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CaMKII is a nodal signal for multiple programmed cell death pathways in heart

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Abstract

Sustained Ca^{2+} /calmodulin dependent kinase II (CaMKII) activation plays a central role in the pathogenesis of a variety of cardiac diseases. Emerging evidence suggests CaMKII evoked programmed cell death, including apoptosis and necroptosis, is one of the key underlying mechanisms for the detrimental effect of sustained CaMKII activation. CaMKII integrates β -adrenergic, Gq coupled receptor, reactive oxygen species (ROS), hyperglycemia, and pro-death cytokine signaling to elicit myocardial apoptosis by intrinsic and extrinsic pathways. New evidence demonstrates CaMKII is also a key mediator of receptor interacting serine/threonine kinase 3 (RIP3) induced myocardial necroptosis. The role of CaMKII in cell death is dependent upon subcellular localization and varies across isoforms and splice variants. While CaMKII is now an extensively validated nodal signal for promoting cardiac myocyte death, the upstream and downstream pathways and targets remain incompletely understood, demanding further investigation.

Keywords

CaMKII; programmed cell death; apoptosis; necroptosis; oxidized CaMKII; RIP3; ROS; mitochondria; mitochondrial permeability transition pore

Introduction

Apoptosis, a process of programmed cell death, is considered a vital component of cardiovascular development [1]. However, inappropriate apoptosis or accelerated apoptosis is one of the major factors contributing to cardiovascular disease [2, 3]. Because of this, prevention of apoptosis has been viewed as a promising therapeutic target. Apoptosis is defined, in part, by distinct morphological characteristics and energy-dependent biochemical mechanisms. Apoptosis yields almost no residual cellular components and is unattended by

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Disclosures

MEA is a cofounder of Allosteros Therapeutics, a company aiming to develop CaMKII inhibitor therapeutics.

inflammatory reaction. An alternative to apoptotic cell death is necrosis, which is considered to be a toxic process where the cell is a passive agent and follows an energy-independent mode of death. However, this paradigm has evolved over the past decade. Emerging evidence has strongly supported a concept that a subset of necrosis is regulated and follows defined signaling mechanisms. This programmed necrosis, often referred as necroptosis, has been recognized to play an important role in variety of cardiovascular diseases and is now considered be a candidate target for therapeutic intervention [4, 5]. Necroptosis appears to play no role in cardiac development. Instead, suppression of necroptosis is important for normal development and tissue homeostasis [6–8]. The essential mechanistic feature of necroptosis is the opening of mitochondrial permeability transition pore (mPTP) [5], which dissipates the inner mitochondrial membrane potential (Ψ_m), disrupts mitochondrial respiratory function and results in metabolic shutdown. Thus, in stark contrast to apoptosis, which requires mitochondrial derived ATP to fuel the multiple processes aiming to disassemble the cell, necroptosis is a consequence of ATP deficiency. Necroptosis yields cellular residua that trigger innate immune inflammatory responses that contribute to the pathology of tissue injury.

The Ca^{2+} and calmodulin dependent protein kinase II (CaMKII) is a pleiotropic signal that regulates cardiomyocyte Ca^{2+} cycling, contractility, inflammation, metabolism, gene expression, and cell survival [9–11]. Although CaMKII plays an important physiological role, such as augmenting the “fight or flight response”, [12] sustained CaMKII activation is now recognized to promote heart failure [13, 14], arrhythmia [15] and sudden cardiac death [16]. Excessive CaMKII activation promotes cardiac myocyte death under conditions of neurohumoral agonist signaling [17, 18], oxidant stress [19–22], hyperglycemia [23, 24], ischemic injury [25–29], doxorubicin toxicity [26, 30], acidosis induced injury and other adverse stimuli associated with increased intracellular $[\text{Ca}^{2+}]$ (e.g. thapsigargin, ionomycin, high extracellular $[\text{K}^+]$) [31]. Thus, improved understanding of the pathophysiology of CaMKII mediated cell death is an important goal for cardiovascular biology with the potential to provide new targets for treating disease.

Molecular physiology of CaMKII

CaMKII is a serine-threonine kinase that is abundant in myocardium and other excitable tissues. CaMKII exists as four distinct isoforms (α , β , γ , δ) that are encoded by four separate genes. Each of these genes resides on a different chromosome, in humans and in mice [19]. The CaMKII isoforms are homologous, consist of the same domains (i.e. association, regulatory and catalytic), operate by shared activation mechanisms, and may assemble heterologously into holoenzymes. CaMKII δ is the major myocardial isoform and CaMKII γ is a minor component [32]. CaMKII α and CaMKII β are absent or scantily represented in heart. CaMKII δ is most clearly linked to myocardial disease because CaMKII δ knock out mice are resistant to transaortic banding surgery, [13, 14] while transgenic mice with myocardial CaMKII δ over-expression develop severe heart failure, arrhythmias and premature death. [33] Although CaMKII is widely distributed throughout cellular compartments, CaMKII δ exists in a splice variant (CaMKII δ_B) where a nuclear localization sequence is part of the hypervariable region (between the regulatory and

association domains). CaMKII δ_B is relatively, but not exclusively, enriched in the nucleus. [34]

Under basal conditions, CaMKII is biased toward inactivation because the catalytic domain is constrained by the pseudosubstrate region of the regulatory domain (Fig 1). When intracellular Ca^{2+} rises it calcifies calmodulin (Ca^{2+}/CaM), a Ca^{2+} sensing protein, to engage the calmodulin binding region of the regulatory domain. Ca^{2+}/CaM binding allosterically displaces the catalytic domain from the inhibition of the regulatory domain, leading to CaMKII activation. Once active, the pseudosubstrate region of the regulatory domain is available for various post-translational modifications that convert CaMKII from a Ca^{2+}/CaM activated enzyme to a Ca^{2+}/CaM autonomous enzyme. These Ca^{2+}/CaM independent forms of CaMKII, due to threonine 287 'autophosphorylation', [35, 36] methionine 281/282 oxidation [20], and serine 280 *O*-GlcNAcylation [37] are linked to myocardial disease, including increased myocardial death, heart failure and arrhythmias. [11] S-nitrosylation of Cysteine 290 in the regulatory domain also induces autonomous activation of CaMKII [38, 39]. This S-nitrosylation dependent activation of CaMKII takes part in β -adrenergic stimulation evoked Ca^{2+} sparks [39]. In neuron, S-nitrosylation induced CaMKII activation is involved in cell death [38]. However, whether this C290 nitrosylation induced CaMKII activation is also involved in β -adrenergic stimulation evoked myocyte death remains unclear. A recent study revealed that, besides cysteine 290, cysteine 273 can also be S-nitrosylated [40]. In contrast to C290 nitrosylation, C273 nitrosylation does not activate CaMKII, but inhibits Ca^{2+} /calmodulin dependent activation of CaMKII. Therefore, nitric oxide plays a dual role in CaMKII signaling. It was suggested in this study that nitrosylation of CaMKII activates or inhibits the kinase activity depending on the timing of CaMKII exposure to nitric oxide relative to the timing of Ca^{2+} /calmodulin binding. Exposure of CaMKII to nitric oxide prior to Ca^{2+} /calmodulin binding, results in inhibition of CaMKII by nitrosylation of C273. Conversely, after Ca^{2+} /calmodulin binding to CaMKII, S-nitrosylation of CaMKII preferentially occurs at the C290 site, leading to activation of CaMKII. Our view is that *in vivo* studies will be required to more completely elucidate the role of nitric oxide regulation of CaMKII in cardiac pathophysiology. Cysteine 290 can also be oxidized by hydrogen peroxide, resulting in Ca^{2+} /calmodulin autonomous CaMKII activation [40]. Further research will be necessary to illuminate how the post-translational modifications in the regulatory domain interact with each other.

The role of CaMKII in myocardial apoptosis

Apoptosis is orchestrated by a series of evolutionarily conserved caspase cysteine proteases [2]. There are two categories of apoptosis pathways, intrinsic and extrinsic. Mitochondria are at the center of the intrinsic pathway. The activation of Bax and Bak proteins, the family members of Bcl2, permeabilize the outer membrane of mitochondria, leading to the release of mitochondrial apoptogenic proteins, including cytochrome C, to the cytosol. Cytosolic cytochrome C activates the execution pathway, consisting of caspase-3, caspase-6, and caspase-7. Extrinsic apoptosis is initiated by ligand-mediated activation of death receptors, including tumor necrosis factor- α (TNF- α), CD95 (Fas/APO-1) and TNF-related apoptosis-inducing ligand (TRAIL) receptor-1. Ligand binding stimulates the assembly of the death-inducing signaling complex (DISC, mainly consists of Fas-associated protein with death

domain (FADD) and FADD-like IL-1 β -converting enzyme)-inhibitory protein (c-FLIP)), which induces caspase-8 activation. After Caspase 8 activation, the extrinsic apoptosis pathway converges with the intrinsic apoptosis pathway at the level of executioner caspases-3, -6, and -7. [2]

CaMKII, Ca²⁺ overload and mitochondria-mediated intrinsic apoptotic pathway

Cytosolic Ca²⁺ overload is one of the hallmarks of heart failure and ischemic heart disease. Augmented L-type Ca²⁺ channel (LTCC) influx by overexpression of LTCC (CaV1.2, beta2a subunit) induced sarcoplasmic reticulum (SR) Ca²⁺ overload, which elicited the mitochondria dependent intrinsic apoptosis pathway [41]. Sustained, excessive CaMKII activation is an upstream signaling event for promoting SR Ca²⁺ triggered arrhythmias [42, 43] and increased LTCC opening probability, [23, 44] which contribute to excitation-contraction coupling dysfunction, myocardial hypertrophy, heart failure, arrhythmia and sudden cardiac death [10]. CaMKII induced loss of cellular Ca²⁺ homeostasis also appears to play a crucial role in cardiac myocyte apoptosis [17]. CaMKII inhibition *in vivo*, by transgenic expression of a myocardial targeted inhibitory peptide (AC3-I), protected against myocardial apoptosis induced by myocardial infarction or isoproterenol administration [18]. This protection appeared to involve reduced SR Ca²⁺ content in AC3-I mice. Interbreeding AC3-I mice with phospholamban knockout (*Pln*^{-/-}) mice restores SR Ca²⁺ content to WT levels. The protection of AC3-I expression against myocardial apoptosis due to isoproterenol injection is reduced, and entirely lost after myocardial infarction in AC3-I transgenic mice interbred into a *Pln*^{-/-} background. These findings highlight the SR Ca²⁺ dependence of CaMKII induced myocardial apoptosis. In contrast, interbreeding mice with transgenic myocardial CaMKII δ over-expression with *Pln*^{-/-} mice caused increased mitochondrial Ca²⁺, augmented cardiomyocyte death, worsened heart failure, and increased mortality [45]. Mutant ryanodine receptor (RyR2) knock-in mice, lacking a validated serine 2814 phosphorylation site (S2814A), were resistant to apoptosis and displayed improved cardiac function after myocardial infarction [46]. In addition, overexpression of mutant voltage-gated Ca(2+) channel (Ca(V)1.2) β subunits, which are resistant to CaMKII binding (L493A) and phosphorylation (T498A), delayed rapid-pacing induced cardiomyocyte death [47]. The final execution pathway for this CaMKII mediated, Ca²⁺ dependent apoptosis appears to be the mitochondria-mediated intrinsic pathway. Overexpression of CaMKII δ in cultured cardiomyocytes led to increased apoptosis in the condition of oxidative stress, accompanied by elevated cytosolic Ca²⁺ and enhanced mitochondrial cytochrome c release [31], which is consistent with LTCC overexpression induced mitochondria dependent apoptosis [41]. However, a clear and comprehensive picture of CaMKII target proteins required for this process has yet to emerge.

CaMKII in p53 mediated apoptosis

p53 is a multi-functional protein, and a pro-apoptotic transcription factor with connections to intrinsic and extrinsic apoptotic pathways. Nuclear p53 accumulation and activation with excessive apoptosis appeared to be essential mechanisms promoting heart failure progression in dilated cardiomyopathy due to mutations in genes encoding cytoskeletal or myofilament proteins [48]. The mutant myofilament often led to decreased Ca²⁺ sensitivity. In apparent compensation for this deficiency, β -adrenergic signaling and Ca²⁺ transients are

increased, which favors sustained CaMKII activation. Inhibition of CaMKII, by transgenic expression of AC3-I or small molecule inhibitors, in a mouse model of dilated cardiomyopathy due to overexpression of a mutated cardiac α -actin (R312H), attenuated p53 activation, reduced apoptosis, and improved cardiac function. It remains to be determined how CaMKII activates p53.

CaMKII in ER stress mediated apoptosis

Endoplasmic reticulum (ER) mediated synthesis and proper folding of multiple proteins, some posttranslational modifications, trafficking of newly synthesized proteins to the Golgi apparatus, lipid biosynthesis, and intracellular Ca^{2+} homeostasis are crucial for normal cellular functioning. However, defects in ER activities (i.e. ER stress) can trigger cell death [5]. The precise molecular mechanisms governing the switch from physiological ER functions to ER stress, and the crosstalk between stressed ER and death machinery remains incompletely understood. ER stress evoked increases in cytosolic Ca^{2+} , and subsequent sustained CaMKII activation was a key mechanism of ER stress induced apoptosis in cholesterol loaded macrophages [22]. Under these conditions, CaMKII was activated by Ca^{2+} overload and oxidation, and contributed to 3 pro-apoptotic pathways. In the first pathway CaMKII directly activated the C-Jun N-terminal kinase (JNK) pathway, resulting in the upregulation of FAS ligand and the activation of the extrinsic apoptotic pathway. The second pathway was facilitated mitochondrial Ca^{2+} uptake by CaMKII that resulted in activation of the intrinsic apoptotic pathway with loss of Ψ_m and release of apoptogens, including cytochrome C. In cholesterol loaded macrophages, oxidized CaMKII γ appeared to selectively partition into mitochondria, and CaMKII $\gamma^{-/-}$ mice were protected from apoptosis by this pathway. The third independent pathway involved the signal transducer and activator of transcription 1 (STAT1) activation, a proapoptotic signal. This CaMKII mediated ER stress elicited cell death is also observed in other tissues and organs, including myocardium. CaMKII inhibition prevents ER stress induced cardiac dysfunction in a tunicamycin induced cardiomyopathy model, although whether the pathways described in cholesterol loaded macrophages are implicated in this model remains to be elucidated [49]. CaMKII appears to link excessive oxidant stress with ER stress and mitochondrial dysfunction in a mouse model of polycystic kidney disease, [50] suggesting this pathway may contribute to multiple diseases. Taken together, CaMKII activation either by Ca^{2+} overload or oxidation, in the setting of ER stress, appears to be a key event driving the transition from ER adaptation to ER stress triggered apoptosis, and CaMKII activation is one of the crucial mediators in the crosstalk between stressed ER and mitochondria.

CaMKII, inflammation and apoptosis

Excessive inflammation following ischemia-reperfusion injury or myocardial infarction exacerbates myocyte death and worsens cardiac function. Several lines of evidence consistently showed CaMKII augments myocardial inflammatory responses after myocardial infarction and ischemia-reperfusion injury [21, 25]. We performed a gene expression profiling study on hearts isolated from mice with myocardial specific transgenic expression of a CaMKII inhibitory peptide (AC3-I) subjected to MI [21]. Interestingly, CaMKII inhibition substantially reduced the myocardial infarction-triggered expression of a constellation of proinflammatory genes. The activity of NF- κ B, a master regulator of

inflammation, is augmented after myocardial infarction and suppressed by CaMKII inhibition. Similarly, nuclear NF- κ B mediated CaMKII activation augmented myocardial ischemia-reperfusion injury [25]. Activated CaMKII in cardiomyocytes following ischemia-reperfusion injury led to I κ B kinase phosphorylation and concomitant increases in nuclear p65, heightened inflammation, marked by increased monocyte infiltration and elevated monocyte chemoattractant protein-1 (MCP-1), and apoptosis [25]. NF- κ B activation, increased inflammatory response and apoptosis following MI are blunted in CaMKII δ knockout mice (*Camk2^{-/-}*) and in mice with myocardial transgenic expression of AC3-I or CaMKIIN. [21, 51] Increased inflammatory response by CaMKII in I/R injury was also observed in another independent study [28]. However, this study showed that CaMKII mediated inflammation played little role in the first five days following I/R injury, but was essential in chronic ventricular remodeling after I/R injury. In this study, *Camk2^{-/-}* mice showed a similar increase in cell death in the acute phase, but displayed improved cardiac function 5 weeks after I/R injury. This improvement was associated with an attenuated inflammatory response, evidenced by decreased leukocyte infiltration and reduced expression of members of the chemokine (C-C motif) ligand family, in particular CCL3 (macrophage inflammatory protein-1 α , MIP-1 α). Overall, CaMKII appears to be an important mediator for integrating neurohormonal signaling and inflammatory responses in MI or I/R injury. This CaMKII mediated inflammatory response is, at least in part, cardiac myocyte dependent, as cardiac specific CaMKII knockout or myocardial transgenic overexpression of CaMKII inhibitory peptide were employed in the studies discussed here.

The role of CaMKII in myocardial necroptosis

Necrosis has been regarded as a passive and unregulated process. However, this concept has been challenged by a growing body of work, strongly suggesting that a subset of necrosis is also regulated or programmed. The recognition of programmed necrosis shifted the focus on cardiac myocyte death in cardiovascular disease, and emerging evidence indicates that programmed necrosis or necroptosis also plays a critical role in the pathogenesis of common cardiovascular diseases [4].

The best characterized necroptosis molecular pathway is partially overlapping with apoptosis, and shares death-domain containing receptors including TNF- α , Fas receptor, and the TRAIL receptor [52, 53]. After the engagement of a pro-necroptotic ligand to its death receptor, the receptor recruits multiple proteins to form a complex mainly consisting of cellular inhibitor of apoptosis proteins (cIAPs), TNFR-associated death domain (TRADD), receptor-interacting protein 1 (RIP1) [54] and TNFR-associated factor 2 (TRAF2), which is different from DISC (Death Inducing Signaling Complex) [55]. This protein complex evolves to the necroptosome after disassociation from the death receptor, and association of proteins, including, RIP1 and RIP3 [52, 53, 56, 57]. Necrostatin, a RIP1 inhibitor, has been shown to protect against myocardial ischemia-reperfusion injury [58, 59]. The downstream signaling pathways of RIP1/RIP3 complex are less understood, but are increasingly studied. Interestingly, CaMKII was recently found to be one of the targets of RIP3 kinase, and RIP3 deletion was broadly protective against acute and chronic ischemia-reperfusion injury, and doxorubicin induced cardiomyopathy. The RIP3 knock out mice (*Ripk3^{-/-}*) displayed attenuated myocyte death, including necrosis and apoptosis, and improved cardiac function

and survival after ischemia-reperfusion injury or doxorubicin challenge [26]. CaMKII phosphorylation, oxidation and activity were blunted in *Ripk3*^{-/-} mice subjected to ischemia-reperfusion injury or doxorubicin. In cultured neonatal myocytes, CaMKII phosphorylation was increased with overexpression of RIP3, and CaMKII inhibition either by dominant-negative CaMKII or KN-93, a small molecule CaMKII inhibitor, decreased cell death in the setting of RIP3 over-expression. Intriguingly, RIP3 directly binds and phosphorylates CaMKII. RIP3 co-localized with CaMKII in myocytes and a co-immunoprecipitation study showed the binding of RIP3 and CaMKII was enhanced in ischemia-reperfusion injury or after doxorubicin challenge. Importantly, in a cell-free recombinant protein system, RIP3 kinase directly phosphorylated recombinant CaMKII protein at the site of T287, the autophosphorylation site [26]. This is the first evidence, to our knowledge, that a kinase, other than CaMKII itself, is able to directly phosphorylate CaMKII. Thus, RIP3 contributes to myocardial necroptosis by acting as a CaMKII kinase.

A growing body of evidence suggests that the opening of mPTP is part of a final pathway in programmed necrosis. siRNA mediated knockdown of Cyclophilin D, a protein that increases the opening probability of the mPTP, markedly protected against RIP3 induced cardiomyocyte necrosis. Overexpression of RIP3 led to depolarization of Ψ_m in a Cyclophilin D-dependent manner, and RIP3 deficiency protected the heart from ischemia-reperfusion injury and doxorubicin-induced loss of Ψ_m . Inhibition of CaMKII blocked RIP3-induced Ψ_m depolarization. Thus, this line of evidence indicated mPTP is a critical downstream effector of the RIP3-CaMKII necroptosis pathway. While this and other studies [29] indicate that CaMKII increases mPTP opening, the identity of the CaMKII targets remains unknown. Taken together, these findings suggest CaMKII importantly contributes to cardiovascular disease by RIP3 activation and that inhibition of the RIP3-CaMKII pathway inhibition may be an attractive candidate therapeutic target.

The role of oxidized CaMKII in myocardial apoptosis

Both calcium and reactive oxygen species (ROS) play a crucial role in pathogenesis of cardiac diseases. CaMKII, a kinase originally identified by Ca^{2+} and calmodulin dependent activation, is persistently activated through the oxidation of a pair of methionines in its regulatory domain (at positions M280/281), independent of Ca^{2+} and calmodulin binding [20]. Oxidative activation of CaMKII (ox-CaMKII) occurs via a variety of upstream ROS resources, including NAPDH oxidases (NOXs) and mitochondria. Angiotensin II [20, 60] and aldosterone [61] are each capable of oxidizing CaMKII by activation of Nox2. This AngII/Nox2/ox-CaMKII pathway is essential to angiotensin II induced cardiomyocyte apoptosis. CaMKII inhibition, over-expression of methionine sulfoxide reductase A (MsrA), a reductase capable of reversing the first oxidation step for methionines (i.e. to a sulfoxide) and loss of p47, a key component of Nox2 in myocardium, all protected against angiotensin II induced apoptosis. MsrA knockout mice (*MsrA*^{-/-}) infused with angiotensin II exhibit elevated ox-CaMKII, extensive apoptosis, rapidly accelerated cardiac dysfunction and increased mortality. The same pattern is observed in *MsrA*^{-/-} mice subjected to myocardial infarction. [20] This angiotensin II/Nox2/ox-CaMKII pathway can contribute to apoptosis of cardiac pacemaker cells, leading to sinus node dysfunction. [60] Our group developed knockin mice where CaMKIIδ M280/281 were replaced by valines (MMVV) in order to

directly determine the impact of ox-CaMKII on cardiovascular disease *in vivo*. Patients with diabetes who suffer myocardial infarction showed increased myocardial ox-CaMKII compared to non-diabetic patients after myocardial infarction. Mice with streptozotocin (STZ)-induced diabetes and subjected to myocardial infarction, displayed increased ox-CaMKII, profound bradycardia secondary to sinoatrial nodal pacemaker cell apoptosis, loss of normal heart rate variability, and markedly increased mortality. STZ-treated, ox-CaMKII resistant MMVV mice showed substantially improved pacemaker cell survival, preserved heart rate and elimination of diabetes attributable increases in mortality after myocardial infarction. [24] Interestingly, in this STZ induced diabetes model [62], and in heart failure model with Gαq protein overexpression [63], CaMKII was also a crucial mediator of ROS production, suggesting the possibility that ox-CaMKII contributes to a ROS induced ROS positive feedback loop. Importantly, pro-oxidant environments can enhance the sensitivity of CaMKII activation by Ca²⁺ and calmodulin, so that CaMKII activation occurs at resting or ambient intracellular Ca²⁺ levels [64].

Many questions arise with the finding of ox-CaMKII induced cell death. Does the ox-CaMKII induced cell death share the same upstream and downstream pathways of cell death linked to autophosphorylation? How do upstream signals, such as ROS, *O*-GlcNAcylation, and Ca²⁺, interact to activate CaMKII? Are these activation pathways hierarchical, and is the fidelity of regulatory pathways (e.g. MsrA for ROS, RIP3 for autophosphorylation and OGA for *O*-GlcNAcylation) preserved in the setting of multiple CaMKII activating post-translational modifications? Do the various post-translationally modified forms of CaMKII have the same subcellular domain localization and protein targets?

ox-CaMKII in inflammation and apoptosis

Oxidative stress and ROS production are associated with inflammation and in the context of disease with excessive inflammatory response. Toll-like receptor (TLR) activation promotes NF-κB-dependent inflammatory transcription and oxidative injury in myocardium after myocardial infarction. Our group demonstrated that ox-CaMKII is a mediator of TLR-4 signaling induced myocardial injury following myocardial infarction [51]. TLR-4 activation by lipopolysaccharide, or endotoxin, induces a ROS burst followed by elevated ox-CaMKII in cardiomyocytes. The myeloid differentiation protein 88 (MyD88) is an adapter protein critical for TLR-4 signaling. MyD88 knockout mice subjected to myocardial infarction or lipopolysaccharide injection showed decreased ox-CaMKII, attenuated pro-inflammatory gene expression, reduced myocyte apoptosis, reduced infarction size and improved cardiac function. Thus, ox-CaMKII may be increased by a diverse array of upstream signals associated with oxidant stress, and contribute to inflammation and apoptosis.

CaMKII activation pathways in myocardial necroptosis

ROS is a critical determinant of necroptosis. TNFα treatment induced necroptosis is associated with increased ROS production and treatment with the ROS scavengers, butylated hydroxyanisole (BHA) or N-acetylcysteine (NAC), delays TNFα-induced necroptosis in several cell types [65]. Inhibition of mitochondrial complexes I and II reduce ROS and protect L929 cells against TNF-induced necroptosis [66]. In addition to mitochondrial ROS, RIP1 and RIP3 also can directly activate NADPH oxidases, which is involved in the

execution of necroptosis [67]. The production of ROS following TNF α treatment is decreased by RIP1 or RIP3 ablation [56]. RIP3 knock out mice subjected to ischemia-reperfusion injury or exposed to doxorubicin were protected from increases in ROS, ox-CaMKII and autophosphorylated CaMKII [26]. In cultured neonatal cardiomyocytes, Nox2 knockdown or Tiron (a ROS scavenger) treatment, ameliorated RIP3 overexpression induced cell necroptosis [26]. CaMKII δ overexpression augmented RIP3 induced necroptosis, but CaMKII mutants resistant to autophosphorylation (T287A) or oxidation (MMVV) attenuated RIP3 induced necroptosis [26]. Cells expressing CaMKII lacking the oxidation and autophosphorylation sites were completely protected against RIP3 mediated necroptosis [26]. Thus, both CaMKII phosphorylation and oxidative activation of CaMKII seem to contribute to RIP3 mediated necroptosis.

Selective participation of subcellular CaMKII compartments in programmed cell death

Under physiological conditions, mitochondrial Ca²⁺ uptake is part of a metabolic response pathway for increasing activity of the tricarboxylic acid cycle to increase reducing equivalents, mostly in the form of NADPH, and thereby enhance oxidative phosphorylation and ATP production. However, excessive mitochondrial Ca²⁺ triggers apoptosis or programmed necrosis through mPTP opening. We identified CaMKII as a component of the mitochondrial matrix, and generated transgenic mice with myocardial and mitochondrial targeted expression of CaMKIIN (mtCaMKIIN) to determine if mitochondrial CaMKII activity could participate in myocardial injury responses [29]. Hearts from mtCaMKIIN mice were resistant to ischemia reperfusion injury, myocardial infarction, and isoproterenol toxicity. These mtCaMKIIN hearts displayed reduced myocardial infarction size and increased myocardial performance after ischemia-reperfusion injury. Isolated cardiomyocytes from mtCaMKIIN mice were protected from mPTP opening and loss of Ψ_m in response to Ca²⁺ challenge. We found that mitochondrial CaMKII bound to the mitochondrial Ca²⁺ uniporter (MCU) and identified 2 CaMKII catalyzed phosphorylation sites of CaMKII on MCU (serine 57 and 92). We developed evidence that CaMKII increased MCU current. However, this last finding is controversial and we are continuing to evaluate mitochondrial targets that explain CaMKII actions. Two recent studies employing cardiac specific MCU knockout mice (*Mcu*^{-/-}) showed MCU deletion prevented mPTP opening and ischemia-reperfusion injury *in vivo*. [68, 69] The *Mcu*^{-/-} mice had deficient exercise responses, consistent with the known metabolic benefits of physiological increases in mitochondrial matrix Ca²⁺. In contrast, mice with constitutive, global loss of MCU showed mild decreases in exercise capacity and strength but no protection from ischemia-reperfusion injury [70]. We developed mice with loss of myocardial MCU activity by α -myosin heavy chain promoter driven expression of a dominant-negative MCU, due to charge reversal mutations in the pore forming domain [71]. These dominant negative MCU mice showed reduced heart rate adaptation to stress [72], increased oxygen consumption and diminished ATP production in response to physiological stress, confirming that MCU mediated mitochondrial Ca²⁺ uptake is essential to metabolic 'fight or flight' responses. Similar to findings in mice with constitutive loss of MCU, the dominant negative MCU transgenic hearts were not protected from ischemia-reperfusion injury despite preservation of the Ψ_m and reduced ROS production. The dominant negative MCU mice had elevations in cytosolic Ca²⁺ that was repaired by cell dialysis of ATP, suggesting that metabolic deficiency, rather

than loss of mitochondrial Ca^{2+} buffering was primarily responsible for defective Ca^{2+} homeostasis and impaired mechanical performance. Chronic loss of MCU led to widespread alterations in gene transcription in dominant negative MCU hearts. Notably, expression of Bax was significantly increased, potentially contributing to the lack of improved survival after myocardial ischemia. Taken together, these findings suggest that mitochondrial CaMKII contributes to cell death and mitochondrial CaMKII inhibition protects against clinically relevant models of myocardial injury and death by incompletely understood mechanisms. Timing and duration appear to be critical variables determining the consequences of loss of MCU current.

Nuclear CaMKII plays a critical role in transcriptional regulation in response to pathological stress. Nuclear CaMKII activation phosphorylates and exports class IIa HDACs [73]. The inhibition of Class IIa HDACs by nuclear CaMKII contributes to hypertrophic gene activation and fetal gene reprogramming in cardiac hypertrophy and heart failure. CaMKII δ has more than 10 splicing variants [74, 75]. Among them, CaMKII δ_B and CaMKII δ_C are present in cardiac myocyte and have been extensively studied. CaMKII δ_B and CaMKII δ_C possess similar catalytic activity and Ca^{2+} and calmodulin binding affinity. CaMKII δ_B contains a nuclear localization sequence that is absent in CaMKII δ_C . Overexpression of CaMKII δ_B and CaMKII δ_C with GFP-Tag Showed that CaMKII δ_B is predominantly localized in the nucleus whereas CaMKII δ_C is localized mainly in cytosol [34]. Although CaMKII δ_B and CaMKII δ_C have distinct distribution in myocytes, it should be kept in mind that the localization of both subtypes is not exclusively restricted to nuclear or cytosol. The lack of high fidelity nuclear targeting by CaMKII δ_B may be because the CaMKII NLS has multiple serines that when phosphorylated prevent nuclear targeting. [76]

CaMKII δ_B and CaMKII δ_C have opposite impact on cardiomyocyte death. Overexpression of CaMKII δ_B protected against doxorubicin induced cardiomyocyte apoptosis and doxorubicin induced cardiomyopathy. CaMKII δ_B induced cardioprotection appears to be related to promotion of GATA4 binding to the promoter of *Bcl2*, an antiapoptotic gene, thus increasing the expression of BCL2 protein [77]. This CaMKII δ_B induced prosurvival signaling pathway may contribute to cardiac progenitor cell survival, commitment and differentiation [78]. CaMKII δ_B can also protect cardiac myocytes against oxidative stress, angiotensin II or hypoxia induced apoptosis and myocardial ischemia/reperfusion injury [79]. The underlying mechanism of this protection appears to be mediated by promotion of inducible heat shock protein 70 (HSP70) expression through phosphorylation and activation of heat shock factor 1 (HSF1) [79]. Increased HSF1/HSP70 was not observed in CaMKII δ_C overexpression. However, CaMKII δ_B over-expression also induces modest myocardial hypertrophy, so is not entirely beneficial [80]. In contrast to CaMKII δ_B over expression models, CaMKII δ_C over expression promotes cell death and sensitizes myocardium to a variety of stress conditions [31].-Despite these opposing actions, it seems clear that the total deletion of CaMKII δ is beneficial in pathological disease, at least in mouse models [13, 14].

CaMKII isoforms may heteromultimerize to form the holoenzyme, and the subcellular distribution of CaMKII δ holoenzymes depends on the ratio of the B and C splice variants [34]. Importantly, the spatial and functional specificity of CaMKII δ appears to be determined by the selective mobilization of subcellular Ca^{2+} stores [34]. For example,

phenylephrine stimulation induced HDAC4 nuclear exportation by activation of CaMKII δ C, the cytosolic isoform of CaMKII δ . Caffeine evoked Ca²⁺ SR release was able to activate CaMKII δ _B, the nuclear isoform of CaMKII δ , and subsequently phosphorylated phospholamban. It will be important to better understand the function of potential subcellular pools of CaMKII. However, there is now broad agreement that CaMKII inhibition, without subcellular targeting, is protective against cardiac injury. Thus, it seems unlikely to us that subcellular targeting of CaMKII inhibition will be necessary to evolve new CaMKII inhibitor therapies.

In summary, emerging evidence shows CaMKII is a nodal signal promoting cardiac myocyte death. CaMKII plays a central role, in part, because it is a convergence point for β -adrenergic receptor signaling, ROS, hyperglycemia, G α q protein coupled signaling (e.g. endothelin-1, angiotensin II), mineralocorticoid receptor signaling, TLR signaling and signaling by pro-death cytokines. Many questions remain to be answered regarding the upstream and downstream pathways and subcellular domains participating in CaMKII induced cell death.

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Highlights

- CaMKII is activated and contributes to cardiomyocyte death by β -adrenergic signaling, Gq protein coupled receptor signaling, ROS, intracellular Ca^{2+} , and pro-death cytokines
- CaMKII elicits cardiac myocyte apoptosis in both intrinsic and extrinsic apoptotic pathway
- CaMKII is an essential mediator of RIP3 induced myocardial necroptosis
- Both Ca^{2+} /calmodulin dependent activation and oxidative activation of CaMKII play a critical role in programmed cell death

Fig 1. Activation pathways and subunit structure of CaMKII
(top right and bottom) CaMKII monomers consist of an N-terminal catalytic domain, a central regulatory domain and a C-terminal association domain. The regulatory domain is bound to the catalytic domain under resting conditions, locking the kinase in the inactive state. The regulatory domain contains a calmodulin binding site, and a rise in intracellular Ca^{2+} promotes binding of Ca^{2+} /calmodulin to this region, releasing the catalytic domain and activating the enzyme, (bottom right) Ca^{2+} /calmodulin autonomous activation of CaMKII occurs by post-translational modifications. There are multiple post translational modification sites in the regulatory domain (table).

Figure 2. Molecular mechanisms of CaMKII mediated myocardial necroptosis

The engagement of tumor necrosis factor alpha (TNF α) with its receptor leads to the formation of necroptosome, mainly consisting of receptor-interacting serine/threonine-protein kinase 1 and 3 (RIP1 and RIP3). RIP3 activation induces CaMKII activation through direct phosphorylation of CaMKII at threonine 287 (P-CaMKII, CaMKII numbering) or oxidation of CaMKII via NOX2 stimulation (ox-CaMKII) at methionines 281 and 282. Both P-CaMKII and ox-CaMKII contribute to myocardial necroptosis by triggering mitochondrial permeability transition pore (mPTP) opening, which leads to the collapse of the inner mitochondrial membrane potential ($\Delta\Psi_m$). The mechanism of CaMKII biased mPTP opening is unclear, but CaMKII induced mitochondrial Ca²⁺ uniporter (MCU) hyperactivity and Ca²⁺ overload may contribute.