CaMKII IN THE CARDIOVASCULAR SYSTEM: SENSING REDOX STATES

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Erickson JR, He BJ, Grumbach IM, Anderson ME. CaMKII in the Cardiovascular System: Sensing Redox States. *Physiol Rev* 91: 889–915, 2011; doi:10.1152/physrev.00018.2010.—The multifunctional Ca^{2+}- and calmodulin-dependent protein kinase II (CaMKII) is now recognized to play a central role in pathological events in the cardiovascular system. CaMKII has diverse downstream targets that promote vascular disease, heart failure, and arrhythmias, so improved understanding of CaMKII signaling has the potential to lead to new therapies for cardiovascular disease. CaMKII is a multimeric serine-threonine kinase that is initially activated by binding calcified calmodulin (Ca^{2+}/CaM). Under conditions of sustained exposure to elevated Ca^{2+}/CaM, CaMKII transitions into a Ca^{2+}/CaM-autonomous enzyme by two distinct but parallel processes. Autophosphorylation of threonine-287 in the CaMKII regulatory domain "traps" CaMKII into an open configuration even after Ca^{2+}/CaM unbinding. More recently, our group identified a pair of methionines (281/282) in the CaMKII regulatory domain that undergo a partially reversible oxidation which, like autophosphorylation, prevents CaMKII from inactivating after Ca^{2+}/CaM unbinding. Here we review roles of CaMKII in cardiovascular disease with an eye to understanding how CaMKII may act as a transduction signal to connect pro-oxidant conditions into specific downstream pathological effects that are relevant to rare and common forms of cardiovascular disease.

I. INTRODUCTION

Protein phosphorylation helps to determine membrane excitability, cellular Ca^{2+} homeostasis, metabolism, vesicle secretion, gene transcription, protein trafficking, and cell survival. The multifunctional Ca^{2+}- and calmodulin-dependent protein kinase II (CaMKII) is a serine/threonine kinase that modulates each of these biological functions in diverse cell types. The CaMKII holoenzyme is configured to coordinate "upstream" Ca^{2+} (98) and reactive oxygen species (ROS) (62) signals into "downstream" responses that are now known to play important roles in cardiovascular physiology and disease. Recently, CaMKII has emerged as a central signal for coordinating ion channels and Ca^{2+} homeostatic proteins involved in excitation-contraction and excitation-transcription coupling in myocardium (45). Ex-

cessively activated CaMKII in myocardium promotes hypertrophic (286) and apoptotic cardiomyopathy (271, 290) and distorts normal excitation-contraction coupling (5, 152). All of these processes can contribute to reduced mechanical performance of myocardium and the clinical syndrome of heart failure. Hyperactive CaMKII leads to proarrhythmic electrical remodeling (286) that is a probable cause of enhanced susceptibility to sudden death in patients with heart failure. Thus the pathological consequences of CaMKII activity in myocardium provide a conceptual framework for understanding why patients with mechanically dysfunctional myocardia are also at risk for arrhythmia-triggered sudden cardiac death. Increased ROS (12, 219) and CaMKII hyperactivity have been understood to occur in patients with atherosclerosis, heart failure, and arrhythmias for some time, but the mechanism for ROS activation of CaMKII is recent. In contrast to a relatively developed understanding of the role of CaMKII in cardiovascular disease, the physiological requirements for CaMKII have been more elusive. Mice lacking a major myocardial CaMKII isoform (CaMKIIδ) (15, 142) and mice with myocardial expression of a CaMKII inhibitory peptide appear to have normal baseline ventricular function and excitation-contraction coupling parameters (282), although CaMKII inhibition reduces the heart rate response to catecholamine stimulation by actions at sinoatrial nodal pacemaker cells (261). We will review the data at hand, which

presently provide more insight into cardiovascular disease than cardiovascular physiology.

ROS are linked to a wide array of processes that lead to heart failure, including myocardial hypertrophy (54, 121), apoptosis (62), increased matrix metalloproteinase activity (121), inflammation (211), fibrosis (54, 121), and left ventricular (LV) cavity dilation (54, 62). However, understanding of discrete molecular pathways and mechanisms by which ROS influences these disease processes is underdeveloped (7). The use of currently available lipid- and water-soluble antioxidant supplements (vitamin E and vitamin C) have not shown therapeutic efficacy (1–3, 51, 187, 200, 277, 278), while antioxidants engineered for local action (109) appear to show benefit in preliminary studies. One potential hypothesis to explain the differential effects of broad-spectrum versus targeted antioxidants is that the geographic or temporal distribution of broad-spectrum supplements does not match the localization of ROS signaling. Indeed, many physiological effects of enhanced oxidative stress are likely to be highly localized. The comparatively greater efficacy of targeted antioxidant treatment motivates studies aiming to improve molecular understanding of ROS-responsive disease pathways.

We recently discovered one such potential pathway in which ROS activates CaMKII, a kinase that is critically linked to structural remodeling, ionic homeostasis, and cell death in the heart. Oxidized CaMKII (ox-CaMKII) is reduced and inactivated by methionine sulfoxide reductase A (MsrA) (62). Identification of the ox-CaMKII/MsrA signaling pathway provides new insights into how ROS may cause cardiovascular injury, and we hypothesize that the balance between ox-CaMKII and Met-reduced CaMKII determines fundamental aspects of myocardial disease related to ROS.

Intracellular Ca^{2+} elevations are sensed by the EF hand domain-containing protein calmodulin (CaM). When calcified CaM (Ca^{2+}/CaM) binds to CaMKII, it releases the catalytic domain from constraint by a pseudosubstrate sequence embedded within the CaMKII regulatory domain. Oxidation and autophosphorylation both convert CaMKII into a Ca^{2+}/CaM-independent enzyme by modification of defined CaMKII regulatory domain amino acids **(FIGURE 1)**. Most evidence suggests that excessive, constitutively active CaMKII contributes to cardiovascular disease. Conditions in which Ca^{2+}/CaM elevations are sustained favor transition of CaMKII to a constitutively active, Ca^{2+}/CaM-independent conformation by a process termed autophosphorylation. Autophosphorylation is favored by intersubunit interactions within the CaMKII holoenzyme. Oxidized methionine residues (Met-281/282) and autophosphorylated threonine-287 (Thr-287, the specific numbering varies slightly between isoforms) reduce the efficacy of pseudosubstrate and kinase domain reassociation that is required for

CaMKII inactivation. However, Thr-287 autophosphorylation is reversed by phosphatases, while Met-281/282 oxidation is reduced by MsrA. Cellular studies show that ROS-activated CaMKII can occur under conditions of low Ca^{2+} (97, 174), suggesting that high ROS conditions may reset the Ca^{2+} dependence of CaMKII activation. Adequate MsrA activity may be required for a normal lifespan in mice (166), while MsrA overexpression increases lifespan in *Drosophila* (197). Additionally, loss of MsrA increases oxidized CaMKII and worsens mortality and myocardial dysfunction after myocardial infarction (MI) (62), suggesting that MsrA activity is necessary to constrain pathological CaMKII hyperactivity. Because a molecular understanding of CaMKII activation by oxidation is new, we will highlight the findings specific to ox-CaMKII. However, we will also consider CaMKII actions at various downstream targets, in some cases where oxidation has not been studied, because there is no evidence (that we know of) to suggest that oxidized and autophosphorylated CaMKII have distinct targets. However, it is possible that ox-CaMKII and autophosphorylated CaMKII participate in distinct functions. For example, ROS may disable phosphatases, disturb CaMKII binding to target proteins, or affect the physiological or pathophysiological consequences of CaMKII-dependent phosphorylation by oxidation of CaMKII target proteins.

II. REGULATION OF CaMKII ACTIVITY

CaMKII represents a particularly elegant example of the interrelationship between protein structure and function. The kinase is a multimeric protein typically composed of 12 subunits. Each subunit contains three distinct domains: an association domain that directs holoenzyme assembly, a regulatory domain that controls activation of the enzyme, and a catalytic domain that associates with substrates and performs the kinase function of CaMKII.

Under resting conditions, the regulatory and catalytic domains are closely associated with one another, blocking substrate binding and resulting in autoinhibition of the kinase. Inspection of the crystal structure of the autoinhibited kinase reveals that neighboring regulatory domains form dimeric coiled-coil pairs that block substrate and ATP binding (195). If intracellular calcium concentration rises (half-maximal activation requires $[Ca^{2+}] \sim 1.0~\mu M$) (196), calcified calmodulin (Ca^{2+}/CaM) binds to CaMKII at the regulatory domain. Ca^{2+}/CaM binding disrupts the association of the regulatory and catalytic domains, causing a conformational shift that exposes the catalytic domain for substrate binding and relieves autoinhibition (189). While other mechanisms of CaMKII activation are discussed in this review, it is critical to note that all known activation mechanisms require this initial interaction with Ca^{2+}/CaM.
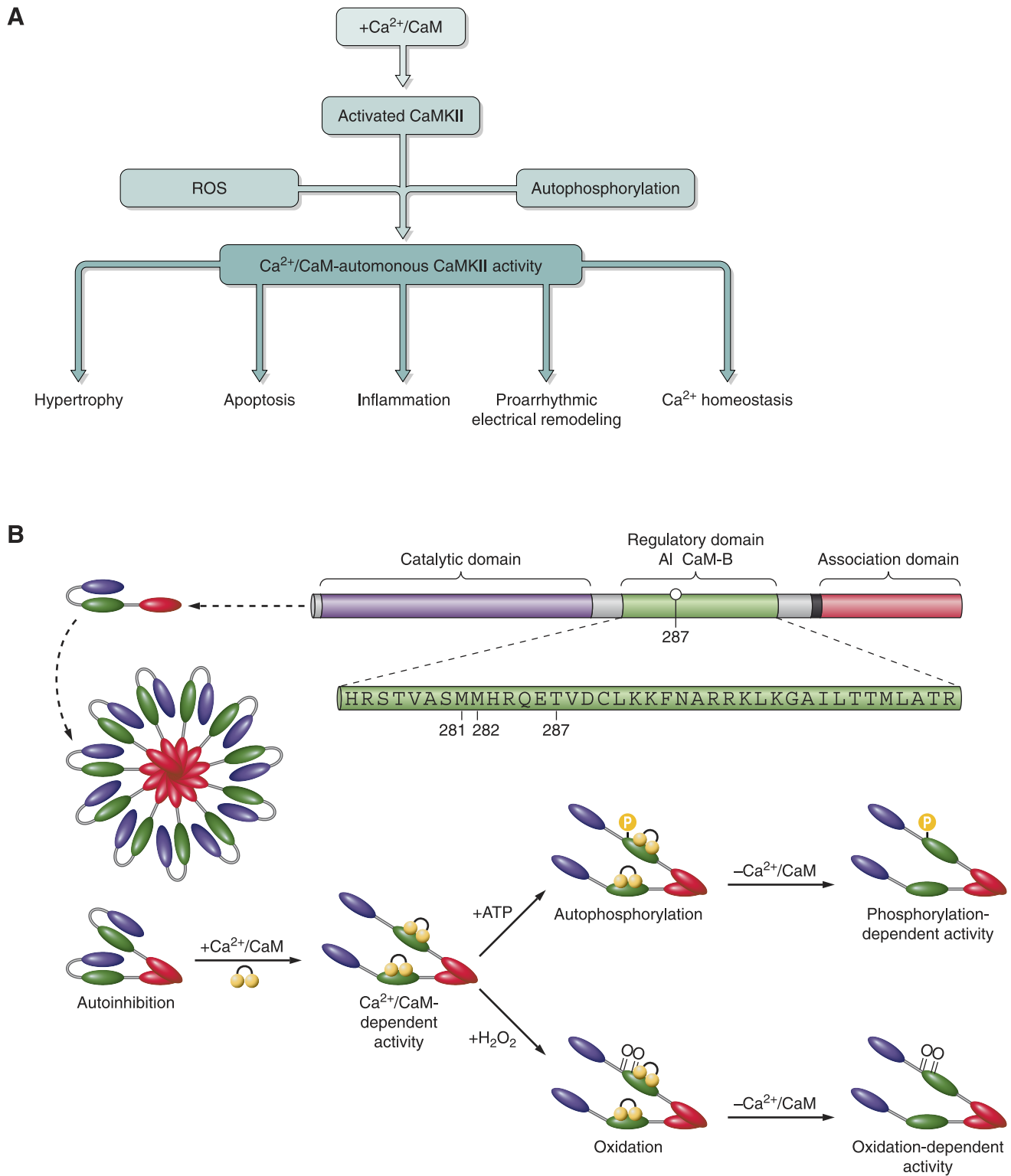
A

B

FIGURE 1 Oxidation and autophosphorylation both convert CaMKII into a Ca^{2+}/CaM-independent enzyme by modification of defined CaMKII regulatory domain amino acids.

If CaMKII activity is sustained by lengthy or frequent calcium transients in the presence of ATP, CaMKII undergoes intersubunit autophosphorylation at Thr-287. The addition of a phosphate group at this residue within the regulatory domain increases the affinity of Ca^{2+}/CaM for CaMKII over 1,000-fold (161). Moreover, phosphorylation at Thr-287 prevents reassociation with the catalytic domain. As a result, Thr-287 phosphorylation can permit persistent CaMKII activity even after the dissociation of Ca^{2+}/CaM from the kinase.

Autophosphorylation at Thr-287 is a critical feature of CaMKII function, as it allows the kinase to translate changes in calcium concentration or transient frequency into sustained enzyme activity. Indeed, CaMKII is known as the "memory molecule" in part because recent calcium conditions within the cell are reflected in the shift between Ca^{2+}/CaM-dependent and Ca^{2+}/CaM-independent activity. Interestingly, new evidence from computer modeling and CaMKII crystal structure indicates that activation via autophosphorylation may be cooperative (34), a function of intersubunit capture of regulatory domains rather than simple coincidence detection. To return to basal inactivation, Thr-287 phosphorylation must be removed by the action of phosphatases, including protein phosphatase (PP)1 and PP2A (220). As protein phosphatases are themselves subject to complex regulation, for instance, by oxidative stress (97), it becomes clear that a number of mechanisms work in tandem to modulate CaMKII activity in vivo.

Recently, our group described a previously unknown mechanism of CaMKII activation by redox (62). Increased levels of ROS cause oxidative modification of the Met-281/282 pair within the regulatory domain, blocking reassociation with the catalytic domain and preserving kinase activity via a similar but parallel mechanism to Thr-287 autophosphorylation. This model is further supported by observations of CaMKII crystal structure, which show the influence of Met-281/282 oxidation on CaMKII dynamics (189). Mutation of this methionine pair ablates redox-dependent CaMKII activity while leaving Ca^{2+}/CaM-dependent and phosphorylation-dependent activation unaltered.

An antiserum designed to detect oxidized Met-281/282 allows visualization of CaMKII redox modification in vitro. Using our custom antiserum, we have shown that *1*) CaMKII oxidation occurs in cardiac tissue, *2*) redox modification is inducible by treatment with specific agonists (e.g., angiotensin II), and *3*) the extent of CaMKII oxidation is directly related to oxidative stress levels in the heart. These observations point to Met-281/282 oxidation as a novel regulatory mechanism for CaMKII in vivo and position redox modification of CaMKII as a sensor of oxidative stress in the heart.

Like phosphorylation, oxidation represents a potential modulatory pathway for CaMKII within the context of cardiac physiology and pathophysiology. We present evidence below that some cellular process are specifically regulated by Met-281/282 oxidation of CaMKII. Methionine sulfoxide reductase, the enzyme that catalyzes the reversal of methionine oxidation, is abundant in cardiac tissue (129). This enzyme has an important cardioprotective role during conditions of increased oxidative stress and may have significant interaction with CaMKII after ROS-mediated activa-

tion. This topic is explored more thoroughly in sections **V** and **VI**.

III. PATHWAYS FOR CaMKII ACTIVATION IN THE HEART

A. Angiotensin

The renin-angiotensin II-aldosterone system (RAAS) is a major pro-oxidant and proinflammatory "stress" neurohormonal system that is overactivated in cardiac disease. For a more thorough discussion of RAAS pathophysiology, the reader is directed elsewhere (76). The octapeptide angiotensin II (ANG II) is the most studied component of the RAAS cascade especially in context of renal, cardiac, and vascular diseases. ANG II is the cleavage product of angiotensin I, a decapeptide product from the enzymatic action of circulating renin. Since its discovery in 1940 as a potent vasoconstrictor (29, 173), ANG II is known to regulate volume and electrolyte balance. ANG II exerts vasopressor action through smooth muscle contraction (46) and contributes to hypertension. In addition to systemic actions, ANG II directly stimulates cardiac cell signaling and affects myocyte contractility. AT1 receptors are implicated in ANG II-induced inotropic effect on cardiac myocytes (106, 207). AT1 receptors couple to phospholipase $C\beta$ ($PLC\beta$) and result in the hydrolysis of phosphatidylinositol 4,5-bisphosphate into diacylglycerol (DAG) and inositol tris-phosphate (IP_3) (205). Both positive and negative inotropic effects have been reported for AT1 receptor stimulation, suggesting complexity in downstream signaling. Positive inotropy is primarily achieved through DAG-stimulated PKC activation and stimulation of L-type Ca^{2+} channels (227). Low ANG II levels can also induce positive inotropy through a different mechanism involving endothelin stimulated ROS and the Na^+/Ca^{2+} exchanger (40). Therefore, ANG II seems to stimulate fluctuations in intracellular calcium and ROS. CaMKII, as a downstream sensor of both signals, can likely mediate a coordinated response to ANG II.

Experimental evidence implicates CaMKII in ANG II-induced pathology, particularly cell death (62, 174) and vascular hypertrophy (137). In cat and rat myocytes, where ANG II exerts opposite inotropic effects, pharmacological CaMKII inhibition attenuated ANG II-induced apoptosis (174). CaMKII seemed to result in p38^{MAPK} activation, and the pathway was dependent on ROS. Our group showed that NADPH oxidase derived ROS directly activated CaMKII through methionine oxidation and that this oxidation-mediated kinase activation was crucial to ANG II-induced cardiac apoptosis (62). Despite ANG II-stimulated changes in intracellular Ca^{2+}, the principle mode for ANG II-directed CaMKII activation, at least in disease states, seems to depend on intracellular ROS. This is in contrast to β-adrenergic receptor-stimulated CaMKII that relies on auto-

phosphorylation but not methionine oxidation. CaMKII exerts proapoptotic effects of ANG II regardless of effects on inotropy.

B. β-Adrenergic Signaling

The major β-adrenergic receptor (βAR) in myocardium is thought to be the βAR1 isoform, with an ~70:30 ratio in nonfailing rat hearts (164). More than a decade ago, Baltas et al. (16) first showed βAR agonists can increase autonomous CaMKII activity using Langendorff-perfused rat hearts. βAR stimulation enhances $[Ca^{2+}]_i$ by several mechanisms including Ca^{2+} entry by L-type Ca^{2+} channels (LTCC), Ca^{2+} release by ryanodine receptors (RyR), and protein kinase A (PKA)-enhanced intracellular Ca^{2+} flux (27). βAR stimulation can also activate CaMKII through the guanine nucleotide exchange factor Epac (exchange protein directly activated by cAMP), which associates with CaMKII and β-arrestin at the type 1 βAR (156). The association of CaMKII with the type 1 βAR and lack of association with the type 2 βAR may explain why catecholamine-mediated myocardial toxicity signals through the type 1 βAR. Whereas short-term βAR agonist exposure evokes PKA activation, long-term treatment predominantly stimulates CaMKII in cardiac myocytes. Likewise, phosphorylation of phospholamban (PLN) switches from the PKA-regulated to CaMKII-regulated site with increasing time of exposure to βAR agonist treatment in vitro (251). βAR-activated PKA may underlie the "fight-or-flight" response. In contrast, CaMKII activation by chronic exposure to a βAR agonist seems to dominate under pathological conditions. Overexpression of a CaMKII inhibitory peptide in transgenic mice affords protection from chronic βAR stimulation (282), supporting the functional importance of CaMKII in pathological βAR signaling. While βAR stimulation increases autonomous CaMKII activity and phosphorylation of CaMKII targets (in particular RyR2 Ser-2815 and PLN Thr-17), the βAR agonist isoproterenol does not seem to have an effect on Met-281/282 oxidation-mediated CaMKII activation (62). βAR contributes to cardiac oxidative stress (280), suggesting that there may be requirements for the source and/or spatiotemporal generation of ROS in oxidizing CaMKII.

IV. DISTRIBUTION OF CARDIAC CaMKII

CaMKII exhibits complex patterns of expression due to multiple encoding genes, splice variants, and posttranslational modifications. CaMKII exists in four known isoforms encoded by different but highly conserved genes (α, β, γ, and δ). Whereas the α and β isoforms are expressed predominantly in neuronal tissue (162), the δ isoform is the major isoform expressed in heart (59). Several alternatively spliced variants exist for each CaMKII isoform, allowing for differential expression and subcellular targeting. Nota-

bly, the δ isoform has the greatest number of splice variants, eight total, making it the major known contributor to CaMKII diversity in the cardiovascular system. In particular, the δB (or δ3) isoform allows for nuclear localization by virtue of an 11-amino acid nuclear localization signal found in the hypervariable region (217). In cotransfection experiments with CaMKI or CaMKIV, it was found that phosphorylation of a serine residue in the nuclear localization signal of CaMKIIδB prevented nuclear targeting (88). In contrast, the δC (or δ2) isoform has predominant cytoplasmic localization, and transgenic mice overexpressing CaMKIIδC have increased RyR phosphorylation, dilated cardiomyopathy, heart failure, and premature death (286). Subcellular targeting likely allows strategic positioning along different signaling pathways and, together with its heteromultimeric construction, CaMKII can transmit a gradient of sensitivity to Ca^{2+}/CaM. Finally, isoform expression changes differentially in diseased states. In failing human hearts, CaMKIIδB isoform expression preferentially increases (93). CaMKII is found in virtually every subcellular locale, including the cytoplasm, the nucleus, and the ER. CaMKII interacts with membrane proteins, allowing it to be in proximity to sites of ROS generation. This ubiquitous expression presumably allows CaMKII to function as a sensor and effective transmitter of cellular oxidant stress signals.

V. CaMKII AND OXIDATION IN CARDIAC EXCITATION-CONTRACTION COUPLING

Excitation-contraction coupling (ECC) is the mechanism where membrane excitation induces release of Ca^{2+} from the sarcoplasmic reticulum (SR) intracellular Ca^{2+} store by a Ca^{2+}-induced Ca^{2+} release process. L-type Ca^{2+} channel (Ca_V1) current enhances the probability of ryanodine receptor (type 2 in myocardium, RyR2) opening. RyR2 opening releases Ca^{2+} stored in the SR into the cytoplasm. The rise in free cytoplasmic Ca^{2+} drives myofilament cross-bridge formation, causing contraction and mechanical systole. Active reuptake of cytoplasmic Ca^{2+} into the SR facilitates a reversal of myofilament interactions, allowing for myocardial relaxation during diastole. CaMKII participates in regulating each component of the ECC process, and excessively activated CaMKII is implicated in ECC dysfunction that underlies heart failure and arrhythmias. The recent recognition that oxidation can directly activate CaMKII provides a potential explanation for earlier studies showing ROS effects on action potential and RyR2 physiology and disease.

Each cardiac action potential is initiated by a voltage-gated inward current that causes cell membrane depolarization. Most myocardial cells rely on the voltage-gated Na^+ channel (mostly $Na_V1.5$) for action potential initiation, but myocardial-derived cells specialized for pacing in the sinoatrial node (SAN) and the atrioventricular node (AVN) depend on voltage-gated Ca^{2+} current (a combination of

$Ca_V1.2$ and $Ca_V1.3$) for initiating action potentials (157, 182). The depolarized cell membrane potential is restored on a millisecond time scale to the physiological diastolic resting potential by coordinated responses of repolarizing (net outward) currents. The electrochemical gradient required to maintain a negative resting cell membrane potential is energetically expensive and is supported by the activity of the Na^+-K^+-ATPase. We are unaware of any evidence linking CaMKII with Na^+-K^+-ATPase activity in heart (133). However, the ion channels that determine membrane depolarization and repolarization are specific to particular cell types in the cardiovascular system, and a growing body of evidence suggests that CaMKII directly and indirectly regulates many, or even most, of these ionic currents. Given the interdependence of ion channels and Ca^{2+} homeostatic proteins in the ECC process, it is appealing to consider that CaMKII is a coordinating signal that helps to orchestrate the interrelationships between membrane excitation and SR Ca^{2+} release. Because ROS are a physiological consequence of working cardiac muscle, a tissue with high metabolic demands, it seems likely (though to our knowledge unproven) that ROS activation of CaMKII is an important, regulated event that enables physiological adjustments to various ECC proteins. CaMKII catalyzes the phosphorylation of several ion channel proteins, including voltage-gated Ca^{2+} channels and RyR2 (45, 151). In general, CaMKII phosphorylation sites increase the probability of ion channel opening. What follows is a discussion of CaMKII and ROS actions at specific ion channels and Ca^{2+} homeostatic proteins involved in myocardial ECC. We refer the reader to recent reviews that focus on CaMKII in heart (45), Ca^{2+} homeostasis and ECC (22, 151), and the role of ROS in myocardial (12) and atherosclerotic vascular disease (219).

A. Action Potentials

Action potentials (AP) embody membrane excitability and repolarization, and so represent the integrated readout of a complicated assembly of inward and outward currents. The open, closed, and inactivated states of ion channels that contribute to action potentials in heart are mostly responsive to membrane potential, and so are designated voltage-gated ion channels. However, various signals add to the effect of membrane voltage, including phosphorylation and oxidation. Furthermore, ion channel trafficking to the cell membrane and assembly into a fully formed macromolecular complex can also be regulated by phosphorylation and oxidation. It is now clear from experimental and computer modeling studies that CaMKII is a pleiotropic transduction signal that couples changes in cellular Ca^{2+} to sculpt action potentials by affecting a wide variety of inward and outward, voltage-gated ion channels in the cardiovascular system (78, 101, 143). Some early clues strongly suggest that ox-CaMKII may be important for transduction of proarrhythmic actions of ROS on action potentials in cells (266) and tissue (39, 266). Surprisingly, the effects of CaMKII

and ROS on the AP duration are not clear. Chronic transgenic overexpression of the primary myocardial CaMKII isoform (CaMKIIδ) causes AP prolongation, proarrhythmic oscillations in membrane potential called afterdepolarizations (ADs), arrhythmias, and sudden death (152, 198). Studies in cultured adult ventricular myocytes overexpressing wild-type CaMKIIδ do not show increased AP duration (125), and modeling suggests that a lack of AP prolongation is due to a net balancing act between competing inward and outward currents (78). Our group recently reported that overexpression of a constitutively active mutant CaMKIIδ (T287D) mimicking autophosphorylation prolonged AP duration and induced ADs in cultured adult rabbit ventricular myocytes. Computer modeling and measurements of ionic currents suggested AP prolongation was due to frequent and prolonged (mode 2) openings of $Ca_V1.2$ and increased SR Ca^{2+} release from RyR2 (128). Taken together, we interpret these various results to suggest that excessive and prolonged CaMKII activity does cause AP prolongation and ADs by actions at a number of target proteins, while shorter exposure to more moderate elevation in CaMKII activity does not necessarily increase AP duration. Like CaMKII, ROS affects multiple processes that influence AP duration. Computational simulation of ROS increases due to ischemia suggest that mitochondrial ROS contributes to ventricular myocyte AP shortening by coupled activation of ATP-sensitive K^+ channels (288). Membrane depolarization and AP duration shortening was also observed in guinea pig papillary muscles in a lipid peroxidation model of increased ROS (235). ROS generated by photoinjury caused acute AP prolongation but later AP shortening in frog atrial cardiomyocytes (229). Application of H_2O_2 to rat and rabbit hearts induced ADs and ventricular fibrillation preferentially in older animals, but without a significant effect on AP duration (165). Arrhythmias were suppressed by the CaMKII inhibitor KN-93 or by the ROS scavenger N-acetylcysteine. H_2O_2 increased AP duration in isolated rabbit ventricular myocytes leading to ADs that were suppressed by CaMKII inhibition (266). Ultimately, simple statements about the effect of CaMKII and/or ROS on AP duration can be unintentionally misleading, because action potential duration is dependent upon many factors, including species (250), age (165), location within the myocardium (e.g., epicardium, endocardium, base, apex), temperature, and stimulation frequency (78).

B. Intracellular Calcium Transients

Disordered intracellular Ca^{2+} homeostasis, reflected in the beat-by-beat intracellular Ca^{2+} transient in myocardial cells, is a hallmark of heart failure in humans (83) and in animal models (282). The heart failure Ca^{2+} transient is reduced in amplitude and prolonged, a phenocopy of the effect of transgenic CaMKIIδ overexpression (152). CaMKII phosphorylates many of the proteins involved in intracellular Ca^{2+} homeostasis, including the main point of Ca^{2+}

entry ($Ca_V1.2$) and proteins involved in SR Ca^{2+} uptake (phospholamban) and release (RyR2) (45). Voltage-clamped ventricular myocytes from mice with CaMKII inhibition due to transgenic expression of a CaMKII inhibitory peptide (AC3-I) show reduced "beat-to-beat" variability of the intracellular Ca^{2+} transient (263). Chronic CaMKII inhibition in AC3-I transgenic mice led to increased peak I_{Ca} (282) and an increased peak intracellular Ca^{2+} transient. Phospholamban is a negative regulator of CaMKII, and CaMKII phosphorylation of phospholamban reduces the negative regulatory effects of phospholamban on the SR Ca^{2+}-ATPase (mostly SERCA2a), allowing increased uptake of cytoplasmic Ca^{2+} into the SR. Loss of phospholamban reduced dynamic variability of I_{Ca} (i.e., facilitation) and of the intracellular Ca^{2+} transient. Thus phospholamban knockout partially mimicked and prevented additional effects of CaMKII inhibition on dynamic properties of the intracellular Ca^{2+} transient. Knock-in mice lacking an important CaMKII phosphorylation site on RyR2 (254) show reduced stimulation frequency-dependent increases in peak intracellular Ca^{2+} (130). We interpret these findings to indicate that both phospholamban and RyR2, key control proteins for SR Ca^{2+} flux, are important for CaMKII effects on intracellular Ca^{2+} transients in ventricular myocytes.

C. Limitations of Available Reagents for Electrophysiological Studies

At this point, it is worth noting that interpretation of much of the published electrophysiological data is complicated due to limitations of available reagents for CaMKII inhibition. The CaMKII inhibitory drugs KN-62 (91) and KN-93 (222) are more specific for CaMKII over other serine-threonine kinases, compared with predecessor inhibitors such as calmidozolium or W-7. These original kinase inhibitory agents were known to have troublesome off-target actions that made them undesirable for electrophysiological studies (9, 79). However, KN-93 and KN-62 are also limited because of CaMKII-independent actions at voltage-gated ion channels important for the cardiovascular system. KN-93 (10) and KN-62 (136, 209) are direct I_{Ca} antagonists. Importantly, the concentrations for off-target and CaMKII inhibitory actions of the KN drugs overlap (10). KN-92 is a KN-93 congener marketed as a control agent. Unfortunately, KN-92 does not share the CaMKII-independent I_{Ca} antagonist actions of KN-93. KN-93 is also a CaMKII-independent antagonist for a variety of voltage-gated K^+ currents (10, 132, 191), including the transient outward current (I_{to}), inward rectifier current (I_{K1}), and the rapid component of the delayed rectifier (I_{Kr}). In our opinion, these limitations mean that electrophysiological studies where KN drugs are the only approaches to establishing a role for CaMKII are inadequate. CaMKII inhibitory peptides, such as AC3-I (282) and CaMKIIN (33), are potent and effective CaMKII inhibitory agents for cellular electro-
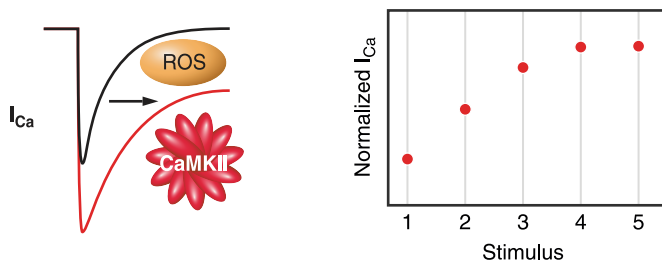
physiology studies. These peptides can be dialyzed via the patch pipette. Unfortunately, cell membrane-permeant formulations of these peptides are effective for transferring peptides across cellular membranes, but result in profound nonspecific disruption of membrane excitability (261). Fortunately, genetic approaches such as transgenic expression of inhibitory peptides (211, 282), CaMKII overexpression (125, 286), gene deletion (15, 142), and siRNAs (62, 237) provide highly selective methods for manipulating CaMKII activity without the off-target actions of currently available small molecule inhibitory agents.

D. Sodium Current

$Na_V1.5$ is the primary voltage-gated Na channel in myocardium. Inward $Na_V1.5$ current (I_{Na}) determines membrane excitability in all atrial and ventricular myocytes, with the exception of specialized pacing and conduction cells in the SAN and AVN where $Ca_V1.2$ and $Ca_V1.3$ serve this purpose. I_{Na} is composed of a massive peak inward current that drives the action potential upstroke and a much smaller slow or noninactivating component that contributes to action potential duration during the action potential plateau **(FIGURE 2)**. Excessive increases in the noninactivating component of I_{Na} are the primary arrhythmia mechanism in long QT syndrome 3 (21) and may contribute substantially to arrhythmia mechanisms in heart failure (155). The increased inward current contributes to AP prolongation and subsarcolemmal Na^+ loading that reduces the efficacy of the Na^+/Ca^{2+} exchanger (NCX), leading to secondary Ca^{2+} overload. Increased I_{Na} during the AP plateau and intracellular Ca^{2+} overload both support ADs. It has recently become clear that $Na_V1.5$ is a target for CaMKII (249). On one hand, transgenic chronic overexpression in mouse cardiomyocytes or transient virally mediated CaMKIIδ overexpression in adult rabbit ventricular myocytes cause a left shift (approximately −6 mV) in I_{Na} availability and slow recovery from inactivation, processes that reduce I_{Na} availability during rapid heart rate and that may contribute to QRS prolongation (249). On the other hand, acute or chronic CaMKII overexpression slows the rate of I_{Na} inactivation and preferentially enhances the persistent noninactivating component of I_{Na}. These properties and increased $Na_V1.5$ expression in mice with transgenic CaMKIIδ overexpression likely contribute to increased cytoplasmic Na^+ loading and increased susceptibility to ventricular arrhythmias due to myocardial CaMKIIδ overexpression (249). Interestingly, the increased cytoplasmic Na^+ and increased noninactivating I_{Na} were reversed by KN-93 and a CaMKII inhibitory peptide in ventricular myocytes with acute, but not with chronic (transgenic) CaMKIIδ overexpression. These data, together with the finding that CaMKII phosphorylates the pore-forming $Na_V1.5$ α subunit (6), support a view that $Na_V1.5$ is an important CaMKII target, but raise questions about the nature and identity of adaptations to $Na_V1.5$ that occur in heart failure due to CaMKIIδ overex-
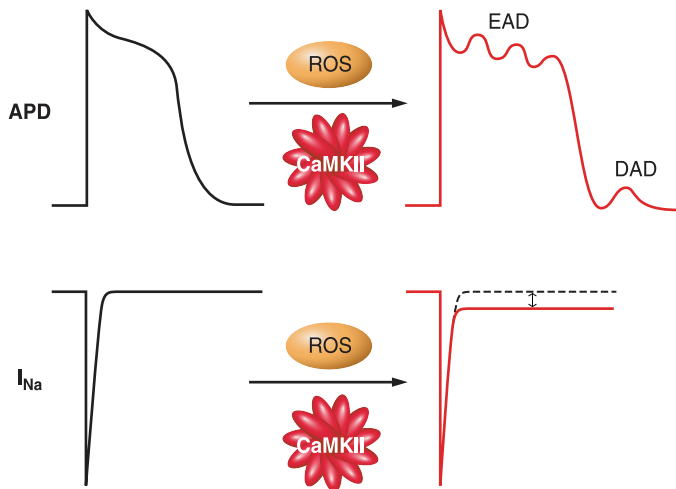
FIGURE 2 ROS and CaMKII both increase the slowly inactivating component of I_{Na} and enhance I_{Ca} facilitation, leading to action potential duration (APD) prolongation and early (EADs) and delayed (DADs) afterdepolarizations.

pression. Our group recently found that CaMKII is targeted to $Na_V1.5$ in myocardium and in neurons by β_{IV} spectrin. β_{IV} Spectrin is necessary for CaMKII to efficiently phosphorylate a key site (S571) on the I-II cytoplasmic linker of $Na_V1.5$ that controls the biophysical effects of CaMKII on I_{Na}. Cardiomyocytes isolated from mice with a mutation in β_{IV} spectrin (qv^{3J}) lacking the CaMKII binding domain were resistant to CaMKII actions on I_{Na} and to isoproterenol-triggered afterdepolarizations (100). These data show that CaMKII effects on $Na_V1.5$ occur by phosphorylation of a specific α-subunit residue and require targeting by a defined molecular platform that appears to be important in diverse excitable tissues. Additionally, elevated intracellular $[Na^+]$, as is observed in heart failure, promotes enhanced mitochondrial ROS production (124), leading to a potential increased in oxidized CaMKII. CaMKII activation and ROS production result in phenotypically similar changes in

I_{Na} in guinea pig, rabbit, and dog ventricular myocytes, including enhancement of the peak and noninactivating components (154, 214). It is interesting that CaMKII inhibition and Ranolazine, a new I_{Na} antagonist with diverse actions, both appear to show antiarrhythmic activity by suppressing the noninactivating component of I_{Na} (214). We interpret the presently available data to suggest that $Na_V1.5$ is a phosphorylation substrate for CaMKII and that at least some of the ROS effects on I_{Na} are due to activation of CaMKII. On the other hand, oxidation of Met residues also affects gating in a variety of Na^+ channels, including $Na_V1.5$ (115), suggesting that oxidation also has CaMKII-independent effects on I_{Na} in the cardiovascular system.

E. Calcium Currents

Voltage-gated Ca^{2+} channels are the primary trans-sarcolemmal pathway for Ca^{2+} entry into the excitable cells of the cardiovascular system. The high voltage-activated or long-lasting, so-called "L-type," Ca^{2+} channels are $Ca_V1.2$ and $Ca_V1.3$. $Ca_V1.2$ is the most important and most abundant voltage-gated Ca^{2+} channel in adult mammalian ventricular myocardium. $Ca_V1.2$ channels are enriched in t-tubular sarcolemmal invaginations, and this localization positions $Ca_V1.2$ to face off against the RyR Ca^{2+} release channels of the SR. $Ca_V1.2$ current ($I_{Ca}1.2$) triggers SR Ca^{2+} release from type 2 RyR (RyR2) as part of the Ca^{2+}-induced, Ca^{2+} release mechanism supporting ECC.

CaMKII is required for a dynamic property of $I_{Ca}1.2$ called facilitation (9, 265, 274) that is thought to be important for contractility. Facilitation consists of a transient increase in peak $I_{Ca}1.2$ and a concomitant slowing of $I_{Ca}1.2$ inactivation **(FIGURE 2)**. Experimental studies (58) and computational modeling (87) suggest that a high-activity $Ca_V1.2$ channel gating mode (mode 2) with frequent and prolonged openings is the biophysical mechanism for $I_{Ca}1.2$ facilitation. Surprisingly, CaMKII activation by Ca^{2+}/CaM that supports $I_{Ca}1.2$ depends on activator Ca^{2+} release from RyR2 instead of $I_{Ca}1.2$, as evidenced by the fact that preventing SR Ca^{2+} release eliminates $I_{Ca}1.2$ facilitation, but expression of a mutant, constitutively active (T286D) CaMKIIδ mutant rescues $I_{Ca}1.2$ facilitation and enhances $Ca_V1.2$ channel mode 2 gating even in the absence of SR Ca^{2+} release (128). CaMKII can phosphorylate the $Ca_V1.2$ pore-forming α subunit, but (in our opinion) the best evidence shows that a CaMKII phosphorylation site that is conserved across the four major β-subunit isoforms is required for CaMKII-dependent $I_{Ca}1.2$ facilitation and mode 2 gating (80, 81, 128).

Increasing ROS by addition of H_2O_2 increases $I_{Ca}1.2$ facilitation in rat ventricular myocytes by a process that is prevented by assiduous Ca^{2+} buffering, KN-93, or a CaMKII inhibitory peptide (215). H_2O_2 also increases peak $I_{Ca}1.2$ and slows $I_{Ca}1.2$ inactivation by a process that is reversed

by KN-93 or a CaMKII inhibitory peptide (266). Similar increases in $I_{Ca}1.2$ with H_2O_2 were reversed or prevented by adenosine receptor or protein kinase C inhibitor drugs (236), and at least one report found that H_2O_2 reduced peak $I_{Ca}1.2$ in isolated adult guinea pig ventricular myocytes (73). Although H_2O_2 is useful as an experimental approach for increasing ROS, the H_2O_2 activity in cells under physiological or disease conditions is not known precisely. However, agonists such as endothelin-1 (279) and aldosterone (248) with the potential to activate NADPH oxidase (see sect. **VII**) and thereby elevate ROS, including H_2O_2, also result in increased $I_{Ca}1.2$ and increased $Ca_V1.2$ channel opening probability. We interpret most published findings to suggest that increasing ROS by direct addition of H_2O_2 or by NADPH oxidase-coupled agonist stimulation causes a phenocopy of CaMKII actions at $Ca_V1.2$. The findings by several groups showing ROS actions are reversed by CaMKII inhibitors are thus consistent with a model where ROS activates CaMKII and oxidized CaMKII increases $Ca_V1.2$ mode 2 gating and $I_{Ca}1.2$ facilitation.

$Ca_V1.3$ is important in atrium and specialized conduction tissue, but little is known about CaMKII or ROS effects on $Ca_V1.3$ in cardiovascular tissue. There is evidence for a functionally important CaMKII phosphorylation site on the $Ca_V1.3$ pore-forming α subunit (at the intracellular COOH terminus near an EF hand domain) that increases peak current (69). Based on the high homology of the β-subunit binding α-interacting domain between $Ca_V1.2$ and 1.3, it is likely that 1.2 and 1.3 share the same β subunits. Thus it is possible (though unproven) that ROS and CaMKII binding and phosphorylation of β subunits will increase $I_{Ca}1.3$ and $I_{Ca}1.2$ by similar biophysical mechanisms. L-type ($Ca_V1.x$) and low voltage-activated or transient, so-called T-type ($Ca_V3.x$), channels are activated over more negative voltage ranges and appear to communicate Ca^{2+} via different microdomains. Transgenic overexpression of $Ca_V1.2$ causes myocardial hypertrophy, heart failure, and apoptosis (168), while overexpression of $Ca_V3.1$ did not contribute to SR Ca^{2+} overload or lead to pathological outcomes (108). $Ca_V1.2$ are preferentially expressed in the t tubules (adjacent to RyR2), while $Ca_V3.1$ expression is relatively more abundant on non-t-tubular sarcolemma. $Ca_V3.1$ Ca^{2+} contributes to antihypertrophic actions by activation of a NOS3-cGMP kinase (type 1) pathway (168). CaMKII increases T-type current by $Ca_V3.2$ during aldosterone stimulation, suggesting that ROS may be a component of this signaling pathway (38). CaMKII increases $Ca_V3.2$, but not $Ca_V3.1$, current by a phosphorylation site on an intracellular domain (II-III linker) of the pore-forming α subunit (255). Taken together, these findings suggest that CaMKII can increase $Ca_V1.2$, $Ca_V1.3$, and $Ca_V3.1$ by distinct mechanisms. ROS effects on $Ca_V1.2$ appear to involve CaMKII, and ROS and CaMKII may also be involved in augmenting $Ca_V3.2$ current. The potential involvement of ROS in CaMKII signaling to $Ca_V1.3$ is un-

certain, while $Ca_V3.1$ does not appear to be a CaMKII target. Thus we anticipate that increased ROS will activate CaMKII and preferentially enhance $Ca_V1.2$, $Ca_V1.3$, and $Ca_V3.2$ currents.

F. Potassium Currents

CaMKII has complex effects on a variety of K^+ currents important for the cardiovascular system (8). CaMKII can affect K currents by phosphorylation (e.g., $K_V4.3$) (60), by effects on SR Ca^{2+} (139) by transcriptional regulation (250), and by decreasing trafficking to sarcolemmal compartments (138). At this point, in our view, there is no simple unifying concept for the effects of CaMKII on repolarizing K^+ currents. ROS effects are well recognized to affect various K^+ currents, and in some cases, these effects potentially overlap with the effects of CaMKII. We found that CaMKII inhibition restricted to myocardium enhanced the efficacy of ischemic preconditioning to reduce cardiomyocyte death resulting from ischemia-reperfusion injury. This beneficial effect of CaMKII inhibition was due, at least in part, to increased membrane expression of the ATP-sensitive K^+ channels, but without changes in ATP-dependent gating or in Kir6.2, SUR1, or SUR2 encoding mRNA or total protein (138). Ventricular myocytes treated with H_2O_2 (73) showed increased I_{KATP} that was reversed by various kinase inhibitory drugs, including KN-62 and KN-93 (268), potentially consistent with a role for oxidized CaMKII in I_{KATP} regulation. However, oxidation also has complex effects, only a portion of which likely involve CaMKII. For example, chloramine-T preferentially oxidizes Met residues and reduces I_{Kr} in HEK cells heterologously expressing the human ether à go-go related gene 1 (hERG1), and chloramine-T effects were reversed by MsrA (221). Chloramine-T also increased availability and slowed deactivation of the K^+ channel hSlo (features that will increase current) even in the near absence of Ca^{2+}. The chloramine-T effects on hSlo were partially reversed by MsrA (228). Because most cells used for heterologous expression studies have low CaMKII, these data suggest ROS effects on I_{Kr} and hSlo current may not require CaMKII. However, oxidation of Met residues suggests the possibility of coordinate regulation of oxidized CaMKII and certain ion channel proteins by MsrA.

K^+ currents are an important determinant of action potential repolarization in myocardium. CaMKII affects AP repolarization, in part by actions at K^+ currents. Action potentials from mice with chronic myocardial CaMKII inhibition due to transgenic expression of an inhibitory peptide (AC3-I) have increased peak I_{Ca}, but shortened AP duration (282), due to upregulation of the repolarizing fast transient outward K^+ current ($I_{to,f}$) and the inward rectifier K^+ current (I_{K1}) (139). These findings suggest that CaMKII may somehow couple intracellular Ca^{2+} and ROS to AP repolarization in myocardium. The hypothesis that CaMKII ef-

fects on repolarization are tied to intracellular Ca^{2+} homeostasis appears to be supported by our finding that AP abbreviation and the increase in $I_{to,f}$ and I_{K1} were reversed to baseline when AC3-I mice were interbred into a phospholamban knockout background. We did not find evidence that CaMKII-mediated increases in $I_{to,f}$ and I_{K1} were due to increased transcription or cellular expression of candidate ion channel proteins, because we found no difference in expression of *KCND2* (encodes $K_V4.2$), *KCND3* (encodes $K_V4.3$), *KCNJ2* (encodes Kir2.1), or *KCNJ12* (encodes Kir2.2) between AC3-I and control mice. Subsequent studies showed that AC3-I mice also had increased expression of I_{KATP}, which contributed to myocardial protection during ischemia-reperfusion injury, without evidence of changes in mRNA (*ABCC8* encodes SUR1, *ABCC9* encodes SUR2, *KCNJ8* encodes Kir6.1, or *KCNJ11* encodes Kir6.2) but that sarcolemmal Kir6.2 protein was selectively increased by CaMKII inhibition, leading us to conclude that CaMKII was important for trafficking of Kir6.2 and, perhaps, other K^+ channels (138). Mice with chronic CaMKIIδ overexpression have delayed ventricular myocyte AP repolarization, compared with wild-type littermates, and downregulation of $I_{to,f}$, $K_V4.2$ and KChIP2, in contrast, $I_{to,slow}$ and $K_V1.4$ are increased. These effects were not reversed by acute CaMKII inhibition, potentially consistent with a differential CaMKII-mediated effect on K^+ channel trafficking. In contrast, CaMKIIδ overexpression (in transgenic mice or virally infected cultured rabbit ventricular myocytes) induced gating changes in both $I_{to,f}$ and $I_{to,s}$ (accelerated recovery from inactivation) that were reversible by acute CaMKII inhibition. Finally, mRNA for I_{K1} and Kir2.1 were both reduced by CaMKII overexpression. Taken together, these data suggest that CaMKII contributes to multiple aspects of K^+ channel biology, including trafficking, gating, and transcription. It remains to be seen which of these processes will be affected by ROS through a CaMKII pathway.

G. Ryanodine Receptors

Increased RyR2 leak is due to increased channel opening probability (P_o) and is associated with arrhythmias in heart failure (159) and in patients with genetically diseased RyR2 (catecholaminergic VT) (134, 184). Both PKA and CaMKII (254) can phosphorylate RyR2 leading to increased P_o and SR Ca^{2+} leak. Although this remains a highly controversial area, recent evidence suggests that CaMKII plays an important and, perhaps, preeminent role in promoting RyR2 leak (5, 48, 64, 231). RyR2 P_o is also increased by H_2O_2 (13, 24, 116, 292). ROS modification of RyR2 increases the steepness of the leak/load relationship and promotes SR Ca^{2+} leak and arrhythmias in dogs with post-MI ventricular fibrillation (19). These findings show that ROS and CaMKII produce phenotypically similar effects on RyR2, suggesting the hypothesis that ROS may affect RyR2 P_o at least partly by activating CaMKII. The relationship between RyR2, the

related inositol 1,4,5-trisphosphate receptors (InsP$_3$R), and CaMKII is further complicated by the fact that CaMKII is uniquely activated in distinct subcellular environments by intracellular Ca^{2+} release. For example, CaMKII at $Ca_V1.2$ in ventricular myocyte t tubules is primarily activated by Ca^{2+} from the SR (128), while CaMKII on the nuclear envelope is primarily activated by Ca^{2+} released from InsP$_3$R (260). At this point, it is unknown if ROS affects local CaMKII signaling to RyR2 or InsP3R.

Recently, RyR2 phosphorylation by CaMKII has been implicated in the myocardial force-frequency relationship (130). The Marks group studied mice lacking a bona fide CaMKII site on RyR2 (S2814A) (254) to show that loss of this site reduced the normally observed enhancement of frequency-dependent intracellular Ca^{2+} release and developed left ventricular pressure, but without providing protection against loss of LV ejection fraction after MI surgery, potentially suggesting that the RyR2 site contributes to physiology, but not disease responses. These same mice show resistance to atrial fibrillation (36) and reduced SR Ca^{2+} leak after MI or isoproterenol toxicity (unpublished data), suggesting that the role of this site may vary depending significantly by model and myocardial cell type.

H. Excitation-Transcription Coupling

Recent evidence supports a concept related to ECC, termed excitation-transcription coupling (260), where increases in intracellular Ca^{2+} (contributed at least in part by Ca_V1 current) lead to activation of Ca^{2+} regulated transcription factors (55, 75). Myocyte enhancer factor 2 (MEF2) is a transcription factor that is required for muscle differentiation and growth, but also contributes to pathological hypertrophy in adult hearts (176). MEF2 activity is repressed by class II histone deacetylases (HDACs) types 4 and 5 under basal conditions (14). CaMKII can phosphorylate HDACs 4 and 5 to create a 14–3-3 protein recognition site. CaMKII-phosphorylated HDAC is removed by 14–3-3 proteins leading to increased MEF2 transcriptional activity. The InsP$_3$R in the nuclear membrane is a source of Ca^{2+} for CaMKII activation and excitation-transcription coupling in heart. InsP$_3$R and RyR2 are homologous proteins. In ventricular myocardium, InsP$_3$R are enriched in the nuclear membranes, while RyR2 expression is most evident in the junctional SR that closely approximates cell membrane invaginations called t tubules. The t tubules are enriched in various ion channel proteins, including those contributing to $Ca_V1.2$. Recent evidence supports a view that the nuclear and SR lumens are continuous (259), suggesting that CaMKII can affect ECC and excitation-transcription coupling by targeting distinct but homologous proteins through macromembrane structures.

VI. HEART FAILURE PATHOPHYSIOLOGY

Ischemic heart disease is the leading cause of death worldwide and poses a significant economic and public health burden (146). Patients with ischemic heart disease usually present with MI, after which the heart undergoes a pathological remodeling process involving functional and morphological changes, namely hypertrophy, cell death, and cardiac dilation. Increasing evidence suggests that CaMKII activation plays a central role in signaling pathways mediating these disease phenotypes. Cardiac CaMKII activity increases in mice with ischemic heart disease induced by MI or isoproterenol. Our group recently showed that methionine oxidation plays an integral component to activated CaMKII after MI, suggesting that cellular redox balance and oxidized CaMKII is part of the pathological response pattern to MI.

A. Apoptosis

Cardiac cell death is a prominent pathological cellular response to injury and stress and underlies the morphological changes seen in dilated cardiomyopathy. Cardiac cell loss is usually considered irreversible because cardiomyocytes are terminally differentiated. Apoptotic cell death contributes to heart disease pathogenesis in patients with MI (202) and end-stage heart failure (169). Triggers to cardiac apoptosis include neurohormones, mechanical stress, and SR Ca^{2+} overload; CaMKII inhibition is beneficial in these situations. An imbalance of phosphatase/kinase activity can also contribute to apoptosis, and CaMKII inhibition can protect from apoptosis induced by phosphatase inhibitors (66). Overexpression of wild-type CaMKII or constitutively active CaMKII induces apoptosis in COS-1 cells, whereas overexpression of a catalytically inactive mutant CaMKII did not. βAR stimulation increases apoptosis in cultured adult ventricular myocytes, but this effect is prevented by pharmacological CaMKII inhibition (290). When CaMKII inhibitory transgenic mice were interbred to $PLN^{-/-}$ mice, the mice became less resistant to ischemia-induced apoptosis (271), while CaMKIIδ overexpressing mice interbred with $PLN^{-/-}$ mice showed markedly enhanced myocardial apoptosis (283). These studies with CaMKII inhibition or overexpression demonstrate the relevance of CaMKII activation in apoptotic cell death.

With the use of isolated feline cardiomyocytes as a model of SR Ca^{2+} overload through overexpression of LTCC, CaMKII activation increased along with myocyte apoptosis through a mitochondrial related process (37). More than one CaMKII isoform may be involved, as Timmins et al. (237) recently showed that activation of CaMKIIγ increases mitochondrial Ca^{2+} and modulates cytochrome c release and apoptosis. In addition, cholesterol-induced ER stress induced CaMKII oxidation. Our group showed that oxidation-mediated CaMKII activation downstream of ANG II

can also increase cardiac apoptosis, with corresponding increase in caspase-3 activity (62). Using Langendorff-perfused mouse hearts with transgenic overexpression of a CaMKII inhibitory peptide targeted to the SR, Salas et al. (199) showed decreased mitochondrial swelling and cytochrome c release after ischemia/reperfusion injury. Adenoviral overexpression of CaMKIIδC appears sufficient to drive mitochondrial activated apoptosis in cultured adult rat cardiomyocytes (289). These observations suggest that CaMKII can stimulate cardiac apoptosis, particularly during pro-oxidant conditions, by a pathway that involves mitochondria.

ROS are strongly implicated in apoptotic pathways (41, 210). ROS can induce DNA damage but can also be effectors of apoptosis. For instance, p53 overexpression leads to enhanced intracellular ROS, a requirement for p53-induced apoptosis (110). Given that CaMKII, an important signal transduction molecule in heart muscle, is sensitive to ROS, ox-CaMKII may potentially relay ROS regulated cellular survival and death triggers into appropriate, cellular responses. The downstream mechanism of CaMKII-dependent apoptosis is not clearly defined but has been linked to activation of proapoptotic proteases. For instance, in a cellular model of apoptosis induced by ultraviolet light or tumor necrosis factor-α, CaMKII leads to activation of AP24, a protease that stimulates DNA fragmentation (258). In a separate model of cultured adult cardiomyocytes, CaMKII inhibition protects from oubain-induced apoptosis and increases in caspase-3 activity (201). CaMKII inhibition can normalize caspase-3 activity levels in other models of cell death such as ischemia/reperfusion injury (199, 245) and ANG II treatment in cultured cardiomyocytes (174). Conversely, CaMKII activation enhances caspase-3 activity (150). CaMKII can also directly phosphorylate the proapoptotic factor Bcl10 (105), which can promote apoptosis when hyperphosphorylated (276). Whether CaMKII-mediated activation of the proapoptotic proteases is correlated or required for CaMKII-induced apoptosis warrants further research.

Although the actions of CaMKII are largely posttranslational, CaMKII also exerts transcriptional control on proapoptotic pathways. CaMKII can activate the stress-activated and proapoptotic mitogen-activated protein kinases (MAPKs). For instance, CaMKII can directly phosphorylate MAPK kinase kinase TAK1 (107) and ASK1 (225), which lead to activation of JNK and p38^{MAPK}, respectively. JNK activates the c-jun/AP-1 pathway (52), known to upregulate proapoptotic genes. CaMKIIδ can also activate the AP-1 transcription factor family independent of the JNK pathway (158), suggesting direct regulation of AP-1-directed gene transcription by CaMKII. CaMKIIγ-dependent phosphorylation of JNK occurs downstream of ER stress and was found to induce the Fas death receptor (237). Alternatively, JNK can directly phosphorylate and stabilize

p53 to induce programmed cell death (67). Likewise, p38^{MAPK} is required for p53-mediated hypoxic cell death (291). CaMKII can also directly modulate the expression of p53. In a transgenic mouse model of dilated cardiomyopathy, there is increased cardiomyocyte apoptosis and elevated p53 protein, normalized by pharmacological or transgenic CaMKII inhibition (239). Therefore, CaMKII can regulate proapoptotic gene transcription either directly or through the JNK pathway.

B. Hypertrophy

A wealth of literature supports a view that CaMKII promotes myocardial hypertrophy. Myocyte hypertrophy is initially a compensatory response to cardiac injury. When hypertrophy becomes decompensated, the heart transitions to heart failure through a process that is not entirely understood. A hallmark feature of pathological hypertrophy is the gene expression switch to a "fetal" profile that is more adaptive to a relatively hypoxic environment (185). CaMKII is implicated in the cellular hypertrophic response through upregulation of the "fetal" gene program. Through cell transfection studies and luciferase reporter assays, Ramierz et al. (186) found that CaMKIIδ_B isoform transcriptionally upregulated the promoter activity of the fetal gene encoding atrial natriuretic factor (ANF). ANF promoter activity increases in vivo in response to CaMKII activation via transgenic overexpression of calmodulin (44). In the Ren-2 transgenic rat model of hypertension-induced hypertrophy, CaMKIIδ isoforms are differentially expressed, with increased SR-associated expression (84). The fetal CaMKIIδ4 isoform also increases in expression (84, 89). In these animal models of spontaneous hypertension or in models of pressure overload (43), it is likely that the increase in CaMKII is both a byproduct and effector of hypertrophy.

ROS production increases concurrently with hypertrophic stimuli and plays a key role in mediating cardiac hypertrophy by relaying mechanical stimuli and stress to second messengers. Studies evaluating the type of ROS important in the hypertrophic response have examined superoxide, hydrogen peroxide, nitric oxide, and hydroxyl radicals (see sect. **VII**) (113). In mouse models of pressure overload-induced cardiac hypertrophy, measures of hypertrophy normalize with several antioxidants including N-2-mercaptopropionyl glycine (50), tetrahydroneopterin (226), dimethylthiourea (121), and a pharmacological mimetic for superoxide dismutase and catalase (243). In a mouse model of impaired expression of major antioxidant enzymes, mice showed enhanced ROS and developed pathological phenotypes including cardiac hypertrophy, which was rescued by superoxide dismutase (SOD) administration (206). Studies have also investigated the subcellular generation of cardiac ROS. Cardiac hypertrophy can be reduced by inhibiting ROS generation at NADPH oxidase (287), nitric oxide synthase (226, 281), or within mitochondria (77).

Studies on CaMKII and pathological hypertrophy have also evaluated the role of the phosphatase calcineurin, a Ca^{2+}-sensing, prohypertrophic signal that recruits nuclear translocation of nuclear factor activated in T cells (NFAT) (163). In cultured primary rat myocytes, the nuclear CaMKIIδ isoform can increase the transcription of the calcineurin A β subunit (147). Whereas the nuclear CaMKIIδ isoform seems to synergize with the prohypertrophic calcineurin pathway, the cytoplasmic isoform can phosphorylate and inhibit calcineurin (150). Our group found that CaMKII inhibition in calcineurin overexpressing mice did not block hypertrophy but improved survival and reduced arrhythmia susceptibility (117). CaMKII appears to cross-talk with the calcineurin pathway to affect myocardial hypertrophy and apoptosis. Calcineurin activation can inhibit H_2O_2-induced apoptosis in neonatal rat ventricular myocytes (114). Since CaMKII is likely activated in this context of enhanced oxidant stress, the protective role of calcineurin may be partially mediated through CaMKII inhibition. Calcineurin itself is redox regulated, and SOD can protect calcineurin from inactivation (252). Several oxidant species can inhibit calcineurin's phosphatase activity including H_2O_2 (32), superoxide, and glutathione disulfide, whereas some antioxidants enhanced but others blocked activity, which may depend on in vivo cellular context (213). ANG II stimulates superoxide production through NADPH oxidase, but ANG II predominantly leads to enhanced calcineurin activity, which is necessary for ANG II-induced myocyte hypertrophy in cultured myocytes (163, 224) and in vivo (74). The role of redox balance in coordinately regulating calcineurin and CaMKII activity will require further investigation.

Whereas earlier studies suggest that the nuclear-targeted CaMKIIδ_B (or CaMKIIδ_3) splice variant of the δ isoform alone directs transcriptional regulation, more recent studies outlined a role for cytoplasmic CaMKIIδ_C isoform. CaMKIIδ_B transgenic mice develop spontaneous cardiac hypertrophy (284), while CaMKIIδ_C transgenic mice exhibit heart failure due to a hypertrophic and dilated cardiomyopathic phenotype (286). However, both isoforms seem capable of initiating hypertrophic gene expression through phosphorylation of the class II HDACs and subsequent derepression of the prohypertrophic transcription factor MEF2 (285). CaMKII inhibition in vivo protects against isoproterenol-induced myocyte hypertrophy (282). Two independent CaMKIIδ knockout mouse models drew different conclusions on the requirement of CaMKIIδ in pathological hypertrophy. While the Olson laboratory showed resistance to pressure overload-induced hypertrophy in their CaMKIIδ knockout mice (15), Ling et al. (142) found their knockout model maintained susceptibility to pressure overload-induced hypertrophy, but later resisted development of chamber dilation and heart failure. These seemingly conflicting findings may be explained by differences in the deleted exons, surgical technique, and/or mouse genetic back-

grounds. When taken together with findings from other groups, both studies reinforce the importance of CaMKII and redox balance in the spectrum of myocardial disease phenotypes leading to heart failure.

C. Arrhythmias

CaMKII mediates ECC, and its hyperactivation is proarrhythmic. Cardiac pacemaking activity sets the rate and rhythm of cardiac contractions. Pacemaker cells have an intrinsic property known as automaticity, which refers to the spontaneous generation of action potentials. These "automatic" cells exhibit a gradual, spontaneous depolarization during diastole. Cells in the SAN, the AVN, and the His bundle and Purkinje system display automaticity. Heart rate is determined by the fastest depolarizing, "automatic" cells, normally the SA nodal cells. Rhythm abnormalities can result from sinus nodal dysfunction or in presence of ectopic pacemaker cells that depolarize faster than sinus nodal cells. Both CaMKII and ROS are implicated in SA nodal firing. In rabbit SA nodal cells, firing rate increases after treatment with hydrogen peroxide (82) and t-butyl hydroperoxide (203). CaMKII activation also contributes to increased SA nodal cell firing frequency. CaMKII inhibition reduces L-type Ca^{2+} current in isolated SA nodal cells, reducing the rate of firing (192, 246). Furthermore, CaMKII mediates SA nodal response to increased β-adrenergic signaling (261). These findings suggest important roles for ROS and CaMKII signaling in sinus node dysfunction and related rhythm abnormalities.

Atrial fibrillation is the most common presenting arrhythmia (256). A self-propagating phenomenon, atrial fibrillation is increasingly linked to redox imbalance. In a porcine model of induced atrial fibrillation, there is a correlational increase in NADPH oxidase- and xanthine oxidase-derived superoxide levels in left atrium and left atrial appendage (57). In patients with atrial fibrillation, the right atrial appendage tissue exhibits more NADPH oxidase-derived superoxide than tissue from patients with sinus rhythm (120). Human atrial tissue from patients with atrial fibrillation shows increased CaMKII expression (232) and autophosphorylation (36). One proposed molecular mechanism for atrial fibrillation is downregulation of ion channels required for cell-to-cell communication and action potential propagation, or so-called proarrhythmic electrical remodeling (275). Redox imbalance can contribute to this phenotype as was recently shown by Smyth et al. (212). Their studies revealed oxidant stress in human and mouse hearts can lead to microtubule dysfunction and decreased delivery of connexon43, the primary gap junction channel in cardiac myocytes, to the cell membrane (212). Another proarrhythmic mechanism in atrial fibrillation models is increased SR Ca^{2+} leak from RyR2. CaMKII increases RyR2 P_o, and SR Ca^{2+} release is a candidate mechanism for CaMKII promoting atrial fibrillation (36, 144). Becuase CaMKII can couple cellular redox state to ECC, CaMKII may mediate redox-dependent arrhythmias, including atrial fibrillation.

When an action potential triggers an additional depolarization inappropriately, this may also lead to an arrhythmia. Two cellular mechanisms underlie these extra depolarizations, called early afterdepolarizations (EADs) and delayed afterdepolarizations (DADs). EADs occur before full repolarization and are more likely in disease states with prolonged action potentials, such as in heart failure and long Q-T syndromes. Sustained EADs may engage reentry pathways and lead to the severe ventricular arrhythmia torsades de pointes (267). In contrast to EADs, DADs occur after full repolarization and under conditions of elevated intracellular Ca^{2+} from an overloaded SR. DADs have been documented in failing heart tissue from dogs (94), rabbits, and humans (244).

Transgenic mice with CaMKIV overexpression have moderate left ventricular hypertrophy, increased myocardial CaMKII expression and activity, and prominent proarrhythmic electrical remodeling, including Q-T interval and AP duration prolongation. CaMKIV transgenic mice had frequent, spontaneous polymorphic ventricular tachycardia. Ventricular myocytes isolated from these mice had spontaneous EADs and enhanced $Ca_V1.2$ channel P_o. CaMKII inhibition prevented arrhythmias, suppressed EADs, and reduced $Ca_V1.2$ P_o to baseline (264). Abnormal Ca^{2+} leak from the SR may also contribute to arrhythmogenesis. Myocytes from nonischemic heart failure show increased CaMKII expression along with enhanced phosphorylation of Ca^{2+} handling SR proteins. CaMKII inhibition by the pharmacological agent KN-93 decreased SR Ca^{2+} leak and increased SR Ca^{2+} content (5). CaMKII regulation of SR Ca^{2+} was studied in vivo through a transgenic mouse model with SR targeted CaMKII inhibitory peptide AIP. These transgenic mice had decreased CaMKII-dependent RyR phosphorylation and SR Ca^{2+} leak compared with wild-type mice or mice overexpressing a nuclear targeted variant CaMKII inhibitory peptide, AIP (181). In addition to Ca^{2+} handling proteins, CaMKII can phosphorylate cardiac sodium channels and alter channel gating properties, leading to enhanced noninactivating current **(FIGURE 2)** prolonged QRS and QT intervals, EADs and predisposing to ventricular arrhythmias (249). Although the complete list of triggers is too long to detail here, it is striking that CaMKII inhibition appears to be beneficial in all known proarrhythmogenic conditions (5, 10, 36, 128, 250, 262, 264). In one recent example, we found that CaMKII was critical for the proarrhythmic properties of a rare genetic disease of the $Ca_V1.2$ channel called Timothy Syndrome (234).

Oxidative stress can trigger EADs in guinea pig and rabbit ventricular myocytes by increasing the delayed sodium current (214). CaMKII inhibition either through KN-93 (or AIP) prevents (or delays) H_2O_2-induced both EADs and DADs in rabbit ventricular myocytes (266). Spontaneous arrhythmias can also be induced during metabolic acidosis. In a confocal microscopy study with fluo-4, acidification in isolated adult mouse myocytes led to SR Ca^{2+} waves that

were abolished with KN-93 (178). In a mouse model expressing a gain-of-function mutation in RyR2, which leads to an enhanced vulnerability to developing atrial fibrillation, either pharmacological or genetic CaMKII inhibition is protective (36). In human right atrial myocytes isolated from patients with atrial fibrillation, Neef et al. (170) found increased RyR2 phosphorylation at the CaMKII site and increased SR Ca^{2+} leak that was reversed with KN-93. Finally, the post-MI state is especially prone to arrhythmias. In a canine model of MI, analysis of the infarct border zones reveals increased CaMKII phosphorylation (99) and oxidation (39). Mathematical modeling further suggested proarrhythmic conduction slowing is redox and CaMKII dependent. Thus CaMKII activation by ROS may be critical to many proarrhythmogenic disease states, and CaMKII inhibition represents a potential alternative to antioxidant approaches seeking to normalize conduction abnormalities.

VII. REDOX SIGNALING IN THE HEART

A. Production of Reactive Oxygen Species in Cardiac Tissue

A number of mechanisms contribute to the production of ROS in cardiac tissue under both baseline and pathophysiological conditions **(FIGURE 3)**. One source of cardiovascular ROS that has received intense scrutiny is the Nox family of NADPH oxidases. While the majority of pro-oxidant cellular processes create ROS as a byproduct, the Nox enzymes are unique in that they produce elevated levels of ROS in the form of superoxide and hydrogen peroxide as their primary function (18). The endogenous activity of NADPH oxidases is low in cardiac tissue at basal conditions but is potently activated by a number of cardiovascu-
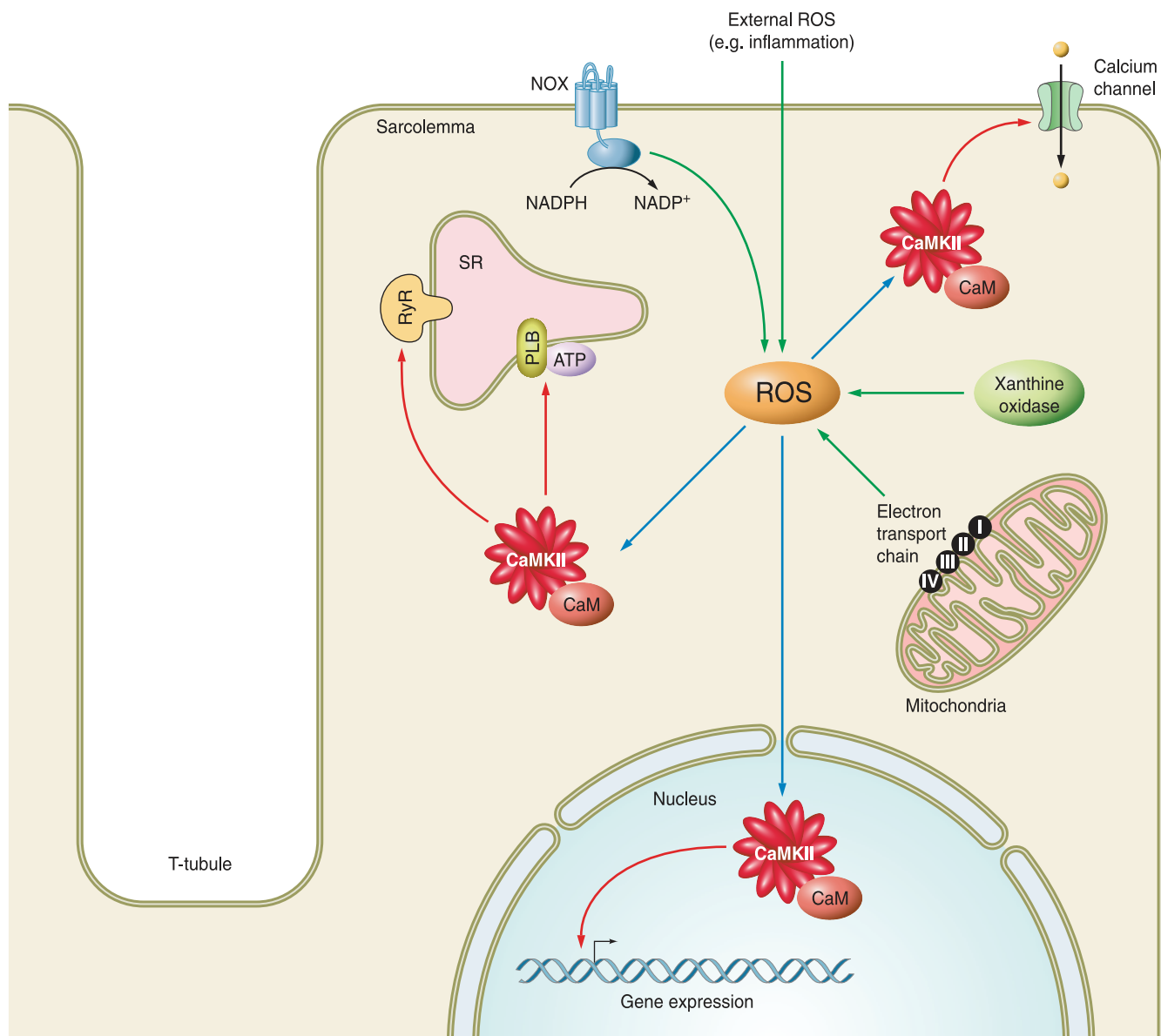

FIGURE 3 ROS and CaMKII in cardiomyocytes.

lar agonists, including ANG II (141, 149), endothelin (56), thrombin (90), and TNF-α (270). Both expression and activity of NAPDH oxidase were increased in a guinea pig model of cardiac hypertrophy and heart failure (140).

Xanthine oxidase, an enzyme in the purine catabolism pathway in mammalian species, constitutes another source of superoxide production in the heart. Contemporaneous coactivation of Nox family enzymes and xanthine oxidase occur in the heart in response to mechanical stress. Furthermore, NAPDH oxidase and xanthine oxidase activities were upregulated in a porcine model of atrial fibrillation (57). While the overall contribution of xanthine oxidase to oxidative stress in the heart remains unclear, these observations indicate that xanthine oxidase may contribute alongside NADPH oxidase as an important source of ROS in pathophysiological conditions.

Mitochondrial-derived ROS is thought to play a critical role in cardiac physiology. Superoxide is generated at several points within the electron transport chain during oxidative phosphorylation (11, 104). Chronic stimulation of mitochondrial activity leads to uncoupling of the electron transport chain and greatly increased production of ROS, a process which contributes to myocardial dysfunction (204). A rat model of right ventricular failure had greatly increased activity of key electron transport enzymes as well as elevated mitochondria-derived ROS (188). Mitochondrial morphology and protein composition are altered significantly during myocardial remodeling and may be partly responsible for progression to heart failure (53).

Complicating matters, several mitochondrial targets are critically sensitive to redox damage in conditions of high oxidative stress, such as during heart failure (131). Mitochondrial DNA (mtDNA) is more susceptible to oxidation than nuclear DNA (72), resulting in reduced mtDNA transcription, reduced metabolic activity, and increased ROS production in strongly pro-oxidant conditions (102). Left unchecked, the cycle of ROS production and mitochondrial damage can induce severe cellular injury (241).

A final source of ROS that has received much attention in the context of cardiovascular health is the family of nitric oxide synthases (NOSs). Nitric oxide plays a key regulatory role in vascular physiology, particularly in vasodilation and vasoprotection (23, 42). Of particular note is the seemingly paradoxical nature of nitric oxide-mediated effects in the induction of heart disease. For example, elevated nitric oxide in the heart promotes caspase activation, a precursor event in the apoptosis pathway, in a dose-dependent manner (257). Conversely, nitric oxide has been shown to inhibit the calcineurin/NFAT signaling pathway, blocking cardiac hypertrophy (65). A comprehensive treatment of the role of nitric oxide is beyond the scope of this review, and this topic has been reviewed recently (112).

It is also important to note in the context of CaMKII, a kinase that is acutely sensitive to both intracellular calcium and ROS, that calcium signaling plays an important role in the modulation of oxidative stress. Indeed, significant cross-talk is believed to exist between calcium and ROS levels in cardiac myocytes **(FIGURE 4)**. We have already noted some of the known effects of increased oxidative stress on calcium handling (see section on RyR, for example). Increased intracellular calcium enhances ROS production by the mitochondria (49, 85), though the mechanism for this enhancement is not well understood. NADPH oxidase activity is also modulated in part by calcium (17). Cross-talk mechanisms linking calcium and ROS signaling in the heart could play a significant role in the regulation of CaMKII activity, particularly in failing myocardium. For further review of this topic, we direct the reader to a pair of recent reviews (63, 269).

B. Oxidative Stress in Heart Failure

A large body of evidence indicates that heart failure is accompanied by increased production of ROS. Patients with heart failure have elevated levels of 8-iso-prostaglandin $F_{2\alpha}$, a key biomarker for oxidative stress, in their pericardial fluid (153). Biopsy samples from human subjects with heart disease showed increased oxidative DNA damage (127) and xanthine oxidase expression (4) in patients suffering from heart disease. Additionally, left ventricular glutathione oxidation and lipid peroxidation were significantly increased in a rat model of heart failure (92), while electron spin resonance spectroscopy has been used to directly demonstrate increased levels of hydroxyl radicals in failing myocardium (103).

Models of heart failure initiated by ANG II are characterized by acutely pro-redox conditions (238). ANG II is known to stimulate ROS production through the NADPH oxidases (149), a critical step in the development of ANG II-induced cardiac hypertrophy (20). In fact, increased levels of oxidized CaMKII were found in immunohistological stains of heart sections from mice after treatment with ANG II and after MI (62), demonstrating that heart failure is accompanied by sufficient generation of ROS to cause biochemical alterations of specific proteins involved in cardiac physiology.

A number of cardiac proteins are functionally sensitive to pro-oxidant conditions. For example, oxidation of thiol residues in the ATP-binding pocket inhibits MEK kinase 1 activity (47). Conversely, increased oxidative stress activates ERK1/2 signaling (61). Redox-dependent changes in protein activity can have broad, indirect effects on cardiac kinase function. Increased generation of ROS in the heart directly inactivates protein phosphatases (240) while simultaneously activating protein kinase kinases, such as IKK-β (190). One key relationship between CaMKII and ROS appears to be one in which NADPH oxidase-derived ROS oxidizes regulatory domain methionines to increase ox-CaMKII and CaMKII activity, with-
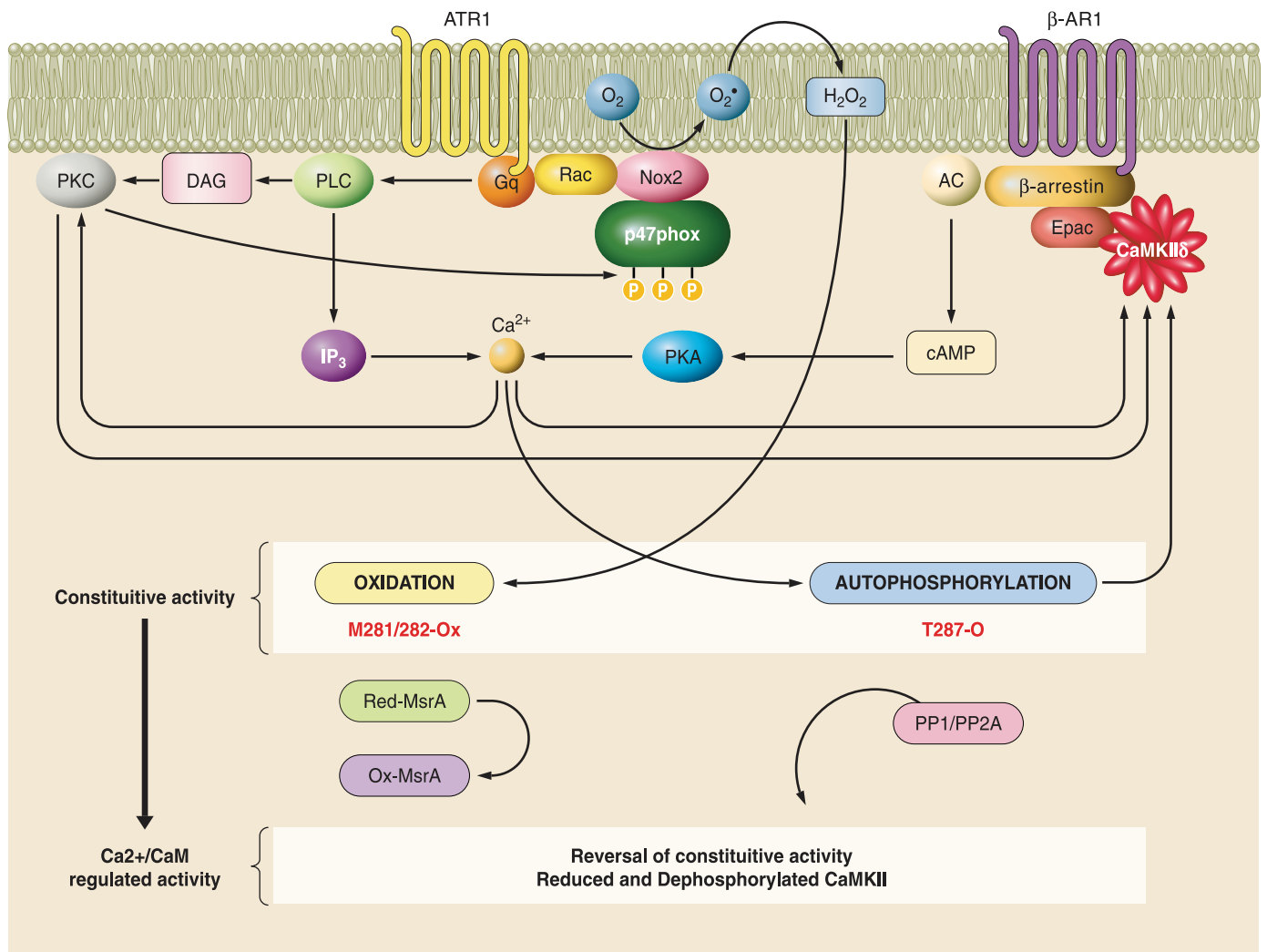
FIGURE 4 CaMKII is a likely participant in complex intercellular cross-talk between calcium and ROS signaling mechanisms.

out evidence for activity disabling actions at other domains.

Moreover, increased oxidative stress contributes directly to cellular damage and remodeling during heart failure. Both apoptosis and hypertrophic remodeling were associated with increased expression and activity of key redox proteins in a model of congestive heart failure (216). Increased NOS activity has been implicated in functional remodeling and β-adrenergic signaling sensitivity in volume overload-induced heart failure (70). Importantly, individual ROS pathways are associated with specific mechanisms of heart failure. For example, activation of the NADPH oxidase isoform Nox2 is implicated in ANG II-induced hypertrophy, while pressure overload-induced hypertrophy is associated with Nox4 activation (30). This finding suggests that clinical treatments tailored to specific redox pathways may be more effective in reducing the pathological processes associated with heart failure than broad-based antioxidant approaches.

C. Biochemical Protection Against Oxidative Stress

The production of ROS is a routine and unavoidable consequence of many physiological processes in aerobic organisms. It is therefore not surprising that complementary defense mechanisms have evolved to protect critical cellular components from irreversible damage by oxidative stress. Two general mechanisms exist to cope with the effects of ROS generation: conversion of ROS to more innocuous waste products and reversal of biomolecular oxidation.

One of the most broadly characterized groups of enzymes involved with ROS conversion is the SOD family. First identified from erythrocyte lysates by Mann and Keilin in 1938, SODs are now known to be a ubiquitous enzyme in eukaryotic cells. Three broad classes of SOD have been described (of which two are present in mammalian species), differentiated by the metal cofactors associated with the enzyme. These include Cu,Zn-SOD (SOD1 and SOD3 in humans), Mn,Fe-

SOD (SOD2), and Ni-SOD, the latter of which is only recently discovered and only present in bacteria (272). All three classes of SOD share a similar catalytic function: the conversion of highly reactive superoxide molecules to molecular oxygen and H_2O_2. Interestingly, SODs have unique structural features that result in near diffusion-limited rate of catalysis (180), evidence of the precise molecular tuning common to this family of enzymes. Indeed, even a single point mutation can be sufficient to drastically reduce the rate of catalysis for Cu,Zn-SOD and result in severe pathophysiology, most notably amyotrophic lateral sclerosis (ALS) (194).

Another group of enzymes involved with the removal of ROS are the catalases. Numbering well over 300 distinct members, this enzyme family is broadly responsible for the conversion of H_2O_2 to water and molecular oxygen. Working in tandem with SOD, these two enzyme families are largely responsible for eliminating oxidative stress in cells (35). Like SOD, catalases contain a variety of metallic cofactors and have been observed to achieve very high turnover rates of catalysis. For further review of the catalase enzyme family, see Reference 122.

Elimination of potentially harmful ROS by SOD and catalase can attenuate a number of physiological and pathophysiological processes. Perhaps the most widely known example is in aging, as simultaneous overexpression of Cu,Zn-SOD and catalase results in significantly increased life span in *Drosophila melanogaster* (172), while deficiency in SOD activity significantly reduces life span in yeast (145). However, the role of SOD/catalase in mammalian aging remains contentious (179).

Many biological targets of oxidation are simply eliminated and replaced to prevent the accumulation of damaged subcellular components. However, turnover of these molecules cannot fully compensate for the frequent modification of proteins and nucleotides by ROS. A number of enzymes are tasked with reversing oxidative damage in cells, including exo- and endonucleases for repairing DNA damage and thioredoxin for reducing peptide oxidation. For this review, we will highlight an enzyme that reverses methionine oxidation, MsrA, due to the observation that CaMKII is susceptible to redox modification at the Met-281/282 residues.

Oxidation of methionine leads to both *S*- and *R*-diastereoisomeric forms of methionine sulfoxide, which can be subsequently reduced by MsrA and MsrB, respectively. Much like CaMKII, Msr is distributed throughout the cell, including in the cytosol, mitochondria, and nucleus (118, 247). Initial observations in bacteria demonstrated the antioxidant properties of Msr, as the addition of MsrA to *Escherichia coli* increased resistance of the bacteria to ROS-mediated growth inhibition (218). Subsequent studies in mammalian models have pointed to a role for MsrA as a

protective agent against redox-mediated brain disorders (68), cancer (135), and aging (123).

In the context of the heart, MsrA activity is strongly linked to resistance against disease phenotypes associated with increased oxidative stress, such as in ischemia-reperfusion and after MI. Cardiac cells overexpressing MsrA were more resistant to cell death after being subjected to hypoxia/reoxygenation than those expressing basal levels of the enzyme (183). Moreover, functional remodeling and mortality after MI were significantly increased in mice lacking MsrA (62). Taken together, these observations point to MsrA as an important cardioprotective enzyme, one that reduces cell death, pathological remodeling, and mortality in conditions of elevated oxidative stress.

VIII. OXIDATION OF CaMKII IN THE HEART

A. CaMKII Oxidation at Met-281/282 Is a Marker for Oxidative Stress

Redox-dependent CaMKII activation is triggered by oxidative modification of the Met-281/282 site located in the regulatory domain of the kinase (62). Thus oxidation at this site serves as a marker for elevated oxidative stress in cardiac tissue. Our group developed an antiserum against a peptide matching the CaMKII regulatory domain that had been subjected to oxidation, allowing us to assess the extent of redox-dependent CaMKII activation after subjecting cardiomyocytes to elevated oxidative stress. Treatment of either purified CaMKII protein or isolated cardiomyocytes with 100 nM H_2O_2 resulted in a significant increase in oxidized CaMKII.

Elevated circulating ANG II stimulates Nox activity and results in increased ROS production (62, 149). It follows that chronic stimulation of cardiomyocytes with ANG II should coincide with a greater proportion of oxidized CaMKII. Indeed, isolated cardiomyocytes treated with 100 nM ANG II for 24 h had unchanged expression of CaMKII but significantly greater oxidized CaMKII compared with cells treated with saline or isoproterenol. Likewise, mice implanted with mini-pumps to administer ANG II had significantly increased oxidized CaMKII after 2 wk compared with animals treated with saline or isoproterenol.

Cardiac pathophysiology after MI is characterized in part by elevated oxidative stress (121). To test the efficacy of CaMKII oxidation as a marker for oxidative stress, we used a custom oxidized CaMKII antiserum to probe heart sections from mice 6 wk after MI or sham treatment. Mice that underwent MI showed significantly increased oxidized CaMKII, consistent with the hypothesis that redox-dependent CaMKII activation may contribute to cell death and functional remodeling in the post-MI heart. Taken together, these observations demonstrate that CaMKII oxida-

tion at Met-281/282 translates a broad range of proredox stimuli and represents a viable marker for elevated oxidative stress in the heart **(FIGURE 5)**.

B. Oxidation of CaMKII Plays a Key Role in Cardiomyocyte Apoptosis

Activation of CaMKII is a critical step for the induction of cell death in the heart by a variety of stimuli (289). To determine whether redox-dependent CaMKII activity plays a role in apoptosis, we used shRNA to silence endogenous CaMKII expression in isolated cardiomyocytes. We then used lentiviral constructs to restore either wild-type CaMKII or a mutant version of the kinase lacking the Met-281/282 pair. While cells expressing the wild-type construct had a normal apoptotic response to ANG II, cells expressing the redox-resistant mutant did not undergo programmed death after 24 h of ANG II treatment. Treatment with isoproterenol, which activates CaMKII through Thr-287 autophosphorylation rather than redox modification, resulted in significant apoptosis regardless of which construct was used.

Elevated oxidative stress is closely associated with the failing human heart (86), and ANG II-induced ROS production has been implicated in promoting heart failure (238). Not surprisingly, ANG II-mediated apoptosis can be reduced by inhibition of ROS formation or CaMKII activity, while we found that isoproterenol-mediated apoptosis is unaffected by blocking oxidative stress (62). These observations support the hypothesis that redox modification of CaMKII activity translates oxidative stress into apoptosis in the heart.

C. MsrA Protects the Heart From Redox-Dependent CaMKII Activity

MsrA plays a critical role in preventing the accumulation of oxidative damage by reversing redox modification of methionine residues. Because redox modification of Met-281/282 on CaMKII is the central mechanism for inducing ROS-depen-

dent CaMKII activity, we hypothesize that MsrA directly regulates CaMKII function during pro-redox conditions. It is not surprising then, that MsrA overexpression protects against cell death after ischemia/reperfusion in the heart (183), a process that has been linked to CaMKII activity (138, 273).

Increased circulating ANG II stimulates production of ROS (149) and induces apoptosis in cardiomyocytes via ox-CaMKII (62). Heart sections from MsrA$^{-/-}$ mice showed more pronounced levels of CaMKII oxidation and greater susceptibility to apoptosis after chronic treatment with ANG II compared with wild-type littermates. Ox-CaMKII and cardiomyocyte apoptosis were increased significantly more in MsrA$^{-/-}$ mice compared with wild-type controls after MI, a condition characterized by greatly increased oxidative stress (95). Of particular note, myocardial apoptosis, adverse functional remodeling, and mortality were significantly increased in MsrA$^{-/-}$ mice post-MI compared with controls, underlining the cardioprotective role of MsrA in pro-redox conditions. We found that oxidation of CaMKII served as a clear marker for elevated oxidative stress and redox-mediated cardiac injury.

IX. CaMKII AND OXIDATION IN VASCULAR TISSUE

Oxidant stress is a common mechanism of injury in vascular tissue (175), and excessive ROS is implicated in the pathogenesis of hypertension, atherosclerosis, and stroke (208). CaMKII is expressed in arterial endothelium and media, but compared with more extensive studies in the myocardium, the function of CaMKII in the vasculature remains relatively less studied. CaMKII mediates smooth muscle cell proliferation (111), hypertrophy (137), migration (160), and contraction (119, 193). While these smooth muscle phenotypes are important mechanisms of atherosclerosis and hypertension, there is to date only emerging evidence for CaMKII as mediator of vascular pathology in vivo (96, 137, 167). Given the important role of ROS in smooth muscle migration (223, 230, 253) and proliferation (126,
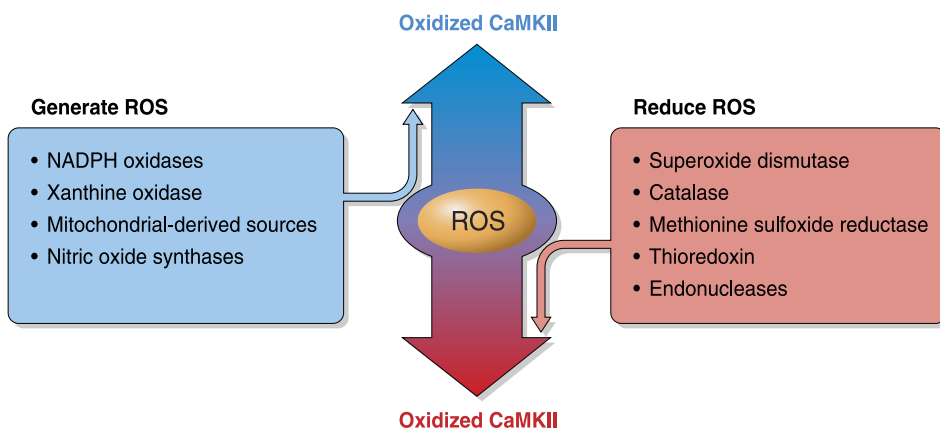
Generate ROS
- NADPH oxidases
- Xanthine oxidase
- Mitochondrial-derived sources
- Nitric oxide synthases

ROS

Reduce ROS
- Superoxide dismutase
- Catalase
- Methionine sulfoxide reductase
- Thioredoxin
- Endonucleases

Oxidized CaMKII

Oxidized CaMKII

CaMKII oxidation state "senses" imbalance in the ROS generation/elimination equation

FIGURE 5 The oxidation state of CaMKII is acutely sensitive to balance between ROS-producing and ROS-ablating processes.

177, 223), it is tempting to speculate that CaMKII is oxidized in the vasculature and contributes to vascular pathology **(FIGURE 6)**.

Oxidized CaMKII is detectable in proliferating smooth muscle cells. However, its function and significance are unknown at this point. In contrast, several studies have reported an increase in autophosphorylated CaMKII in response to oxidative stress in vascular smooth muscle (148, 242). After endothelial oxidative injury, the L-type calcium channels were activated and CaMKII maximally phosphorylated after 5 min in the medial smooth muscle cells in murine posterior cerebral arteries (71). CaMKII inhibitors KN-62 and -93 prevented CaMKII autophosphorylation and decreased the expression of the vascular cell adhesion molecule-1 (VCAM-1) (148). As the binding of Ca^{2+}/CaM is also a prerequisite for oxidation of CaMKII Met-281/282 (62), it seems likely that oxidized CaMKII is present in these models (148, 242). Another study provided indirect evidence for CaMKII activation as mediator of H_2O_2-induced proliferative responses in vascular smooth muscle. Application of H_2O_2 increased the phosphorylation of protein kinase B and Erk1/2, Pyk-2 and insulin-like growth factor. Pretreatment with the calmodulin inhibitor calmidazolium, the CaMKII inhibitor KN-93, or CaMKII knock down with siRNA decreased these phosphorylations (28).

CaMKII is present in vascular endothelium. However, our understanding of endothelium-specific CaMKII signaling is nascent. In aortic endothelial cells, H_2O_2 induced CaMKII autophosphorylation and increased its enzymatic activity.

H_2O_2 is an extremely potent stimulus for endothelial NOS (eNOS) gene expression, and both the CaMKII inhibitor KN-93 and the calmodulin antagonist W-7 can attenuate eNOS mRNA induction by H_2O_2 (31).

Treatment of bovine aortic endothelial cells with H_2O_2 increased ERK1/2 and p38 MAPK phosphorylation and activity. This effect was attenuated by KN-93, and transfection with a CaMKII inhibitory peptide modeled on the CaMKII regulatory domain. Furthermore, CaMKII inhibition reduced the H_2O_2-mediated activation of HSP27 and attenuated H_2O_2-induced formation of actin stress fibers (171).

Thrombin induces NADPH oxidase-dependent ROS production in endothelial and smooth muscle cells (233) and increased CaMKII activity (25, 26). Pretreatment with KN-93 attenuated both thrombin-induced increases in monolayer permeability to albumin and decreased transendothelial electrical resistance (26). Thrombin caused translocation and significant phosphorylation of nonmuscle filamin (ABP-280), which was attenuated by KN-93. Together, these studies indicate that oxidation, CaMKII activation, and filamin phosphorylation may participate in thrombin-induced cytoskeletal reorganization and endothelial barrier dysfunction. These accumulating data demonstrate that CaMKII is activated by oxidative stress in vascular tissue and that ox-CaMKII regulates the mitogen-activated protein kinases Erk1/2 and p38 that are central to many ROS-induced cellular activities. A detailed dissection of the activation of CaMKII by oxidation of Met-281/282 versus autophosphorylation of Thr-287 is missing at this time.
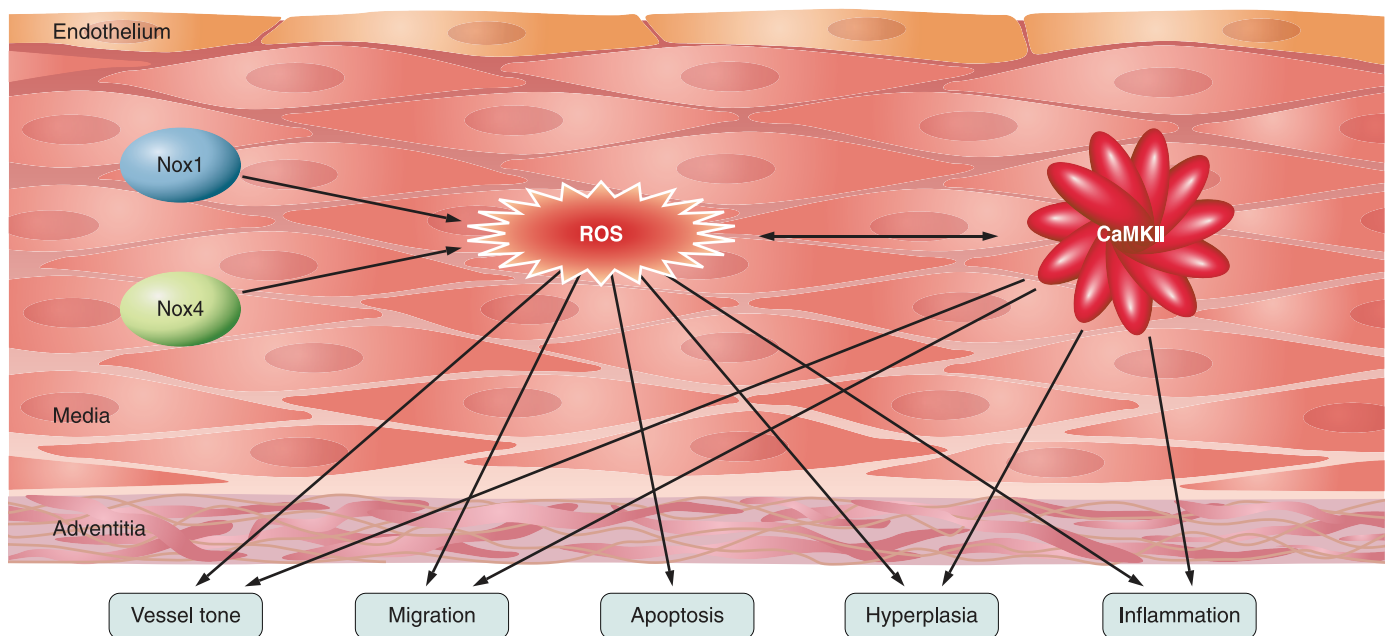
FIGURE 6   Mechanical and biological factors increase the production of ROS and activate CaMKII in vascular smooth muscle cells, resulting in impaired vessel tone, enhanced inflammatory response, and increased smooth muscle cell migration, proliferation, and apoptosis.

X. CONCLUSIONS AND FUTURE DIRECTIONS

The discovery that CaMKII is configured to coordinate and transduce upstream Ca^{2+} and ROS signals into physiological and pathophysiological downstream responses has potentially broad implications for understanding cardiovascular biology and disease. Because the identification of the ox-CaMKII/MsrA pathway is new, there remains much to do before we know to what extent ox-CaMKII and autophosphorylated CaMKII behave similarly. What is clear is that in many cases there is an intriguing overlap between ROS and CaMKII responses at Ca^{2+} homeostatic proteins, ion channels, signaling molecules, and gene transcription. In a much smaller number of studies, direct evidence supports the concept that ROS modification of CaMKII is the molecular mechanism for ROS-triggered cardiovascular disease phenotypes. Because ROS and CaMKII are important in neurobiology, cell survival, and cell cycle control, it is possible that the relationship between ROS and CaMKII will be important for understanding and treating neurological diseases and cancer. Future research in this area will allow us to parse the contributions of the various CaMKII activation mechanisms to specific physiological processes in the heart and beyond.

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DISCLOSURES

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