MINIREVIEW

This paper is available online at www.jbc.org

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 286, NO. 12, pp. 9921–9927, March 25, 2011

Redox Signaling and Cardiac Sarcomeres*

Published, JBC Papers in Press, January 21, 2011, DOI 10.1074/jbc.R110.175489

Marius P. Sumandea[‡1] **and Susan F. Steinberg**[§]

From the [‡]*Department of Physiology, Center for Muscle Biology, University of Kentucky, Lexington, Kentucky 40536 and the* [§]*Department of Pharmacology, College of Physicians and Surgeons, Columbia University, New York, New York 10032*

Oxidative stress is common in many clinically important cardiac disorders, including ischemia/reperfusion, diabetes, and hypertensive heart disease. Oxidative stress leads to derangements in pump function due to changes in the expression or function of proteins that regulate intracellular Ca^{2+} homeostasis. There is growing evidence that the cardiodepressant actions of reactive oxygen species (ROS) also are attributable to ROS-dependent signaling events in the sarcomere. This minireview focuses on myofilament protein post-translational modifications induced by ROS or ROS-activated signaling enzymes that regulate cardiac contractility.

Cardiac myofilament activation is tightly regulated by protein-protein interactions that convert the chemical energy of ATP into the mechanical energy of muscle contraction. In the healthy heart, these protein-protein interactions are precisely tuned (largely through a series of orchestrated phosphorylations on myofilament proteins) to accommodate differences in hemodynamic load during rest and exercise. Disease-specific alterations in the post-translational modification of myofilament proteins lead to miscommunication between sarcomeric proteins and to contractile dysfunction.

Direct Reactive Oxygen Species-dependent Modifications of Sarcomeric Proteins

Many models of oxidative stress lead to heart failure syndromes that are not associated with changes in Ca^{2+} homeostasis and are likely attributable to reactive oxygen species (ROS)[2]-dependent modifications of sarcomeric proteins (1–6). ROS-dependent protein modifications typically map to reactive cysteines (cysteines flanked by basic or aromatic residues that form thiolate anions and are susceptible to redox modifications at physiological pH).

Studies in detergent-permeabilized cardiac fibers show that ROS reduce Ca^{2+}-activated force, with no immediate effect on fibers in rigor (with inactive cross-bridges) (7), suggesting that ROS act on regions of the myofilaments exposed by Ca^{2+} activation and inaccessible in attached cross-bridges (and that ROS do not induce nonspecific effects that disrupt the integrity of the sarcomeric lattice). Some studies identify the myosin heavy chain (MHC) as a redox sensor in the sarcomere because redox modifications at Cys^{697} and Cys^{707} decrease myosin ATPase activity and lead to myofilament dysfunction (6, 8–10). Other redox modifications of proteins in the thin filament (actin and tropomyosin (Tm)) also lead to defects in actin-myosin cross-bridge formation and thin filament activation by Ca^{2+} (11). Cys^{374} in actin (which indirectly interacts with myosin) may be particularly important because Cys^{374} oxidation results in changes in maximum actomyosin ATPase activity and actin filament sliding velocity (12). Cys^{374} also is the likely target of a glutathionylation reaction that decreases Tm-actin binding cooperativity and maximum developed force in permeabilized trabeculae (13). Redox modification of Tm (in this case, dimerization due Cys^{190} oxidation) is detected in ischemic pig hearts and also may contribute to ROS-induced myofilament dysfunction because it alters Tm flexibility and Tm-thin filament interactions (14, 15).

Titin (the giant sarcomeric protein that controls passive tension and functions as a molecular scaffold to recruit signaling proteins that regulate mechanotransduction) also is ROS-sensitive. Oxidative stress leads to the formation of one or more disulfide bonds involving the titin N2-B domain (which contains six Cys residues). This redox modification decreases the extensibility of titin and increases its passive tension (16, 17). Desmin is the main intermediate filament protein that forms a network around sarcomeric Z-discs, links neighboring myofibrils, and connects myofilaments to other cellular structures (nuclei, cytoskeleton, and mitochondria) (11). Redox-modified (oxidized or nitrated) forms of desmin accumulate in insoluble aggregates that disrupt the sarcomeric lattice, have a toxic effect on the proteasome, and may contribute to contractile dysfunction (18, 19).

MHC, cardiac troponin (cTn) T, Tm, actin, cardiac myosin-binding protein C (cMyBP-C), myofibrillar bound creatine kinase, and α-actinin are Tyr-nitrated following treatment with peroxynitrite (20, 21). Actin, cTnC, cTnI, desmin, myosin light chain, and Tm also are Tyr-nitrated in the aging heart in association with a decrease in contractile function (22). With the exception of α-actinin (where Tyr nitration alters longitudinal force transmission between adjacent sarcomeres) (21), the functional consequences of individual sarcomeric protein Tyr nitration remain unclear.

Sarcomeric Protein Phosphorylation

Myofilament activation is modulated through sarcomeric protein phosphorylation. Because several sarcomeric proteins are phosphorylated by ROS-sensitive enzymes, stimuli that alter the intracellular redox state and shift the balance of cellular kinase *versus* phosphatase activity are predicted to alter cardiac contractility. Sarcomeric proteins cTnI, cTnT, cMyBP-C,

* This work was supported, in whole or in part, by National Institutes of Health Grants AG032009 and HL77860. This is the fourth article in the Thematic Minireview Series on Signaling in Cardiac Sarcomeres in Health and Disease. This minireview will be reprinted in the 2011 Minireview Compendium, which will be available in January, 2012.

[1] To whom correspondence should be addressed. E-mail: mariussumandea@uky.edu.

[2] The abbreviations used are: ROS, reactive oxygen species; MHC, myosin heavy chain; Tm, tropomyosin; cTn, cardiac troponin; cMyBP-C, cardiac myosin-binding protein C; β-AR, β-adrenergic receptor; PTM, post-translational modification; Trx1, thioredoxin-1; C, catalytic; R, regulatory; DAG, diacylglycerol; PMA, phorbol 12-myristate 13-acetate.

TABLE 1
ROS-induced modifications of cardiac sarcomeric proteins

Protein	Modification type	Major target site	Functional effect	Ref.
Actin	Oxidation	Cys^{374}	↓ Myosin ATPase activity	11
			↓ Actin filament sliding velocity	12
			↑ F-actin depolymerization	13
			↓ Tm-actin binding	10
			↓ Maximum force	13
		Tyr	↓ Contractile function	20, 22, 96
α-Actinin	Oxidation	Tyr	↓ Longitudinal force transmission	21
Desmin	Oxidation	Cys	↓ Proteasome degradation	18, 19
			↑ Aggregate formation	18, 19
			↑ Myofibrillar disarray	18, 19
		Tyr	↓ Contractile function	22
MHC	Oxidation	Cys^{697}/Cys^{707}	↑ Myosin inhibition	8, 10
			↓ Maximum force	7, 9, 10
		Tyr	↓ Contractile function	20
cMyBP-C	Oxidation	Cys, Tyr	↓ Contractile function	9, 20
	Phosphorylation	Ser^{302} (PKA, PKCϵ, PKCδ, PKD)	↓ Thin-thick filament interactions, ↑ force generation	32, 39, 40
Titin	Oxidation	N2-B Cys	↓ Extensibility	16
			↑ Passive tension	16
	Phosphorylation	Ser^{469} (PKA, PKG)	↓ Passive tension	41, 42
		Ser^{11878}/Ser^{12022} (PKCα)	↑ Passive tension	43
Tm	Oxidation	Cys^{190}	↓ Contractile function	11, 14
			↓ Flexibility	13, 15
			↓ Binding to actin	14
			↓ Assembly of actin·Tm complexes	15
		Tyr	↓ Contractile function	20, 22
cTnI	Phosphorylation	Ser^{23}/Ser^{24} (PKA, PKC, PKG, PKD, $p90^{RSK}$)	↑ Ca^{2+} dissociation from TnC	23–32
			↓ Ca^{2+} sensitivity	
			↑ Rate of relaxation	
		Ser^{43}/Ser^{45} (PKC)	↓ Maximal force	33
		Thr^{144} (PKCβ, tyrosine-phosphorylated PKCδ, Mst1)	↑ Myofilament Ca^{2+} sensitivity	28
		$Thr^{32}/Thr^{52}/Thr^{130}$ (Mst1)	Altered conformation	71
		Ser^{151} (PAK-3)	↑ Myofilament Ca^{2+} sensitivity	23
cTnT	Oxidation	Tyr	↓ Contractile function	22
	Phosphorylation	Thr^{206} (PKC, Raf)	↓ Maximal force	34, 36
			↓ Myofilament Ca^{2+} sensitivity	
		Thr^{197}/Ser^{201} (PKC, ASK-1)	May exacerbate the effect of Thr^{206}	34, 37
		Ser^{274}/Thr^{287} (PKC)	May exacerbate the effect of Thr^{206}	34, 36

and titin are phosphorylated by ROS-sensitive enzymes. cTnI contains functionally important phosphorylation clusters at Ser^{23}/Ser^{24}, Ser^{43}/Ser^{45}, and Thr^{144} (as well as additional phosphorylation sites at Thr^{32}, Thr^{52}, Ser^{76}/Ser^{77}, Thr^{130}, and Ser^{150} that are less well characterized) (23–25). cTnI Ser^{23}/Ser^{24} phosphorylation is traditionally attributed to the β-adrenergic receptor (β-AR)/cAMP pathway involving PKA. cTnI Ser^{23}/Ser^{24} phosphorylation reduces myofilament Ca^{2+} sensitivity and is required for the β-AR-dependent lusitropic response. In some settings, cTnI Ser^{23}/Ser^{24} phosphorylation also is attributable to PKG, various PKC isoforms, or PKC-activated enzymes such as $p90^{RSK}$ and PKD (26–32). Ser^{43}/Ser^{45} and Thr^{144} are traditionally viewed as sites for phosphorylation by PKC. Although recent studies show that Thr^{144} is a good *in vitro* substrate for PKCβ and Tyr-phosphorylated PKCδ (27, 28), PKCs with cTnI Ser^{43}/Ser^{45} kinase activity have not been identified. In fact, several laboratories have reported that Ser^{43}/Ser^{45} is a relatively poor *in vitro* substrate for many PKC isoforms. Nevertheless, WT cTnI replacement with a cTnI mutant harboring phosphomimetic substitutions at Ser^{43}, Ser^{45}, and Thr^{144} leads to pronounced changes in contractile function in transgenic mice, suggesting that myofilaments may be very sensitive to PKC-dependent changes in cTnI phosphorylation (33).

cTnT phosphorylation at Thr^{206} (by PKC or Raf-1 but not by PKA or PKG) (34, 35) results in decreased maximum force and myofilament Ca^{2+} sensitivity. PKC and ASK-1 also phosphorylate cTnT at other sites (Table 1) (36, 37). Because phosphorylation (or phosphomimetic substitutions) at sites other than Thr^{206} does not lead to gross changes in mechanical function, some have speculated that other post-translational modifications (PTMs) on cTnT might regulate its scaffolding function (particularly because enzymes such as PKA and PKG phosphorylate cTnI only when anchored to cTnT) (38, 80).

cMyBP-C phosphorylation at Ser^{273}, Ser^{282}, and Ser^{302} is generally attributed to PKA and viewed as a mechanism that decreases thick-thin filament interactions and increases force generation (39). There is recent evidence that Ser^{302} (but not Ser^{273} or Ser^{282}) also is phosphorylated by PKCϵ, PKCδ, and PKD and that Ser^{302} phosphorylation alone may be sufficient to regulate contractile function (32, 40).

The titin elastic region (consisting of serially linked immunoglobulin-like domains, the N2-B element, and a PEVK domain) is phosphorylated by PKA or PKG. PKA- or PKG-dependent phosphorylation of human titin at Ser^{469} in the N2-B element decreases the passive tension of titin (41, 42). PKG (but not PKA) also phosphorylates titin at other sites that do not influence its mechanical properties but could in theory control docking interactions on the titin scaffold (42). PKCα phosphorylates titin at two highly conserved sites in the PEVK region (Ser^{11878} and Ser^{12022}), leading to an increase in the passive tension of titin (an effect opposite to the actions of PKA or PKG) (43).

ROS-dependent Regulation of Sarcomeric Protein Phosphorylation—Oxidative stress typically increases protein phosphorylation by inhibiting protein phosphatases and stimulating protein kinases. The invariant Cys in the active sites of protein-tyrosine phosphatases is highly susceptible to ROS-dependent inactivation (44). ROS-dependent inactivation of protein-tyrosine phosphatases is sufficient to increase protein Tyr phosphorylation. However, ROS-dependent increases in Src activity also are detected in cardiomyocytes and some other cell types (45–47). Changes in protein Tyr phosphorylation typically influence cell growth, survival, and differentiation rather than sarcomeric protein phosphorylation. However, ROS-dependent changes in protein Tyr phosphorylation indirectly influence sarcomeric protein phosphorylation by activating Ser/Thr kinases such as PKC and PKD (see below).

Oxidative stress also inactivates the Ser/Thr phosphatase calcineurin (or protein phosphatase 2B) (48). Redox regulation of calcineurin may impact transcriptional programs that regulate cardiac hypertrophy, but direct effects on the sarcomere are unlikely because myofilament protein phosphorylation is not disordered in transgenic mouse models of altered calcineurin activity (49). Rather, myofilament protein dephosphorylation is generally attributed to PP1 (protein phosphatase 1) or PP2A. Most studies have focused on PP2A, which co-immunoprecipitates with cTnT and cTnI, co-localizes to the Z-disc with ROS-sensitive enzymes (PKCϵ, PKCζ, PAK-1, and p38 MAPK), and contributes to dynamic changes in cTnI and cMyBP-C phosphorylation (50–53). The role of PP1-dependent sarcomeric protein dephosphorylation seems more tenuous because transgenic mouse models of altered PP1 activity display changes in phospholamban (but not cTnI) phosphorylation (54, 55). In fact, the assumption that ROS inactivate PP1 and PP2A is not supported by *in vitro* biochemical studies or cell-based studies, which show that oxidative stress increases PP1 and/or PP2A activity (56–59). This may be via an indirect mechanism because several ROS-activated kinases (PKCζ, PAK-1, and p38 MAPK) increase PP1 and/or PP2A activity (50, 52, 60). The functional consequences of ROS-dependent changes in PP1 or PP2A activity are difficult to predict because most studies have scrutinized kinase (and not phosphatase)-mediated mechanisms that regulate sarcomeric protein phosphorylation. This minireview focuses on the ROS-dependent mechanisms that regulate the various kinases that phosphorylate sarcomeric proteins.

ASK-1—ASK-1 (apoptosis signal-regulating kinase-1) is a ROS-regulated stress-activated MAPK kinase kinase that is abundant in cardiomyocytes and acts as a redox sensor to activate effector pathways that regulate apoptotic/necrotic cell death (61–65). ASK-1 contains a central kinase domain flanked by N- and C-terminal regulatory domains. In resting cells, ASK-1 activity is maintained at low basal levels as a result of inhibitory interactions between the Ser^{967}-phosphorylated C-terminal regulatory domain and 14-3-3 proteins and between the N-terminal regulatory domain and reduced thioredoxin-1 (Trx1). Oxidation of Trx1 leads to the dissociation of ASK-1·Trx1 complexes. Oxidative stress also leads to the dissociation of the ASK-1·14-3-3 complex due to ASK-1 Ser^{967} dephosphorylation (presumably due to the activation of a ROS-sensitive phosphatase) and/or 14-3-3 phosphorylation by a ROS-regulated kinase (PKD, Mst (mammalian sterile 20-like kinase) family kinases, or the catalytic fragment of PKCδ) (66–68). Once released from these inhibitory constraints, ASK-1 is activated as a result of oligomerization and activation loop (Ser^{845}) autophosphorylation.

ASK-1 is activated by H_2O_2 or agonists for G-protein-coupled receptors that increase ROS accumulation *in vitro* in cardiomyocyte cultures and by pressure overload or myocardial infarction *in vivo* in the intact heart (37, 68, 69). This increase in ASK-1 activity contributes to ventricular remodeling by activating pathways involving JNK or NF-κB (69). Recent evidence indicates that ASK-1 co-localizes with sarcomeric structures, where it phosphorylates cTnT at Thr^{197} and Ser^{201} (37). Overexpression of the constitutively active ASK-1ΔN deletion mutant leads to increased cTnT phosphorylation and decreased fractional shortening in cardiomyocyte cultures (37). However, the role of cTnT phosphorylation in the cardiodepressant actions of ASK-1 remains uncertain because (*a*) cTnT phosphorylation at Thr^{206} (not Thr^{197} or Ser^{201}) has been implicated in the control of thin filament function, and (*b*) ASK-1ΔN overexpression also decreases the Ca^{2+} transient amplitude (34, 37).

Mst1—Mst1 is another ROS-activated Ser/Thr kinase that activates p38 MAPK/JNK and caspase-dependent mechanisms that amplify apoptosis (70, 71). Mst1 also phosphorylates cTnI and cTnT; cTnT phosphorylation is detected only when Mst1 is anchored to cTnI. Mst1-dependent cTnI phosphorylation has been mapped to Thr^{144} as well as novel sites (Thr^{32}, Thr^{52}, and Thr^{130}) that may influence the conformation of cTnI and its binding affinity for cTnT and cTnC (71).

PKA—PKA holoenzyme is a heterotetramer composed of two catalytic (C) subunits kept in an inactive conformation by two cAMP-binding regulatory (R) subunits. PKA activation is generally attributed to the β-AR/cAMP pathway; cAMP binding to the R subunit frees the C subunit to phosphorylate target substrates. However, ROS-dependent mechanisms that regulate PKA also influence myofilament protein phosphorylation.

PKA holoenzymes are classified as type I or II based upon the identity of their R subunit (RI or RII) that targets PKA to different subcellular compartments through interaction with PKA-anchoring proteins. Both PKA RI and RII subunits are ubiquitously expressed in cardiac myocytes and were shown to interact with myofilaments (38). The presence of two distinct PKA isoforms anchored at the sarcomeres could impart a more dynamic modulation of myofilament function in response to varying cAMP levels, and the combined regulation could provide a more refined physiological response.

Oxidative modifications of the RI subunit (at a pair of reactive cysteines not found in the RII subunit) result in the formation of interprotein disulfide dimers that display increased affinity for α-MHC, translocate to the myofibrillar fraction, and phosphorylate cTnI and cMyBP-C (72). This ROS-dependent (cAMP-independent) mechanism involving type I PKA has been linked to an increase in cardiac contractility.

Oxidative modification of the C subunit at Cys^{199} (one of two highly conserved Cys residues in the active sites of PKA and other Ser/Thr kinases such as PKC, PKG, and AKT) has the

opposite effect to decrease PKA activity (73). Mutagenesis studies suggest that Cys^{199} does not directly influence catalytic activity. Rather, the thiol modification at Cys^{199} indirectly decreases catalytic activity by rendering the C subunit susceptible to phosphatase-mediated dephosphorylation at Thr^{197}, a stable PTM at an adjacent site in the activation loop that is required for kinase activity (74). Cell-based studies suggest that similar thiol modifications may decrease activation loop phosphorylation and inactivate related kinases (such as PKC) (74).

PKG—PKG is activated by autocrine/paracrine stimuli that increase NO and cGMP. Mammalian PKGs are homodimers of identical subunits; each PKG monomer contains an N-terminal regulatory domain (consisting of an autoinhibitory pseudosubstrate sequence, tandem cGMP-binding cassettes, and a leucine zipper dimerization domain) and a C-terminal catalytic domain. An autoinhibitory interaction between the pseudosubstrate domain and the catalytic pocket maintains PKG in an inactive/resting state; cGMP binding induces a conformational change that relieves autoinhibition and permits activation.

Three molecular forms of mammalian PKG have been identified: PKGIα and PKGIβ arise through alternative mRNA splicing and differ only at their extreme N-terminal ~100 amino acids (the dimerization domain), whereas PKGII is the product of a different gene locus. Because PKGI is the major isoform in cardiomyocytes, it is the focus of this discussion (75, 76). The distinct N-terminal homodimerization domains of PKGIα and PKGIβ underlie isoform-specific interactions with docking proteins and cell substrates. Elements within the dimerization domain influence the kinetics of cGMP binding and PKG activation; PKGIα and PKGIβ have identical cGMP-binding cassettes, but PKGIα binds cGMP with 10-fold higher affinity than PKGIβ (77, 78). Native PKGI has traditionally been viewed as a constitutive dimer. However, a recent study challenged this assumption and showed that PKGIα dimerization is a ROS-regulated mechanism; this study concluded that the PKGIα dimers identified in previous studies are artifacts of oxidation during sample preparation (79). PKGIα dimerization results from disulfide bond formation between reactive Cys^{42} residues that abut in the enzyme homodimer; PKGIβ does not contain a reactive Cys at this position and is not ROS-sensitive. The PKGIα dimers that accumulate during oxidative stress display a high level of cGMP-independent catalytic activity (79). Moreover, whereas cGMP activates PKGIα by increasing its maximum velocity (V_{max} and not the K_m for substrate), ROS-activated (disulfide-linked) PKGIα dimers display a marked (>10-fold) increase in K_m for substrate. Some have speculated that this ROS-induced increase in PKGIα affinity for substrate underlies the ROS-induced change in PKGIα subcellular compartmentation in smooth muscle cells; ROS-activated/disulfide-linked PKGIα translocates to membrane and myofilament fractions (which contain functionally important PKG substrates). Future studies that examine the subcellular compartmentation and substrates of ROS-activated PKGIα in cardiomyocytes may be quite revealing, given evidence that a PKGIα-docking interaction (via its homodimerization domain) with cTnT is required for the rapid/efficient phosphorylation of cTnI (80). PKG-targeting mechanisms may be critical for substrate phosphorylation in cardiomyocytes, where PKG expression is quite low (~10-fold lower compared with PKA expression) (81), and the V_{max} for cTnI phosphorylation by PKG is 12-fold lower than that for cTnI phosphorylation by PKA (82).

PKCs—PKCs are Ser/Thr kinases that are activated by growth factor-dependent pathways that mobilize Ca^{2+} and promote diacylglycerol (DAG) accumulation. PKC isoforms contain a highly conserved C-terminal catalytic domain and are subdivided into three classes based on differences in their N-terminal regulatory domains. The regulatory domains of conventional or Ca^{2+}-sensitive PKCs (α, βI/βII, and γ) and novel PKCs (in cardiomyocytes, PKCδ and PKCϵ) contain a C1 domain (consisting of tandem Cys-rich sequences) that binds lipid cofactors such as DAG and phorbol 12-myristate 13-acetate (PMA). Atypical PKCs (ζ and i/l) contain an abbreviated C1 domain (with only one Cys-rich motif) that binds phosphatidylinositol 1,4,5-trisphosphate or ceramide but not DAG or PMA. PKC activation is generally attributed to stimuli that promote DAG accumulation and anchor the enzyme in its active conformation to membranes. However, ROS-dependent mechanisms that activate PKCs by oxidizing C1 domain Cys residues (which disrupts autoinhibitory intramolecular constraints) also have been identified (83, 84).

A ROS-dependent mechanism involving Tyr phosphorylation by Src specifically activates PKCδ (and not other PKC isoforms). We recently demonstrated that Src phosphorylates PKCδ at Tyr^{311} *in vitro* and in H_2O_2-treated cardiomyocytes and that Tyr^{311}-phosphorylated PKCδ accumulates in the soluble fraction as a constitutively active lipid-independent enzyme; this form of the enzyme is poised to phosphorylate proteins in the sarcomere, not just on lipid membranes (85). We showed that 1) allosterically activated PKCδ phosphorylates cTnI at Ser^{23}/Ser^{24}, 2) Tyr^{311}-phosphorylated PKCδ phosphorylates cTnI at Ser^{23}/Ser^{24} and Thr^{144}, and 3) a PKCδ mutant harboring a Y311F substitution selectively phosphorylates cTnI at Ser^{23}/Ser^{24} but not Thr^{144} (28). Functional studies in detergent-skinned cardiomyocytes show that allosterically activated PKCδ depresses tension at submaximum but not maximum Ca^{2+}, as predicted for cTnI Ser^{23}/Ser^{24} phosphorylation. Src-phosphorylated PKCδ (which phosphorylates cTnI at both Ser^{23}/Ser^{24} and Thr^{144}) depresses maximum tension and cross-bridge kinetics; under these conditions, the effect of cTnI Thr^{144} phosphorylation predominates.

Stimulus-specific differences in PKCδ phosphorylation of sarcomeric proteins have been identified in H_2O_2- and PMA-treated cardiomyocytes (86). Here, PMA and H_2O_2 increase cTnI and cMyBP-C phosphorylation via a PKC-dependent mechanism, but only the H_2O_2-dependent increase in cTnI and cMyBP-C phosphorylation requires Src, presumably reflecting a role for Tyr-phosphorylated PKCδ. PMA and H_2O_2 elicit distinct cTnI and cMyBP-C phosphorylation patterns in PKCδ-overexpressing cardiomyocytes, providing further evidence that stimulus-specific differences in the PTM of PKCδ impact its enzymology and PKCδ-mediated sarcomeric protein phosphorylation. In a more general sense, these results caution against extrapolations regarding the cellular actions of ROS-activated enzymes based upon studies that examined myofilament protein regulation during signaling by G-protein-coupled receptors. Stimulus-specific differ-

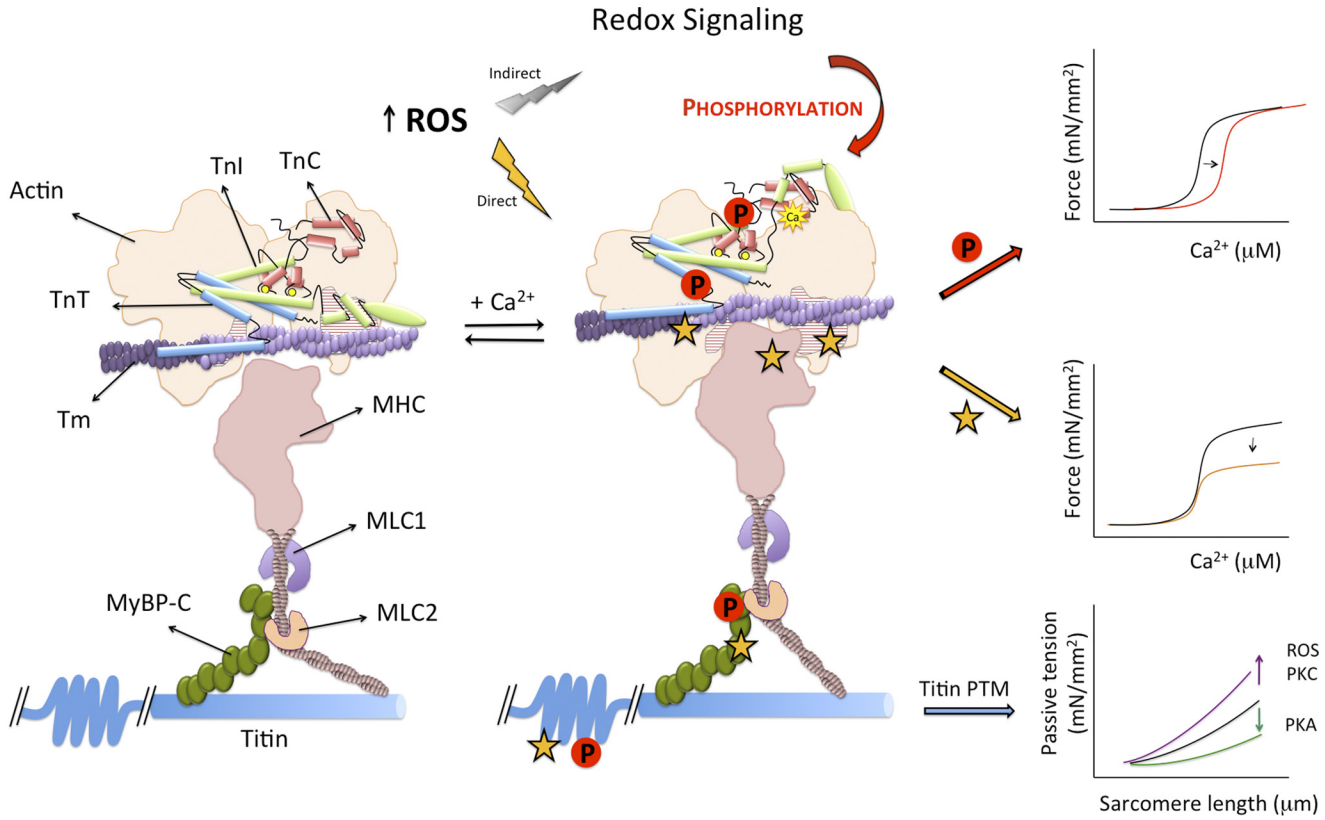

FIGURE 1. **Schematic of Ca^{2+}-dependent cardiac myofilament activation and ROS-induced PTMs that alter this process.** At low Ca^{2+}, actin-myosin cross-bridges are inhibited (*left*). At high Ca^{2+}, Ca^{2+} binding to the regulatory site of cTnC induces a conformational change in cTnI causing disinhibition of actin. This conformational change is transmitted to cTnT, which moves Tm and exposes a weak myosin-binding site on actin. Myosin binding to actin enhances activation by pushing Tm farther away, leading to strong cross-bridge formation. This activation mechanism is regulated in a highly specific manner by phosphorylation reactions on individual sarcomeric proteins. Myofilament activation and contractile function also are altered during oxidative stress due to direct oxidative modifications (*yellow stars*) of specific sites on contractile proteins or ROS-induced changes in the activity of kinases or phosphatases that regulate sarcomeric protein phosphorylation (*P*). *MLC*, myosin light chain; *mN*, millinewtons.

ences in the subcellular compartmentalization, binding partners, and enzymology of many signaling enzymes may impact sarcomeric protein phosphorylation.

PKD—PKD consists of a family of Ser/Thr kinases that exert important cardiac actions (87). PKDs contain an N-terminal regulatory C1 domain (which targets the enzyme to DAG- or phorbol ester-enriched membranes), an autoinhibitory pleckstrin homology domain, and a C-terminal kinase domain. Agonists that promote DAG accumulation activate PKD via a novel PKC-dependent pathway that leads to PKD phosphorylation at Ser^{744}/Ser^{748} in the activation loop. Ser^{744}/Ser^{748}-phosphorylated PKD then autophosphorylates at Ser^{916}, and it displays a high level of activity toward heterologous substrates such as cTnI and cMyBP-C (30, 31). PKD decreases myofilament Ca^{2+} sensitivity by increasing cTnI Ser^{23}/Ser^{24} phosphorylation (similar to the actions of PKA). PKD also accelerates cross-bridge cycle kinetics. This effect does not require cTnI Ser^{23}/Ser^{24} phosphorylation (because it is preserved in cardiomyocytes that express cTnI S23A/S24A in place of WT cTnI); it has been attributed to cMyBP-C phosphorylation at Ser^{302}, the only site in cMyBP-C that is targeted by PKD (32).

PKD is activated during oxidative stress via a mechanism involving the ROS-activated form of PKCδ and the tyrosine kinases Src and c-Abl; Src and c-Abl are not required for growth factor-dependent PKD activation (88, 89). The current model holds that c-Abl phosphorylates PKD at Tyr^{463} (in the pleckstrin homology domain), leading to a conformational change that permits Src-dependent PKD phosphorylation at Tyr^{95} (90). This generates a consensus binding motif for the C2 domain of PKCδ, which activates PKD. ROS-activated PKD phosphorylates CREB (cAMP-responsive element-binding protein) in cardiomyocytes (91) and controls ASK-1, NF-κB, and apoptotic cell death in other cell types (66, 92). A potential role for PKD in redox regulation of sarcomeric protein phosphorylation has not been considered.

Conclusions

This minireview has summarized recent studies that identify ROS-induced PTMs of sarcomeric proteins that lead to contractile dysfunction (Fig. 1). Studies to date suggest that direct oxidative modifications of sarcomeric proteins lead to a decrease in force generation, whereas sarcomeric protein phosphorylation by ROS-activated enzymes decrease myofilament Ca^{2+} sensitivity. However, these conclusions are based largely on experiments that rely on reductionist approaches to resolve the functional consequences of myofilament protein phosphorylation by a single ROS-activated enzyme or ROS-dependent PTMs of a single contractile protein. Extrapolations to the *in vivo* context must be made with caution for several reasons.

1) An inherent assumption of studies in reductionist models is that kinases act in a stereotypical fashion to phosphorylate a fixed set of sarcomeric proteins (or consensus phosphorylation sites within a given sarcomeric protein). The stimulus-specific differences in cTnI phosphorylation by PKCδ identified in our studies emphasize that standard *in vitro* approaches may not necessarily incorporate protocols that capture all regulatory phosphorylations that control *in vivo* enzyme activity (or substrate specificity). This approach also will not detect changes in phosphorylation due to ROS-dependent modifications of myofilament proteins that alter the accessibility of phosphorylation sites on individual substrate proteins.

2) ROS-dependent modifications of sarcomeric proteins do not occur in isolation. Rather, ROS-activated kinases typically phosphorylate multiple proteins in the sarcomere; many ROS-activated enzymes also sit upstream in signaling cascades that regulate effectors with Ser/Thr kinase activity. For example, PKC isoforms activate PKD, $p90^{RSK}$, and Raf-1; ROS-activated pathways involving PKD, PKC, or Mst phosphorylate 14-3-3 proteins, leading to decreased inhibitory interactions with ASK-1. Progress in understanding ROS-induced changes in contractile performance *in vivo* must consider the ensemble actions of multiple ROS-activated enzymes on multiple sarcomeric proteins.

3) Oxidative stress may induce a spectrum of responses that vary according to the identity of the free radical species, the location of the ROS signal, and/or the level of oxidative stress. This issue has been addressed directly in studies of redox regulation of PKA, where ROS shift the balance of cellular kinase *versus* phosphatase activity in a dose-dependent manner. Low levels of oxidant stress amplify PKA responses by inactivating phosphatases that counteract PKA-dependent phosphorylations; high levels of oxidative stress inactivate PKA (presumably due to direct Cys oxidation of the C subunit) and decrease substrate phosphorylation (93).

4) The importance of a particular ROS-dependent protein phosphorylation or oxidative modification may be context-dependent; it may be influenced by PTMs elsewhere in that particular protein or in other proteins in the sarcomere. For example, cTnI Thr^{144} phosphorylation alone has little effect on force generation or Ca^{2+} sensitivity, but Thr^{144} phosphorylation prevents Ca^{2+} desensitization due to cTnI Ser^{23}/Ser^{24} phosphorylation (*i.e.* Thr^{144} phosphorylation becomes functionally important in a Ser^{23}/Ser^{24}-phosphorylated background) (94). Similarly, the functional consequences of PKC-dependent cTnI $Ser^{43}/Ser^{45}/Thr^{144}$ phosphorylation are amplified during acidosis (95). PTMs of sarcomeric proteins that are inert under normal physiological conditions and become functionally important only in a pathologic microenvironment may represent novel therapeutic targets.

REFERENCES

1. Ytrehus, K., Myklebust, R., and Mjøs, O. D. (1986) *Cardiovasc. Res.* **20,** 597–603
2. Vaage, J., Antonelli, M., Bufi, M., Irtun, O., DeBlasi, R. A., Corbucci, G. G., Gasparetto, A., and Semb, A. G. (1997) *Free Radic. Biol. Med.* **22,** 85–92
3. Wang, L., Lopaschuk, G. D., and Clanachan, A. S. (2008) *J. Mol. Cell. Cardiol.* **45,** 787–795
4. Schulz, R., Dodge, K. L., Lopaschuk, G. D., and Clanachan, A. S. (1997) *Am. J. Physiol.* **272,** H1212–H1219
5. Gao, W. D., Liu, Y., and Marban, E. (1996) *Circulation* **94,** 2597–2604
6. Luo, J., Xuan, Y. T., Gu, Y., and Prabhu, S. D. (2006) *J. Mol. Cell. Cardiol.* **40,** 64–75
7. MacFarlane, N. G., and Miller, D. J. (1992) *Circ. Res.* **70,** 1217–1224
8. Passarelli, C., Petrini, S., Pastore, A., Bonetto, V., Sale, P., Gaeta, L. M., Tozzi, G., Bertini, E., Canepari, M., Rossi, R., and Piemonte, F. (2008) *J. Muscle Res. Cell Motil.* **29,** 119–126
9. Rao, V. S., La Bonte, L. R., Xu, Y., Yang, Z., French, B. A., and Guilford, W. H. (2007) *Am. J. Physiol. Heart Circ. Physiol.* **293,** H654–H659
10. Tiago, T., Simão, S., Aureliano, M., Martín-Romero, F. J., and Gutiérrez-Merino, C. (2006) *Biochemistry* **45,** 3794–3804
11. Canton, M., Neverova, I., Menabò, R., Van Eyk, J., and Di Lisa, F. (2004) *Am. J. Physiol. Heart Circ. Physiol.* **286,** H870–H877
12. Crosbie, R. H., Miller, C., Cheung, P., Goodnight, T., Muhlrad, A., and Reisler, E. (1994) *Biophys. J.* **67,** 1957–1964
13. Chen, F. C., and Ogut, O. (2006) *Am. J. Physiol. Cell Physiol.* **290,** C719–C727
14. Canton, M., Skyschally, A., Menabò, R., Boengler, K., Gres, P., Schulz, R., Haude, M., Erbel, R., Di Lisa, F., and Heusch, G. (2006) *Eur. Heart J.* **27,** 875–881
15. Williams, D. L., Jr., and Swenson, C. A. (1982) *Eur. J. Biochem.* **127,** 495–499
16. Grützner, A., Garcia-Manyes, S., Kötter, S., Badilla, C. L., Fernandez, J. M., and Linke, W. A. (2009) *Biophys. J.* **97,** 825–834
17. Singal, P. K., Khaper, N., Palace, V., and Kumar, D. (1998) *Cardiovasc. Res.* **40,** 426–432
18. Janué, A., Odena, M. A., Oliveira, E., Olivé, M., and Ferrer, I. (2007) *J. Neuropath. Exp. Neur.* **66,** 711–723
19. Maloyan, A., Osinska, H., Lammerding, J., Lee, R. T., Cingolani, O. H., Kass, D. A., Lorenz, J. N., and Robbins, J. (2009) *Circ. Res.* **104,** 1021–1028
20. Snook, J. H., Li, J., Helmke, B. P., and Guilford, W. H. (2008) *Free Radic. Biol. Med.* **44,** 14–23
21. Borbély, A., Tóth, A., Edes, I., Virág, L., Papp, J. G., Varró, A., Paulus, W. J., van der Velden, J., Stienen, G. J., and Papp, Z. (2005) *Cardiovasc. Res.* **67,** 225–233
22. Kanski, J., Behring, A., Pelling, J., and Schöneich, C. (2005) *Am. J. Physiol. Heart Circ. Physiol.* **288,** H371–H381
23. Buscemi, N., Foster, D. B., Neverova, I., and Van Eyk, J. E. (2002) *Circ. Res.* **91,** 509–516
24. Zabrouskov, V., Ge, Y., Schwartz, J., and Walker, J. W. (2008) *Mol. Cell. Proteomics* **7,** 1838–1849
25. Noland, T. A., Jr., Raynor, R. L., and Kuo, J. F. (1989) *J. Biol. Chem.* **264,** 20778–20785
26. Lee, D. I., Vahebi, S., Tocchetti, C. G., Barouch, L. A., Solaro, R. J., Takimoto, E., and Kass, D. A. (2010) *Basic Res. Cardiol.* **105,** 337–347
27. Wang, H., Grant, J. E., Doede, C. M., Sadayappan, S., Robbins, J., and Walker, J. W. (2006) *J. Mol. Cell. Cardiol.* **41,** 823–833
28. Sumandea, M. P., Rybin, V. O., Hinken, A. C., Wang, C., Kobayashi, T., Harleton, E., Sievert, G., Balke, C. W., Feinmark, S. J., Solaro, R. J., and Steinberg, S. F. (2008) *J. Biol. Chem.* **283,** 22680–22689
29. Itoh, S., Ding, B., Bains, C. P., Wang, N., Takeishi, Y., Jalili, T., King, G. L., Walsh, R. A., Yan, C., and Abe, J. (2005) *J. Biol. Chem.* **280,** 24135–24142
30. Haworth, R. S., Cuello, F., Herron, T. J., Franzen, G., Kentish, J. C., Gautel, M., and Avkiran, M. (2004) *Circ. Res.* **95,** 1091–1099
31. Cuello, F., Bardswell, S. C., Haworth, R. S., Yin, X., Lutz, S., Wieland, T., Mayr, M., Kentish, J. C., and Avkiran, M. (2007) *Circ. Res.* **100,** 864–873
32. Bardswell, S. C., Cuello, F., Rowland, A. J., Sadayappan, S., Robbins, J., Gautel, M., Walker, J. W., Kentish, J. C., and Avkiran, M. (2010) *J. Biol. Chem.* **285,** 5674–5682
33. Kirk, J. A., MacGowan, G. A., Evans, C., Smith, S. H., Warren, C. M., Mamidi, R., Chandra, M., Stewart, A. F., Solaro, R. J., and Shroff, S. G. (2009) *Circ. Res.* **105,** 1232–1239
34. Sumandea, M. P., Pyle, W. G., Kobayashi, T., de Tombe, P. P., and Solaro, R. J. (2003) *J. Biol. Chem.* **278,** 35135–35144
35. Pfleiderer, P., Sumandea, M. P., Rybin, V. O., Wang, C., and Steinberg, S. F. (2009) *J. Muscle Res. Cell Motil.* **30,** 67–72
36. Sumandea, M. P., Vahebi, S., Sumandea, C. A., Garcia-Cazarin, M. L., Staidle, J., and Homsher, E. (2009) *Biochemistry* **48,** 7722–7731
37. He, X., Liu, Y., Sharma, V., Dirksen, R. T., Waugh, R., Sheu, S. S., and Min, W. (2003) *Am. J. Pathol.* **163,** 243–251

38. Sumandea, C. A., Garcia-Cazarin, M. L., Bozio, C. H., Sievert, G. A., Balke, C. W., and Sumandea, M. P. (2011) *J. Biol. Chem.* **286,** 530–541
39. Barefield, D., and Sadayappan, S. (2010) *J. Mol. Cell. Cardiol.* **48,** 866–875
40. Xiao, L., Zhao, Q., Du, Y., Yuan, C., Solaro, R. J., and Buttrick, P. M. (2007) *Biochemistry* **46,** 7054–7061
41. Yamasaki, R., Wu, Y., McNabb, M., Greaser, M., Labeit, S., and Granzier, H. (2002) *Circ. Res.* **90,** 1181–1188
42. Krüger, M., Kötter, S., Grützner, A., Lang, P., Andresen, C., Redfield, M. M., Butt, E., dos Remedios, C. G., and Linke, W. A. (2009) *Circ. Res.* **104,** 87–94
43. Hidalgo, C., Hudson, B., Bogomolovas, J., Zhu, Y., Anderson, B., Greaser, M., Labeit, S., and Granzier, H. (2009) *Circ. Res.* **105,** 631–638
44. Meng, T. C., Fukada, T., and Tonks, N. K. (2002) *Mol. Cell* **9,** 387–399
45. Aikawa, R., Komuro, I., Yamazaki, T., Zou, Y., Kudoh, S., Tanaka, M., Shiojima, I., Hiroi, Y., and Yazaki, Y. (1997) *J. Clin. Invest.* **100,** 1813–1821
46. Giannoni, E., Buricchi, F., Raugei, G., Ramponi, G., and Chiarugi, P. (2005) *Mol. Cell. Biol.* **25,** 6391–6403
47. Kemble, D. J., and Sun, G. (2009) *Proc. Natl. Acad. Sci. U.S.A.* **106,** 5070–5075
48. Namgaladze, D., Hofer, H. W., and Ullrich, V. (2002) *J. Biol. Chem.* **277,** 5962–5969
49. Wilkins, B. J., and Molkentin, J. D. (2002) *J. Physiol.* **541,** 1–8
50. Ke, Y., Wang, L., Pyle, W. G., de Tombe, P. P., and Solaro, R. J. (2004) *Circ. Res.* **94,** 194–200
51. Robia, S. L., Kang, M., and Walker, J. W. (2005) *Am. J. Physiol. Heart Circ. Physiol.* **289,** H1941–HH1950
52. Wu, S. C., and Solaro, R. J. (2007) *J. Biol. Chem.* **282,** 30691–30698
53. Vahebi, S., Ota, A., Li, M., Warren, C. M., de Tombe, P. P., Wang, Y., and Solaro, R. J. (2007) *Circ. Res.* **100,** 408–415
54. Yang, F., Aiello, D. L., and Pyle, W. G. (2008) *Biochem. Cell Biol.* **86,** 70–78
55. Nicolaou, P., Hajjar, R. J., and Kranias, E. G. (2009) *J. Mol. Cell. Cardiol.* **47,** 365–371
56. Sommer, D., Coleman, S., Swanson, S. A., and Stemmer, P. M. (2002) *Arch. Biochem. Biophys.* **404,** 271–278
57. Cicchillitti, L., Fasanaro, P., Biglioli, P., Capogrossi, M. C., and Martelli, F. (2003) *J. Biol. Chem.* **278,** 19509–19517
58. Pham, F. H., Sugden, P. H., and Clerk, A. (2000) *Circ. Res.* **86,** 1252–1258
59. Deshmukh, P. A., Blunt, B. C., and Hofmann, P. A. (2007) *Am. J. Physiol. Heart Circ. Physiol.* **292,** H792–H799
60. Liu, Q., and Hofmann, P. A. (2003) *Am. J. Physiol. Heart Circ. Physiol.* **285,** H97–H103
61. Tobiume, K., Inage, T., Takeda, K., Enomoto, S., Miyazono, K., and Ichijo, H. (1997) *Biochem. Biophys. Res. Commun.* **239,** 905–910
62. Tobiume, K., Matsuzawa, A., Takahashi, T., Nishitoh, H., Morita, K., Takeda, K., Minowa, O., Miyazono, K., Noda, T., and Ichijo, H. (2001) *EMBO Rep.* **2,** 222–228
63. Yamaguchi, O., Watanabe, T., Nishida, K., Kashiwase, K., Higuchi, Y., Takeda, T., Hikoso, S., Hirotani, S., Asahi, M., Taniike, M., Nakai, A., Tsujimoto, I., Matsumura, Y., Miyazaki, J., Chien, K. R., Matsuzawa, A., Sadamitsu, C., Ichijo, H., Baccarini, M., Hori, M., and Otsu, K. (2004) *J. Clin. Invest.* **114,** 937–943
64. Liu, Q., Wilkins, B. J., Lee, Y. J., Ichijo, H., and Molkentin, J. D. (2006) *Mol. Cell. Biol.* **26,** 3785–3797
65. Liu, Q., Sargent, M. A., York, A. J., and Molkentin, J. D. (2009) *Circ. Res.* **105,** 1110–1117
66. Zhang, W., Zheng, S., Storz, P., and Min, W. (2005) *J. Biol. Chem.* **280,** 19036–19044
67. Zhou, J., Shao, Z., Kerkela, R., Ichijo, H., Muslin, A. J., Pombo, C., and Force, T. (2009) *Mol. Cell. Biol.* **29,** 4167–4176
68. Hirotani, S., Otsu, K., Nishida, K., Higuchi, Y., Morita, T., Nakayama, H., Yamaguchi, O., Mano, T., Matsumura, Y., Ueno, H., Tada, M., and Hori, M. (2002) *Circulation* **105,** 509–515
69. Yamaguchi, O., Higuchi, Y., Hirotani, S., Kashiwase, K., Nakayama, H., Hikoso, S., Takeda, T., Watanabe, T., Asahi, M., Taniike, M., Matsumura, Y., Tsujimoto, I., Hongo, K., Kusakari, Y., Kurihara, S., Nishida, K., Ichijo, H., Hori, M., and Otsu, K. (2003) *Proc. Natl. Acad. Sci. U.S.A.* **100,** 15883–15888
70. Yamamoto, S., Yang, G., Zablocki, D., Liu, J., Hong, C., Kim, S. J., Soler, S., Odashima, M., Thaisz, J., Yehia, G., Molina, C. A., Yatani, A., Vatner, D. E., Vatner, S. F., and Sadoshima, J. (2003) *J. Clin. Invest.* **111,** 1463–1474
71. You, B., Yan, G., Zhang, Z., Yan, L., Li, J., Ge, Q., Jin, J. P., and Sun, J. (2009) *Biochem. J.* **418,** 93–101
72. Brennan, J. P., Bardswell, S. C., Burgoyne, J. R., Fuller, W., Schröder, E., Wait, R., Begum, S., Kentish, J. C., and Eaton, P. (2006) *J. Biol. Chem.* **281,** 21827–21836
73. Humphries, K. M., Juliano, C., and Taylor, S. S. (2002) *J. Biol. Chem.* **277,** 43505–43511
74. Humphries, K. M., Deal, M. S., and Taylor, S. S. (2005) *J. Biol. Chem.* **280,** 2750–2758
75. Feil, R., Lohmann, S. M., de Jonge, H., Walter, U., and Hofmann, F. (2003) *Circ. Res.* **93,** 907–916
76. Lukowski, R., Rybalkin, S. D., Loga, F., Leiss, V., Beavo, J. A., and Hofmann, F. (2010) *Proc. Natl. Acad. Sci. U.S.A.* **107,** 5646–5651
77. Ammendola, A., Geiselhöringer, A., Hofmann, F., and Schlossmann, J. (2001) *J. Biol. Chem.* **276,** 24153–24159
78. Surks, H. K., Mochizuki, N., Kasai, Y., Georgescu, S. P., Tang, K. M., Ito, M., Lincoln, T. M., and Mendelsohn, M. E. (1999) *Science* **286,** 1583–1587
79. Burgoyne, J. R., Madhani, M., Cuello, F., Charles, R. L., Brennan, J. P., Schröder, E., Browning, D. D., and Eaton, P. (2007) *Science* **317,** 1393–1397
80. Yuasa, K., Michibata, H., Omori, K., and Yanaka, N. (1999) *J. Biol. Chem.* **274,** 37429–37434
81. Lincoln, T. M., Hall, C. L., Park, C. R., and Corbin, J. D. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73,** 2559–2563
82. Blumenthal, D. K., Stull, J. T., and Gill, G. N. (1978) *J. Biol. Chem.* **253,** 324–326
83. Gopalakrishna, R., and Jaken, S. (2000) *Free Radic. Biol. Med.* **28,** 1349–1361
84. Korichneva, I., Hoyos, B., Chua, R., Levi, E., and Hammerling, U. (2002) *J. Biol. Chem.* **277,** 44327–44331
85. Rybin, V. O., Guo, J., Sabri, A., Elouardighi, H., Schaefer, E., and Steinberg, S. F. (2004) *J. Biol. Chem.* **279,** 19350–19361
86. Avner, B. S., Hinken, A. C., Yuan, C., and Solaro, R. J. (2010) *Am. J. Physiol. Heart Circ. Physiol.* **299,** H723–H730
87. Avkiran, M., Rowland, A. J., Cuello, F., and Haworth, R. S. (2008) *Circ. Res.* **102,** 157–163
88. Waldron, R. T., and Rozengurt, E. (2000) *J. Biol. Chem.* **275,** 17114–17121
89. Waldron, R. T., Rey, O., Zhukova, E., and Rozengurt, E. (2004) *J. Biol. Chem.* **279,** 27482–27493
90. Döppler, H., and Storz, P. (2007) *J. Biol. Chem.* **282,** 31873–31881
91. Ozgen, N., Guo, J., Gertsberg, Z., Danilo, P., Jr., Rosen, M. R., and Steinberg, S. F. (2009) *Mol. Pharmacol.* **76,** 896–902
92. Storz, P., and Toker, A. (2003) *EMBO J.* **22,** 109–120
93. Humphries, K. M., Pennypacker, J. K., and Taylor, S. S. (2007) *J. Biol. Chem.* **282,** 22072–22079
94. Lu, Q. W., Hinken, A. C., Patrick, S. E., Solaro, R. J., and Kobayashi, T. (2010) *J. Biol. Chem.* **285,** 11810–11817
95. Engel, P. L., Hinken, A., and Solaro, R. J. (2009) *J. Mol. Cell. Cardiol.* **47,** 359–364
96. Mihm, M. J., Yu, F., Reiser, P. J., and Bauer, J. A. (2003) *Biochimie* **85,** 587–596