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A review of calcineurin biophysics with implications for cardiac physiology

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Calmodulin is a prevalent calcium sensing protein found in all cells. Three genes exist for calmodulin and all three of these genes encode for the exact same protein sequence. Recently mutations in the amino acid sequence of calmodulin have been identified in living human patients. Thus far, patients harboring these mutations in the calmodulin sequence have only displayed an altered cardiac related phenotype. Calcineurin is involved in many key physiological processes and its activity is regulated by calcium and calmodulin. In order to assess whether or not calcineurin contributes to calmodulinopathy (a pathological state arising from dysfunctional calmodulin), a comprehensive search of relevant literature has been performed. Herein, the physiological roles of calcineurin and consequences of dysfunction have been reviewed for literature focused on the heart.

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CHAPTER I

AN OVERVIEW OF CARDIAC PHYSIOLOGY

1.1 General mechanisms of heart function.

The heart is essential for human life. Dysfunction of the heart can reduce blood flow throughout the body, which will impair delivery of nutrients such as oxygen. Notably, loss of oxygen to the brain can result in irreversible damage within five minutes and fatality in approximately ten minutes. The heart is comprised of four main chambers, two atria and two ventricles. The ventricles are responsible for generating the force that pumps the blood throughout the body. The atria serve to refill the ventricle after they are depleted. The timing of atrial and ventricular contractions are governed by electrical currents that are generated by ion channel proteins contained in specialized cells throughout the heart. Dysfunction or loss of function to these ion channels can alter or disrupt the timing or contractile properties of the different chambers in the heart [1].

1.2 An overview of excitation-contraction coupling.

Ion channels function by allowing ions to pass across a cellular membrane. Some ion channels allow ions to flow into the cell, while others allow passage out of the cell. Ions flow through the ion channels due to differences in ion concentration between the inside and outside of cells. When ions pass through the membrane this alters the electrical potential on the membrane surface. In the specialized muscle cells, known as cardiomyocytes, electrical currents are used to manipulate the concentration of calcium inside the cardiomyocyte. This mechanism is

referred to as excitation contraction coupling. Briefly, in response to membrane depolarization voltage-gated sodium channels open. Further depolarization is achieved by opening of the voltage-sensitive calcium channels, which allows Ca^{2+} ions to flow into the cell. This influx of calcium triggers calcium-induced calcium release from the sarcoplasmic reticulum through the intracellular Ca^{2+} ion channel referred to as ryanodine receptor type II. The resulting effect is an elevation of intracellular [Ca^{2+}] from 100 nM to approximately 1 μM. Changes in intracellular [Ca^{2+}] primarily do three things inside the cell. First, an increase in [Ca^{2+}] alters the conformation of troponin C. Restructuring the troponin complexes allosterically moves tropomyosin out of the way so the myosin and actin proteins are able to interact. Myosin utilizes ATP to generate a pulling force along actin. Cooperativity between the sarcomeric proteins and cardiomyocytes generate the necessary force required for heart contraction. Cardiac contraction can be enhanced by increasing the amplitude of calcium transient, or changes in [Ca^{2+}], inside a cardiomyocyte. This allows for a greater population of myosin to engage actin and thereby generates a larger force. In a typical human resting heart approximately 30% of myosin is used to engage actin and generate the force for contraction. This allows the heart to have a tunable range to support extra physical exertion when needed [2].

1.3 Other roles of calcium in a ventricular cardiomyocyte.

Second, calcium also provides feedback to many of the ion channels that are used for generating electrical currents of the cardiac action potential. While the functional implications of these feedback systems have been the subject of extensive investigation, the mechanistic details and physiological consequences are widely debated and largely unknown.

Third, changes in intracellular [Ca^{2+}] can exert an influence on protein function through post translational modification. Ca^{2+}-sensitive enzymes such as calmodulin dependent kinase

(CaMKII) and the calmodulin enhanced phosphatase calcineurin, modify a myriad of proteins. Despite extensive cellular and physiological characterization, the mechanisms underlying target protein selection as well as post translational functional modification are insufficiently described, and thus present knowledge gaps that limit our ability to connect atomic resolution structure with cellular and physiological outcomes [2].

For my thesis, I have explored the relevant literature surrounding calcineurin structure and function in ventricular cardiomyocytes. In this thesis, I highlight the seminal contributions that have shaped our current understanding of calcineurin cardiac function in health and disease. With the recent identification of mutations in the calcium sensing protein calmodulin, I seek to understand if there are unique functions of calcineurin in a cardiomyocyte that could render the heart susceptible to calmodulinopathy disease.

CHAPTER II

A REVIEW OF CALCINEURIN BIOPHYSICS WITH IMPLICATIONS IN CELLUAR PHYSIOLOGY

2.1 Identification and nomenclature of calcineurin.

Calcineurin (CaN) was initially identified by two independent research laboratories [3,4]. In 1976, Wang *et al.* demonstrated that bovine brain cyclic nucleotide phosphodiesterase (likely PKA and/or PKG) required a higher concentration of a "calcium regulated protein modulator" (one of the original names for calmodulin) for activation compared to heart tissue. This was due to the presence of an "inhibitory factor" (CaN) in the brain preparations that counteracted the activation of the phosphodiesterase [4]. Concurrently, in 1976 Antoniw *et al.* demonstrated that two distinct enzymes dephosphorylated the independent subunits of phosphorylase kinase [3] (phosphorylase kinase activates glycogen phosphorylase to release glucose-1-phosphate from glycogen [5]). β phosphorylase kinase phosphatase is now known as Protein phosphatase 1 glycogen-associated regulatory subunit or protein phosphatase type-1 glycogen targeting subunit. The α-phosphorylase kinase phosphatase (later discovered to be comprised of two similar enzymes, one of these being CaN, see below) was shown to be an inhibitor of: cyclic nucleotide phosphodiesterase [3,6], adenylate cyclase [7,8], turkey gizzard myosin light chain kinase [9], erythrocyte calcium magnesium ATPase [10,11], and phosphorylase b kinase [12]. While the mechanisms were not explicitly identified; inhibition likely occurs by either: CaN outcompeting

and exhausting calmodulin (CaM) availability, and/or dephosphorylation of the enzyme substrate.

In 1979 the name calcineurin was coined by Klee *et al.* due to the "inhibitory factor's" calcium binding properties and prevalence in the nervous system [9]; though it took a few years before the name was widely adopted. In 1981, Helmreich *et al.* renamed alpha phosphorylase kinase phosphatase to protein phosphatase 2 [13]. Shortly after, Stewart *et al.* resolved Protein Phosphatase 2 into two fractions (PP2A and PP2B). Both PP2A and PP2B (CaN) can dephosphorylate the α and β subunits of phosphorylase kinase, however there are enzymatic differences between these two proteins. PP2A has broad substrate specificity and dephosphorylates the phosphorylase kinase α subunit more rapidly (4-5 fold) than the β subunit. Conversely, PP2B has narrow substrate specificity, but also dephosphorylates the phosphorylase kinase α subunit more rapidly (100 fold) compared to the β subunit [13].

2.2 Metal binding and calcineurin activity.

In 1982, Stewart *et al.* demonstrated that CaN (isolated from bovine neuronal tissue) has high affinity ($K_d \leq 10^{-6}$ M) for calcium in the presence of physiological concentrations of other divalent cations (*i.e.* Mg^{2+} and Mn^{2+}). Moreover, 8-12% of maximal CaN activity can be obtained by calcium saturation (~ pCa 5.8). An additional 10-fold increase in CaN activity was observed for the same calcium titration in the presence of CaM [14]. In 1984, two separate labs investigated the activity of CaN in the presence of other divalent cations. Li and Chan demonstrated that full activity of brain CaN requires Ca^{2+}, CaM, and another divalent cation (Mg^{2+}, Mn^{2+}, Co^{2+}, or Ni^{2+}) [15]. Using a p-Nitrophenyl Phosphate (pNPP) assay Pallen and Wang demonstrated that Ni^{2+} and Mn^{2+} can activate CaN in the absence of CaM, and addition of CaM further enhanced this CaN activity. Other divalent cations ($Co^{2+} >> Ca^{2+} > Sr^{2+}$, Ba^{2+}) require the

presence of CaM to exert significant enhancement of CaN activity. Cu^{2+} is a better activator of CaN in the absence, rather than the presence, of CaM. Moreover, Be^{2+}, Cd^{2+}, Fe^{2+}, Mg^{2+}, Al^{3+}, Fe^{3+} do not stimulate CaN activity in the absence or presence of CaM. Lastly, Zn^{2+} inhibits the activation of CaN by other cations in both the absence and presence of CaM [16]. In 2004, Ping *et al.* investigated specific components of CaN in order to identify a mechanism for divalent cation activity enhancement. Their results revealed that the mechanism of divalent cation CaN activity enhancement is complex and likely involves several domains of calcineurin [17]. It is worth noting that an iron atom was found in the active site of the crystal structure determined by Kissinger *et al.* in 1995 (reviewed below). The iron is utilized for hydrolytic cleavage of phosphate from CaN substrates [18].

2.3 Kinetic descriptions of the calcium-calmodulin-calcineurin interaction.

A decade after identification, quantitative biophysical properties of CaN were reported. In 1987, Hubbard and Klee provided insight into the CaM-CaN complex lifetime by determining the association ($k_{on} = 8.9 \times 10^3\ M^{-1}\ s^{-1}$) and dissociation ($k_{off} = 8.5 \times 10^{-5}\ s^{-1}$) rates. Moreover, they calculated a dissociation constant for the CaM-CaN interaction ($K_d \leq 0.1$ nM). The dissociation rate (k_{off}) of the CaM-CaN complex was shown to be dependent on free calcium concentration ($[Ca^{2+}]_{free}$). A half maximal k_{off} was achieved at 700 nM $[Ca^{2+}]$ [19]. This suggests that CaN has potential to function as a cardiac modification protein on a beat-to-beat basis, as this $[Ca^{2+}]$ would provide a maximal calcium-sensing ability in between diastolic (0.1 μM) and systolic (1.0 μM) $[Ca^{2+}]$ (K_d defines the inflection point, which has the steepest slope *i.e.* the most sensitive response within the sigmoidal calcium binding curve). In 2005, Quintana *et al.* utilized stopped-flow fluorescence spectroscopy to determine the association and dissociation rates of the full length CaN-CaM interaction in the presence of calcium ($4.6 \times 10^7\ M^{-1}\ s^{-1}$, 1.2 x

10^{-3} s^{-1}, and calculated K_d of 28 pM). Moreover, a dissociation rate for the isolated CaN A subunit-CaM interaction in the presence of calcium was determined (2.6 x 10^{-4} s^{-1}) [20]. In 2009, Kilka *et al.* reported CaN dephosphorylation activity (K_M and k_{cat}) NFAT, DARP-32, Elk-1, Tau, and RII peptide by α, β, and γ calcineurin using ^{33}P-labeled substrates [21]. These results indicate that substrate processing is likely the rate limiting step of the CaN dephosphorylation process, as the rapid calcium-calmodulin kinetics occur on a much more rapid timescale.

2.4 Isoforms of calcineurin.

There are three isoforms of the CaN A subunit (CaN A) (α, β, and γ) [22,23]and two isoforms of the B subunit (CaN BI and CaN BII) [24]. In the heart, the CaN A subunit isoforms (α and β) can interact with CaN BI [24]. The CaN Aγ isoform is predominantly found in the testis and can interact with CaN BII [22,25,26]. It is worth noting that early CaN studies likely characterized the α isoform of CaN A due to its abundance in neuronal tissue, which was the primary source of CaN before recombinant protein expression.

2.5 Autoinhibition of calcineurin activity.

In 1979 Klee *et al.* demonstrated that calcineurin was comprised of two polypeptide chains (CaN A, MW = 61,000 Da and CaN B, MW = 15,000 Da) and these two subunits form a 1:1 heterodimer (Figure 2.1). The A subunit interacts with CaM in a calcium-dependent manner, and the B subunit directly binds Ca^{2+} [9]. In 1983, Manalan and Klee demonstrated that, similar to other CaM-stimulated enzymes, CaN can be activated and rendered Ca^{2+}-CaM independent by limited proteolysis. Moreover, they found CaM can protect CaN from degradation in the presence of Ca^{2+}. Based on their tryptic digest data, Manalan and Klee concluded that CaN contained a long intrinsically disordered region connected to a small-structured domain (Auto

Inhibitory Domain (AID)) that was required for blunting enzyme activity. This was the first report of an autoinhibitory mechanism that described CaN auto-inhibition [27]. Shortly after, Aitken *et al.* posited the CaN B subunit shares structural similarity with the four Ca^{2+} binding regions of CaM and troponin C based on sequence analysis and garners method of secondary structure prediction [28,29].

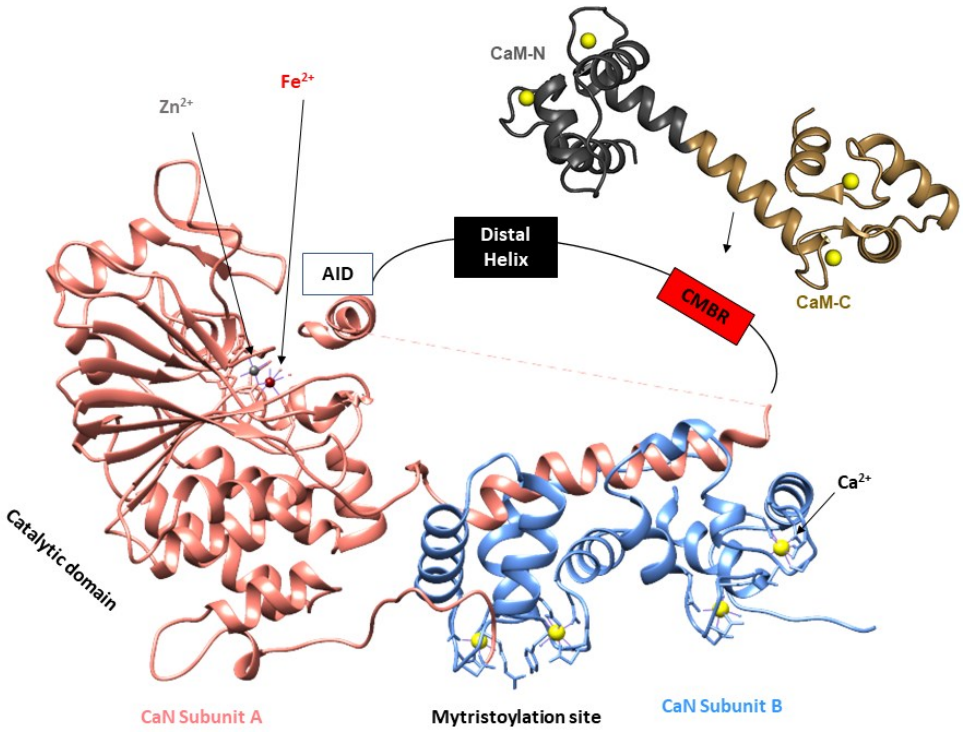


Figure 2.1 Diagram illustrating the key components of the CaN heterodimer (developed from PDB ID: 1AUI [18]). CaN A shown in salmon, CaN B shown in blue, Auto-Inhibitory Domain (AID), Calmodulin-Binding Region (CMBR). Due to the disordered nature of the regulatory domain, this region has been represented by a line and boxes for the distal helix and CMBR. Zn and Fe are located at the active site for dephosphorylation of CaN substrates. The myristoylation site located at the N-terminal region of the CaN B subunit is cleaved so the two CaN subunits can form a heterodimer [30]. Ca^{2+} binding to the CaN B subunit can evoke a small enhancement of enzymatic activity in the absence of CaM. Ca^{2+}–CaM–CaN interaction enriches CMBR α-helical content resulting in translocation of the autoinhibitory domain which leads to enhancement of CaN activity [31].

2.6 Enhancement of calcineurin activity.

CaN activity may be modified by several mechanisms. In 1994, Stemmer and Klee demonstrated that CaN B and CaM activate the phosphatase by different yet complementary mechanisms [31]. In the absence of CaM, Ca^{2+} can stimulate a small but significant level of CaN activity. At less than 10 nM [Ca^{2+}], isolated CaN is inactive; while at approximately 500 nM [Ca^{2+}], 10% of maximal activation is achieved. CaM binding in the presence of Ca^{2+} further stimulates CaN activity an additional 10-fold. Intriguingly, Ca^{2+} binding to CaN B is a prerequisite for CaM enhancement of CaN activity. Stemmer *et al.* also posited that CaN B and CaM play two different roles in calcium stimulation of CaN activity. Ca^{2+} binding to CaM results in the disruption of the CaN AID from the active site and an increase in V_{max}. Ca^{2+} binding to CaN B increases the affinity of CaN for its substrate [31].

In 2006, Ye *et al.* reported a crystal structure displaying a 2:2 stoichiometry between calmodulin and a peptide corresponding to the calmodulin binding domain of calcineurin. Notably, the protein construct used for crystallization was comprised of only 25 amino acids of calcineurin (corresponding to the calmodulin binding site [32]) covalently attached to calmodulin by a 5-glycine linker [33]. In 2008, Ye *et al.* repeated this investigation without the 5-glycine linker and reported a nearly identical 2:2 stoichiometric structure [34]. In 2014, Dunlap *et al.* also reported an x-ray crystallography structure of the calmodulin-calcineurin interaction. The stoichiometry of calmodulin binding to this construct (regulatory domain of the catalytic subunit of calcineurin (residues 373-468)) revealed a 1:1 stoichiometry which was also confirmed by time-resolved fluorescence and size exclusion chromatography [35].

In 2012, Rumi-Masante *et al.* directly demonstrated that the autoinhibitory domain of calcineurin is connected to the main catalytic subunit by an intrinsically disordered region using

hydrogen-deuterium exchange mass spectrometry. Based on calmodulin's interaction with this region, the region has been referred to as the "regulatory domain" (Figure 2). Ca^{2+}-CaM binding to the regulatory domain enriches the alpha helical content resulting in subsequent enzyme activation. This is strikingly similar to the activation mechanism of calmodulin dependent kinase type II [36]. Notably, CaM binding to CaN allosterically enriches alpha helical content of more than 45 amino acids [37]. This highlights CaM's ability to transmit long range signals. In 2013, Dunlap *et al.* confirmed these findings and further demonstrated that the Ca^{2+}-CaM-CaN interactions reduce the distance between the two termini of the regulatory domain [38].

In 2016, Cook and Creamer demonstrated that molecular crowding increases α-helical melting temperature of the calcineurin distal helix by measuring its thermal denaturation using circular dichroism. Correspondingly, they observed that molecular crowding enhanced enzyme activity [39], as increasing the α-helical content of the regulatory domain likely facilitates dislocation of the autoinhibitory domain. In 2017, Yadav *et al.* reported solution state NMR resonance assignments of the regulatory domain of CaN. These chemical shift values were consistent with the CaN regulatory domain containing a high percentage of disordered content. In depth analysis revealed that a small amount of secondary-structure helical propensity may be present in the CaN regulatory domain in the absence of Ca^{2+}-CaM [40]. In 2018, Sun *et al.* proposed a mechanism of CaM enhancement of CaN activity. Specifically, they posited that residues outside of the CaM binding region may contribute to CaN activation based on molecular dynamics simulation data [41]. The complete mechanistic details governing CaN enhancement by Ca^{2+} and CaM are still the subject of active inquiry [41,42].

2.7 External inhibition of calcineurin activity.

Cyclosporin A (CsA) is commonly used to prevent rejection of transplanted organs (kidney, heart, bone marrow, and liver) in humans. Prior to the early 1990s, the molecular mechanism was largely unknown and therefore has been the subject of much investigation. Many scientists also use CsA and Tacrolimus (a.k.a. FK506) to inhibit CaN activity *in vivo*. In 1989, Kay *et al.* demonstrated that lymphocyte activation induced by Ca^{2+} ionophores alone are completely suppressed by 0.1 nM FK506 [43]. In 1989, Tropschug *et al.* observed that cyclophilin mediates cyclosporin A effect and posited that binding of cyclosporin A to cyclophilin lead to the formation of a complex that interacts with an "unidentified cellular component" (later shown to be CaN) [44]. Shortly after, Randak *et al.* found that CsA treated cell lack an interaction between "lymphocyte-specific factors" (later shown to be CaN and NFAT, see section 2.10) and the interleukin-2 enhancer [45].

In 1991, Lin *et al.* demonstrated that FK506 exerts its inhibitory effect during early events of T-cell activation in a manner indistinguishable from CsA. Moreover, the pathways inhibited by FK506 and CsA required measurable rise in intracellular calcium concentration [46]. Concurrently, Friedman *et al.* and Liu *et al.* demonstrated that CsA-cyclophilin and FK506-FKBP12 complexes directly bind to and inhibit calcineurin *in vitro* [47,48]. Fruman *et al.* built upon these findings and demonstrated that CsA and FK506 lead to abolished CaN activity within Thymus (T)-cells [49]. These studies were the first to report a common mechanism of CaN inhibition. It is important to note that the cyclophilin and FKBP12 proteins have other roles in cellular physiology [50] and these require further investigation.

2.7.1 Other mechanisms of calcineurin-NFAT inhibition.

CaN activity may be impaired by several mechanisms, such as modification of the active site metal properties or the substrate binding interface. In 2001, Namgaladze *et al.* demonstrated that superoxide can inhibit CaN activity by reducing Fe^{3+} at the active site. Moreover, they found that nitric oxide can block superoxide's ability to inhibit CaN activity [51]. In 2004 Roehrl *et al.* identified several small organic molecules that inhibit CaN's ability to activate the Nuclear Factor of Activated T-cells (NFAT) by directly acting on the CaN-NFAT interaction [52]. This was unique as the commonly used CsA-cyclophilin and FK506-FKBP12 complexes achieve CaN inhibition by blocking the calcineurin active site [47,48]. Roehrl's innovative strategy achieved inhibition of the CaN-NFAT pathway without inhibiting other known cellular functions of CaN [52].

2.7.2 A structural basis of calcineurin inhibition and activity.

Shortly after the molecular mechanism of CsA and FK506 was identified, the structural underpinnings of inhibition were described. In 1995, Griffith *et al.* determined a high-resolution x-ray crystal structure (2.5 Å) of neuronal bovine CaN bound to FKBP12-FK506 complex. To obtain a well diffracting crystal, samples were subjected to proteolysis for 3-4 days in order to remove disordered regions of the protein complex. The resulting structure captured the well-structured regions of the CaN A and B subunits interacting with the FKBP12-FK506 complex. This revealed the structural details underlying FK506-calcineurin inhibition. In this structure, the FKBP12-FK506 complex does not contact the phosphatase active site on CaN A. Instead, it was posited that the FK506-FKBP12 complex inhibits dephosphorylation by sterically occluding substrate approach to the active site [53].

In 1995, Kissinger *et al.* determined multiple high-resolution x-ray crystal structures; one of full-length human CaN (2.1 Å) and a second of full-length human CaN bound to FKBP12-FK506 (3.5 Å). SDS-PAGE electrophoresis indicated that the protein crystals contained intact CaN without proteolytic degradation. In the absence of the FKBP12-FK506 complex, the CaN autoinhibitory domain sterically occluded the Zn/Fe active site. The well-resolved structure suggested a mechanism for CaN substrate dephosphorylation. Specifically, dephosphorylation could occur by a catalytic nucleophilic attack on the substrate phosphate by a metal activated water molecule (Figure 2.2) [18].

In 2005, Kang *et al.* demonstrated that several small molecules can allosterically achieve inhibition of calcineurin-Nuclear Factor of Activated T-cells (NFAT) association through interactions outside of the consensus NFAT binding site *(see calcineurin substrate binding section below)* [54]. In 2014, Qian *et al.* produced a novel high affinity (K_d = 2.6 nM) inhibitor of the calcineurin NFAT interaction and showed that this peptide prevented NFAT translocation to the nucleus using confocal microscopy [55].

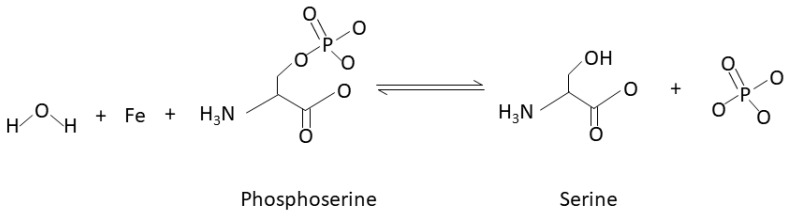


Figure 2.2 Chemical equation of dephosphorylation of a serine residue by an iron atom located in the CaN-active site adapted from Kissinger *et al.* (1995). A water molecule is deprotonated by the iron atom, followed by a hydrolysis reaction between the deprotonated (activated) water molecule and phosphorylated serine residue (phosphoserine). This reaction yields serine and phosphate. Threonine substrates follow a similar mechanism of water activation and dephosphorylation [18].

2.8 Endogenous regulators of calcineurin activity.

In 2009 Mehta *et al.* demonstrated that the human protein RCAN (Regulator of Calcineurin) can either stimulate or inhibit CaN activity. There are three types of RCAN proteins (RCAN1, RCAN2, and RCAN3) [56]. RCAN stimulation of CaN required Glycogen Synthase Kinase 3 (GSK-3) and the E3 ubiquitin ligase SCF^{Cdc4}. RCAN inhibition of CaN requires a conserved calcineurin docking site (PxIxIT-like amino acid motif, described below). Moreover, Glycogen Synthase Kinase 3 (GSK-3) and the E3 ubiquitin ligase SCF^{Cdc4} were required for calcineurin stimulation by RCAN [56]. In 2009, Mulero *et al.* developed an *in vitro* high-throughput fluorescence polarization assay that utilizes an RCAN1 peptide for identifying molecules that have immunosuppressant potential [57]. In 2015, Kim *et al.* demonstrated that protein kinase A indirectly inhibits CaN activity through RCAN1 using an NFAT luciferase assay [58]. Recently, Li *et al.* utilized a combination of structural, biophysical, and biochemical techniques to describe RCAN1 inhibition of CaN activity. Specifically they found that RCAN1-CaN inhibition occurs by multiple mechanisms: block of substrate recruitment sites and block of the CaN active site [59].

In 1998, two independent laboratories identified an endogenous inhibitor of CaN. They named the inhibitory protein calcineurin inhibitor (cain) and calcineurin binding protein 1 (Cabin 1). Sun *et al.* demonstrated that the CaN-cain/Cabin 1 interaction is dependent on protein kinase C (PKC) and $[Ca^{2+}]$. Specifically, they demonstrate that PKC and Ca^{2+} lead to hyperphosphorylation of cain/Cabin 1. Furthermore, they observe that in the presence of a Ca^{2+} ionophore (ionomycin) and a PKC activator (PMA) CaN-dependent luciferase activity is almost completely abolished [60]. Concurrently, in 1998 Lai *et al.* demonstrated that cain/Cabin 1 noncompetitively inhibits CaN [61].

2.9 Calcineurin substrate binding.

In 1982, Aitken *et al.* demonstrated that the N-terminus of the CaN B subunit contains a myristoyl group [30]. It is now known that myristoylation is widely utilized by many proteins for membrane targeting [62]. It remains to be determined if and how myristoylation influences or alters the localization and/or substrate specificity of CaN. Localization of CaN to specific cellular compartments could influence substrate selection. In 1994, Donella-Deana *et al.* demonstrated (using small 6-32 amino acid peptides) that the sequence surrounding the substrate's phosphate does not always provide a clear signature for CaN activity [63].

2.9.1 Calcineurin binding region 1 (CNBR1) and the PxIxIT motif.

Over the past several decades, there has been a growing interest in CaN substrate recognition. Thus far, two binding motifs have been identified. In 2004, Li *et al.* demonstrated that several CaN targets (such as NFAT) utilize a common amino acid sequence for interaction, the PxIxIT motif (also termed CaN Binding Region 1 (CNBR1) in 1998 [64]) [65]. In 2007, Roy *et al.* found that CaN binds with varying weak affinities to small peptides containing variations of the PxIxIT motif. CaN, like other signaling enzymes, does not typically form stable complexes with substrates. Intriguingly, weak CaN-substrate affinity conflicts with achieving selective target protein post-translational modification [66].

In 2007, Li *et al.* provided a structural characterization that yielded insight into the molecular requirements of CaN-PxIxIT motif interactions. An x-ray crystal structure of CaN bound to a 14 amino acid peptide containing the PVIVIT sequence provided an atomic resolution description of a CaN-substrate interaction and revealed the important side chains used for binding. Alterations to the substrate amino acids significantly impaired CaN binding *in vitro* which confirmed the observed crystal contacts. Additionally, this investigation found that the

PVIVIT sequence generated the strongest of the series of PxIxIT interaction affinities. Lastly, they reported that substrate affinity influenced the response of CaN to $[Ca^{2+}]$[67]. This is reminiscent of Ca^{2+}-calmodulin binding affinity being tuned by substrate interaction [68].

2.9.2 Calcineurin binding region 2 (CNBR2) and the LxVP motif.

In addition to the PxIxIT motif, CaN utilizes a second binding motif that differs in key binding properties from the PxIxIT motif. In 1999, Liu *et al.* identified a second binding region for CaN-NFAT interaction (CaN Binding Region 2 (CNBR2)) using a GST-pulldown assay with western blot analysis [69]. Concurrently, Park *et al.* also reported a second binding site for calcineurin in NFAT using a Secreted Alkaline Phosphatase (SEAP) assay [70]. Soon after, Liu *et al.* demonstrated that a shorter 16 residue peptide (Pep3 derived from CNBR2) was responsible for the CNBR2 interaction [71]. In 2006, Martı´nez-Martı´nez *et al.* provided a detailed comparative analysis of CaN binding activity between PxIxIT and CNBR2 (now known as the LxVP docking site). Consistent with data from Liu *et al.* (1999), Park *et al.* (2000), and Liu *et al.* (2001), Martı´nez-Martı´nez *et al.* demonstrated that the NFATc1 LxVP motif binds CaN more efficiently than PxIxIT motifs [72]. It is important to note that these investigations utilized NFAT binding sites. Further investigation is required to delineate if other CaN substrates have PxIxIT and/or LxVP binding preference.

2.10 An overview of calcineurin function in cardiac physiology.

The most prominent and well-recognized CaN signaling pathway is attributed to the activation of nuclear factor of activated T cells (NFAT). While not the focus of this review, a brief description of the CaN-NFAT interaction has been provided to understand many of the investigations that are focused on the roles of CaN in cellular functions. Dephosphorylation of

cytosolic NFAT ($NFAT_c$) facilitates translocation of the CaN-$NFAT_c$ complex to the nucleus, which promotes synthesis of nuclear NFAT ($NFAT_N$) protein [73]. An interaction between $NFAT_C$ and $NFAT_N$ proteins lead to production of interleukin-2 (IL-2) (cytokine that is used for an immune response). For a focused review of CaN's role in NFAT signaling, see Hogan 2017 [74].

2.10.1 Calcineurin is essential for cardiac development, cardiomyocyte stress response, and cardiac physiology.

CaN has a prominent role in cardiac development. In 2010, Maillet *et al.* demonstrated that cardiac-specific deletion of CaN was lethal for mice one day after birth. Specifically, they found defects in right ventricular development, reduced ventricular trabeculation septal defects, and valvular overgrowth [75]. CaN also appears to have a role in adult cardiac physiology. CaN deletion (alpha myosin heavy chain Cre-expression dependence) reduced cardiac contractility, increased incidence of arrhythmia, and reduced cardiac myocyte content [75]. Moreover, in 2006, Bukowska *et al.* demonstrated that CaN A (β isoform) was significantly upregulated in human atrial tissue from patients with sinus rhythm and chronic persistent atrial fibrillation [76].

CaN has also been shown to have a variety of other roles in cardiomyocytes as well as other cell types. Moreover, several lines of evidence suggest that hypertrophy impairs physiological CaN-ion channel regulation (discussed below). For a comprehensive overview of direct interactions between CaN and cardiac proteins, see Table 2.3. Interestingly, CaN is also an effector for several proapoptotic kinases such as Apoptosis Signaling Kinase 1 (ASK1) and c-Jun N-terminal protein kinase (JNK1/2) [77–79]. These kinases are important for physiological viability of cells. ASK1 is an upstream promotor of apoptosis that activates in response to proapoptotic stimuli. Upon dephosphorylation of ASK1 by CaN, ASK1 disassociates with 14-3-3 protein [80]. Downstream to this dephosphorylation event, a variety of other kinases are activated such as

JNK1/2 [81]. At the end of this signaling cascade, apoptosis is induced. For a review of ASK1 signaling with insights on prolonged JNK activation, see Ogier *et al.* (2020) [82].

2.10.2 Calcineurin, cardiac ion channels, and ion pumps

Given data presented by Bukowska *et al.* [76] and Maillet *et al.* [75], I have surveyed the literature to understand the potential of CaN regulating or modifying cardiac ion channels and pumps. While many of the investigations have explored CaN-ion channel modification relationships for systems outside of a cardiomyocyte, there are several lines of evidence that suggest CaN-ion channel regulation contributes to cardiac physiology.

In 2011, Prasad and Inesi demonstrated that CaN A can alter production of SERCA 2 leading to changes in cytosolic [Ca^{2+}]. Neonatal rat ventricular cardiomyocytes with silenced genes for either the α or β isoform of CaN A displayed reduced SERCA 2 expression. Application of thapsigargin (SERCA 2 inhibitor) rescued SERCA 2 expression (mRNA and protein) and restored Ca^{2+} transport. Importantly, these effects were reproduced using ionomycin in place of thapsigargin. This is consistent with a mechanism where an increase in diastolic Ca^{2+} activates CaN and restores SERCA 2 production. Additionally, there is evidence that CaN influences production of other important cardiomyocyte proteins. Cardiomyocytes exposed to CsA displayed lower mRNA content for phospholamban and Na^{+}/Ca^{2+} exchanger [83].

CaN has been shown to modify the function of the cardiac L-type calcium channel in excitable cells. This channel has a pivotal role in establishing the cytosolic [Ca^{2+}] through the calcium-induced-calcium-release mechanism. Changes in cytosolic [Ca^{2+}] activate or inactivate many calcium-sensitive proteins and enzymes within a cell. In 1997, Schuhmann *et al.* observed that CaN alters the function of the L-type calcium channel in human umbilical vein smooth muscle cells [84]. Others have shown that CaN activity and ion channel function are correlated in

diseased cardiomyocytes. In 2000, Saito *et al.* demonstrated that CaN and the L-type calcium channel play a critical role in isoproterenol-induced apoptosis of rat cardiomyocytes [85]. Further investigation of the physiological CaN-L-type calcium channel relationship is required as the molecular and cellular functions are likely different in a diseased or non-stressed state [86]. Much of the work surrounding CaN-L-type calcium channel modification relies on the CaN inhibitor CsA, and conflicting results are obtained from several laboratories. For further descriptions of these disparate results, the reader is referred to Wang *et al.*, 2014 [86].

The heart the Na^+/K^+ ATPase may also be modified by CaN. In renal tubule cells, Aperia *et al.* demonstrated that stimulation of α-adrenergic receptors increased the activity of the Na^+/K^+ ATPase. Addition of FK506, a CaN inhibitor, was shown to blunt the effects of α-adrenergic receptor stimulation to the Na^+/K^+ ATPase [87]. The α1and β1 isoforms contained in proximal convoluted tubules appear to be present in cardiomyocytes [88]. This raises the possibility that CaN may have a role in stimulating Na^+/K^+ ATPase activity in a cardiomyocyte.

An interaction between CaN and the ryanodine receptor (RyR2) has also been posited for modulating RyR2 Ca^{2+} release. In 2000, Bandyopadhyay *et al.* demonstrated CaN coimmunoprecipitates with RyR2 in a calcium-dependent manner [89]. Intriguingly, the RyR2 amino acid sequence contains 5 LxVP motifs which suggests CaN may contribute to RyR2 regulation. Moreover, CaN inhibitors (CsA and FK506) can modify RyR2 function, as Bandyopadhyay *et al.* report they can reduce the frequency of spark calcium release [89]. This data is further supported by Savoia *et al.* as they demonstrated in 2014 that application of a CaN autoinhibitory peptide reduces RyR2 calcium release frequency in airway smooth muscle cells [90].

2.10.3 Calcineurin can impart different effects to ion channel function.

It has been demonstrated that CaN is involved in the restoration of the resting membrane potential by dephosphorylating the Na^+/K^+ ATPase, resulting in Na^+ retention [87,91]. Conversely, dephosphorylation of L-type calcium channel by CaN reduces ion channel conduction. The mechanistic details of this process are largely unknown [84,85]. In the case of the RyR2, it has been suggested that CaN anchors to the FKBP12 accessory protein associated to the receptor and then inactivates RyR2 by dephosphorylation. To the best of our knowledge, there is one report of this mechanism, and further investigation is required [89].

2.11 Calcineurin's roles and contribution to cardiac disease.

An extensive number of investigations have demonstrated that improper CaN activity can induce and/or contribute to heart disease. Inhibition or reduction of CaN activity is becoming an attractive approach for intervention [92,93]. Table 2.2 provides a comprehensive summary of CaN's roles in cardiac pathophysiology. In addition to seminal findings, below some of the less appreciated mechanisms of CaN signaling are highlight. Lastly, CaN activity with consideration for hormone signaling and ion channel dysfunction is discuss.

2.11.1 Inhibition of calcineurin reduces cardiac hypertrophy.

One of the most prominent pathological outcomes related to CaN is cardiac hypertrophy (thickening of the heart muscle). In 1998, Molkentin *et al.* demonstrated that addition of phenylephrine or angiotensin II with constitutively active CaN or NFAT increased brain natriuretic peptide (BNP) promoter activity (located in the heart). This increased BNP activity was shown to be dependent on the presence of CaN, NFAT, and GATA4 (a transcription factor involved in proper cardiac muscle development). This led to heart dilation and hypertrophy.

Inhibition of CaN (CsA or FK506) prevented these effects [94]. The role of CaN in cardiac hypertrophy has also been investigated using other CaN inhibition strategies. In 2001, De Windt *et al.* demonstrated that genetic inhibition of CaN by cain/cabin1 or the AKAP79 inhibitory domain reduces cardiac hypertrophy in mouse models [95]. In addition to preventing hypertrophy, reducing CaN activity may also be advantageous for treating hypertrophic disease. In 2000, Taigen *et al.* demonstrated that CaN activity is increased upon the addition of hypertrophic stimuli (angiotensin II and phenylephrine) and application of CsA or FK506 rescued cardiomyocytes from hypertrophy [96]. This was unique as previous investigations from Molkentin *et al.* had explored the effects of overactive CaN without consideration for variable CaN activity [94].

While NFAT is the predominant mechanism identified for generating hypertrophy, there are many proteins that can trigger this process. Moreover, CaN modification can have different functional consequences in physiological and diseased states. In 2002, Sah *et al.* demonstrated that overexpression of the Kv4.2 N-terminus increased CaN activity and heart to body weight ratio (changes in heart to body weight ratio are often attributed to cardiac dilation and/or hypertrophy). Addition of Verapamil (L-type calcium channel inhibitor that reduces cytosolic Ca^{2+} concentration [97]) reduced the heart to body weight ratio. CaN's contribution to the phenotype was verified by application of CsA which removed the effects. Neither CsA nor verapamil altered heart to body weight ratio in wild-type mice, suggesting that physiological heart to body weight ratio is not reliant on CaN activity [98].

2.11.2 Constitutively active CaN or NFAT can adversely affect ion channels and cardiac function.

In 2001, Yatani *et al.* demonstrated that models of constitutively active CaN exhibit enhanced L-Type calcium channel conduction. This led to cardiac hypertrophy and heart failure [99]. The mechanisms for this process remain to be elucidated, as CaN inhibitors such as CsA have yielded disparate results [86]. Under disease conditions, there is also evidence that CaN can modify potassium channels. In 2006, Gong *et al.* demonstrated that excessive CaN activity (constitutively active CaN) increases $K_V4.2$ channel density through interactions with the $K_V4.2$ gene. Addition of cain/Cabin 1 removed this effect [100]. Interestingly, contrary effects have been observed in other investigations, where hypertrophy impaired CaN-$K_V4.2$ regulation. Specifically, Zobel *et al.* observed that stimulation of CaN (phenylephrine) resulted in a decreased $K_V4.2$ current density [101]. This was subsequently supported by Panama *et al.* who demonstrated that CaN inhibition (via cain) can reduce mRNA of KChIP2 and Kv4.2 and the combination of phenylephrine/propranolol exacerbated this effect [102]. Further investigation is required to understand how excessive CaN activity imparts different effects to $K_V4.2$ channel density.

CaN also modifies the production of other cardiac ion channel proteins. In 2006, Kuwahara *et al.* demonstrated that activation of CaN in a pressure-overloaded system can upregulate transient receptor potential proteins (TRPC6) in mouse and failing human hearts [103]. It has also been reported that CaN can form a complex with IP_3R or RyR receptors through interactions with FKBP12. Moreover, inhibition of CaN with CsA alters the Ca^{2+} flux produced by ligand (IP_3) activation of the ion channel [104].

In 2002, Petrashevskaya *et al.* demonstrated that constitutively active CaN or NFAT can create several adverse physiological effects. Critical modifications such as increased action

potential duration, decreased heart rate, and decreased β- adrenergic stimulation have been observed [105]. Moreover, De Windt *et al.* demonstrated that genetic inhibition of CaN (overexpression of cain/Cabin 1 or the AKAP79 inhibitory domain) reduced isoproterenol induced hypertrophy [95]. Lastly, excessive inhibition of CaN during transaortic constriction leads to modification of the function of several key proteins involved in cardiac calcium cycling (phospholamban, RyR2, SERCA2a, and NCX) and promotes an increase in left ventricular end diastolic pressure and myocardial stiffness [106]. Given the abundance of ion channel targets that are modified by CaMKII, under disease conditions (hypertrophic and constitutively active CaMKII), it has been demonstrated that CaMKII can phosphorylate CaN (S197) leading to reduced CaN activity [107]. Further work is required to understand the relationships between CaMKII and CaN function under physiological conditions.

2.11.3 CaN may also protect cardiomyocytes from DNA-damage-induced apoptosis.

CaN activity stimulated by phenylephrine protected cardiomyocytes from staurosporine and 2-deoxyglucose induced cell death [108]. This appears to conflict with other reports that have implicated CaN in facilitating apoptosis [77–79]. These investigations interrogate CaN using different adrenergic pathways (phenylephrine = α-adrenergic stimulation, isoproterenol = β-adrenergic stimulation). It is possible that similar to health and diseased states, the different adrenergic pathways may utilize CaN activity uniquely. For a depiction of the α-adrenergic stimulation pathway that results in pathophysiological hypertrophy, the reader is referred to Cotecchia *et al.*, 2015 (Figure 1) [109].

2.11.4 Hormonal connections to calcineurin.

Hormone signaling is an important modifier of cardiac physiology. Of the several hormones involved in regulating cellular homeostasis [110], estrogen has been demonstrated to modify CaN signaling in pathological T cells, neuronal cells, and heart cells. Mechanisms include estrogen receptor stimulation, alteration of CaN mRNA levels, and alterations to CaN compartmentalization or localization. For a more in-depth review of hormonal regulation of the heart, see Gordan *et al.*, 2015 [111].

With recent reports identifying estrogen modification of CaN in cardiomyocytes [112,113], estrogen-CaN findings in T-cells are summarized as this may shed light on estrogen-CaN cardiomyocyte signaling. In 1998, Rider *et al.* revealed that estrogen can alter the levels of CaN mRNA in human female lupus T cells. The effects were found to be dose- and time-dependent [114]. In 2000, Rider *et al.* also demonstrated that the estrogen receptor can impart gender-specific effects. Blocking female estrogen receptors (ICI 182,780) inhibited an increase in CaN mRNA and CaN activity in female patients. Moreover, this effect was not observed in male patient T cells [115], indicating that CaN has gender-specific physiological and diseased contributions. In 2008, Don Yi and Simpkins observed that in primary cortical neurons, glutamate (glutamate-induced apoptosis) decreases CaN and PP2A activity. Moreover, 17β-estradiol dampened glutamate's ability to alter CaN activity [116].

In 2008, Pedram *et al.* used a cardiac fibrosis model (angiotensin II-induced) to demonstrate that 17β-estradiol indirectly inhibits CaN and prevents angiotensin II-induced fibrosis. Specifically, knockout of the estrogen receptor (β isoform) impaired 17β-estradiol's ability to reduce angiotensin II enhancement of CaN activity. This data confirmed that the estrogen receptor β isoform is required for estrogen reduction of CaN activity as well as

prevention or rescue of cardiac hypertrophy and fibrosis. Moreover, RCAN1 protein expression was found to be stimulated by 17β-estradiol [117], hinting at a possible mechanism for estrogen-mediated CaN inhibition in a cardiomyocyte. In 2009, Donaldson *et al.* demonstrated that estrogen hinders the rise in CaN A subunit expression that is induced by transaortic constriction or phenylephrine. Based on this data, they posit that 17β-estradiol and estrogen receptors reduce pressure overload cardiac hypertrophy by increasing CaN A degradation [118]. Further work is required to delineate the molecular and cellular mechanisms that underly hormonal CaN regulation in cardiomyocytes.

2.12 There is still much work to be done.

While cellular and physiological descriptions of CaN function have been well described, there are several knowledge gaps that limit our understanding of CaN's role in cardiac physiology and disease (Table 2.3). It will be of significant interest to characterize biophysical details of CaN complexes. Quantitative descriptions such as kinetic on and off rates, binding energies, and thermal stability of the CaN-substrate interactions will provide further insight into CaN-mediated signaling pathways. Elucidation of these properties will illuminate potential therapeutic targets and novel strategies for small molecule modification and intervention

Table 2.1 Roles of calcineurin in cardiac physiology

Ref	Cellular Function	System	Results	Conclusion	Conditional Effect	Mouse Line
73	Adaptive response	Jurkat cells	NFATc translocates → nucleus and + newly synthesized $NFAT_N$; CsA and FK506 block translocation of NFATc without altering synthesis of $NFAT_N$	CsA and FK506 block NFAT translocation	No stimulants required	N/A
87	Adaptive response	Jurkat cell	synergistic activation of α1-α2 adrenergic receptors → ↑ Na^+/K^+ ATPase activity CaN inhibitors abolish this ATPase enhancement	α-adrenergic stimulation → CaN dephosphorylates Na^+/K^+ ATPase → ↑Na^+/K^+ ATPase activity	No stimulants required	N/A
119	Apoptosis	BHK-21	CaN transfected cells + 4hr serum deprivation + calcium ionophore = 60% apoptosis	CaN plays an important role in mediating upstream events in calcium activated cell death	+Ca^{2+} and - growth factors	N/A
120	Adaptive response	Jurkat cell	Indirect evidence suggests CaN + PMA = inactive IκB via unknown kinase	CaN activation → inactivated IκB = activate NF-κB	No stimulants required	N/A
77	Adaptive response	B lymphocytes	>5min @ 200-400 nM $[Ca2+]_i$ = activation NFATc alone >2min @ 1 µM $[Ca2+]_i$ = IκB degradation or JNK1 + PO_4	Amplitude and duration of $[Ca2+]_i$ = activation of NFAT, NF-κB, or c-Jun N-terminal kinase	No stimulants required	N/A
121	Adaptive response	Jurkat cells and dominant negative NFAT Tg mouse model	dominant negative NFAT = ↓expression of IL-2 protein Overexpression of CaN = restored IL-2 expression	NFAT = critical component of signaling pathway that regulates IL-2 expression	No stimulants required	B10.BR /SGSNJ
89	Adaptive response	Rat ventricular CM	+Ca^{2+} = RyR2-CaN coimmunoprecipitation +20 mM EGTA = no interaction CaN inhibitors inhibit RyR2 Ca^{2+} release in CM	CaN-RyR2 interaction may modulate calcium release in rat hearts	No stimulants required	Sprague - Dawley

Table 2.1 Roles of calcineurin in cardiac physiology (Continued)

Ref	Cellular Function	System	Results	Conclusion	Conditional Effect	Mouse Line
122	Differentiation	skeletal muscle cells (C2C12 and Sol8)	+CaN or +NFATc3 → ↑MyoD → myogenesis CaN inhibitor (CAIN) blocks differentiation	CaN signaling contributes to initial myogenic myocyte differentiation through NFATc3 dependent mechanism	No stimulants required	N/A
85	Apoptosis	cultured CM from 1-day Wistar rats and dominant negative CaN Tg mice	Isoproterenol induces apoptosis Cav1.2 blocker (1 μM nifedipine) inhibit apoptosis CsA and FK506 inhibit apoptosis	CaN and LTCC play a critical role in iso-induced apoptosis of rat CM	Isoproterenol	Wistar
78	Adaptive response	Neonatal rat CM primary cultures 10T12/2 fibroblasts and COS-7 cells	constitutively active CaN = ↑MAKP1 expression and p38-MAPK inactivation in CM	CaN indirectly inactivates p38 MAPK CaN + MEK1, MKK6, or MKK7 = ↑MKP-1 protein levels	Phenylephrine	Harlan Sprague - Dawley
123	Differentiation	chonrogenic cells (RCJ3.1C5.18)	Ionomycin induces limb buds in mouse embryos CsA → ↓cartilage development (both +/- ionomycin) + ionomycin → dephosphorylated NFAT4 = activated NFAT4	CaN/NFAT4 activates bone morphogenetic protein expression → chondrogenesis (cartilage formation)	Ionomycin	N/A
79	Apoptosis	neonatal rat CM with Adenovirus modification	CaN dephosphorylates ASK1 = dissociation from 14-3-3 protein → activates ASK1 CaN and ASK1 cooperatively regulate CM apoptosis	CaN and ASK1 contribute to a feedback regulatory mechanism involved with signaling CM apoptosis	No stimulants required	Sprague - Dawley
124	Apoptosis	CaNβ double knockout mouse model	Removal of CaNβ → ↑spontaneous T-cell apoptosis IL-7 and/or IL-15 treatment ↓spontaneous T-cell apoptosis Constitutively active CaN → ↑Bcl-2 reporter activity	CaN → ↑B-cell lymphoma expression ↑[CaNβ] → restored Bcl-2 protein expression = ↑T-cell survival	No stimulants required	C57BL/ 6

Table 2.1 Roles of calcineurin in cardiac physiology (Continued)

Ref	Cellular Function	System	Results	Conclusion	Conditional Effect	Mouse Line
[125]	Growth	neonatal rat ventricular myocytes PS120	Na^+/H^+ Exchanger 1 (NHE1) interacts with CaN NHE1 → activated CaN → NFAT translocates nucleus	NHE1 activity → ↑local pH → ↑CaN activity → NFAT signaling → cardiomyocyte enlargement (hypertrophy)	Overexpression of NHE1	Sprague - Dawley
[126]	Adaptive response	Bovine pulmonary atrial endothelial cells Human pulmonary atrial endothelial cells HEK-tsA201	CaN dephosphorylates MYPT1 MYPT1 interacts with CaN (pulldown) $K_a = 1.06*10^7$ CsA + thrombin → sustains MYPT1 and myosin phosphorylation → ↓endothelial electrical resistance	CaN dephosphorylation of cofilin and Myosin Phosphatase → improve endothelial barrier function (i.e. increased electrical resistance)	Thrombin	

Descriptions of the results and conclusions of relevant literature investigating the roles of calcineurin in cardiac physiology.

Table 2.2 Roles of calcineurin in cardiac disease physiology

Ref	System	Animal Model	Conditional requirement	Results	Conclusion
94	Rat	Tg expression of constitutively active CaN Tg expression of constitutively active NFAT3 (nuclear)	Angiotensin II or Phenylephrine	NFAT3 interacts with GATA4 (transcription factor) CaN + GATA4 + NFAT3 activate (~150x) brain natriuretic peptide promoter CsA and FK506 → ↓induced hypertrophy (Angiotensin II and Phenylephrine) constitutively active CaN or NFAT → heart dilation and hypertrophy	Constitutively active CaN → upregulated NFAT3 → cardiac dilation and hypertrophy
96	Rat	Neonatal Wistar-Kyoto	Angiotensin II, Phenylephrine, or 1% Fetal Bovine Serum	CaN (β-isoform) activity increased by hypertrophic stiumli ↑expression of Cain or AKAP79 inhibitory domain → ↓stimulated enlargement of CM surface area CsA and FK506 → ↓stimulated enlargement of CM surface area	↑CaN activity → ↑CM surface area ↓CaN activity can prevent ↑CM surface area
127	Mouse	Sprague-Dawley mouse model of Load-induce hypertrophy	Abdominal aortic banding (AAB)	↑CaN activity → ↑coimmunoprecipitation of CaM-CaN complex CsA prevented AAB-induced ↑heart to body weight ratios	In pressure overload hypertrophy: CaN-CaM interaction is enhanced CsA can ↓induction of hypertrophy and/or partially reverse hypertrophy
108	Rat and mouse	Sprague-Dawley rat neonate mouse model of ischemia/reperfusion mouse model of constitutively active CaN	DNA damage inducing agent (Saturosporine or 2-deoxyglucose)	constitutively active CaN = ↑ CM surface area + protection from induced DNA damage CaN (α-isoform) reduces DNA damage by multiple mechanisms Both NFAT3 and protein kinase B can contribute to CaN driven protection from DNA damage	CaN can protect CM from apoptosis in vitro and in vivo CaN inhibition is not sufficient to induce apoptosis in vivo
95	Mouse	Tg expression of Cain/Cabin-1 Tg expression of AKAP79 inhibitory domain	Isoproterenol	genetic inhibition of CaN activity in the heart reduces load-induced hypertrophy	CaN is an important contributor to pressure-overload hypertrophy

Table 2.2 Roles of calcineurin in cardiac disease physiology (Continued)

Ref	System	Animal Model	Conditional requirement	Results	Conclusion
99	Mouse	Tg expression of constitutively active CaN Tg expression of CAIN Tg expression of AKAP79 inhibitory domain	No stimulant required	↑CaN activity → cardiac hypertrophy → quickly progression to heart failure ↑CaN activity → altered LTCC conduction altered LTCC due to changes Ca^{2+} signaling NOT changes in LTCC gating	CaN induced hypertrophy is associated with ↑LTCC activity
128	Mouse	C57BL/6 Tg removal of NFATc3 Tg removal of NFATc4	Abdominal aortic banding or Angiotensin II	NFATc3 deletion → ↓pathological CaN induced hypertrophy NFATc4 deletion does not reduce pathological CaN induced hypertrophy	mice lacking NFATc3 are partially protected from cardiac hypertrophy in response to CaN activation
98	Mouse	Tg overexpression Kv4.2 N-terminus	Kv4.2 N-terminus overexpression	Reduced I_{to} → ↑CaN activity, ↑AP duration, and ↓SERCA2a expression Overexpression of Kv4.2N-terminus leads to ↑heart to body weight ratio CsA or verapamil removed this effect and restored SERCA2a expression	↓I_{to} → ↑Ca^{2+} cycling and hypercontractility
105	Mouse ventricle	Tg expression of constitutively active CaN Tg expression of constitutively active NFAT3 (nuclear)	Overexpression of chronically active CaN and Isoproterenol	↑CaN activity or ↑NFAT3 = ↑I_{Ca} current density, ↑I_{Ca} inactivation kinetics, ↑heart contractility, ↓$K_v2.1$ expression, ↑AP duration, ↓heart rate, and ↓β-adrenergic stimulation	CaN overexpression causes hyperdynamic cardiac remodeling
129	Rat ventricle	Sprague-Dawley	Phenylephrine or Verapamil and PKG over-expression	↑Phenylephrine → ↑LTCC activity → ↑NFATc3 translocation → ↑ transcriptional activity ↑Nitric Oxide → activation of PKG I → ↓phenylephrine effect Nitric Oxide/cGMP diminish CaN induced CM size increase	Nitric Oxide/cGMP activation of PKG I inhibits hypertrophic CaN-NFAT pathway in CM

Table 2.2 Roles of calcineurin in cardiac disease physiology (Continued)

Ref	System	Animal Model	Conditional requirement	Results	Conclusion
[130]	Mouse	Tg expression of dominant negative JNK1/2 (C57BL/6 background, FVB/N strain)	TAC	↓JNK1/2 activity = ↑heart to body weight ratio, ↑induced hypertrophy, and ↑CaN-NFAT signaling	dominant negative JNK → ↑CaN-NFAT signaling → cardiac hypertrophy
[131]	Mouse	Tg expression of dominant negative p38α Tg expression of dominant negative MKK3 Tg expression of dominant negative MKK6	Aortic banding, angiotensin II, isoproterenol infusion, or phenylephrine infusion	dnp38α, or dnMKK3, or dnMKK6 = ↑ heart to body weight ratio, ↑heart dilation, ↑cross sectional area Angiotensin II, phenylephrine, or isoproterenol effects are enhanced in dominant negative animal models with aortic banding ↓p38α activity + CaN A transient transfection = ↑ NFAT translocation to nucleus	dnp38α, or dnMKK3, or dnMKK6 → ↑CaN-NFAT translocation and transcription
[132]	Mouse	Tg knockout of guanylyl cyclase A (C57BL/6 and 129SVj)	No stimulants required	K.O. Guanylyl Cyclase A = ↑CaN activity, ↑NFATc3 translocation, ↑GATA4 DNA binding, ↑ANP and BNP mRNA, ↑collagen I and III, and ↑Fibronectin K.O. Guanylyl Cyclase A + FK506 → ↓observed modifications	Disruption of guanylyl cyclase A activates cardiac CaN-NFAT pathway FK506 → ↓fibrosis and hypertrophy
[100]	Rat	Overexpression of constitutively active CaN (Sprague-Dawley)	Overexpression of CaN	↑CaN activity = ↑cell capacitance, ↑^{3}H-leucine uptake, ↑ K_V4.2 mRNA, ↑K_V4.2 current, and ↑chord conductance CAIN = removal of all enhancement effects	Constitutively active CaN → ↑cardiac K_V4.2 current

Table 2.2 Roles of calcineurin in cardiac disease physiology (Continued)

Ref	System	Animal Model	Conditional requirement	Results	Conclusion
[106]	Mouse	Tg overexpression of ZAKI-4β	TAC	TAC + overexpression of ZAKI-4β = ↓PLB-PO_4, ↓SERCA2a protein, ↓ NCX protein, ↓RyR2-PO_4, and ↑Left ventricle end diastolic pressure → ↑myocardial stiffness	In TAC mouse model: CaN inhibition = ↓hypertrophy but does not prevent diastolic dysfunction
[107]	Rat and feline	Tg expression of constitutively active CaMKII Tg expression of dominant negative CaMKII	No stimulants required	↑CaMKII activity = ↓NFATc3 in nucleus, ↓fractional shortening, ↑DNA damage ↑Ca^{2+} → ↑NFAT in nucleus ↑CaMKII activity → ↑NFAT-PO_4 CaN inhibitors remove these effects	↑CaMKII activity→ phosphorylation of CaN → ↓NFAT in nucleus
[75]	Mouse	Tg K.O. of CaN B-subunit (C57BL/6 background)	No stimulants required	Deletion of CaN B-subunit = fatal < 1 day after birth, ↓NFAT activity, ↓mRNA of ion-handling genes, ↓capillaries per myocyte, ↓active force generation, and ↑CM size	CaN signaling is linked to control of cardiac contractility, rhythm, and expression of Ca^{2+} handling proteins

Descriptions of the roles that calcineurin has in contributing to heart disease.

Table 2.3 Calcineurin substrates

Substrate	K_D	Molecular Effect	Cellular Effect	Physiological Effect(s)	Ref.
Nuclear Factor of Activated T-cells 1-4 (NFAT1-4)	N/A	Dephosphorylation of $NFAT_c$	Relocalization of CaM-CaN-NFAT complex to nucleus → bind to $NFAT_N$ → expression of interleukin	Initiation of inflammatory immune response and/or Cardiac Hypertrophy	61,94,127,133–135
ATP sensitive K^+ channel	N/A	↑ Ca^{2+} = ↓ $K_{ir}6.1$ current Constitutively active CaN Aα= ↓ $K_{ir}6.1$ current	N/A	N/A	136,137
Myosin Phosphatase (MYPT1)	94 nM	CaN dephosphorylates ^{32}P-MYPT1 MYPT1-CaN interaction supported by pulldown, colocalization, and SPR experiments	N/A	N/A	126
L-Type Ca^{2+} Channel (LTCC)	N/A	Inside-out patch + CaN inhibits LTCC conduction	N/A	N/A	84,85
Na^+/K^+ ATPase	N/A	+FK506 or CaN peptide inhibitor = no effect of Oxymetazoline (Na^+/K^+ ATPase activator)	N/A	N/A	87
IκB	N/A	Constitutively active CaN + ionomycin = ↑NF-κB reporter activity	N/A	N/A	120
RyR2	N/A	Ca^{2+}-dependent CaN binding to RyR2 → inactivation of RyR2	N/A	N/A	89
ASK1	N/A	CaN B dephosphorylates ASK1 (S967) → ASK1 dissociation from 14-3-3 protein → ASK1 activation	Apoptosis	N/A	79,80
Myopodin	N/A	CaN dephosphorylates myopodin → hindered myopodin binding to 14-3-3β Prevention of myopodin binding to importin α	N/A	N/A	138
Calsarcin1	N/A	Calsarcin 1 binds to CaN A → formation of trimer with α-actinin	Localization of CaN to z-line of cardiomyocyte	Inhibition of calcineurin in hypertrophic signaling	139,140

Table 2.4 Calcineurin inhibitors

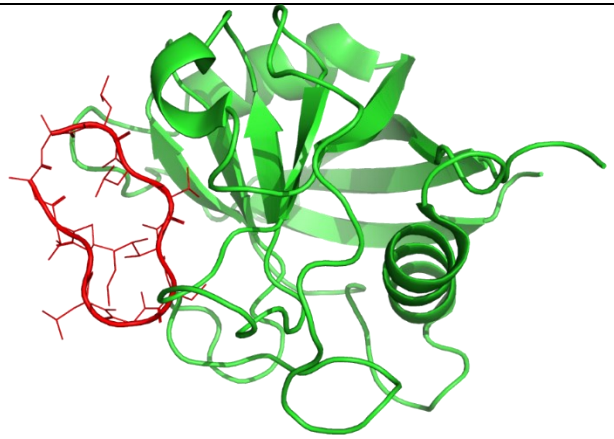
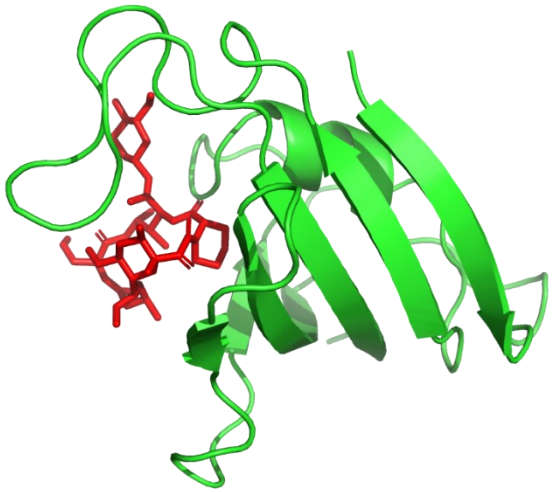
Inhibitor	Structure	Molecular Effect	Usage	Ref
Cyclosporin A-Cyclophillin A complex (CsA-CyA)	 PDBID: 1CWA; Red = Cyclosporin A, Green = Cyclophillin A	CsA binding to cyclophilin A → Binding/inhibition of CaN	Clinical and Experimental	48,141
Tacrolimus-FKBP12 complex (FK506-FKBP)	 PDBID: 1FKF; Red = FK506; Green = FKBP12	FK506 binding to FKBP12 → Binding/inhibition of CaN	Clinical and Experimental	46,142

Table 2.4 Calcineurin inhibitors (Continued)

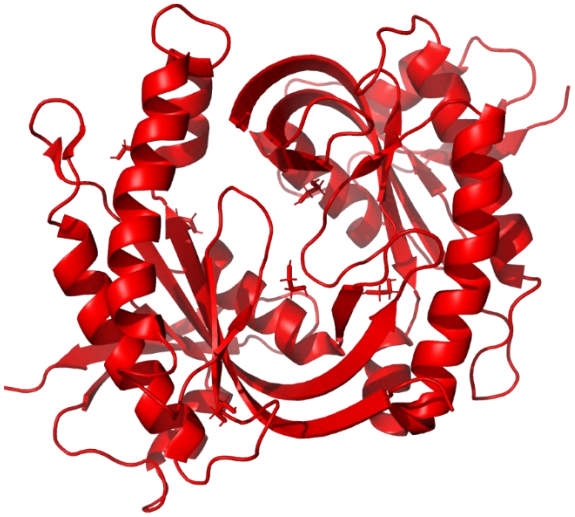
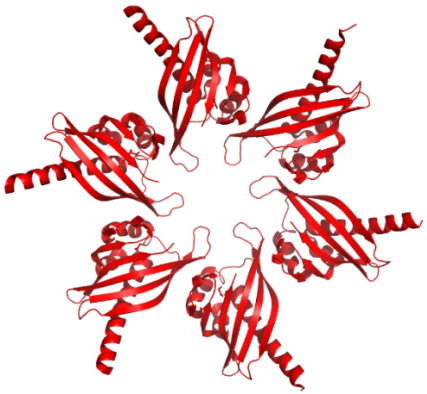
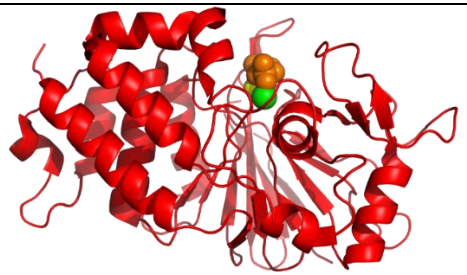
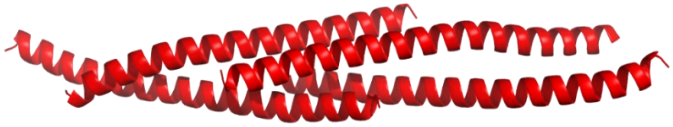
Inhibitor	Structure	Molecular Effect	Usage	Ref
Calcineurin Inhibitor (CAIN) protein/Cabin1	 PDBID: 1N6J; CABIN1 in complex with Myocyte-specific enhancer factor 2B (MEF2B) *MEF2B removed for clarity	PKC hyperphosphorylation of CAIN/Cabin1 →Inhibition of CaN	Experimental	60,61
Guanylyl Cyclase A	 PDBID: 4NI2	Posited that GCA/cGMP/PKG signaling inhibits CaN	Experimental	132

Table 2.4 Calcineurin inhibitors (Continued)

Inhibitor	Structure	Molecular Effect	Usage	Ref
Calmodulin Dependent Kinase II (CAMKII)	 PDBID: 2UX0	Posited that rise in $[Ca^{2+}]_i$ → CaMKII activation → phosphorylation/inhibition of CaN	Experimental	107
Calcipressin 1 (RCAN1)	 PDBID: 6UUQ; Orange = PO_4, Green = Fe, Yellow = Zn	Phosphorylation of RCAN1 → binding/inhibition of CaN	Experimental	143
Muscle-specific RING finger protein 1 (MuRF1)	 PDBID: 4M3L	MuRF1 ubiquitinates CaN → CaN degradation	Experimental	144

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