade of NFAT activation by the second calcineurin binding site. J Biol Chem 2006;281:6227-35.

149. Xiang BQ, Jia Z, Xiao FX, Zhou K, Liu P, Wei Q. The role of loop 7 in mediating calcineurin regulation. Protein Eng Des Sel 2003;16:795-8.

150. Aramburu J, Yaffe MB, Lopez-Rodriguez C, Cantley LC, Hogan PG, Rao A. Affinity-driven peptide selection of an NFAT inhibitor more selective than cyclosporin A. Science 1999;285:2129.

151. Li H, Rao A, Hogan PG. Structural delineation of the calcineurin-NFAT interaction and its parallels to PP1 targeting interactions. J Mol Biol 2004;342:1659-74.

152. Rodriguez A, Martinez-Martinez S, Lopez-Maderuelo MD, Ortega-Perez I, Redondo JM. The linker region joining the catalytic and the regulatory domains of CnA is essential for binding to NFAT. J Biol Chem 2005;280:9980-4.

153. Takeuchi K, Roehrl MH, Sun ZY, Wagner G. Structure of the calcineurin-NFAT complex: defining a T cell activation switch using solution NMR and crystal coordinates. Structure 2007;15:587-97.

154. Li SZ, McDill BW, Kovach PA, Ding L, Go WY, Ho SN, Chen F. Calcineurin-NFATc signaling pathway regulates AQP2 expression in response to calcium signals and osmotic stress. Am J Physiol Cell Physiol 2007;292:C1606-16.

155. Liu JO, Nacev BA, Xu J, Bhat S. It takes two binding sites for calcineurin and NFAT to tango. Mol Cell 2009;33:676-8.

156. Mehta S, Li H, Hogan PG, Cunningham KW. Domain architecture of the regulators of calcineurin (RCANs) and identification of a divergent RCAN in yeast. Mol Cell Biol 2009;29:2777-93.

157. Martinez-Martinez S, Genesca L, Rodriguez A, Raya A, Salichs E, et al. The RCAN carboxyl end mediates calcineurin docking-dependent inhibition via a site that dictates binding to substrates and regulators. Proc Natl Acad Sci U S A 2009;106:6117-22.

158. Dell'Acqua ML, Dodge KL, Tavalin SJ, Scott JD. Mapping the protein phosphatase-2B anchoring site on AKAP79. Binding and inhibition of phosphatase activity are mediated by residues 315-360. J Biol Chem 2002;277:48796-802.

159. Kashishian A, Howard M, Loh C, Gallatin WM, Hoekstra MF, Lai Y. AKAP79 inhibits calcineurin through a site distinct from the immunophilin-binding region. J Biol Chem 1998;273:27412-9.

160. Kang S, Li H, Rao A, Hogan PG. Inhibition of the calcineurin-NFAT interaction by small organic molecules reflects binding at an allosteric site. J Biol Chem 2005;280:37698-706.

161. Mulero MC, Aubareda A, Orzaez M, Messeguer J, Serrano-Candelas E, et al. Inhibiting the calcineurin-NFAT (nuclear factor of activated T cells) signaling pathway with a regulator of calcineurin-derived peptide without affecting general calcineurin phosphatase activity. J Biol Chem 2009;284:9394-401.

162. Ghosh MC, Wang X, Li S, Klee C. Regulation of calcineurin by oxidative stress. Methods Enzymol 2003;366:289-304.

163. Vincent JB, Averill BA. Sequence homology between purple acid phosphatases and phosphoprotein phosphatases. Are phosphoprotein phosphatases metalloproteins containing oxide-bridged dinuclear metal centers? FEBS Lett 1990;263:265-8.

164. Yu L, Golbeck J, Yao J, Rusnak F. Spectroscopic and enzymatic characterization of the active site dinuclear metal center of calcineurin: implications for a mechanistic role. Biochemistry 1997;36:10727-34.

165. Flint DH, Tuminello JF, Emptage MH. The inactivation of Fe-S cluster containing hydro-lyases by superoxide. J Biol Chem 1993;268:22369-76.

166. Sommer D, Fakata KL, Swanson SA, Stemmer PM. Modulation of the phosphatase activity of calcineurin by oxidants and antioxidants in vitro. Eur J Biochem 2000;267:2312-22.

167. Ullrich V, Namgaladze D, Frein D. Superoxide as inhibitor of calcineurin and mediator of redox regulation. Toxicol Lett 2003;139:107-10.

168. Stemmer PM, Wang X, Krinks MH, Klee CB. Factors responsible for the Ca(2+)-dependent inactivation of calcineurin in brain. FEBS Lett 1995;374:237-40.

169. Namgaladze D, Shcherbyna I, Kienhofer J, Hofer HW, Ullrich V. Superoxide targets calcineurin signaling in vascular endothelium. Biochem Biophys Res Commun 2005;334:1061-7.

170. Heisel O, Heisel R, Balshaw R, Keown P. New onset diabetes mellitus in patients receiving calcineurin inhibitors: a systematic review and meta-analysis. Am J Transplant 2004;4:583-95.

171. Sankaranarayanan TK, Sethi BK, Subramanyam C. Serum calcineurin activity in relation to oxidative stress and glycemic control in type II diabetes mellitus. Clin Biochem 2005;38:218-22.

172. Agbas A, Hui D, Wang X, Tek V, Zaidi A, Michaelis EK. Activation of brain calcineurin (Cn) by Cu-Zn superoxide dismutase (SOD1) depends on direct SOD1-Cn protein interactions occurring in vitro and in vivo. Biochem J 2007;405:51-9.

173. Tokheim AM, Martin BL. Association of calcineurin with mitochondrial proteins. Proteins 2006;64:28-33.

174. King MM. Modification of the calmodulin-stimulated phosphatase, calcineurin, by sulfhydryl reagents. J Biol Chem 1986;261:4081-4.

175. Bogumil R, Namgaladze D, Schaarschmidt D, Schmachtel T, Hellstern S, Mutzel R, Ullrich V. Inactivation of calcineurin by hydrogen peroxide and phenylarsine oxide. Evidence for a dithiol-disulfide equilibrium and implications for redox regulation. Eur J Biochem 2000;267:1407-15.

176. Carruthers NJ, Stemmer PM. Methionine oxidation in the calmodulin-binding domain of calcineurin disrupts calmodulin binding and calcineurin activation. Biochemistry 2008;47:3085-95.

177. Lee JE, Kim H, Jang H, Cho EJ, Youn HD. Hydrogen peroxide triggers the proteolytic cleavage and the inactivation of calcineurin. J Neurochem 2007;100:1703-12.

178. Lee JE, Jang H, Cho EJ, Youn HD. Down syndrome critical region 1 enhances the proteolytic cleavage of calcineurin. Exp Mol Med 2009;41:471-7.

179. Zakany R, Szijgyarto Z, Matta C, Juhasz T, Csortos C, et al. Hydrogen peroxide inhibits formation of cartilage in chicken micromass cultures and decreases the activity of calcineurin: implication of ERK1/2 and Sox9 pathways. Exp Cell Res 2005;305:190-9.

180. Reiter TA, Abraham RT, Choi M, Rusnak F. Redox regulation of calcineurin in T-lymphocytes. J Biol Inorg Chem 1999;4:632-44.

181. Furuke K, Shiraishi M, Mostowski HS, Bloom ET. Fas ligand induction in human NK cells is regulated by redox through a calcineurin-nuclear factors of activated T cell-dependent pathway. J Immunol 1999;162:1988-93.

182. Huang C, Ding M, Li J, Leonard SS, Rojanasakul Y, et al. Vanadium-induced nuclear factor of activated T cells activation through hydrogen peroxide. J Biol Chem 2001;276:22397-403.

183. Choi SE, Min SH, Shin HC, Kim HE, Jung MW, Kang Y. Involvement of calcium-mediated apoptotic signals in H2O2-induced MIN6N8a cell death. Eur J Pharmacol 2006;547:1-9.

184. Maziere C, Morliere P, Louandre C, Conte MA, Gomilla C, et al. Low UVA doses activate the transcription factor NFAT in human fibroblasts by a calcium-calcineurin pathway. Free Radic Biol Med 2005;39:1629-37.

185. Huang C, Mattjus P, Ma WY, Rincon M, Chen NY, Brown RE, Dong Z. Involvement of nuclear factor of activated T cells activation in UV response. Evidence from cell culture and transgenic mice. J Biol Chem 2000;275:9143-9.

186. Flockhart RJ, Diffey BL, Farr PM, Lloyd J, Reynolds NJ. NFAT regulates induction of COX-2 and apoptosis of keratinocytes in response to ultraviolet radiation exposure. FASEB J 2008;22:4218-27.

187. Smit N, Musson R, Romijn F, van Rossum H, van Pelt J. Effects of ultraviolet A-1 radiation on calcineurin activity and cytokine production in (skin) cell cultures. Photochem Photobiol 2010;86:360-6.

188. Stewart AA, Ingebritsen TS, Manalan A, Klee CB, Cohen P. Discovery of a Ca2+- and calmodulin-dependent protein phosphatase: probable identity with calcineurin (CaM-BP80). FEBS Lett 1982;137:80-4.

189. Klee CB, Crouch TH, Krinks MH. Calcineurin: a calcium- and calmodulin-binding protein of the nervous system. Proc Natl Acad Sci U S A 1979;76:6270-3.

190. Merat DL, Hu ZY, Carter TE, Cheung WY. Subunit A of calmodulin-dependent protein phosphatase requires Mn2+ for activity. Biochem Biophys Res Commun 1984;122:1389-96.

191. Stewart AA, Ingebritsen TS, Cohen P. The protein phosphatases involved in cellular regulation. 5. Purification and properties of a Ca2+/calmodulin-dependent protein phosphatase (2B) from rabbit skeletal muscle. Eur J Biochem 1983;132:289-95.

192. King MM, Huang CY. Activation of calcineurin by nickel ions. Biochem Biophys Res Commun 1983;114:955-61.

193. Pallen CJ, Wang JH. Regulation of calcineurin by metal ions. Mechanism of activation by Ni2+ and an enhanced response to Ca2+/calmodulin. J Biol Chem 1984;259:6134-41.

194. Pallen CJ, Wang JH. Stoichiometry and dynamic interaction of metal ion activators with calcineurin phosphatase. J Biol Chem 1986;261:16115-20.

195. Martin BL, Graves DJ. Isotope effects on the mechanism of calcineurin catalysis: kinetic solvent isotope and isotope exchange studies. Biochim Biophys Acta 1994;1206:136-42.

196. Wolff DJ, Sved DW. The divalent cation dependence of bovine brain calmodulin-dependent phosphatase. J Biol Chem 1985;260:4195-202.

197. Li HC, Chan WW. Activation of brain calcineurin towards proteins containing Thr(P) and Ser(P) by Ca2+, calmodulin, Mg2+ and transition metal ions. Eur J Biochem 1984;144:447-52.

198. Ping L, Ke Z, Benqiong X, Qun W. Effect of metal ions on the activity of the catalytic domain of calcineurin. Biometals 2004;17:157-65.

199. Martin BL, Jurado LA, Hengge AC. Comparison of the reaction progress of calcineurin with Mn2+ and Mg2+. Biochemistry 1999;38:3386-92.

200. Martin BL, Rhode DJ. Effect of substitution inert metal complexes on calcineurin. Arch Biochem Biophys 1999;366:168-76.

201. Martin BL, Li B, Liao C, Rhode DJ. Differences between Mg(2+) and transition metal ions in the activation of calcineurin. Arch Biochem Biophys 2000;380:71-7.

202. Dolmetsch RE, Lewis RS, Goodnow CC, Healy JI. Differential activation of transcription factors induced by Ca2+ response amplitude and duration. Nature 1997;386:855-8.

203. Barchowsky A, O'Hara KA. Metal-induced cell signaling and gene activation in lung diseases. Free Radic Biol Med 2003;34:1130-5.

204. Salnikow K, Davidson T, Kluz T, Chen H, Zhou D, Costa M. GeneChip analysis of signaling pathways effected by nickel. J Environ Monitor 2003;5:206-9.

205. Huang C, Li J, Costa M, Zhang Z, Leonard SS, et al. Hydrogen peroxide mediates activation of nuclear factor of activated T cells (NFAT) by nickel subsulfide. Cancer Res 2001;61:8051-7.

206. Saripalli YV, Gadzia JE, Belsito DV. Tacrolimus ointment 0.1% in the treatment of nickel-induced allergic contact dermatitis. J Am Acad Dermatol 2003;49:477-82.

207. Takahashi K, Akaishi E, Abe Y, Ishikawa R, Tanaka S, Hosaka K, Kubohara Y. Zinc inhibits calcineurin activity in vitro by competing with nickel. Biochem Biophys Res Commun 2003;307:64-8.

208. Tanaka S, Akaishi E, Hosaka K, Okamura S, Kubohara Y. Zinc ions suppress mitogen-activated interleukin-2 production in Jurkat cells. Biochem Biophys Res Commun 2005;335:162-7.

209. Politino M, King MM. Calcineurin-phospholipid interactions. Identification of the phospholipid-binding subunit and analyses of a two-stage binding process. J Biol Chem 1990;265:7619-22.

210. Kennedy MT, Brockman H, Rusnak F. Contributions of myristoylation to calcineurin structure/function. J Biol Chem 1996;271:26517-21.

211. Kennedy MT, Brockman H, Rusnak F. Determinants of calcineurin binding to model membranes. Biochemistry 1997;36:13579-85.

212. Kessen U, Schaloske R, Aichem A, Mutzel R. Ca(2+)/calmodulin-independent activation of calcineurin from Dictyostelium by unsaturated long chain fatty acids. J Biol Chem 1999;274:37821-6.

213. Martin BL. Inhibition of calcineurin by the tyrphostin class of tyrosine kinase inhibitors. Biochem Pharmacol 1998;56:483-8.

214. Spannaus-Martin DJ, Martin BL. In vitro effect of retinoids on calcineurin activity. Biochem Pharmacol 2000;60:803-8.

215. Tokheim AM, Martin BL. Inhibition of calcineurin by polyunsaturated lipids. Bioorg Chem 2006;34:66-76.

216. Yin Y, Xie M, Wu H, Jiang M, Zheng J, Wei Q. Interaction of calcineurin with its activator, chlorogenic acid revealed by spectroscopic methods. Biochimie 2009;91:820-5.

217. Wang H, Zhou CL, Lei H, Zhang SD, Zheng JQ, Wei Q. Kaempferol, a new immunosuppressant on calcineurin. IUBMB Life 2008;60:549-54.

218. Wang H, Zhou CL, Lei H, Wei Q. Inhibition of calcineurin by quercetin in vitro and in Jurkat cells. J Biochem 2010;147:185-190.

219. Lei H, Qi Y, Jia ZG, Lin WL, Wei Q. Studies on the interactions of kaempferol to calcineurin by spectroscopic methods and docking. Biochim Biophys Acta 2009;1794:1269-75.

220. Tatlock JH, Angelica Linton M, Hou XJ, Kissinger CR, Pelletier LA, et al. Structure-based design of novel calcineurin (PP2B) inhibitors. Bioorg Med Chem Lett 1997;7:1007-12.

221. Baba Y, Hirukawa N, Sodeoka M. Optically active cantharidin analogues possessing selective inhibitory activity on Ser/Thr protein phosphatase 2B (calcineurin): implications for the binding mode. Bioorg Med Chem 2005;13:5164-70.

222. Erdmann F, Weiwad M, Kilka S, Karanik M, Patzel M, et al. The novel calcineurin inhibitor CN585 has potent immunosuppressive properties in stimulated human T cells. J Biol Chem 2010;285:1888-98.