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Calcineurin

Kamaldeen A. Muili, Abrahim I. Orabi, and Sohail Z. Husain

From the Department of Pediatric Gastroenterology, Children's Hospital of Pittsburgh of UPMC,

Pittsburgh, Pennsylvania, 15224

e-mail: abekemi@gmail.com, abrahim.orabi@chp.edu, sohail.husain@chp.edu

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Protein symbols: PP2B, Cn

Gene Symbol: PPP3CA (CnAα); PPP3CB, (CnAβ); PPP3CC (CnAγ); PPP3R1 (CnB1); PPP3R2 (CnB2)

Abstract

Calcineurin (CN) is a Ca^{2+}/calmodulin dependent serine/threonine protein phosphatase first identified in brain and also known as protein phosphatase 2B (PP2B). It has two subunits, A and B, each of which has several isoforms and is inhibited by the immunosuppressant drugs FK506 and cyclosporine. A number of endogenous inhibitors have also been identified. CN has a number of targets but its most prominent are the NFAT (nuclear factor of activated T cells) transcription factors. In the pancreatic acinar cell CN plays a role in mediating the action of elevated Ca^{2+} to stimulate cell division, pancreatic growth and protein synthesis. Pathophysiologically, it is involved in mediating experimental pancreatitis induced by bile salts and caerulein.

1. General Function

Calcineurin (Cn) is a Ca^{2+}/calmodulin (CaM)-dependent serine/threonine phosphatase first identified in extracts of mammalian brain (68, 94). Its name was further derived from its ability to bind Ca^{2+}. Its importance has been documented in a number of physiologic and pathologic conditions including neuronal and muscle development, lymphocyte activation, cardiac hypertrophy, switching of skeletal muscle fiber type, and expression of ion channels. Cn (also known as PP2B) is part of a family of type 2 protein phosphatases that include PP2A and PP2C. They are classified according to their dependence on certain divalent metal ions for phosphatase activity and Cn is uniquely dependent upon Ca^{2+}. PP2A and PP1, but not Cn, are inhibited by the exogenously administered phosphatase inhibitors okadaic acid, microcystin, and calyculin, as well as the endogenous inhibitors inhibitor-1 and DARPP-32 (dopamine- and cAMP-regulated phosphoprotein of 32 kDa). Cn is specifically inhibited by the immunosuppressant drugs FK506 (tacrolimus) and cyclosporine A (CsA) (44).

There are several comprehensive reviews on Cn (4, 68), more recent brief updates (3, 37, 44), information on Cn inhibitors (42, 55), and disease- or organ/tissue-specific reviews relating to Cn in the neurosciences (29), muscle (65), islet cells (35).

Cn isoforms, structure, and function

Cn consists of two subunits, CnA and CnB, which form a heterodimer in order to conduct phosphatase activity. CnA (**Figure 1**) contains the catalytic domain, which is homologous to other serine/threonine protein phosphatases (4). A dinuclear metal center composed of one iron and one zinc molecule each lies next to a β sandwich on the active site (**Figure 2**). CnA also has 3 regulatory domains: a binding domain for its partner subunit CnB, a CaM-binding domain, and an autoinhibitory domain. CnB, the regulatory subunit, contains 4 Ca^{2+}-binding EF hand motifs that regulate (through a conformational change) the catalytic function of Cn. CnB binding to CnA may also facilitate proper folding of the active enzyme (21). CnB resembles CaM in that both bind to an extended α helix on their respective CnA domains. The primary sequence of the Cn subunit is highly conserved. CnA and CnB are tightly bound ($k_d \leq 10^{-13}$ M) even in the total absence of Ca^{2+}. Two short α-helices form the inhibitory domain and block the catalytic center under basal low Ca^{2+} conditions. The CaM binding domain is flexible. Binding of CaM along with Ca^{2+} binding to CnB induces displacement of the inhibitory domain, thus exposing the catalytic domain.

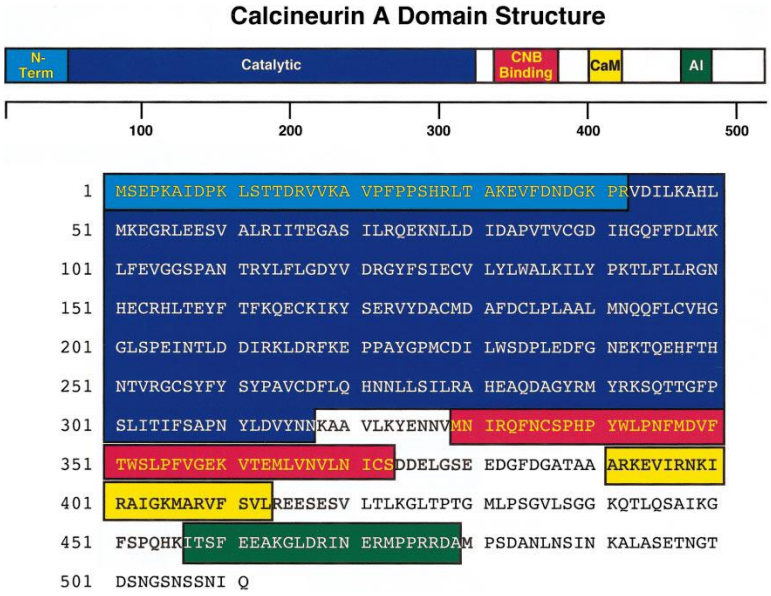

Figure 1. Primary sequence and domain structure of CnA. The amino acid sequence represents rat CnAα. Note the regulatory domains that bind CnB and CaM as well as the autoinhibitory domain (AI). Modified from (68).

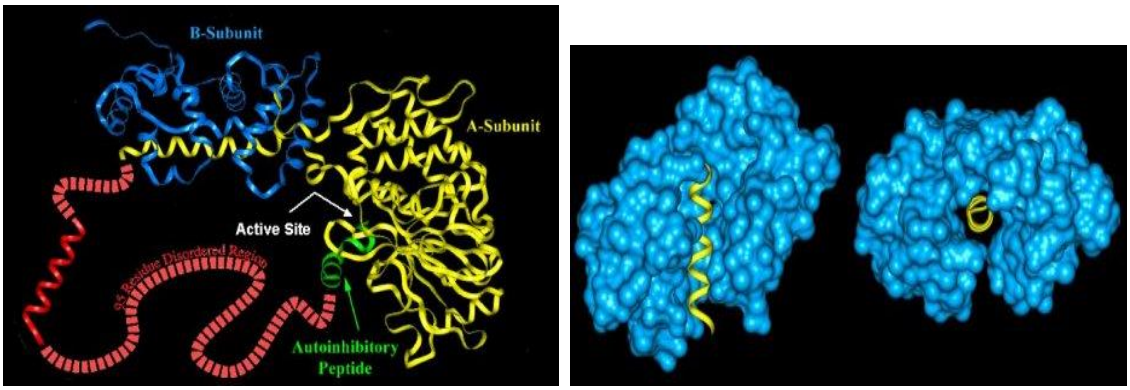

Figure 2. Cn structure. (left panel) Ribbon diagram of CnA (yellow) and CnB (blue). The former has several disordered regions (DRs) , including a long stretch of 95 residues (red); the CaM binding α helix is contained within the this DR. (right panel) X-ray crystallography of CaM (blue) demonstrates binding to the α helix of CnA (colored yellow). Source: http://www.pondr.com/pondr-tut1.html

Cn isoforms and tissue distribution

CnA has 3 isoforms CnAα, CnAβ, and CnAγ. CnB has two isoforms CnB1 and CnB2. CnAβ also has two splice variants which differ in their C-terminal domain (30). CnAα and CnAβ appear to interact interchangeably with CnB1. Cn is highly enriched in brain; it constitutes 1% of total protein content and there are 20-30 fold greater amounts than in other tissues. However, Cn is ubiquitously expressed and has differential isoform distribution. CnAγ and CnB2 are primarily found in testis. There is greater abundance of CnAα over CnAβ in brain and heart, but the reverse is true in spleen, thymus, and lymphocytes. CnAβ is considered a stress-responsive isoform (12, 84).

The subcellular distribution of Cn is also distinct in certain cell types. Although Cn is localized to the cytoplasm in most systems, in spermatids, for example, it is localized to the nucleus; levels are most abundant during the initial stage of nuclear elongation with almost no signal present in the cytoplasm (57). In some systems, Cn co-translocates with NFAT (nuclear factor of activated T cells) to the nucleus upon activation by Ca^{2+} (76). In chicken forebrain, Cn is highly enriched in cytoplasmic, microsomal, and synaptosomal fractions (1). Cn is also co-localized with the cytoskeleton in cultured neurons (22) and the T-tubules of ventricular myocytes (70). The molecular mechanism of this targeting is not fully clear. However, calsarcin-1 and -2 tether Cn to α-actinin and may couple Cn activity with muscle contraction (23).

Regulation of Cn

Several factors regulate Cn. The most potent activators are Ca^{2+} and CaM. As mentioned earlier, even at low cytosolic Ca^{2+} concentrations, CnA is tightly bound to CnB (49). However, a sustained rise in Ca^{2+} causes the dual recruitment of CaM to CnA and the binding of Ca^{2+} to CnB (89). The type of Ca^{2+} signature necessary for Cn activation is unclear. Using NFAT nuclear translocation as a measure of Cn activation, Timmerman et al. demonstrated in lymphocytes that sustained, but not transient elevations were required to maintain NFAT in the nucleus (89). However, Dolmetsch et al. demonstrated in the same cell type that rapid oscillations in Ca^{2+} could induce NFAT translocation (19). Nonetheless, the rise in cytosolic Ca^{2+} results in a conformational change in CnA that forces its autoinhibitory domain to dissociate from its catalytic groove, thereby permitting Cn activity. Of the two initiating components, it is thought that CaM is the critical activator. Cn is also reversibly inactivated by oxidation of its Fe^{2+} molecule (95). In fact, there is some thought that a Ca^{2+}/CaM-induced conformational change in Cn exposes the Fe^{2+} to

oxidation, thus providing negative feedback for Cn activation.

A number of endogenous Cn inhibitors have been identified. They include AKAP79 (A-kinase anchoring protein of 79 kDa) which as its name implies also anchors PKA with Cn (17). Another protein with this dual anchoring and inhibitory action on Cn in cardiac and skeletal muscle is calsarcin (23). A family of proteins called modulatory Cn interacting proteins (MCIPs) serves as feedback inhibitors of Cn (67). In humans, its gene was initially identified as DSCR1 (Downs's syndrome critical region 1) (81). A recent consensus was reached to call these proteins regulators of Cn (RCANs) (15). They are upregulated by Cn-mediated activation of the transcription factor NFAT. They can inhibit Cn and interestingly also directly inhibit NFAT through binding a highly conserved ISPPxSPP motif found on both proteins. Other inhibitors include Cn homologous protein (CHP) and Cain/Cabin1 (46). The latter is a 240 kDa nuclear protein that inhibits Cn in a Ca^{2+}- and PKC-dependent manner, likely by binding to the same site as FK506-FKBP. Other factors contributing to Cn activation include polyunsaturated fatty acids (43).

Cn targets

Several phospho-protein targets of Cn have been identified. The best known is NFAT, which resides in the cytoplasm during basal conditions, but translocates to the nucleus upon dephosphorylation by Cn (52). Other targets of Cn are listed in **Table 1**.

Cn in physiology and disease

As mentioned earlier, Cn is highly enriched in neurons, but it is ubiquitously expressed in all tissues and cells. In the brain, it functions to activate a series of phosphatases by dephosphorylating the endogenous inhibitors of PP-1: inhibitor-1 and DARPP-32. Cn thus intricately regulates synaptic plasticity and long term memory (53, 103). Cn also regulates synaptic vesicle endocytosis by dephosphorylating the dephosphins (14). In T cells, sustained cytosolic Ca^{2+} release leads to Cn/NFAT activation and the induction of T cell-activating genes, notably interleukin-2 (52). The reason why the Cn inhibitors FK506 and CsA are such effective chronic immunosuppressive drugs is the blockade of T cell Cn. In heart, the Cn/NFAT pathway may protect against dilated cardiomyopathy (34). However, it plays a pathologic role in cardiac hypertrophy (56, 83, 100), through either activation of NFAT3 (along with GATA4), co-activation of NFAT and MEF2, or PKC activation. In skeletal muscle, Cn regulates, again through NFAT3 and MEF2, switching of muscle fiber subtype (65, 102). In islet cells Cn/NFAT regulates beta cell growth (36, 41) and survival (6, 79).

2. Cn in the exocrine pancreas

Investigations in the exocrine pancreas relating to Cn have focused on the acinar cell. Cn was reported to inhibit acinar cell exocytosis of pancreatic enzymes (18, 27, 97). Initial work in both pancreatic lobules as well as dispersed acini demonstrated that CsA and FK506 each reduced caerulein- and carbachol-stimulated amylase secretion (18, 27, 97). However, later studies could reproduce only a modest reduction using FK506 (27, 40). Further work showed that Cn is required for translational control of acinar cell protein synthesis. In isolated acinar cells stimulated with either CCK, bombesin, or carbachol, FK506 reduced methionine incorporation into protein. FK506 modulated factors in the translation machinery: it reduced the phosphorylation of mRNA cap binding protein eukaryotic initiation factor (eIF) 4E binding protein, reduced the formation of the eIF4F complex, and increased the phosphorylation of eukaryotic elongation factor 2 (69). In a series of elegant studies using an experimental model of adaptive growth in which mice were fed the trypsin inhibitor camostat in order to stimulate endogenous CCK release, pancreatic growth was shown to be dependent

upon Cn (32, 33, 87). This was initially demonstrated using CsA and FK506 (87). Cn pathways could explain several important aspects of pancreatic growth, such as c-Jun NH2-terminal kinase activation (86). In a subsequent study, overexpression of the endogenous Cn inhibitor Rcan1 selectively within acinar cells also led to reduced adaptive growth of the pancreas (32). Because Rcan1 is a transcriptional target of NFAT, validated in the acinar cell by chromatin immunoprecipitation, it was suggested that Cn modulates growth through NFAT activation (32). Indeed, using an NFAT-luciferase reporter, CCK activated NFAT signaling (33).

Cn has also been shown to mediate experimental pancreatitis (40, 60, 73). FK506 administration *in vivo* attenuated the severity of pancreatitis induced by intra-ductal bile acid infusion or hyperstimulation with the CCK analog caerulein. Further, the Cn inhibitors FK506, CsA, and Cn inhibitory peptide (CiP) reduced pathologic intra-acinar protease activation, NF-κB activation, and cell injury (58-60). Similarly, NF-κB activation due to thapsigargin-induced Ca^{2+} signals was dependent on Cn (31). CsA was shown to attenuate pancreatic inflammation in an experimental model of chronic pancreatitis that was induced in the MRL/MP mouse strain by injecting poly IC (polyinosinic:polycytidylic acid) (72). In this model, it was suggested that the effect of Cn in chronic pancreatitis was mediated through auto-activated T cells.

There are also reports that NFAT is involved in pancreatic injury (5). Mice deficient in NFATc3 had reduced trypsinogen activation, pancreatic inflammation, and were protected against intra-ductal bile infusion or L-arginine-induced pancreatitis. Other Cn substrates that mediate non-transcriptional events may also play a role in the exocrine pancreas. Notably, the Cn substrate CRHSP-24 was first identified in the exocrine pancreas (28, 47).

3. Tools for the study of Cn

a. Molecular constructs

Several Cn clones and adenoviral constructs have been created. A major tool employed to identify a role for Cn in a cellular process has been the overexpression of a CaM-independent derivative of CnAα residues (1-392), in effect producing a constitutively active Cn (ΔCnA) (64). A host of plasmids are available for purchase at Addgene (www.addgene.org). Adenoviral vectors are available from Seven Hills Bioreagents. siRNA for Cn is available through Ambion.

b. Antibodies

In our experience, commercially available Cn antibodies are not particularly specific for their labeled Cn isoforms, particularly the CnAβ antibody. However, they are available from Santa Cruz, Upstate Biotech, BD Transduction Labs, and Chemicon. Most of them can be used for immunofluorescence in addition to western blotting.

c. Transgenic mice

CnAβ knockout mice were made by Dr. Jeff Molkentin (12). They live to adulthood, breed well, and have no gross phenotypic defects. CnAα knockout mice were made Dr. Jon Seidman (104). CnB1 knockout mice do not live beyond the embryonic period due to fatal defects in vascular patterning. However, floxed CnB1 mice were made by Dr. Gerald Crabtree (62). To our knowledge, CnAγ-/- or CnB2-/- are not available. An NFAT luciferase reporter mouse has been used to monitor Cn activation (99).

d. Cn Activity

Cn activity is primarily measured *in vitro* using a 19 residue synthetic peptide corresponding to residues 81-99 of the RII subunit of cAMP-dependent protein kinase (8). As an alternative Biomol Labs carries a colorimetric assay kit. *In vivo* measurements can be performed by monitoring NFAT nuclear translocation. In pancreas the

dephosphorylation of CRHSP-24 has also been used (48). As mentioned earlier, there are adenoviral NFAT-reporter constructs and transgenic NFAT-luciferase mice (99).

e. Pharmacologic inhibitors

The two prototypic Cn inhibitors, FK506 and CsA, form a complex with FK506 binding protein (FKBP12) and cyclophilin, respectively (55). The complex then binds Cn and blocks access of substrates to its catalytic site. The junction between CnB and CnA has been identified by crystallography to be the binding site for FK506 (**Figure 3**). The two inhibitors are widely used in clinical practice as immunosuppressants after organ transplantation or for the treatment of autoimmune disorders because they diminish Cn-dependent T cell activation. Several novel variations of these inhibitors are in testing (77). FK506 is more specific than CsA. The latter can bind cyclophilin D and thereby inhibit the mitochondrial permeability transition pore (101). It should also be noted that both FK506 and CsA have worrisome side effects with prolonged, chronic use, such as hypertension, neurotoxicity and diabetes (91, 92).

CiP is a short peptide that mimics the auto-inhibitory domain of Cn. A cell permeant form of CiP, made by covalently attaching an arginine tail to the peptide is available through Calbiochem (88). As a negative control, however, it will be important to synthesize a scrambled peptide of equal length that also has an arginine tail.

Phosphatase inhibitors that do not inhibit Cn can be used as negative controls to determine the selectivity of an effect for Cn. In particular, the serine/threonine phosphatases PP1 and PP2A can be inhibited by okadaic acid (IC_{50} 20 nM and 0.2 nM, respectively), calyculin-A (IC_{50} 1 nM for both), and microcystin-LR (IC_{50} 0.1 nM for both) (75). The former two inhibitors have been used in pancreatic acinar cells (71), and none inhibit Cn (PP2B) at the noted concentrations.

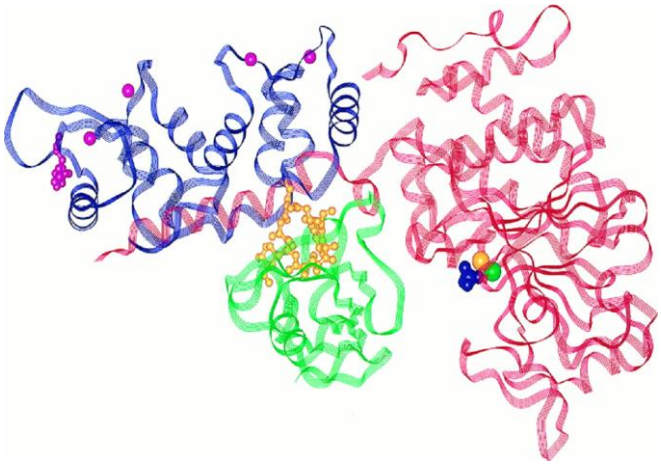

Figure 3. Ribbon diagram of truncated Cn complexed with FK506-FKBP12. CnA is shown in red and CnB in purple, with myristic acid covalently linked to the N-terminal glycine shown in pink. Iron and zinc are contained within the active site of CnA (yellow and green spheres, respectively), and the bound phosphate is shown in purple. Four molecules of Ca^{2+} on their respective CnB binding sites are shown as pink spheres. The FK506 (yellow)-FKBP12 (green) complex blocks entry of Cn substrates to the active site on CnA. (Protein Data Bank code 1TCO(26)). Modified from (45).

Table 1: Cn targets			
Abbreviation	**Full Name**	**Primary Function**	**References**
NFAT	nuclear factor of activated t-cells	transcription factor	(52)
DARP32	dopamine- and camp-regulated neuronal phosphoprotein	dopaminergic pathway transducer	(63)
DYN1 & 2	dynamin-2	GTPase	(50)
α-chrystallin	α-chrystallin	structural protein	(11, 54)
BAD	bcl-2-associated death promoter (bad)	pro-apoptotic protein	(93)
TAU	tau proteins	microtubule-associated protein	(25)
FLNA	filamin a, alpha	structural protein	(24)
DRP1	dynamin related protein 1	mitochondrial fission	(66, 78)
CNX	calnexin	chaperone	(9)
CKI-epsilon	casein kinase 1 epsilon	protein kinase	(7)
GAD65 & 67	glutamic acid decarboxylase 65 & 67	decarboxylation enzyme	(38)
HSP27 & 70	heat shock protein 27 & 70	differentiation & signal transduction	(47)
NFH	neurofilaments	structural protein	(80, 85)
IP3R & RyR	inositol 1,4,5-trisphosphate receptor & ryanodine receptor	intracellular Ca^{2+} release	(13)
NTCP	Na+ taurocholate co-transporting polypeptide	bile acid transporter	(98)
RII-α	regulatory subunit-ii alpha	PKA regulatory subunit	(8)
SSH-1	slingshot 1	protein phosphatase	(96)
VASP	vasodilator-stimulated phosphoprotein	structural protein	(90)
Tubulin	tubulin	microtubule-associated protein	(25)
KSR2	kinase suppressor of RAS	ERK cascade activation	(20)
C-JUN	cellular-JUN	cell cycle progression/anti-apoptosis	(39)
ELK1	ETS domain-containing protein	transcription factor	(82)
GSK3β	glycogen synthase kinase 3 beta	serine-threonine kinase	(74)
CRHSP-24	ca^{2+}-regulated heat-stable protein of 24 kDa	protein translation	(28, 71)
Inhibitor-1	inhibitors of protein phosphatase 1	phosphatase inhibitor	(61)
NOS	nitric oxide synthase	signaling molecule	(16)
adenylate	adenylate cyclase	signal transducer, catalyzes camp	(2)
MEF2	myocyte enhancer factor 2	transcription factor	(102)
Gap43	growth associated protein 43 (neuromodulin)	neuronal growth and development	(10, 51)
Map2	microtubule associated protein-2	microtubule-associated protein	(25)

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