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Review

Chasing cardiac physiology and pathology down the CaMKII cascade

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Mattiazzi A, Bassani RA, Escobar AL, Palomeque J, Valverde CA, Vila Petroff M, Bers DM. Chasing cardiac physiology and pathology down the CaMKII cascade. *Am J Physiol Heart Circ Physiol* 308: H1177–H1191, 2015. First published March 4, 2015; doi:10.1152/ajpheart.00007.2015.—Calcium dynamics is central in cardiac physiology, as the key event leading to the excitation-contraction coupling (ECC) and relaxation processes. The primary function of Ca^{2+} in the heart is the control of mechanical activity developed by the myofibril contractile apparatus. This key role of Ca^{2+} signaling explains the subtle and critical control of important events of ECC and relaxation, such as Ca^{2+} influx and SR Ca^{2+} release and uptake. The multifunctional Ca^{2+}-calmodulin-dependent protein kinase II (CaMKII) is a signaling molecule that regulates a diverse array of proteins involved not only in ECC and relaxation but also in cell death, transcriptional activation of hypertrophy, inflammation, and arrhythmias. CaMKII activity is triggered by an increase in intracellular Ca^{2+} levels. This activity can be sustained, creating molecular memory after the decline in Ca^{2+} concentration, by autophosphorylation of the enzyme, as well as by oxidation, glycosylation, and nitrosylation at different sites of the regulatory domain of the kinase. CaMKII activity is enhanced in several cardiac diseases, altering the signaling pathways by which CaMKII regulates the different fundamental proteins involved in functional and transcriptional cardiac processes. Dysregulation of these pathways constitutes a central mechanism of various cardiac disease phenomena, like apoptosis and necrosis during ischemia/reperfusion injury, digitalis exposure, post-acidosis and heart failure arrhythmias, or cardiac hypertrophy. Here we summarize significant aspects of the molecular physiology of CaMKII and provide a conceptual framework for understanding the role of the CaMKII cascade on Ca^{2+} regulation and dysregulation in cardiac health and disease.

Ca^{2+}; CaMKII; ischemia/reperfusion; cell death; arrhythmias; hypertrophy

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Calcium signaling is central in cardiac physiology and the link of the different steps of excitation-contraction coupling (ECC) and relaxation mechanisms. Moreover, the multifunctional Ca^{2+}-calmodulin-dependent protein kinase II (CaMKII) is a signaling molecule that regulates ECC in the heart but is also involved in several cardiac diseases. The present review proffers an outline of intracellular Ca^{2+} handling in the heart, as well as on CaMKII localization, regulation, and targets in the cardiac myocyte. The review also focuses on Ca^{2+} dysregulation and CaMKII activation in ischemia/reperfusion (I/R), as well as the dual role of CaMKII in this condition (i.e., beneficial in the stunned heart, but detrimental in irreversible I/R injury, leading to apoptosis and necrosis). We also address the importance of ryanodine receptors (RyR2) and their regulation by CaMKII in different proarrhythmic processes, such as those occurring during reperfusion, after acidosis, atrial fibrillation, heart failure (HF), and digitalis intoxication. Finally, the role of CaMKII in cardiac transcriptional regulation is described. We hope that this review may help to provide a solid basis for understanding the importance of the CaMKII cascade in cardiac health and disease.

Overview of Intracellular Ca^{2+} Dynamics

Calcium is a remarkably ubiquitous and versatile intracellular signal, since not only does it trigger and regulate a number of physiological processes but also may play an important role in regulation of its own fluxes among cell organelle and plasma membranes. The primary function attributed to Ca^{2+} in muscle

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cells is to control mechanical activity developed by the myofibril contractile apparatus. In the cardiac muscle, membrane depolarization during the action potential (AP) triggers a transient rise in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) that leads to a phasic contraction. Thus Ca^{2+} dynamics in the cytosol is the most critical event in the ECC process.

In the mature mammalian ventricular myocardium, most of the Ca^{2+} that contributes to an AP-triggered Ca^{2+} transient is released from the sarcoplasmic reticulum (SR) (27), upon binding of Ca^{2+} to RyR2 (Fig. 1), a mechanism identified as Ca^{2+}-induced Ca^{2+} release (38). These channels are homotetramers and are assembled in a macromolecular complex that includes RyR2 regulatory molecules, such as calmodulin (CaM), FK-506 binding proteins (FKBP12/12.6), and protein phosphatases and kinases, including CaMKII (64, 135, 144). Thus Ca^{2+} can regulate the channels by both, direct binding to them and via CaM and CaMKII. During the AP, Ca^{2+} influx through voltage-dependent L-type Ca^{2+} channels (LTCC) causes a rapid and large increase in subsarcolemmal $[Ca^{2+}]$, which results in the synchronized opening of RyR2 clusters and massive Ca^{2+} release to the dyadic space, from which Ca^{2+} diffuses to the bulk cytosol and reaches the myofilaments (Fig. 1). It is accepted that SR Ca^{2+} release during ECC is terminated by closure of the RyR2, attributed to regulation of the channels by intra-SR local free $[Ca^{2+}]$ and proteins directly or indirectly associated with RyR2, such as calsequestrin, triadin, and junctin (47, 59, 158). Because the release is not sustained, the cytosolic Ca^{2+} transient is self-limiting, since the rise in $[Ca^{2+}]_i$ not only activates contraction but also increases the transport rate of mechanisms that remove Ca^{2+} from the cytosol, thus causing $[Ca^{2+}]_i$ to fall and mechanical relaxation to occur. Among these transporters, the most prominent is the SR Ca^{2+}-ATPase (SERCA2a), located in the extra-dyadic SR, which allows refilling the SR Ca^{2+} store (8, 89). SERCA2a is negatively regulated by phospholamban (PLN; Fig. 1). However, this inhibition may be relieved by PLN phosphorylation, which decreases the PLN-SERCA interaction (29, 61). Moreover, the effects of PLN on cardiac function are subjected to additional regulation by its interacting partners (61). The most important Ca^{2+} efflux mechanism is the Na^+-Ca^{2+} exchanger (NCX), which, at steady-state cyclic activity, removes most of the Ca^{2+} that enters the cell during the AP (27, 33), whereas slower mechanisms (the sarcolemmal Ca^{2+}-ATPase and mitochondrial Ca^{2+} influx pathways) do not seem to play a significant role in the decline of electrically triggered Ca^{2+} transients in the mature myocardium (7, 19).

Because the SR is the major Ca^{2+} source for ECC, it is plausible to consider that contraction amplitude largely depends on how much Ca^{2+} is released from it. During a twitch, this organelle releases only a fraction (50–70%) of its total content (6, 10, 117, 118). Several factors seem to determine the fractional SR Ca^{2+} release: *1*) the amplitude of the release trigger, i.e., typically L-type Ca^{2+} current (I_{CaL}) (6); *2*) the SR Ca^{2+} content, especially the free $[Ca^{2+}]$ in the SR lumen (6, 117), since evidence indicates that the RyR2 activity is regulated not only by cytosolic but also by intra-SR Ca^{2+} (26, 47, 60, 65); and *3*) the RyR2 functional state, which can be altered by interaction with proteins (e.g., FKBP12.6, CaM, luminal proteins), divalent cations, ATP and other compounds, by phosphorylation, and by post-translational modifications by reactive oxygen species (ROS) and reactive nitrogen species (30, 47, 64, 91, 144).

The SR Ca^{2+} content available for release during ECC basically depends on the balance between uptake and release rates during the decline of the Ca^{2+} transient and diastole. The rate of diastolic SR Ca^{2+} release (SR Ca^{2+} leak) is low in myocardial cells (9, 14, 74, 157). Diastolic SR Ca^{2+} leak may be augmented by an increase in SR Ca^{2+} load and by other factors that increase the RyR2 activity state, e.g., phosphorylation (14).

SR Ca^{2+} release is not only involved in determining cardiac contractility, but it also can modify the sarcolemmal electrical

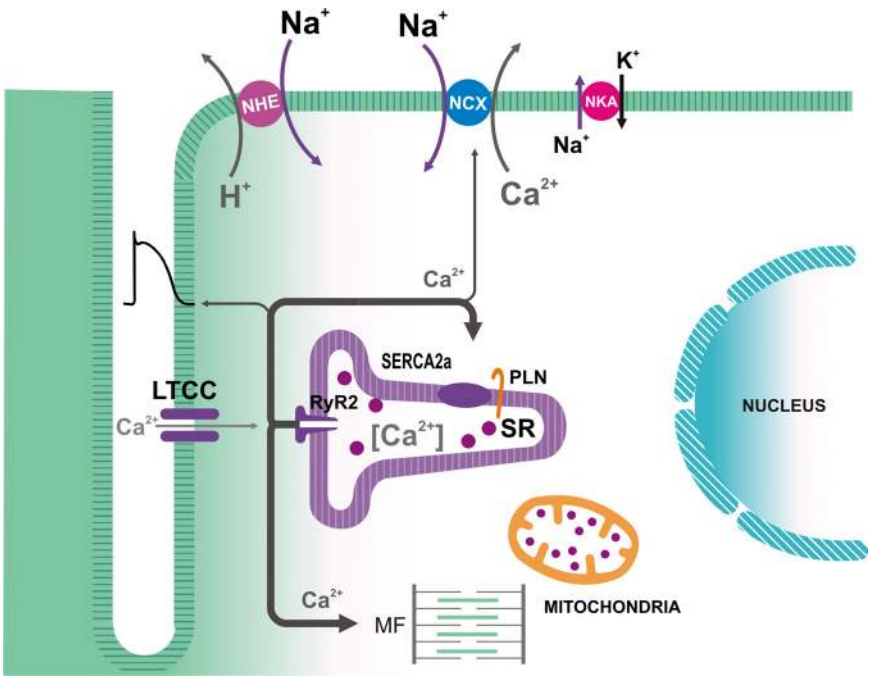

Fig. 1. Ca^{2+} fluxes associated with excitation-contraction coupling in mammalian cardiac myocytes. During the action potential, Ca^{2+} influx via L-type Ca^{2+} channels (LTCC) triggers Ca^{2+} release from the sarcoplasmic reticulum (SR) by Ca^{2+} binding to the ryanodine receptors (RyR2) in the SR membrane. In addition to interacting with the myofilaments (MF), Ca^{2+} is removed from the cytosol mainly by the SR Ca^{2+}-ATPase (SERCA2a), which is regulated by phospholamban (PLN), and by the electrogenic sarcolemmal Na^+/Ca^{2+} exchanger (NCX1), which is driven by the Na^+ electrochemical gradient across the membrane. This gradient is maintained by the Na^+-K^+-ATPase (NKA). Intracellular $[Na^+]$ may also be affected by the operation of the Na^+-H^+ exchanger (NHE).

properties, affecting the AP shape. One obvious mechanism involves Ca^{2+}-dependent inactivation of the LTCC (37, 124): a large Ca^{2+} release will reduce sarcolemmal Ca^{2+} influx, shortening the AP. However, mechanisms involved in activation of Cl^- (66) and/or K^+ (68, 131) channels by Ca^{2+} have also been postulated to modify the AP repolarization phase. Schouten and ter Keurs (115) first showed that the late and relatively negative AP plateau seen in rat ventricular myocytes is driven by Ca^{2+}-dependent inward NCX current. Shattock and Bers (119) detected NCX-dependent Ca^{2+} extrusion by measuring transient interstitial $[Ca^{2+}]_o$ elevation during the $[Ca^{2+}]_i$ transient. More recently, Ferreiro et al. (39) demonstrated that in the intact mouse heart under physiological conditions, Ca^{2+} release from the SR can cause an AP phase 2 that is more positive than that traditionally reported in rat or mouse ventricular myocytes, but that is still mediated by inward NCX current that is driven by SR Ca^{2+} release. Moreover, this more prominent AP plateau in mouse ventricle resembles that observed in both human atrial myocytes and the ventricular epicardium of other mammals (i.e., dog, cat, pig, etc.).

CaMKII Structure, Function, and Targets

Molecular physiology and localization. CaMKII is a multimeric holoenzyme complex consisting of a pair of hexameric assembled rings (Fig. 2*A*). There are four CaMKII gene products: α, β, γ, δ (52). These genes show differential tissue expression, with CaMKIIδ being the predominant isoform in the heart (32), although CaMKIIγ is also present (120). In the adult myocardium, two major splice variants of CaMKIIδ are expressed: CaMKIIδ_B and CaMKIIδ_C (32). CaMKIIδ_B possesses an 11-amino acid nuclear localization signal that is responsible for its preferential nuclear localization, whereas the splice variant δ_C (lacking only this 11-amino acid nuclear localization signal) is preferentially localized in the cytosol (122). It is also well known that most CaMKII isoforms readily form stable hetero-oligomers, such that the ratio of δ_B to δ_C in a multimer could regulate the localization of the holoenzyme (100, 122). However, even when only one splice variant (CaMKIIδ_B or CaMKIIδ_C) is expressed, CaMKIIδ_B is not exclusively nuclear and CaMKIIδ_C is not exclusively cytosolic. Interestingly, the relative expression of CaMKIIδ_B can be altered in vitro by phosphorylation/dephosphorylation processes and has been shown to be modified under different physiological and pathological conditions, suggesting that CaMKIIδ splicing is a highly regulated dynamic process (44). Indeed, recent experimental evidence suggests that CaMKIIδ splice variants are selectively susceptible to autophosphorylation/oxidation, providing CaMKII with a mechanism for target signaling specificity (12).

Mechanisms of CaMKII activation and regulation. As shown in Fig. 2*A*, each CaMKII monomer that composes the holoenzyme consists of three domains: an NH_2-terminus catalytic domain, a COOH-terminus association domain, and a core regulatory domain. Under basal conditions, the catalytic domain is restrained by the pseudo substrate region within the regulatory domain, which hampers the CaMKII catalytic activity. The regulatory domain binds CaM with a K_D of 10–70 nM (41), when intracellular Ca^{2+} concentration is elevated (102). CaM binding to CaMKII generates a conformational shift that releases the association between the catalytic and regulatory domains, exposing the catalytic domain for substrate binding and phosphorylation. If a sustained increase in Ca^{2+}/CaM interaction occurs, the already active CaMKII monomers catalyze the autophosphorylation of the kinase at Thr^{286} (or Thr^{287}, depending on isoform). CaMKII phosphorylation increases the binding affinity of the enzyme for Ca^{2+}/CaM (79), preventing the re-association of the catalytic and regulatory domains (63) and retaining residual Ca^{2+}/CaM-independent or autonomous activity (52). CaMKIIδ can be also oxidized at $MetMet^{281/282}$ ($CysMet^{280/281}$ in CaMKIIα), which induces a similar Ca^{2+}/CaM-independent form of CaMKII (35). Interestingly, oxidation of CaMKII resets its Ca^{2+} sensitivity in such a way that activation of the kinase may occur at very low levels of intracellular Ca^{2+} (93). Indeed, activation of the renin-angiotensin-aldosterone signaling pathway, which promotes enhanced oxidative stress in the heart (87), induces CaMKII-dependent apoptosis of cardiac myocytes in the absence of significant increases in cytosolic Ca^{2+} in vitro (93) and in vivo (136). Moreover, apoptosis induced by the hormone is prevented in isolated neonatal mouse myocytes expressing the oxidation-resistant mutant CaMKII (35). Similarly, in the prediabetic stage induced in a model of impaired glucose tolerance, it has been described that the increase in oxidative stress contributed to CaMKII activation, SR Ca^{2+} leak, and the generation of arrhythmias and apoptosis (94, 121). These findings suggest that conditions of high ROS production may lead to increased CaMKII activity, even in the absence of changes in the basal levels of Ca^{2+}/CaM. Interestingly, recent experiments have described that ROS production may also occur downstream CaMKII activation (92, 116). These experiments suggest that under conditions of high oxidative stress, a vicious cycle of CaMKII activation and ROS production may occur. Further experimental evidence is required to confirm this possibility.

Two additional posttranslational modifications of CaMKII have been recently reported. Erickson et al. (36) described, using overt diabetes cellular and animal models, a novel mechanism for CaMKII activation during hyperglycemia, different from that produced by the oxidation of CaMKII, typical of diabetic patients (71). This mechanism occurs through the addition of an O-linked N-acetylglucosamine (O-GlcNAc) at the Ser^{280} site, which similarly to oxidation and phosphorylation, creates molecular memory after the decline in Ca^{2+} concentration. It has also been shown that nitric oxide production by β-adrenergic stimulation is sufficient, by itself, to activate CaMKII and increase SR Ca^{2+} leak, leading to arrhythmogenic spontaneous Ca^{2+} waves (22, 46, 56). Zhang et al. (153) further showed that nitric oxide-PKG signaling augmented CaMKII activity in rabbit ventricular myocytes. Moreover, in vitro studies showed that CaMKII contains *S*-nitrosylated cysteine residues, and computational prediction of *S*-nitrosylation sites on CaMKII indicates different potential target sites, including the Cys^{290} site in the CaMKIIδ regulatory domain (46). Coultrap and Bayer (21) recently demonstrated that nitrosylation of CaMKIIα at the analogous Cys^{289} and also Cys^{280} (Met^{281} in CaMKIIδ) sites was critical to autonomous CaMKII activation by nitric oxide donors. The cluster of the different regulatory sites at the regulatory/autoinhibitory CaMKII-domain suggests that these sites are part of a hotspot region for post-translational regulation of the kinase.

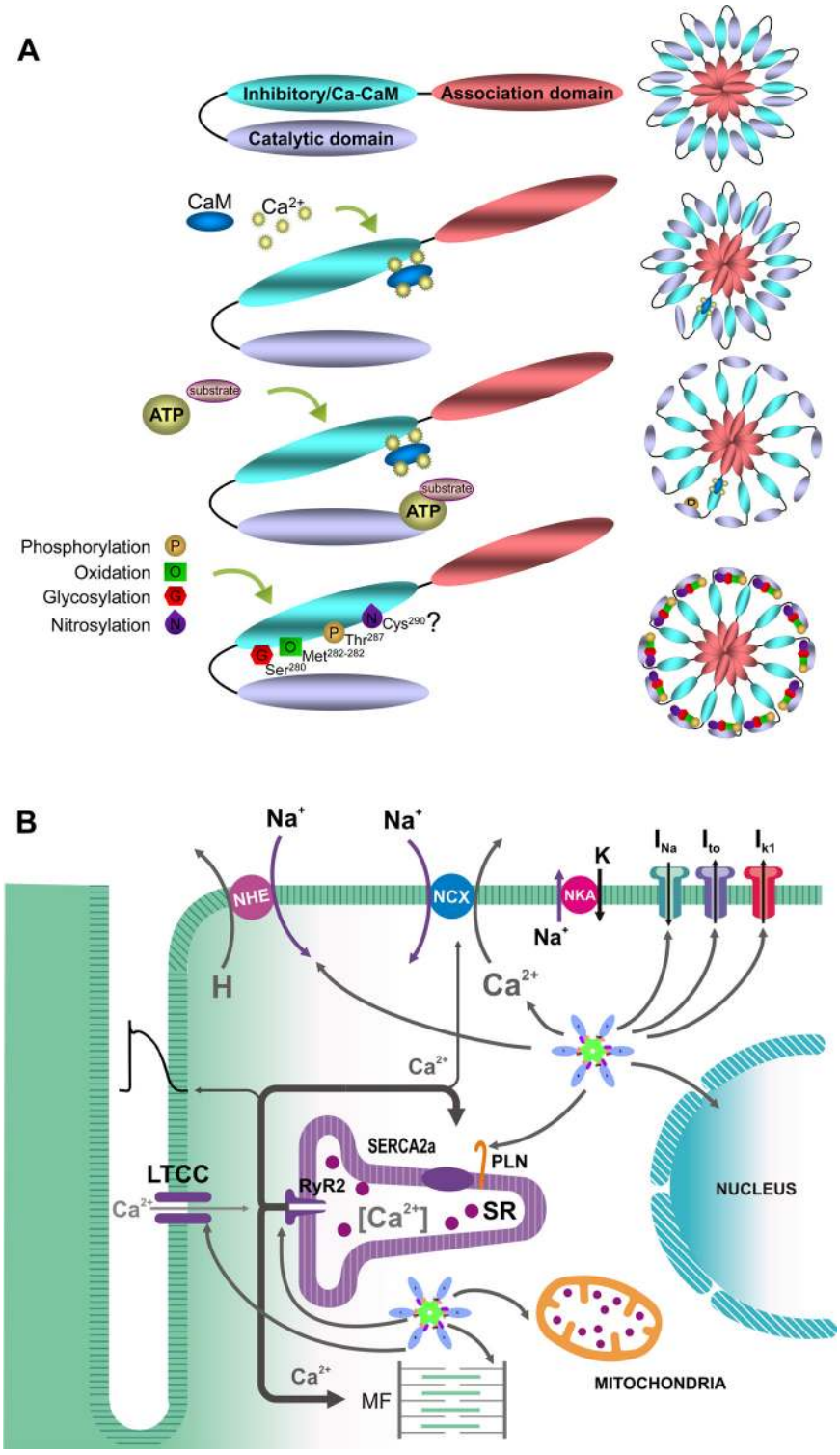

Fig. 2. *A*: schematic representation of CaMKII structure and regulation. See text for description. Note the proximity of sites involved in the sustained regulation of CaMKII. The question mark in Cys^{290} indicates a computationally predicted site of CaMKII nitrosylation [modified from Erickson et al. (35)]. *B*: effects of CaMKIIδ_C on excitation-contraction coupling (ECC). See text for description. I_{Na}, Na^+ current; I_{to}, transient outward K^+ current; I_{K1}, inward rectifier K^+ current.

CaMKII targets in the heart. CaMKII regulates different ion channels and transport proteins involved in cardiac ECC (Fig. 2*B*). CaMKII-dependent phosphorylation of LTCC potentiates I_{CaL} and slows its inactivation (152). Experimental evidence indicates that, in the long term, both δ_B and δ_C CaMKII isoforms decrease the expression of LTCC pore-forming α1c-subunit (101). CaMKII-dependent phosphorylation of PLN increases SR Ca^{2+} uptake, whereas phosphorylation of RyR2 increases diastolic SR Ca^{2+} leak and systolic SR Ca^{2+} release (25, 40, 43, 45, 108, 134, 145). CaMKII also catalyzes phosphorylation of the Na^+-H^+ exchanger (NHE-1) (139), and of the voltage-gated ion channels responsible for Na^+ current (I_{Na}), transient outward K^+ current (I_{to}), and inward rectifier K^+ current (I_{K1}) (67, 142, 143). Persistent (late) inward Na^+ current (I_{NaL}) is enhanced (gain-of-function effect). In contrast, Na^+ channel availability is reduced, intermediate inactivation

is enhanced, and recovery from inactivation of rapid I_{Na} is slowed by CaMKII-dependent phosphorylation (loss-of-function effects) (142). The effects of CaMKII on I_{to} and I_{K1} are complex (67, 143). Acute and chronic CaMKII overexpression increases $I_{to,slow}$ amplitude and expression of the underlying channel protein $K_{V1.4}$. On the other hand, chronic but not acute CaMKII overexpression causes downregulation of $I_{to,fast}$, as well as of $K_{V4.2}$ and KChIP2. Interestingly, these amplitude changes were not reversed by acute CaMKII inhibition, consistent with CaMKII-dependent regulation of channel expression and/or trafficking (143).

It has also been shown that the overexpression of CaMKIIδ_B led to an increase in NCX abundance and disruption of the NCX/SERCA2 expression balance via class IIa histone deacetylase (HDACs)/myocyte enhancer factor-2 (MEF2)-dependent signaling (70). Moreover, available data indicates that NCX upregulation induced by β-adrenoceptor stimulation is dependent on CaMKII activation in the adult heart (72).

Further work demonstrated that CaMKII interacts with the mitochondrial Ca^{2+} uniporter (MCU) and promotes Ca^{2+} entry into the mitochondria, probably by catalyzing phosphorylation of serine residues 57 and 92 (57). Finally, two cardiac myofilament proteins are known to be phosphorylated by CaMKII. Cardiac myosin binding protein C can be phosphorylated at Ser^{282} and Ser^{302} [which are also protein kinase A (PKA) targets], although the functional effects of this phosphorylation remain to be resolved (82, 105). The spring region of the giant sarcomeric protein titin, a main determinant of diastolic stiffness, is also a target of CaMKIIδ. Interestingly, it has been shown that phosphorylation of this protein (possibly at the N2B element) increased during ischemia/reperfusion (49). As we further discuss next, it is now known that alterations in the phosphorylation of most of these proteins and transporters are crucially involved in the genesis of myocardial injury and arrhythmias.

Ca^{2+} and CaMKII in I/R

Ca^{2+} dysregulation in I/R. Ischemic heart disease, a leading cause of mortality worldwide, is invariably characterized by impaired cardiac function and disturbed Ca^{2+} homeostasis. Earlier experiments revealed an increase in diastolic $[Ca^{2+}]_i$ during ischemia (73, 80, 103, 132). This increase has been related to a diversity of concurrently altered Na^+-dependent (55, 85, 99, 128, 129) and independent mechanisms (88, 123, 125). The core of these changes essentially lies on the oxygen deprivation produced by blood flow reduction and the consequent shift from aerobic to anaerobic metabolism (86). Fluorescent detection of cytosolic and SR Ca^{2+} transients at the epicardial layer of the intact beating heart (78) demonstrated

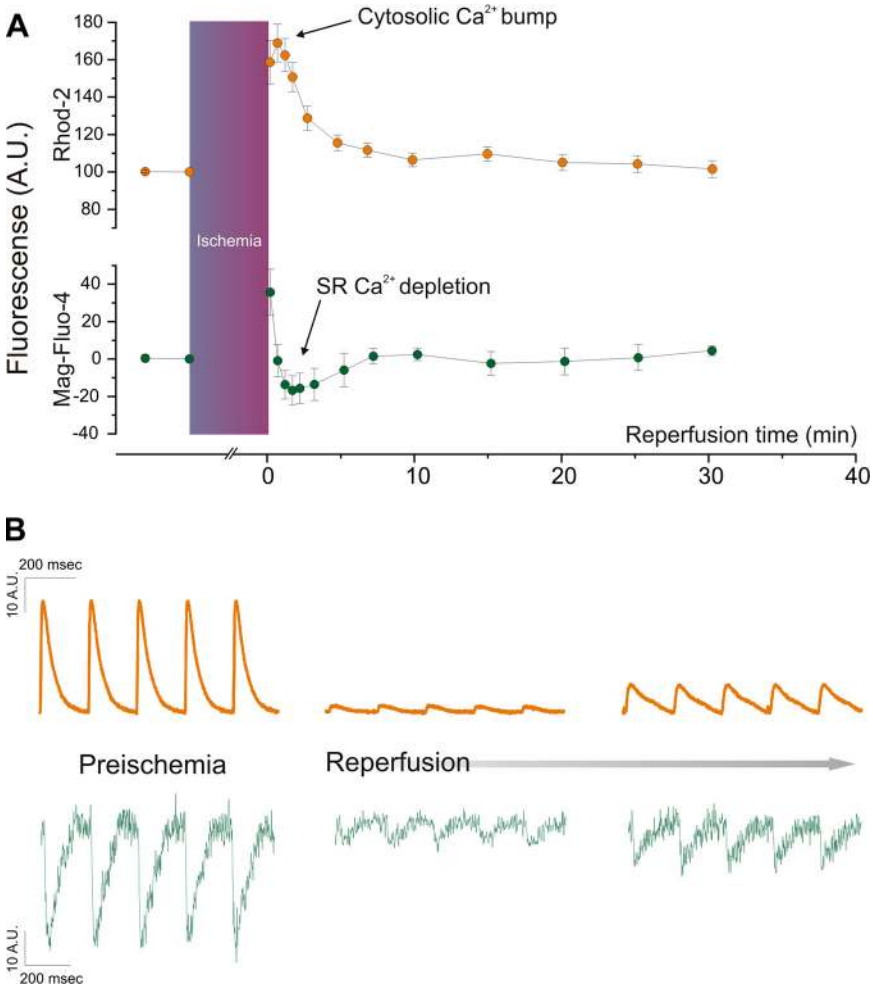

Fig. 3. *A*: increase in diastolic $[Ca^{2+}]_i$ at the onset of reperfusion. At the onset of reperfusion, there is an abrupt increase in diastolic $[Ca^{2+}]_i$ (Ca^{2+} bump) associated with a mirror-like image of the decrease in SR Ca^{2+} content. Mean values are from individual signals recorded at the epicardial layer of intact hearts loaded with Rhod-2 and Mag-Fluo-4, respectively [modified from Valverde et al. (132)]. *B*: typical records showing the decrease in SR Ca^{2+} content associated with a diminished Ca^{2+} transient amplitude, after the Ca^{2+} bump. AU, arbitrary units.

that the increase in cytosolic $[Ca^{2+}]_i$ during ischemia is associated with an enhancement of SR Ca^{2+} load (132). The increased SR Ca^{2+} content was released at the onset of reperfusion, producing an abrupt rise in cytosolic $[Ca^{2+}]_i$ (Ca^{2+} *bump*) (Fig. 3*A*), and the subsequent decrease in SR Ca^{2+} content was associated with a diminished Ca^{2+} transient amplitude (132) (Fig. 3*B*). More recent experiments further showed that a major mechanism for the increase in diastolic $[Ca^{2+}]_i$ during ischemia is an increase in the frequency of Ca^{2+} sparks. Notably, the increase in Ca^{2+} sparks during ischemia switched to an increase in arrhythmogenic Ca^{2+} waves during reperfusion (74) (Fig. 4).

Activation of CaMKII in I/R. Previous studies showed the time course of phosphorylation of Thr^{17} of PLN, used as a marker of CaMKII activation, during I/R. This initial work showed a significant increase in Thr^{17} phosphorylation at the beginning of ischemia and at the onset of reflow (141). Experimental evidence reveals that Ca^{2+} influx through LTCC and phosphatase inhibition, due to the ischemia-induced intracellular acidosis, play a central role in the activation of CaMKII at the beginning of ischemia (83, 84).

The increase in Thr^{17} phosphorylation at the onset of reperfusion may be produced by the transient increase in cytosolic $[Ca^{2+}]$ that occurs at this time (86). This $[Ca^{2+}]_i$ increase has been usually attributed to the influx of Ca^{2+} through the reverse mode of the NCX (54, 86, 95). However, the abrupt Ca^{2+} release from the SR recently described at the onset of reperfusion (132) may also be greatly involved in the increase in CaMKII activation and PLN Thr^{17} phosphorylation at this moment.

Reperfusion is also associated with ROS generation (15). As described earlier in this review, both Ca^{2+} mishandling and ROS production set an ideal intracellular milieu for activation of CaMKII. Further studies have shown that other CaMKII substrates are also phosphorylated at the beginning of reperfusion, e.g., the Ser^{2814} site of RyR2, which have been shown to play a significant role in reperfusion injury (28, 108), as it will be discussed next.

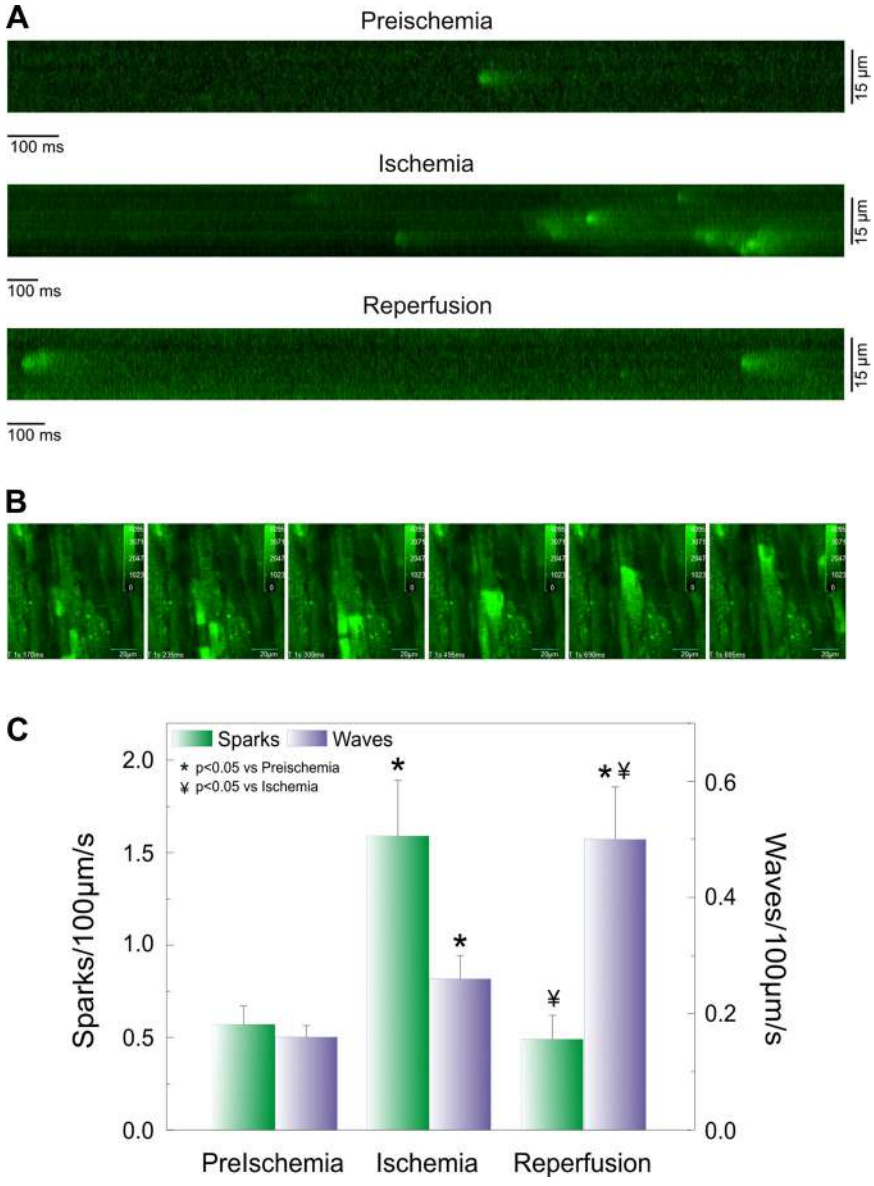

Fig. 4. *A–C*: Ca^{2+} sparks increase during ischemia and turn into Ca^{2+} waves during reperfusion. Typical examples and overall results are shown [modified from Mattiazzi et al. (74)].

Dual role of CaMKII in I/R. In the last few years, a dual effect of CaMKII-dependent protein phosphorylation (beneficial and detrimental) has been described in the scenario of I/R in the intact heart. The beneficial effect of CaMKII refers to the recovery of intracellular Ca^{2+} and contractile function that occurs during stunning (109, 133), a fully reversible postischemic dysfunction (20). It has been shown that phosphorylation of PLN-Thr^{17} is essential for the recovery of Ca^{2+} transients and contractility in the stunned heart (109, 133), offering a mechanism that helps to limit cytosolic Ca^{2+} overload, by accelerating SR Ca^{2+} reuptake and ameliorating intracellular Ca^{2+} handling.

The detrimental effect of CaMKII refers to the role of CaMKII in reperfusion arrhythmias, which occur even after a short ischemic period (11, 108), and to the necrosis and apoptosis typical of the irreversible I/R injury (28, 110, 140). Targeted inhibition of CaMKII at the level of cardiac SR-membranes in mice (SR-AIP) clearly indicated that most of reperfusion arrhythmias are triggered by CaMKII-dependent mechanisms (108). Moreover, prevention of CaMKII-dependent phosphorylation of RyR2 was able to significantly reduce reperfusion arrhythmias (108), but failed to completely prevent them. These findings indicate that other CaMKII targets may be involved in reperfusion arrhythmias. A possible candidate is Thr^{17} of PLN, which is phosphorylated at the beginning of reperfusion in association with the increase in CaMKII phosphorylation of Ser^{2814} of RyR2 (108). Phosphorylation of Thr^{17} and the consequent increase in SR Ca^{2+} reuptake could produce two opposite effects, which are actually inherent to the characteristics of SR Ca^{2+} uptake itself. On one hand, increasing SERCA2a activity would increase the rate of resequestration of the Ca^{2+} released through RyR2. This would reduce cytosolic Ca^{2+} levels, increasing the availability of free cytosolic buffer sites able to bind Ca^{2+} (increase in dynamic cytosolic buffer capacity). This may limit Ca^{2+} wave propagation and reperfusion arrhythmias (4, 53). On the other hand, increasing Ca^{2+} sequestration would necessarily increase SR Ca^{2+} content, favoring diastolic Ca^{2+} leak. This situation would be exacerbated if the increase in SR Ca^{2+} uptake coexists with an increase in the open probability of RyR2, as that produced by CaMKII-dependent phosphorylation (134), and may contribute to favor a futile circle of increased SR Ca^{2+} uptake and leak with an additional metabolic cost. Thus the beneficial effects of the increase in SR Ca^{2+} uptake in I/R may turn to be deleterious under conditions in which the balance between SR Ca^{2+} uptake and leak is lost. Finally, although in the experiments in SR-AIP mice with inhibition of CaMKII targeted to the SR, reperfusion arrhythmias virtually disappeared, phosphorylation of LTCC by CaMKII was also inhibited in these mice (98). Thus the contribution of CaMKII-dependent LTCC phosphorylation to reperfusion arrhythmias cannot be excluded.

After a prolonged ischemic period, reperfusion evokes irreversible cardiac injury. Under these conditions, myocytes die by apoptosis, autophagy, and necrosis. Experimental evidence indicates that CaMKII inhibition is protective in the irreversible I/R injury (28, 110, 140, 154). Although the mechanisms of this protection are still unclear, it has been established that CaMKII is clearly involved in the intrinsic (mitochondrial) cell death pathway (110). This signaling pathway involves CaMKII-dependent phosphorylation of SR protein(s), mitochondrial Ca^{2+} overload, cytochrome *c* release, and caspase-3 activation (28, 110, 140). Notably, this cascade of events mediates not only the programmed cell death known as apoptosis but also a CaMKII-dependent programmed necrosis (110). These deleterious effects appear to be associated with both RyR2 phosphorylation and caspase-mediated degradation of this protein, which in turn would favor an increase in SR Ca^{2+} leak. Supporting and extending the signaling cascade described, Joiner et al. (57) showed that CaMKII-dependent phosphorylation of MCU increases Ca^{2+} entry through it and favors cell death.

Phosphorylation of Thr^{17}, the CaMKII site of PLN, was also transiently enhanced at the onset of reperfusion (110, 140). However, the functional consequences of PLN phosphorylation and of the increase in SR Ca^{2+} uptake after prolonged ischemia are controversial and remain uncertain (90, 126, 127, 151). As discussed for reperfusion arrhythmias, the inconsistent results may reflect the opposite effects of accelerating SR Ca^{2+} reuptake, which diminishes the diastolic $[Ca^{2+}]_i$ elevation produced by increased SR Ca^{2+} leak, but simultaneously increases SR Ca^{2+} load, favoring SR Ca^{2+} leak.

Recent experiments by Di Carlo et al. (28) addressed this puzzle by using mice expressing nonphosphorylatable PLN (i.e., Ser^{16} and Thr^{17} mutated to Ala), submitted to I/R. In these mice, cardiac damage was significantly enhanced, suggesting that increasing Thr^{17} phosphorylation to the level observed at the onset of reperfusion (when phosphorylation of Ser^{16} did not occur) has protective effects. However, when CaMKII-dependent RyR2 phosphorylation was selectively precluded, prevention of PLN phosphorylation failed to increase cardiac injury. Thus the results from Di Carlo et al. (28) strongly suggest that CaMKII-dependent inhibition of RyR2 phosphorylation is necessary and sufficient to prevent CaMKII-dependent cardiac damage that originates at the SR level in I/R. Taken together, these findings indicate that the progression toward a beneficial or detrimental effect of CaMKII activation and PLN phosphorylation in I/R would critically depend on the balance between the extent of SR Ca^{2+} reuptake and the SR Ca^{2+} leak, largely given by the status/characteristics of other proteins also involved in SR Ca^{2+} handling, such as RyR2.

CaMKII, Ryanodine Receptors, and Arrhythmias

CaMKII has been shown to contribute to arrhythmogenesis in cardiac pathologies of different etiology. Although CaMKII-dependent arrhythmogenesis was originally attributed to its impact on sarcolemmal LTCC and the development of early afterdepolarizations, which may indeed occur (1), intense ongoing investigation has provided evidence of multiple additional targets through which CaMKII may exert its arrhythmogenic action. Among these, RyR2 seem to be one of particular functional importance. A CaMKII-dependent increase in RyR2 open probability has been shown to increase SR Ca^{2+} leak, which would enhance Ca^{2+} extrusion via the electrogenic NCX. This electrogenic transport generates a depolarizing current (I_{ti} or transient inward current) (34, 114), which, when sufficiently large, leads to delayed afterdepolarizations (DADs), that may reach the threshold and trigger spontaneous AP, resulting in extra-systoles and ventricular arrhythmias (146) (Fig. 5). Indeed, extensive experimental evidence demonstrates that CaMKII-induced SR Ca^{2+} leak is associated

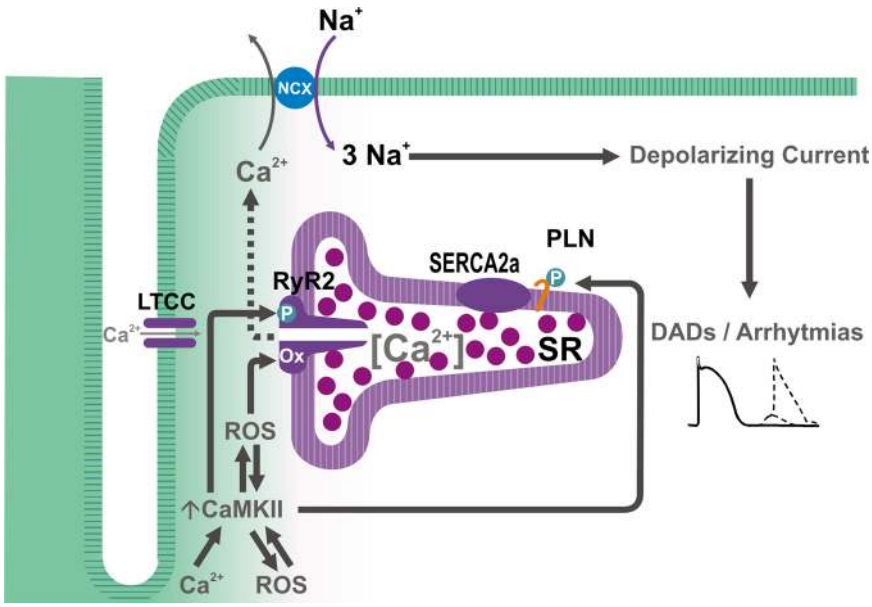

Fig. 5. Scheme of the possible mechanisms of CaMKII-dependent arrhythmias. Increases in $[Ca^{2+}]_i$ and/or reactive oxygen species (ROS) can activate CaMKII, which in turn result in further increase in ROS, leading to the phosphorylation and oxidation of the RyR2 and phosphorylation of phospholamban, which, in concert, would enhance SR Ca^{2+} load and favor SR Ca^{2+} leak, resulting in an NCX-dependent depolarizing current (transient inward current, or I_{ti}), which generates arrhythmogenic delayed afterdepolarization (DADs).

with cardiac arrhythmias. Elegant studies from Wehrens′ laboratory showed that genetic inhibition of CaMKII-dependent RyR2 phosphorylation could prevent atrial fibrillation and lethal ventricular arrhythmias (25, 76). In addition, several studies have suggested that CaMKII-dependent SR Ca^{2+} leak also mediates reperfusion arrhythmias, as already discussed in the context of I/R (13, 108), heart failure-induced arrhythmias (106), digitalis-induced arrhythmias (43, 50), and even arrhythmias of genetic origin, such as catecholaminergic polymorphic tachycardia (69) and those associated with Duchenne muscular dystrophy (2).

Mechanisms underlying CaMKII-dependent regulation of SR Ca^{2+} leak. The magnitude of SR Ca^{2+} leak depends on two main factors: *1*) SR Ca^{2+} load and *2*) RyR2 open probability. CaMKII can modulate SR Ca^{2+} load through the phosphorylation of PLN at site Thr^{17}, which relieves the inhibition of PLN on SERCA2a and increases SR Ca^{2+} uptake (58). CaMKII can also phosphorylate the RyR2 and activate the channel. Indeed, CaMKII was originally shown to phosphorylate the Ca^{2+} release channel at the site Ser^{2809} (148), and more recent studies revealed another phosphorylation site at Ser^{2814} (40, 145). Although the impact of CaMKII phosphorylation on RyR2 function is still a matter of debate, the general consensus indicates that CaMKII increases RyR2 open probability. Whether an independent increase in SR Ca^{2+} load or in RyR2 open probability is able to produce sufficient SR Ca^{2+} leak to induce arrhythmogenic diastolic Ca^{2+} release, is controversial. Ca^{2+} overload of the SR has been reported to trigger spontaneous Ca^{2+} release, at least in part, via the activation of the RyR2 luminal Ca^{2+} sensor (26). However, several lines of evidence suggest that increased SR Ca^{2+} load by itself, is not sufficient to promote arrhythmogenic SR Ca^{2+} release. For example, PLN knock-out mice, which have a fully loaded SR, have not proven to be prone to arrhythmias under basal conditions (111, 155). In addition, Venetucci et al. (138) showed that increasing RyR2 open probability alone does not produce arrhythmogenic diastolic Ca^{2+} release because of the intrinsically accompanying decrease of SR Ca^{2+} content. Thus, although CaMKII-dependent RyR2 phosphorylation may sensitize the RyR2, at basal conditions this can be roughly offset by the lower SR Ca^{2+} content. However, when SR Ca^{2+} content is driven up (e.g., by heart rate, sympathetic activation, or post-ischemic Ca^{2+} overload), the propensity for triggering SR Ca^{2+} sparks and waves leading to DADs and arrhythmias can be dramatically increased.

In addition to phosphorylation, RyR2 function may be enhanced by oxidation at the level of specific methionine residues (16, 130). RyR2 contains multiple thiols (150) that can be affected by redox modification. RyR2 thiol oxidation increases the sensitivity of the channel to luminal Ca^{2+}, thus lowering the critical SR Ca^{2+} content at which spontaneous Ca^{2+} release occurs (130). As mentioned above, CaMKII can also be activated by oxidation. In addition, recent data suggests that CaMKII can enhance ROS production (116). Thus CaMKII may promote arrhythmogenic RyR2 Ca^{2+} leak not only by enhancing RyR2 phosphorylation but also by promoting its oxidation. Consistent with this, Ho et al. (51) showed that arrhythmogenic adverse effects of cardiac glycosides involve alterations in RyR2 function caused by oxidative changes in the channel structure. More recently, Gonano and Vila Petroff (42) demonstrated that cardiac glycoside-induced arrhythmias require CaMKII activation, suggesting that CaMKII-dependent RyR2 oxidation could also participate in the development of the arrhythmogenic substrate. Although the relevance of CaMKII-dependent oxidation of RyR2 remains to be further explored, experiments in knock-in mice in which the site Ser^{2814} of the RyR2 could not be phosphorylated (S2814A mice) provided unequivocal evidence of the importance of phosphorylation of this site in cardiac glycoside-induced arrhythmias (50). Myocytes isolated from S2814A mice did not show enhanced SR Ca^{2+} leak when exposed to digitoxin, compared with myocytes from wild-type mice. These experiments demonstrate that phosphorylation, rather than oxidation of RyR2, is required for the increase in channel spontaneous activity and arrhythmogenesis in the context of digitalis toxicity.

CaMKII has also been shown to be involved in cardiac arrhythmias associated with acidosis. This is important in the clinical setting since substantial changes in pH may occur in

disorders of different origin, such as sleep apnea/hypopnea syndrome, diabetic ketoacidosis, or during episodes of myocardial ischemia. Said et al. (107) showed that ectopic activity produced upon returning to normal pH after acidosis could be prevented by pharmacologic inhibition of CaMKII and did not occur in a transgenic mouse model with the inhibition of CaMKII targeted to the SR. The authors concluded that CaMKII activation during acidosis favors an increase in SR Ca^{2+} load by phosphorylation of PLN Thr^{17}, which, on the one hand, is responsible for the mechanical recovery observed with sustained acidosis, but may also increase spontaneous SR Ca^{2+} leak and produce arrhythmias during the return to normal pH. This effect was attributed to the increase in the opening probability of RyR2 due to the pH increase after acidosis and the acidosis-induced increase in SR Ca^{2+} content, still present at the beginning of post-acidosis period. The return to normal pH also leads to recovery of the acidosis-induced inhibition of NCX (97), favoring Ca^{2+} extrusion and Na^{+} gain into the cell, membrane depolarization, and eventually triggered arrhythmias. Together, these results indicate that post-acidosis CaMKII-dependent DADs are triggered by two concurrent factors: *1*) acidosis-induced increase in SR Ca^{2+} content and *2*) relief of acidosis-induced inhibition of RyR2 and NCX.

The evidence provided herein demonstrates the critical role played by CaMKII and RyR2 in arrhythmogenesis and suggests the potential therapeutic benefit of CaMKII inhibition for the treatment of arrhythmias. However, the ubiquitous nature of CaMKII and its effects on different protein targets challenge the use of CaMKII inhibitors as a therapeutic tool. Moreover, pharmacological CaMKII inhibition would probably require compounds selective toward cardiac-specific CaMKII isoforms, which are not currently available. In addition, a target-specific therapy would be desirable, taking into account the existence of multiple targets for CaMKII activity. For example, the phosphorylation of the PLN site Thr^{17} plays a key role in the β-adrenergic inotropic response and mediates the recovery of contractility after cardiac acidosis (107).

The demonstration of RyR2 as a crucial player in the development of CaMKII-induced arrhythmias allows us to postulate an alternative therapeutic approach, which involves the concept of RyR2 stabilization. The term "stabilization" refers to the possibility to reduce RyR2 spontaneous diastolic opening without affecting systolic release. Thus compounds that are able to stabilize the RyR2 could be used to prevent arrhythmias without the undesirable effects of global CaMKII inhibition. Indeed, using the multi-channel blocker JTV-519 (K201), which has been shown to stabilize the RyR2, Sacherer et al. (104) showed, in mouse myocytes and in nonfailing human myocardium treated with the cardiac glycoside ouabain, that JTV-519 was able to reduce the ouabain-induced SR Ca^{2+} leak. Similarly, additional reports showed that alternative RyR2 stabilizers such as VKII86 or tetracaine could reduce DAD-triggered arrhythmias (137, 156). Further work is warranted to find the ideal RyR2 stabilizer, which should reduce spontaneous RyR2 openings during diastole without inhibition of the normal Ca^{2+}-induced Ca^{2+} release that triggers contraction. This is the case for dantrolene (75), although dantrolene is now only used for acute treatment of malignant hyperthermia. Nevertheless, novel RyR2 stabilizers could be a promising approach for the treatment of arrhythmias of different etiology.

CaMKII in cardiac transcriptional regulation. The foregoing discussion has focused on acute modulatory effects of CaMKII on ion channels, transporters, and myofilaments, but activation of CaMKII can also have major effects on gene transcription. This should be considered as a slower response to certain stress-related signals in which the acute regulatory CaMKII-dependent effects may not be sufficient. This type of transcriptional regulation can be beneficial but can also contribute to maladaptive signals in hypertrophy and HF. Indeed, when myocyte CaMKII is chronically activated, as in all of the above autonomous states (autophosphorylation, oxidation, O-GlcNAcylation, nitrosylation), it appears to be largely maladaptive by worsening arrhythmogenic diastolic SR Ca^{2+} leak and altering expression and gating of ion channels in ways that contribute to arrhythmogenesis. Moreover, this situation seems to occur in HF or upon CaMKII overexpression (either genetically induced or as an intrinsic part of the hypertrophy/HF phenotype). Thus both acute and transcriptional actions of CaMKII can contribute to acute dysfunctions of the type discussed above.

Ca^{2+}-dependent signaling can lead to transcriptional regulation, and we call this process excitation-transcription-coupling (ETC), by analogy to ECC. One ETC pathway that is known to directly involve CaMKII is the CaMKII-dependent phosphorylation of class II histone deacetylases, HDACs, of which HDAC4 and HDAC5 have been the best studied (3, 77, 96, 149). As illustrated in Fig. 6, at baseline when these HDACs are dephosphorylated, they bind to and repress hypertrophic transcription factors, such as myocyte enhancer factor 2 (MEF2). Although these particular HDACs have weak histone deacetylase activity, their presence at MEF2 also prevents histone acetyl transferase localization, resulting in more condensed de-acetylated chromatin structure in this situation. When these HDACs are phosphorylated by CaMKII or protein kinase D (PKD), translocation out of the nucleus via binding to 14-3-3 chaperone proteins is induced. This translocation relieves MEF2 repression, allows histone acetyl transferase binding, and favors transcriptional activation (Fig. 6). We focus here on the upstream side of these ETC pathways.

Calcineurin (CaN) is an additional Ca^{2+}-dependent ETC pathway that works in parallel with the CaMKII-HDAC pathway (Fig. 6) (62, 81, 147). When the phosphatase CaN is activated by Ca-CaM, it dephosphorylates nuclear factor of activated T cells (NFAT), and dephosphorylated NFAT is translocated to the nucleus where it interacts with transcription factors (e.g., GATA4) and activates the transcription of genes involved in hypertrophic signaling and HF. So, two questions that come up are where CaN and CaMKII are localized with respect to ETC and whether the same types of Ca-CaM signals are likely to drive these two Ca-CaM-dependent ETC pathways.

Regarding subcellular localization, at baseline in adult ventricular myocytes, both CaN and CaMKII seem to be preferentially concentrated at the Z-line, at or near the SR T-tubule junctions involved in ECC (but also exist elsewhere). This localization has four important implications. First, if one measures global CaN or CaMKII activation state, it may be biased by the quantitatively large amount of CaN and CaMKII at these sites. Second, the local $[Ca^{2+}]_i$ in this junctional cleft domain is very different from the global or nuclear $[Ca^{2+}]_i$, because of the close proximity to both L-type Ca^{2+} channels and RyR2

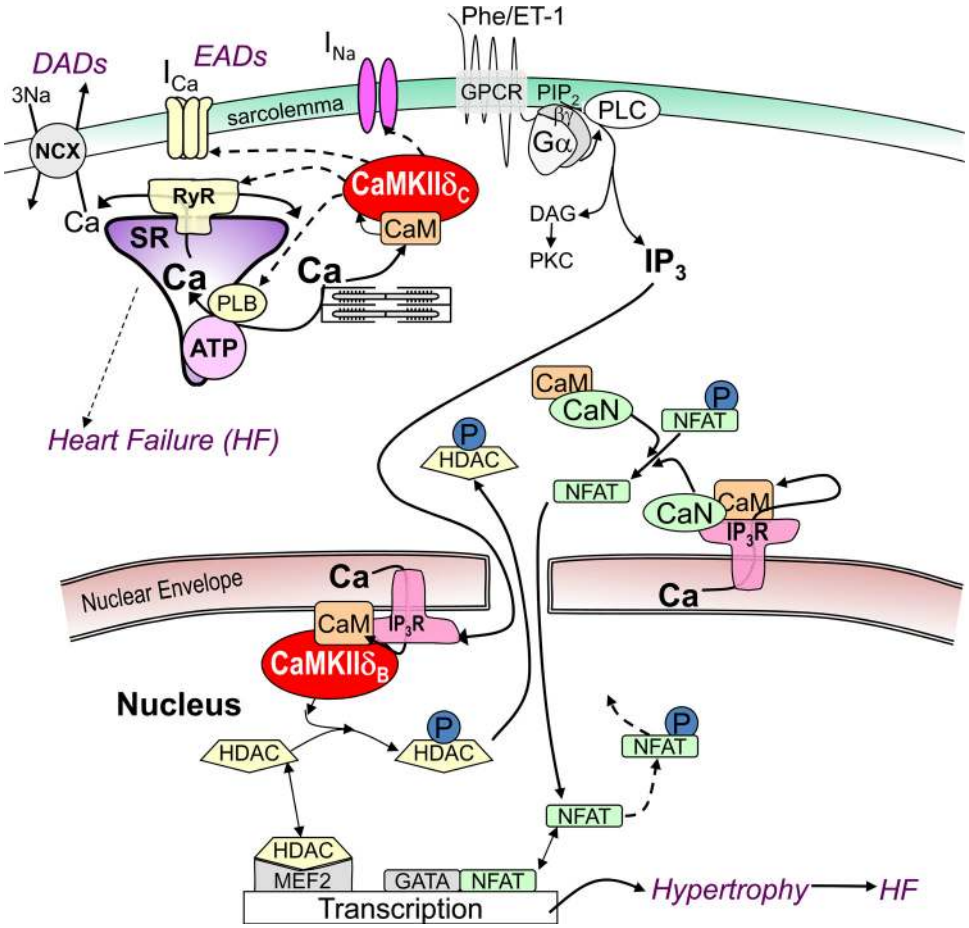

Fig. 6. Ca^{2+}-dependent signaling in excitation-transcription coupling via Ca-CaM. CaMKIIδ can acutely regulate ion channels (that carry I_{Na} and I_{Ca}) and Ca^{2+} handling proteins (RyR2, IP_3R, PLN), contributing to triggered arrhythmias such as early and delayed afterdepolarization (EADs and DADs). G protein-coupled receptor (GPCR) agonists endothelin-1 (ET-1) and phenylephrine (PE) activate Gαq/βγ and phospholipase C (PLC) to produce diacylglycerol (DAG), which can activate protein kinases C and D. PKC, CaMKII, and PKD can phosphorylate (P) HDAC, and calcineurin (CaN) can dephosphorylate nuclear factor of activated T cells (NFAT), altering nuclear MEF2- and GATA-dependent transcription.

channels (i.e., peak and even diastolic $[Ca^{2+}]_i$ here can be much higher than anywhere else in the myocyte). Third, there may be significant translocation of CaN or CaMKII or their downstream targets (e.g., NFAT or HDAC), that could allow Ca^{2+}-dependent signaling in this ECC domain to have longer distance effects on ETC. Fourth, CaN and CaMKII in different subcellular domains (cleft, perinuclear, intranuclear) may have more selective and locally dictated signaling to the ETC machinery. This will be discussed further below.

CaN and CaMKII have very different Ca-CaM affinities, which may be very important functionally. CaMKII has a relatively low Ca-CaM affinity (K_d = 10–50 nM), whereas CaN has a very high Ca-CaM affinity ($K_d \ll 1$ nM) (112, 113). CaMKII activation requires relatively high local $[Ca^{2+}]_i$ and will tend to de-activate more rapidly when $[Ca^{2+}]_i$ declines. Thus CaMKII activation works especially well in environments where large local Ca^{2+} transients occur, such as near the mouths of Ca^{2+} channels. One place that this occurs is in the ECC cleft near L-type Ca^{2+} channels and RyR2, and this environment is expected to be more sensitive to CaMKII activation (31). A second location is at the nuclear envelope, where the 1,4,5-inositol-trisphosphate ($InsP_3$) receptor ($InsP_3R$) type 2 (the main isoform in adult ventricular myocytes) is concentrated. Both CaM and CaMKII appear to directly associate with the $InsP_3R$ (5). Thus Ca^{2+} released by the nuclear envelope $InsP_3R$ can cause a high local $[Ca^{2+}]_i$ that is sufficient to activate CaMKII. That activated nuclear CaMKII can then phosphorylate the $InsP_3R$ and inhibit channel gating, constituting a local negative feedback loop that may limit the duration of local $InsP_3R$ Ca^{2+} release. However, this local CaMKII activation may also be critical for CaMKII-dependent nuclear signaling to HDACs in ETC (149). Note also that CaMKII activation has memory in the form of autonomous activation; that is, if local $[Ca^{2+}]_i$ is sufficiently high for a long enough time, there is much greater likelihood for a neighboring CaMKII monomer in the dodecameric structure to become autophosphorylated, oxidized, O-GlcNAcylated, or nitrosylated, all of which would prolong the active autonomous state. The other side of this issue is that bulk cytosolic CaMKII (e.g., near PLN or myofilament sites) may not be substantially activated during beat to beat global Ca^{2+} transients (112). So it is less clear how CaMKII that is not near Ca^{2+} channels is normally activated.

CaN activation is very different from that of CaMKII, because of its very high Ca-CaM affinity and slow off-rate (112). CaN that is very near Ca^{2+} channels that open at each beat (e.g., SR-T-tubule clefts) could be nearly fully activated at each beat, and the slow deactivation could result in nearly fully activated local CaN at all relevant heart rates. In contrast, cytosolic CaN that is far away from Ca^{2+} channels could still be activated in a way that is intrinsically integrating because of the slow off-rate of Ca-CaM; that is, each Ca^{2+} transient would slightly increase the CaM-CaN level, but the slow off-rate would mean that it does not relax back before the next Ca^{2+} pulse drives a bit more CaM onto CaN. So both CaN and

CaMKII exhibit molecular memory, but the molecular basis differs considerably.

Olson's laboratory was the first to demonstrate both the important role of class II HDACs in cardiac ETC, as well as the fact that CaMKII can be an HDAC kinase (3, 96). Indeed, both HDAC4 and 5 in cardiac myocytes are phosphorylated by CaMKII and PKD (which is another member of the CaMK kinome family). That HDAC phosphorylation induced HDAC nuclear export, and that could be induced by neurohumoral stimuli [e.g., by endothelin-1 (ET-1) and α-adrenergic activation by phenylephrine (PE)], that are known to be parts of the hypertrophic signaling pathway, and also the neurohumoral storm associated with the vicious cycle of HF. The HDAC4 protein contains a specific CaMKII docking, since that CaMKII activation is very tightly linked to HDAC4 nuclear export.

HDAC5 knockout mice exhibit baseline cardiac hypertrophy and have an exaggerated hypertrophic response to pressure overload or cardiac CaN activation (24), suggesting that HDAC5 might be a particularly important ETC pathway in mammalian heart. HDAC5 does not have the CaMKII docking site as in HDAC4, and in HEK cells or cultured neonatal myocytes, HDAC5 nuclear export is controlled by PKD rather than CaMKII. However, in adult ventricular myocytes, where PKD expression is dramatically lower than in the neonate (48), and where CaMKII expression is higher, CaMKII and PKD appear to be equal partners in HDAC5 phosphorylation, nuclear export, and MEF2 driven transcription in response to ET-1 (149). Moreover, ET-1-induced HDAC5 nuclear export was entirely dependent on Ca^{2+} release through $InsP_3R$ type 2 at the nuclear envelope, since it was abolished in $InsP_3R2$ knockout mice or by $InsP_3R$ inhibitors, and could be quantitatively mimicked by selective $InsP_3R$ activation in adult ventricular myocytes (149). HDAC5 translocation driven by the α-adrenergic agonist PE is like that induced by ET-1 at the endothelin receptor in that it is mediated by the G-protein G_{aq} (17). Surprisingly, PE-induced HDAC5 nuclear export was completely independent of Ca^{2+}, $InsP_3R$, or CaMKII activity and instead was completely dependent on PKC- and PKD-dependent signaling (note that PKC did not seem to be important in ET-1-induced HDAC5 nuclear export). Bossuyt et al. (17) used confocal, targeted fluorescence resonance energy transfer (FRET)-based reporters and total internal reflectance fluorescence (TIRF) microscopy to elucidate the mechanism for this ET-1 versus PE difference. For PE activation, PKD was rapidly recruited and activated at the sarcolemma, but then it was very rapidly translocated to the nucleus, where it could phosphorylate HDAC5. For ET-1, PKD was also rapidly recruited to the sarcolemma where it was activated, but in this case, PKD largely remained at the sarcolemma and did not shuttle to the nucleus. So although PKD can powerfully drive HDAC5 nuclear export, it depends on how PKD is activated. For ET-1, which activates CaMKII via an $InsP_3$-dependent nuclear pathway but activates PKD preferentially at the sarcolemma, nuclear HDAC5 is substantially CaMKII dependent.

During stress, there is often co-activation of the G_q-coupled receptors (ET-1 and α-adrenergic) with β-adrenergic receptors (β-AR). Chang et al. (23) evaluated this cross-talk in adult ventricular myocytes. β-AR activation caused an acute PKA-dependent HDAC5 nuclear import that was mediated by PKA-dependent phosphorylation of HDAC5 at Ser^{279}, between the two PKD/CaMKII sites (Ser^{259} and Ser^{498}), which are responsible for driving ET-1 and PE-induced HDAC5 nuclear export. This effect could be mimicked by pseudo-phosphorylation of HDAC5 (S279D) and prevented by a nonphosphorylatable S279A mutant HDAC5. Moreover, this β-AR effect was dominant over the G_q-coupled receptor effect in that the ET-1 or PE-induced nuclear export was blocked in the S279D HDAC5 mutant or after pretreatment of myocytes with isoproterenol or forskolin. However, chronic β-AR activation (over 24 h) allowed the usual ET-1 and PE effects to occur. Thus acute β-AR activation may suppress genetic reprogramming driven by this HDAC5 system during the acute fight-or-flight response. However, in chronic activation, as in HF, this β-AR-induced suppression may be overcome, such that the Gq-coupled signaling drives chronic HDAC5 nuclear export and activation of transcription, which reinforce the genetic HF phenotype (18).

In conclusion, CaMKII signaling in ETC is complex, and much additional work will be needed to fully understand the overall integrated Ca-CaM dependent signaling, even by just the CaMKII-HDAC and CaN-NFAT pathways discussed here. But these longer term ETC effects, which can change the expression levels of numerous ion channels and transporters as well as modulate their acute functional behavior in ECC, have to be integrated in the long run.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS

Author contributions: A.M. conception and design of research; A.M., J.P., C.A.V., M.V.P., and D.M.B. prepared figures; A.M., R.A.B., A.L.E., J.P., M.V.P., and D.M.B. drafted manuscript; A.M., R.A.B., C.A.V., M.V.P., and D.M.B. edited and revised manuscript; A.M., R.A.B., A.L.E., J.P., M.V.P., and D.M.B. approved final version of manuscript.

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