CaMKII Negatively Regulates Calcineurin–NFAT Signaling in Cardiac Myocytes

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Rationale: Pathological cardiac myocyte hypertrophy is thought to be induced by the persistent increases in intracellular Ca2+ needed to maintain cardiac function when systolic wall stress is increased. Hypertrophic Ca2+ binds to calmodulin (CaM) and activates the phosphatase calcineurin (Cn) and CaM kinase (CaMK)II. Cn dephosphorylates cytoplasmic NFAT (nuclear factor of activated T cells), inducing its translocation to the nucleus where it activates antipapoptotic and hypertrophic target genes. Cytoplasmic CaMKII regulates Ca2+ handling proteins but whether or not it is directly involved in hypertrophic and survival signaling is not known.

Objective: This study explored the hypothesis that cytoplasmic CaMKII reduces NFAT nuclear translocation by inhibiting the phosphatase activity of Cn.

Methods and Results: Green fluorescent protein–tagged NFATc3 was used to determine the cellular location of NFAT in cultured neonatal rat ventricular myocytes (NRVMs) and adult feline ventricular myocytes. Constitutively active (CaMKII-CA) or dominant negative (CaMKII-DN) mutants of cytoplasmic targeted CaMKII were used to activate and inhibit cytoplasmic CaMKII activity. In NRVM CaMKII-DN (48.5±3%, \( P<0.01 \) versus control) increased, whereas CaMKII-CA decreased (5.9±1%, \( P<0.01 \) versus control) NFAT nuclear translocation (Control: 12.3±1%). Cn inhibitors were used to show that these effects were caused by modulation of Cn activity. Increasing \( \text{Ca}^{2+} \) increased Cn-dependent NFAT translocation (to 71.7±7%, \( P<0.01 \)) and CaMKII-CA reduced this effect (to 17.6±4%). CaMKII-CA increased TUNEL and caspase-3 activity (\( P<0.05 \)). CaMKII directly phosphorylated Cn at Ser197 in CaMKII-CA infected NRVMs and in hypertrophied feline hearts.

Conclusion: These data show that activation of cytoplasmic CaMKII inhibits NFAT nuclear translocation by phosphorylation and subsequent inhibition of Cn. (Circ Res. 2009;105:316-325.)

Key Words: CaMKII ■ calcineurin ■ NFAT ■ myocytes ■ heart disease

Intermittent changes in the amplitude and duration of the systolic Ca2+ transient are the principle mechanism for regulating the strength of contraction (contractility) of the heart in health. Cardiovascular diseases that cause persistent increases in systolic wall stress require sustained increases in Ca2+ influx and sarcoplasmic reticulum uptake, storage and release to produce the necessary increases in [Ca2+] required to maintain the pump function of the heart under these conditions.1 The persistent increases in [Ca2+] that are required to maintain cardiac pump function in pathological cardiovascular stress also activate complex signaling pathways that lead to cardiac hypertrophy, structural and functional remodeling, and cell death.2 The signaling cascades that link changes in myocyte Ca2+ to activation of hypertrophic and survival signaling are the topic of this study.

Increases in myocyte [Ca2+] activate both the type 2B Ca2+/calmodulin (CaM)-dependent phosphatase, calcineurin (Cn), and the Ca2+/CaM-dependent protein kinase II (CaMKII). Activation of these signaling pathways is linked to electric and contractile disturbances in pathological cardiac hypertrophy.3,4 Activated Cn induces pathological hypertrophy by dephosphorylation and subsequent nuclear translocation of transcription factors associated with the NFAT family (nuclear factor of activated T cells) that activates specific hypertrophic target genes.3,5–8 In mice, transgenic cardiac overexpression of a constitutively active form of Cn has been shown to cause hypertrophy, mechanical dysfunction, arrhythmias, and premature death.3,9 In larger animal models10 and in patients,11 increased Cn activity has been linked to structural heart disease and the development of heart failure.
CaMKII activity is both constitutively active and inducibly inactive. In addition, CaMKII activity inhibits the myocyte apoptosis induced by phenylephrine stimulation. Importantly, although inhibition of CaMKII reduces cardiac hypertrophy, it fails to reduce mortality or improve left ventricular function. Collectively, these studies show that CaMKII nuclear translocation induces prohypertrophic and prosurvival signaling.

CaMKII signaling is complex because there are a number of CaMKII isoforms with different cellular locals. CaMKII\(\delta\) is the predominant isoform in the heart, with \(\delta_h\) and \(\delta_c\) being the major splice variants. The major difference between these 2 isoforms is that \(\delta_h\) contains a nuclear localization signal, and \(\delta_c\), does not. Therefore CaMKII\(\delta_h\) subunits localize to the nucleus, whereas \(\delta_c\) localize to the cytoplasm. Activation of the nuclear CaMKII\(\delta_h\) is linked to the activation of hypertrophic signaling by phosphorylating nuclear histone deacetylases (repressors of gene expression) and inducing their export from the nucleus. Activation of cytoplasmically restrained CaMKII\(\delta_c\) causes well-known effects on Ca\(^{2+}\) handling proteins, but its role in the induction of pathological hypertrophy or survival signaling is not known.

CaMKII\(\delta_c\) regulates the activity of Ca\(^{2+}\) handling proteins in the heart, and excess CaMKII\(\delta_c\) activity could mediate cardiomyopathic signaling in patients with end-stage heart failure by disrupting myocyte Ca\(^{2+}\) handling. In animal models, transgenic overexpression of CaMKII causes myocardial dilation, dysfunction, and abnormal myocyte Ca\(^{2+}\) homeostasis. CaMKII-mediated phosphorylation of Ca\(^{2+}\) regulatory proteins, such as the L-type Ca\(^{2+}\) channel, the ryanodine receptor, and phospholamban (PLB), are likely to be involved in the pathogenesis of dilated cardiomyopathy, cardiomyocyte apoptosis, arrhythmias, and heart failure. The fact that inhibition of CaMKII protects against cardiac structural and functional defects after myocardial infarction supports that it plays a critical role in these disturbances. Although both Ca\(^{2+}\) and CaMKII\(\delta_c\) are activated by changes in cytoplasmic Ca\(^{2+}\), the idea that these 2 signaling cascades directly interact to modulate hypertrophic and survival signaling has not been well studied. The activity and expression of CaMKII are increased in hearts from Ca\(^{2+}\)-overexpressing mice and CaMKII inhibition improves left ventricular function. These results suggest that CaMKII might mediate some of its cardiomyopathic effects by modulating Ca\(^{2+}\) signaling. Further support for this idea comes from studies in human T Cells where an active form of CaMKII was shown to decrease NFAT activity by nearly 35%. The idea that activation of cytoplasmic CaMKII\(\delta_c\) modulates cardiomyopathic signaling by regulating CaMKII-mediated NFAT nuclear translocation is the central topic of this study.

The working hypothesis of this study is that constitutive activation of the cytoplasmically localized isoform of CaMKII\(\delta_c\) regulates myocyte stress responses (hypertrophy and cell survival signaling) by modulating (inhibition) CaMKII-mediated NFAT nuclear translocation. We specifically tested the idea that the autophosphorylated, constitutively (Ca\(^{2+}\)-independent) active (CA) form of cytoplasmic CaMKII\(\delta_c\) regulates Cn-mediated NFAT nuclear translocation. Our studies show that CaMKII\(\delta_c\)-CA reduces myocyte contractility, inhibits NFAT nuclear translocation and hypertrophic signaling and induces myocyte apoptosis. These findings demonstrate a previously unappreciated role of cytoplasmic CaMKII in cardiac myocytes and suggest that inhibition of the autophosphorylated form of CaMKII\(\delta_c\) may be a viable strategy for improving myocardial function in heart disease.

Methods

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

Construction of Viral Vectors

Hemagglutinin (HA)-tagged constitutively active CaMKII\(\delta_c\) (CaMKII-CA) and dominant negative CaMKII\(\delta_c\) (CaMKII-DN) were kindly provided by Dr Joan Heller Brown. Green fluorescent protein (GFP)-tagged NFATc3 (adNFATc3) was used to determine cytoplasmic versus nuclear NFAT localization. Nuclei with NFATc3-GFP fluorescence that was 2× above cytoplasmic NFAT-GFP fluorescence were deemed to be NFAT positive.

Cell Culture and Adenoviral Gene Transfer

Primary cultures of neonatal rat cardiomyocytes (NRVMs) were prepared from hearts of 1- to 2-day-old Sprague–Dawley rat pups as...
previously described. Feline left ventricular myocytes were isolated as described previously. Myocytes were infected with adNFATc3 and subsets were coinfected with CaMKII-CA or CaMKII-DN for 6 hours at a multiplicity of infection of 100.

**TUNEL and Caspase Assays**
The TUNEL assay was performed with NFATc3-GFP–expressing NRVMs attached to coverslips using the In Situ Cell Death Detection Kit (TMR Red) (Roche Applied Science, Indianapolis, Ind). The number of TUNEL-positive nuclei from GFP-positive NRVMs as a percentage of the total nuclei was counted in a minimum of 10 random fields per isolation.

Caspase 3 activity was measured with EnzChek Caspase-3 (Molecular Probes) assay kit according to the instructions of the manufacture, with minor modifications.

**Immunoprecipitation and Western Blotting**
NRVMs were infected with or without specific adenoviruses for 48 hours, protein was collected, and immunoprecipitation and Western blotting performed.

Tissue protein abundance and phosphorylation in isolated protein were analyzed using Western blot analysis as previously described.

**Feline Physiological Measurement**
All experiments were performed on single rod–shaped myocytes with clear sarcomeric cross striations as previously described.

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**Results**

**Adenoviral Gene Transfer of adNFATc3-GFP and CaMKII in Neonatal Cardiac Myocytes**
Infection rates of 96% were achieved with adNFATc3-GFP and >99% for both CaMKII-CA and CaMKII-DN (Figure 1). Anti-HA staining confirmed the cytoplasmic localization of both CaMKII isoforms and expression was quantified by Western blotting (Figure 1). PLB phosphorylation at Thr17, a known CaMKII phosphorylation site, was increased only in CaMKII-CA, demonstrating that CaMKII activity was increased in these myocytes.

**NFAT Translocation in Neonatal Ventricular Myocytes**
In our control conditions, NFATc3 was primarily localized in the cytoplasm but had been translocated into the nucleus in 12.4% of NRVMs (Figure 2A and 2B). Coinfection with CaMKII-CA reduced nuclear NFATc3 accumulation (6%, P<0.01 versus control) (Figure 2). In contrast, coinfection with CaMKII-DN increased nuclear NFATc3 accumulation (48.5%, P<0.01 versus control). The coexpression of both CA and DN produced an intermediate effect (21.3%). Inhibition of Cn with cyclosporin A (CsA) (2 μmol/L), FK506
(2 μmol/L), or calcineurin inhibitor (CAIN) (2.5 μmol/L) all significantly reduced NFAT translocation in control and CaMKII-DN myocytes, but not in myocytes infected with CaMKII-CA. These results support the idea that NFATc3 nuclear translocation in control and CaMKII-DN myocytes is dependent on Cn activation and that basal Cn signaling is regulated by the activation state of CaMKII. To further support a role for CaMKII in Cn-mediated NFATc3 nuclear translocation, CaMKII-CA infected NRVMs were treated with the CaMKII inhibitor autocamtide 2–related inhibitory peptide (AIP) (Online Figure I). AIP induced a dose-dependent increase in NFATc3 nuclear translocation, supporting the idea that CaMKII directly modulates Cn signaling, even under basal conditions.

**Calcium-Dependent NFAT Translocation**

NFAT translocates from the cytoplasm to the nucleus in stress states when myocyte Ca^{2+} is increased. To prove that our system was also sensitive to changes in myocyte Ca^{2+}, we tested the effects of 4 mmol/L bath [Ca^{2+}] on NFATc3 nuclear translocation (Figure 3 and Online Figure II). NFATc3 translocation was significantly increased in control myocytes (to 71.7±6.6%, P<0.01) when the bath [Ca^{2+}]o was increased to 4 mmol/L (Figure 3A through 3C). This Ca^{2+}-mediated NFATc3 translocation was abolished by CsA and FK506, supporting the idea that translocation was Ca^{2+}-dependent (Figure 3B). [Ca^{2+}]o-induced (4 mmol/L) NFATc3 nuclear translocation was significantly reduced by CaMKII-CA (Figure 3C). A similar percentage of control and CaMKII-DN myocytes had nuclear NFATc3 translocation in 4 mmol/L bath [Ca^{2+}] (Figure 3C). These results show that Ca^{2+}-mediated NFATc3 nuclear translocation requires Cn activation and suggests that activated CaMKII is a negative regulator of this process.

**CaMKII-Mediated Cardiomyocytes Apoptosis**

Cardiovascular stress states that involve increased myocyte [Ca^{2+}] are associated with activation of CaMKII and an increased rate of myocyte death.27 We explored the idea that activation of cytoplasmic CaMKII(δc) promotes myocyte death signaling, at least partially by regulating NFAT nuclear translocation. The TUNEL assay was used to measure the percentage of myocytes undergoing apoptosis. In control conditions, the percentage of TUNEL-positive myocytes was greater in CaMKII-CA versus both adNFATc3 and CaMKII-DN myocytes (Figure 4A). Increasing the bath [Ca^{2+}]o to 4 mmol/L increased the percentage of TUNEL-positive cells in both adNFATc3 and CaMKII-CA myocytes, but not in CaMKII-DN–infected cells (Figure 4A). Caspase-3 activity was increased in myocytes expressing CaMKII-CA, whereas no differences were observed with control or CaMKII-DN (Figure 4B and 4C). Coexpression of CA and DN was able to blunt the increased caspase activity observed in CaMKII-CA (Figure 4B and 4C). These studies show that Ca^{2+} induced myocyte apoptosis is enhanced by CaMKII(δc)-CA and is reduced by CaMKII(δc)-DN.

**CaMKII-Mediated Cn Phosphorylation**

Our experiments have shown that CaMKII(δc) activity influences Cn mediated NFATc3 nuclear translocation. To define the mechanism for this effect we next tested if activated CaMKII maintains NFATc3 in the cytoplasm by directly phosphorylating it or by phosphorylation and inhibition of Cn.
The phosphorylation status of NFAT in myocytes expressing CaMKII-CA or CaMKII-DN, was determined by immunoprecipitation of NFAT-GFP (IP:GFP) followed by Western analysis with antibodies against phospho-serine or phospho-threonine (Figure 5A). Phosphorylation (serine and threonine) of NFAT was greatest in CaMKII-CA and lowest in CaMKII-DN, consistent with the cytoplasmic and nuclear NFATc3 localization displayed in Figure 2. These results strongly support the idea that cytoplasmic CaMKII activity regulates NFAT phosphorylation state, although they do not discern between direct phosphorylation of NFAT by CaMKII or an effect mediated by alterations in Cn activity. Indeed, although glycogen synthase kinase 3, p38, and c-Jun N-terminal kinase are kinases that directly phosphorylate NFAT,37–39 resulting in nuclear export/cytoplasmic localization, it is not known whether CaMKII directly phosphorylates NFAT. To explore direct CaMKII-NFAT or CaMKII-Cn interactions, in vitro kinase assays were performed (Figure 5B). We could not identify direct CaMKII phosphorylation of NFATc3 in any of 3 recombinant protein fragments that together comprise the entire protein (N1, N2, or N3). However, in multiple experiments, we were able to show that recombinant CaMKII directly phosphorylated purified Cn (Figure 5B).

CaMKII phosphorylation of Cn at Ser197 is known to inhibit its activity.40–42 We generated an antibody with some ability to detect Cn-Ser197 phosphorylation. With this antibody, we found CaMKII phosphorylation in NRVMs expressing CaMKII-CA (Figure 6A) but with little detectable levels under our other conditions. PLB phosphorylation at Thr17 was also increased in CaMKII-CA myocytes, consistent with increased CaMKII activity.

Cn-mediated translocation of NFAT from the cytoplasm to the nucleus is known to activate hypertrophic gene expression. We measured the hypertrophic marker atrial natriuretic peptide to determine whether the nuclear NFATc3 translocation induced by CaMKII-CA and -DN activated hypertrophic signaling. Atrial natriuretic peptide protein abundance was significantly (P<0.01) reduced in myocytes expressing CaMKII-CA and significantly increased (P<0.01) in myocytes expressing CaMKII-DN, consistent with our contention that CaMKII reduces hypertrophic and cardioprotective signaling by opposing Cn-mediated NFATc3 nuclear translocation.

Cn-Ser197-Phosphorylation in Feline Hypertrophy
Pathological signaling involving Ca2+ may not be similar in rodents and larger mammals because of fundamental differences in Ca2+ handling in large and small mammals.43,44 To determine whether increased phosphorylation at Cn-Ser197 is present in the intact heart of a large mammal (with Ca2+...
handling like that in humans) subjected to chronic myocardial stress, samples were collected from left ventricular tissue from aortic banded felines and analyzed via Western blot analysis. Greater phosphorylation of Cn at Ser197 was observed in hypertrophied feline left ventricular tissue (Figure 6B). In these animals, we were able to detect Ser197 phosphorylation in control hearts. These data strongly support the idea that the observations in NRVMs are relevant to cardiac disease states.

**Adult Ventricular Myocytes: NFAT Nuclear Translocation and Functional Analysis**

The effects of CaMKII on NFATc3 nuclear translocation were also studied in adult feline ventricular myocytes to ensure that our results in NRVMs are relevant to the adult heart of a large mammal. In addition, these myocytes were used to examine the effects of CaMKII activity on contractile function. In feline myocytes, an infection rate of 97% was achieved with adNFATc3 and 98% for both CaMKII-CA and CaMKII-DN. CaMKII was localized to the cytoplasm and PLB-Thr17 was highest in myocytes expressing CaMKII-CA (Figure 7A and 7B), as we have shown for NRVMs. NFATc3 nuclear localization was greatest in myocytes expressing CaMKII-DN (55.1±3.8%, P<0.01) versus adNFATc3-GFP (24.8±1.9%) and was lowest in CaMKII-CA (18.9±2.1%). These results are similar to those in NRVMs. Pacing feline myocytes, which are normally quiescent in culture, induced NFATc3 nuclear translocation that was frequency-dependent.

**Figure 4.** A, Increased rate of apoptosis was observed in myocytes expressing CA-CaMKII (18.1±2.9%, P<0.05 vs control and CamKII-DN). In 4 mmol/L [Ca2+]o, increased apoptosis was observed in both adNFAT and CaMKII-CA, whereas cell viability was preserved in CaMKII-DN. This Ca2+-mediated apoptosis was significantly greater in myocytes expressing CaMKII-CA compared to both control and CaMKII-DN. B, Representative caspase-3 activity for NRVMs from each experimental condition. The slope of the line was used as an index of caspase-3 activity. C, Average caspase-3 activity data are presented (n=5/group). Increased caspase-3 activity (control: 1.81±0.2) was observed in myocytes expressing CaMKII-CA (3.3±0.4), whereas no differences were observed with CaMKII-DN (2.02±0.2). The coexpression of CA and DN (2.1±0.2) blunted the increase observed in CaMKII-CA. *P<0.05 vs adNFAT and CaMKII-DN; †P<0.01 vs respective control.

**Figure 5.** A, To demonstrate the phosphorylation status of NFAT, protein extracts were made from cultured neonatal myocytes expressing CaMKII-CA or CaMKII-DN, whereas β-galactosidase acted as a control. Immunoprecipitation was performed (IP: NFAT-GFP) and subsequent Western blots for phospho-serine, phospho-threonine, or GFP as a loading control. Representative blots are presented in A. An increase in phosphorylated NFAT (Ser, Thr) was observed in myocytes expressing CaMKII-CA, whereas CaMKII-DN resulted in reduced phosphorylation (Ser, Thr), consistent with reduced and enhanced nuclear translocation, respectively. B, An in vitro kinase showed no phosphorylation of NFAT after incubation with activated CaMKII; however, CaMKII did phosphorylate calcineurin. Blots from 2 separate experiments are presented.
Pacing-induced NFATc3 nuclear translocation was inhibited by CsA (Figure 7C), demonstrating the role of Cn. These experiments show that CaMKII-CA and DN have similar effects on NFATc3 nuclear translocation in neonatal and adult myocytes.

Feline ventricular myocytes expressing CaMKII-CA had smaller fractional shortening and dL/dt values than myocytes expressing either control (adNFATc3) or CaMKII-DN (Figure 7D and 7E). These studies suggest that in spite of the known effects of CaMKII to increase Ca\(^{2+}\) influx and

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**Figure 6.** A, To demonstrate protein abundance and phosphorylation state of proteins involved in this signaling cascade, protein extracts were made from cultured NRVMs expressing CaMKII-CA or CaMKII-DN, whereas GFP acted as a control. The phosphorylation of Cn at Ser197 was detectable only in NRVMs expressing CaMKII-CA (Figure 6A), whereas PLB phosphorylation was increased at Thr17, consistent with increased CaMKII activity. A reduction in atrial natriuretic peptide (ANP) abundance was observed in myocytes expressing CaMKII-CA, consistent with reduced hypertrophic signaling. B, An increase in phosphorylation of Cn at Ser197 was observed in hypertrophied feline left ventricular tissue.

**Figure 7.** A and B, Adult feline ventricular myocytes were infected with either GFP (control), CaMKII-CA, or CaMKII-DN mutants of cytoplasmic-targeted CaMKII\(_d\) (multiplicity of infection, 100). Expression and localization of CaMKII\(_d\) was confirmed by immunostaining with GFP-conjugated antibodies against HA (Sigma-Aldrich). CaMKII\(_d\) was localized to the cytoplasm and PLB-Thr17 was highest in myocytes expressing CaMKII-CA. C, Pacing-induced (0.5 or 1.0Hz) NFAT nuclear translocation that was frequency and CsA-dependent. D and E, Functionally, reduced fractional shortening was observed in cells expressing CaMKII-CA. *P<0.05.
promote sarcoplasmic reticulum Ca\(^{2+}\) uptake, the net effect is to reduce myocyte contractility, at least under our conditions (see Discussion).

**Discussion**

Cardiovascular diseases such as hypertension and myocardial infarction raise the contractile stress on the heart. This requires reflex responses (primarily via the sympathetic nervous system) that increase cardiac systolic stress generation by producing increases in myocyte [Ca\(^{2+}\)]. When excess contractile stress is persistent, it leads to cardiac hypertrophy and over time is associated with arrhythmias, myocyte death, and eventually congestive heart failure.\(^2\,46\) The links among persistent increases in myocyte Ca\(^{2+}\), pathological hypertrophy, cardiac arrhythmias, and myocyte contractile abnormalities are still not well established.\(^3\,9\,15\)

Increased myocyte Ca\(^{2+}\) activates Cn, a phosphatase that dephosphorylates and thereby induces nuclear translocation of NFAT, which is involved in the induction of pathological cardiac hypertrophy and prosurvival signaling.\(^3\,5\,8\,12\,13\) Increased myocyte Ca\(^{2+}\) also activates cytoplasmic CaMKII\(_\delta\), which phosphorylates a number of critical target proteins involved in Ca\(^{2+}\) regulation. Although the effects of CaMKII on individual Ca\(^{2+}\) regulatory proteins is fairly well described, its role in the regulation of cardiac contractility is complex and its role as a regulator of NFAT mediated cardiac gene expression and cell death are not, to our knowledge, known.

The central question of the present research was whether activation of (cytoplasmic) CaMKII\(_\delta\) was involved in the regulation of NFAT nuclear translocation and, if so, to determine the molecular signaling pathway(s) involved. Our results clearly show that, at least under our experimental conditions, activation of CaMKII\(_\delta\) (with ad-CaMKII-CA) increases NFAT phosphorylation state (indirectly) and reduces NFAT nuclear translocation, whereas inhibition of CaMKII\(_\delta\) (with ad-CaMKII-DN or AIP) reduces NFAT phosphorylation (indirectly) and induces NFAT translocation from the cytoplasm to the nucleus. The novel aspect of our study is that we show that CaMKII\(_\delta\) induces these differences in NFAT phosphorylation, by modulating the activity of Cn. Other kinases that alter Cn-NFAT signaling (glycogen synthase kinase, p38m, c-Jun N-terminal kinase, protein kinase A, MEKK1, CKII) do so by directly phosphorylating the N terminus of NFATc1-c4, within the regulatory domain that controls nuclear shuttling by masking and unmasking a NLS.\(^37\,\,39\) However, in our hands, CaMKII did not directly phosphorylate NFAT, but, instead, we showed it directly phosphorylated Cn. These data were confirmed using a phosphorylation specific antibody against the Ser197 on Cn, a site previously shown to be phosphorylated by the autophosphorylated form of CaMKII and this interaction results in and partial inactivation of Cn.\(^40\,\,42\) Our results suggest that phosphorylation of Cn by CaMKII inhibits its phosphatase activity, allowing NFAT to be more phosphorylated and maintained within the cytoplasm. We found that CaMKII-CA/DN, as well as Cn and CaMKII inhibitors, altered NFAT translocation to the nucleus at baseline. These results suggest that both CaMKII and Cn are functionally active under basal conditions and that CaMKII is a modulator of this basal activity. Our results further support the idea that increases in myocyte Ca\(^{2+}\) activate 2 cytoplasmic signaling cascades with opposite effects on Cn activity, and, together, they determine NFAT nuclear translocation, hypertrophy and prosurvival signaling (Figure 8). Clearly, increased Cn-mediated NFAT nuclear translocation was the dominant

**Figure 8.** A representative schematic of the proposed CaMKII–calcineurin interaction. The phosphorylation of calcineurin by CaMKII results in reduced calcineurin activity, leading to reduced basal and calcium mediated NFAT translocation.
effect of increased Ca\(^{2+}\), with CaMKII acting as a negative regulator.

CaMKII-mediated phosphorylation of Cn has been described previously,\(^{40–42}\) and we confirmed that CaMKII\(\delta\) directly phosphorylates calcineurin in vitro. This phosphorylation occurs on the Ser197 residue which is located in the C-terminal boundary of the putative Ca\(^{2+}\)/CaM binding domain.\(^{41}\) This phosphorylation appears to be blocked when Ca\(^{2+}\)/CaM is bound to Cn, suggesting interaction between this phosphorylation site and the CaM binding domain.\(^{41}\) Phosphorylation of Cn-Ser197 by CaMKII results in partial inactivation of Cn as evidenced by an increase in \(K_m\) for protein substrates.\(^{41}\) Phosphorylation and partial inactivation of Cn in vivo would enhance phosphorylation of those proteins normally dephosphorylated by Cn such as tubulin,\(^{47}\) protein phosphatase inhibitor 1,\(^{48}\) apoptosis signal-regulating kinase 1,\(^{39,49,50}\) and NFAT. We showed that NFAT phosphorylation was increased in myocytes expressing the CaMKII-CA and in hypertrophied feline hearts, consistent with the central hypothesis of this study.

Increasing Ca\(^{2+}\) in either neonatal or adult ventricular myocytes induced NFAT nuclear translocation and this translocation was abolished by Cn inhibitors. These results suggest that when Ca\(^{2+}\) is increased in normal myocytes the net effect is to activate Cn such that NFAT is dephosphorylated and translocates into the nucleus. Activation of CaMKII appears to negatively regulate this signaling cascade providing additional control. When we increased the activity of CaMKII with CaMKII-CA, the effects of increased Ca\(^{2+}\) on NFAT nuclear translocation were significantly smaller, and when CaMKII activity was reduced with CaMKII-DN or AIP, the Ca\(^{2+}\) effects were enhanced. Collectively, these results show that cytoplasmic CaMKII is a critical negative regulator of Cn and NFAT nuclear translocation.

CaMKII activity is significantly increased in cardiovascular stress states that eventually lead to structural and functional remodeling. The excessive adrenergic activity needed to maintain cardiac pump function in the face of increased contractility demands, overtime, causes significant changes in adrenergic and CaMKII signaling, with \(\beta\)-adrenergic signaling downregulated\(^{18,24}\) and CaMKII signaling enhanced.\(^{18,24}\) Recent studies by Anderson and colleagues\(^{16,28}\) have shown that inhibition of CaMKII in this context can have beneficial effects on cardiac function and reduces myocyte apoptosis. The bases of these beneficial effects are not yet clear and could involve both cytoplasmic and nuclear CaMKII. Some of the beneficial effects of CaMKII inhibition in heart disease could also be attributable to removal of excess phosphorylation of Ca\(^{2+}\)-regulated proteins through inhibition of cytoplasmic CaMKII. In addition, inhibition of nuclear CaMKII could reset hypertrophic signaling cascades. Our data suggest that an additional explanation for the beneficial effects of CaMKII inhibitors in the failing heart is by removing the inhibitory effect of cytoplasmic CaMKII on Cn, thereby increasing NFAT nuclear translocation and unleashing its cardioprotective features. Many additional studies are required to sort through these issues.

Collectively, our results demonstrate that the constitutively activate form of CaMKII\(\delta\) directly phosphorylates Cn and inhibits its activity, resulting in reduced NFAT nuclear translocation and increased myocyte apoptosis. In cardiac disease states in which CaMKII activation is known to be increased, its effects on Cn could disrupt NFAT signaling and contribute to cardiac structural and functional defects. Thus, as suggested by others,\(^{28,51}\) inhibition of the excessive CaMKII activity of the failing heart may be an effective strategy for improving myocardial function in heart disease.

**Limitations**

We studied the relationship between CaMKII and Cn using in vitro–forced overexpression of either constitutively active of dominant negative mutant proteins. The mechanistic insights defined will need to be explored further in genetically modified mouse models with normal levels of expressed protein. The sources of Ca\(^{2+}\) to selectively activate CaMKII in the nucleus and cytoplasm and cytoplasmic Cn also need to be studied further.

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**Disclosures**

None.

**References**


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Cell Culture and Immunostaining: Cell cultures were treated with cyclosporine A (CsA, Santa Cruz Biotechnology), FK506 (Sigma-Aldrich), Cn autoinhibitory peptide (CAIN, Calbiochem), or autocamtide 2-related inhibitory peptide (AIP - Sigma-Aldrich) for 24 hours. In separate experiments, cultures were exposed to 4 mM bath [Ca\(^{2+}\)] for 4-hours. Myocytes were fixed (4% paraformaldehyde) or utilized for functional assays 48 hours after infection.

Immunostaining was performed as previously described.\(^1\) Antibodies included: Anti-HA (Sigma-Aldrich), \(\alpha\)-Actinin (Sigma-Aldrich), and DAPI (Molecular Probes). All animal protocols were approved by the Animal Care and Use Committee of Temple University. All animals received humane care in compliance with Temple University standards and “Principles of Laboratory Animal Care” formulated by the National Society for Medical Research and the “Guide for the Care and Use of Laboratory Animals” prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH Publication No. 85-23, revised 1996).

TUNEL and Caspase Assays: Caspase 3 activity was measured with EnzChek® Caspase-3 (Molecular Probes) assay kit according to the manufactures instructions with minor modifications. In brief, adherent and detached cells were washed with ice-cold PBS pH 7.4 and scraped in ice-cold Cell Lysis buffer (10mM Tris, pH 7.5, 100mM NaCl, 1mM EDTA, 0.01% Triton X-100) with one freeze thaw cycle. The lysates were then sonicated and centrifuged. Z-DEVD-AMC substrate solution was added to the supernatants from the samples in a Costar 96-well white plate (Corning). An enzyme-less control was included in each assay in order to determine the background fluorescence of the substrate. Fluorescence was measured at an
excitation and emission wavelength of 342 and 441, respectively, using a Fluostar Optima spectrofluorometer (BMG Technologies), with reading taken every minute for 2-hours.

**Immunoprecipitation and Western Blotting:** Cells were lysed at 4°C in immunoprecipitation buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.5% Triton X-100) containing protease inhibitors (10 µg/mL Aprotinin, 10 µg/mL Leupeptin, 5 µg/mL Pepstatin, 200 µg/mL Benzamidine). Lysates were cleared by centrifugation at 15,000 × g for 10 min and then incubated with a GFP antibody (Cell Signaling) for 12-hours at 4°C followed by the addition of protein A/G agarose (Santa Cruz) and another hour of incubation at 4°C. The samples were washed 3 times with 200 µL of immunoprecipitation buffer and subjected to SDS-PAGE.

Target antigens were probed with phosphorylation-specific polyclonal antibodies: Phospho-Serine (Chemicon), Phospho-Threonine (Cell signaling), total phospholamban (PLBt) (Upstate), phosphorylated phospholamban at Threonine 17 (PLB-Thr^{17}) and Serine 16 (PLB-Ser^{16}) (Badrilla), ANP (Abcom), sarcomeric actin (Sigma), GAPDH (Biogenesis), phosphorylated Cn at Serine^{197} (Cn-Ser^{197}) (YenZym), and Anti-HA (Sigma-Aldrich).

**Kinase Assay:** Kinase assay buffer included: 20 mM Tris-Cl [pH 7.5], 150 mM NaCl, 10 mM MgCl₂, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride plus 1 mM cold ATP, calcineurin was incubated with active CaMKII (Millipore Corporation) in 25 µl of kinase assay buffer with 0.3 µCi of [³²P]ATP for 10 min at 30°C. Samples were subjected to SDS-PAGE (8%) and visualized by PhosphorImager analysis (Amersham Pharmacia Biotech).
Data Analysis and Interpretation: Data were compared with one-way ANOVA followed by a Tukey post hoc analysis. Significance was set at an alpha level of P<0.05. Data are reported as the mean ± SEM.
Supplemental Figure Legends

**Online Figure I.** Neonatal ventricular myocytes expressing GFP-tagged NFATc were co-infected with a constitutively active (CaMKII-CA) mutant of cytoplasmic targeted CaMKII\(\delta_c\) (m.o.i. 100). In myocytes expressing CaMKII-CA, reduced nuclear NFAT accumulation (5.98%, P<0.01 vs. control) was observed. In an additional set up experiments, myocytes expressing both GFP-tagged NFATc and CaMKII-CA were treated with autocamtide 2-related inhibitory peptide (AIP - Sigma-Aldrich), a potent CaMKII inhibitor, for 24 hours. An increase in NFAT nuclear translocation was observed in myocytes treated with AIP suggesting that CaMKII plays a fundamental role in the reduced translocation observed in myocytes expressing the constitutively active form of CaMKII\(\delta_c\).

**Online Figure II.** Representative images are displayed from control conditions and after a 4-hour incubation at 4mM \([Ca^{2+}]_o\). GFP tagged NFATc3 expressing NRVMs were co-stained with \(\alpha\)-Actinin and DAPI to confirm the documented translocation was occurring in cardiac myocytes. Individual staining patterns and the merged images are presented.

**REFERENCE**

Online Figure I

![Bar chart showing NFAT translocation (%)]

- **NFAT translocation (%):**
  - adNFAT: 10%
  - AIP – 5 uM: 20%
  - AIP – 10 uM: 25%
  - AIP – 20 uM: 30%

Significance levels:
- P<0.05: Between adNFAT and AIP – 5 uM
- P<0.01: Between adNFAT and AIP – 10 uM, AIP – 20 uM
Online Figure II

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