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Ryanodine Receptor Phosphorylation, Calcium/Calmodulin-dependent Protein Kinase II, and Life-threatening Ventricular Arrhythmias

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Abstract

RyR2 dysfunction, which may result from a variety of mechanisms, has been implicated in the pathogenesis of cardiac arrhythmias and heart failure. In this review, we will discuss the important role of Ca²⁺/calmodulin-dependent protein kinase II (CaMKII) in the regulation of RyR2-mediated Ca²⁺ release. In particular, we will examine how pathological activation of CaMKII can lead to an increased risk of sudden arrhythmic death. Finally, we will discuss how reduction of CaMKII-mediated RyR2 hyperactivity might reduce the risk of arrhythmias, and may serve as a rationale for future pharmacotherapeutic approaches.

Keywords

Cardiac arrhythmias; Ca²⁺/calmodulin kinase II; ryanodine receptor

Ventricular arrhythmias are a common cause of cardiac death and lead to over 300,000 deaths in the United States annually (Rubart and Zipes 2005). Ventricular arrhythmias have been historically difficult to treat because of their sudden onset, unpredictable clinical course, and diverse mechanisms underlying both trigger and maintenance. These diverse mechanisms have perplexed researchers and clinicians alike, and have made the medical treatment of arrhythmias complex. However, recent insights into the molecular physiology underlying cardiac rhythmicity have yielded new information on how arrhythmias may initiate, and have suggested new targets for the prevention of lethal arrhythmias. In this review, we examine the emerging role of Ca²⁺ as a major signaling molecule within the cardiomyocyte, and how the modulation of its release by Ca²⁺/calmodulin-dependent protein kinase II (CaMKII) is integrally associated with the risk for both arrhythmias and sudden cardiac death.

Ca²⁺ is the major signaling molecule involved in excitation-contraction coupling, the process by which an electrical depolarizing impulse is transduced into a mechanical cardiac

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contraction (Bers 2002). Ca^{2+} is primarily an extracellular molecule, and is also sequestered in the sarcoplasmic reticulum (SR), a specialized organelle, which stores Ca^{2+} at a resting (diastolic) state. During systole, electrical depolarization of the cardiomyocyte plasma membrane causes the voltage-gated Ca^{2+} channel (Cav1.2) to open, allowing Ca^{2+} to enter the cytoplasm, and inducing a ten-fold greater release of Ca^{2+} from the SR via the cardiac ryanodine receptor (RyR2). The acute rise of cytoplasmic $[\text{Ca}^{2+}]$ facilitates interdigitation of myosin and actin in an ATP-dependent process that causes cardiac contraction during systole (Bers and Guo 2005). Diastolic fall in $[\text{Ca}^{2+}]$ occurs as Ca^{2+} is returned into the SR via SERCA2a and exchanged across the plasma membrane for Na^+ by the $\text{Na}^+/\text{Ca}^{2+}$ -exchanger (NCX). The drop in $[\text{Ca}^{2+}]$ causes a conformational change of tropomyosin, and subsequent relaxation of actin and myosin filaments. Thus, the rhythmic rise and fall of $[\text{Ca}^{2+}]$ is an essential signaling event necessary for cardiac contraction and normal cardiac rhythmicity. Conversely, alterations in Ca^{2+} handling dynamics may result in disruption of heart rhythm and promote lethal arrhythmias.

RyR2 is one of the largest ion channels in the human body, and plays a significant role as the main SR Ca^{2+} release molecule in the cardiomyocyte. RyR2 is part of a larger macromolecular complex of proteins that govern excitation-contraction coupling, and which includes FK506-binding protein 12.6 (FKBP12.6), calsequestrin (CSQ), and calmodulin (CaM), among others (Lanner et al. 2010). These molecules influence the rate and extent of Ca^{2+} release through RyR2 by regulating Ca^{2+} -induced Ca^{2+} release (Cav1.2), RyR2 open probability (FKBP12.6, CaM), and intraluminal SR Ca^{2+} buffering (CSQ). Additionally, RyR2 is integrally involved with modulating heart rate and rhythm response to a variety of intracellular signaling mechanisms. These pathways include sympathetic nervous system stimulation (leading to protein kinase A phosphorylation of RyR2), intracellular $[\text{Ca}^{2+}]$ (activating Ca^{2+} /calmodulin binding protein kinase II), and mitochondrial metabolism and oxidation (Anderson 2005). The importance of RyR2 in the regulation of SR Ca^{2+} release is highlighted by the potentially lethal complications of arrhythmias and heart failure, which may result from its dysregulation. Detailed below are key aspects of how inherited and acquired RyR2 dysfunction may result in pathological arrhythmias and sudden death.

Genetic Mutations in RyR2 Predispose to Lethal Ventricular Arrhythmias

Systolic SR Ca^{2+} release is a highly regulated event, which in the physiologic state signals systolic contraction of myofilaments and cardiac relaxation during diastole. However, dysregulation of RyR2-mediated Ca^{2+} release has been shown to be associated with cardiac arrhythmias such as atrial fibrillation and ventricular tachycardia/fibrillation, sudden cardiac death, and also heart failure (Chelu et al. 2009; Netticadan et al. 2000; van Oort et al. 2010a). One example of pathological SR Ca^{2+} “leak” may be found in the rare genetic syndrome known as ‘catecholaminergic polymorphic ventricular tachycardia’ (CPVT), which confers susceptibility to lethal cardiac arrhythmias primarily during episodes of exercise or emotional stress (Gyorke 2009). These episodes are primarily triggered by the release of catecholamines such as epinephrine and norepinephrine. In 2001, it was discovered that mutations in human RyR2 underlie a significant number of instances of CPVT-mediated arrhythmias (Laitinen et al. 2001; Priori et al. 2001). Subsequent analysis of human RyR2 mutations in knock-in mouse models has validated that these RyR2 mutations recapitulate the human bidirectional VT phenotype under exercise stress conditions (Cerrone et al. 2005; Kannankeril et al. 2006). Interestingly, epicardial optical mapping studies suggested that arrhythmias in mice with CPVT might originate from the His-Purkinje portion of the cardiac conduction system (Cerrone et al. 2007).

Current experimental pharmacogenetic approaches to patients with CPVT include RyR2-stabilizing drugs, such as flecainide and carvedilol. Watanabe *et al.* (Watanabe et al. 2009)

showed that flecainide binds to and stabilizes RyR2, and prevents RyR2-mediated SR Ca^{2+} release. RyR2 stabilization was further characterized by data showing that flecainide reduces RyR2 open probability, rather than stabilizing its closed state (Hilliard et al. 2010). Additional data from Zhou *et al.* (Zhou et al. 2011), show that stabilization of RyR2 by the beta blocker carvedilol prevents store overload-induced calcium release in a mouse model of CPVT, and that carvedilol is the only beta blocker (of 14 tested) that has this property. Thus, it is becoming apparent that in the rare disease CPVT, drugs that directly bind to and stabilize RyR2 have a beneficial effect on SR Ca^{2+} release, and may suppress further arrhythmias.

Gain-of-Function Defects in RyR2 may Promote Cardiomyopathy

Investigations by our and other laboratories have revealed that certain gain-of-function mutations in RyR2, for example CPVT-linked mutation R176Q, can promote or predispose to the development of structural heart disease (Kannankeril et al. 2006). Whereas R176Q/+ knock-in mice were found to have structurally normal hearts at a young age (Mathur et al. 2009), analysis of older R176Q/+ mice revealed that long-term pathologic SR Ca^{2+} leak from RyR2 can promote development of mild right ventricular cardiomyopathy (Kannankeril et al. 2006). Moreover, it was shown that pressure overload in R176Q/+ mice led to accelerated development of dilated cardiomyopathy, likely due to excessive induction of the calcineurin-NFAT signaling pathway due to increased RyR2-mediated Ca^{2+} leak (van Oort et al. 2010b).

In addition to genetic mutations, post-translational modifications of RyR2 (such as phosphorylation, nitrosylation, and oxidation) have been shown to increase channel open probability. Knock-in mouse models of constitutive hyperphosphorylation of the S2808 or S2814 sites, respectively, both develop an age-dependent dilated cardiomyopathy. The onset of heart failure is early (1–2 months of age) in S2808D knock-in mice (Shan et al. 2010), and occurs later (9–12 months of age) in S2814D mice (van Oort et al. 2010a). These studies suggest that chronic Ca^{2+} ‘leak’ from the SR might induce cardiac remodeling associated with development of cardiomyopathy and heart failure.

CaMKII Phosphorylation of RyR2 Promotes Lethal Arrhythmias

Heart failure is characterized by a chronic hyperadrenergic state, which in turn, may activate intracellular kinases such as protein kinase A (PKA) and CaMKII. Of the CaMKII isoforms, the CaMKII δ has been shown to be the predominant cardiac isoform, with CaMKII δ_C being the cytoplasmic splice variant, and CaMKII δ_B comprising the nuclear envelope (Anderson et al. 2005). CaMKII δ_C over-activity is generally attributed to post-translational modifications of RyR2, phospholamban, and the L-type calcium channel (among other Ca^{2+} handling proteins) and is associated with pathological phosphorylation of key Ca^{2+} -handling proteins that may result in arrhythmias (Bers 2011). On the other hand, CaMKII δ_B activity is associated with GATA-4-mediated co-activation and binding to the Bcl-2 promoter within the nucleus, which may actually be an anti-apoptotic pathway (Little et al. 2009). Thus, it is becoming apparent that selective activation of CaMKII δ_C by elevations of intracytosolic Ca^{2+} may be a critical determinant of arrhythmias independent of other splice variant activity.

Several lines of evidence suggest that CaMKII, rather than PKA, can reverse arrhythmogenic SR Ca^{2+} leak in animal models of heart failure (Ai et al. 2005; Wagner et al. 2011). In a rabbit model of heart failure, Ai *et al.* (Ai et al. 2005) demonstrated that increased CaMKII activity was responsible for increased SR Ca^{2+} “leak” through RyR2. In this study, enhanced SR Ca^{2+} release was reversible by pharmacologic CaMKII inhibition, but not by PKA inhibition (Ai et al. 2005). Similar findings were obtained calcineurin

transgenic (TG) mice with heart failure, in which CaMKII was found to be upregulated with increased kinase activity (Khoo et al. 2006). Interestingly, genetic inhibition of CaMKII by overexpression of CaMKII-inhibitory peptide AC3-I was sufficient to significantly reduce both LV dilation and arrhythmias in this mouse model.

Although these prior studies suggested involvement of CaMKII in disruption of SR Ca^{2+} release associated with ventricular arrhythmias in failing hearts, the specific downstream phosphorylation targets affected by CaMKII upregulation remained unclear. To examine the specific role of RyR2 as a target of CaMKII in greater detail, we created a knock-in (S2814D) mouse model that mimics constitutively phosphorylation of S2814 on RyR2 (van Oort et al. 2010a), the main CaMKII phosphorylation site (Wehrens et al. 2004b). Our finding revealed that CaMKII phosphorylation of RyR2 leads an increased frequency of subcellular Ca^{2+} release events (Ca^{2+} sparks) (van Oort et al. 2010a). Single channel recordings in planar lipid bilayers provided additional and direct evidence for enhanced RyR2 open probability in hearts from S2814D knock-in mice.

Constitutive activation of the S2814 phosphorylation site on RyR2 by CaMKII increased the risk for ventricular arrhythmias in S2814D mice, even in the absence of structural heart disease in young mice (van Oort et al. 2010a). Following rapid ventricular pacing or pharmacological stimulation of the β -adrenergic pathway, triggered ventricular arrhythmias were commonly observed in S2814D mice, suggesting that increased SR Ca^{2+} load under those conditions promotes pro-arrhythmogenic SR Ca^{2+} release events via phosphorylated RyR2. Inhibition of CaMKII in the heart by cross-breeding with AC3I transgenic mice (see above) did not result in a significant suppression of arrhythmogenesis, suggesting that enhanced RyR2 activity due to CaMKII phosphorylation is an independent risk factor for lethal arrhythmias.

Recent studies have shed more light on the mechanisms by which aberrant SR Ca^{2+} release via CaMKII phosphorylated RyR2 cause arrhythmias (Li et al. 2011). Diastolic SR Ca^{2+} release events, if sufficient in amplitude, can trigger spontaneous Ca^{2+} waves at the cellular level. Such a global rise in cytosolic Ca^{2+} , in turn, can activate the plasmalemmal $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX), which extrudes Ca^{2+} from the cell. However, the exchange of Ca^{2+} is accompanied by the import of 3 Na^+ molecules, which generates a net inward current that can result in a delayed afterdepolarization (Bers and Guo 2005).

Additional insights were obtained in knock-in (S2814) mice impervious to phosphorylation of the S2814 site (Chelu et al. 2009; van Oort et al.). CaMKII-induced increases in Ca^{2+} spark frequency were blocked in ventricular myocytes isolated from these mice, suggesting that S2814 is the major functional CaMKII site on RyR2 (van Oort et al. 2010a). Genetic inhibition of the S2814 phosphorylation site also protects mice both from excess SR Ca^{2+} release and also ventricular ectopy in an experimental mouse model of heart failure (van Oort et al. 2010a). Thus, CaMKII phosphorylation of RyR2 is a critical downstream effect of pathological CaMKII over-activity, and may propagate lethal ventricular arrhythmias in the failing heart. This data suggests that the RyR2-CaMKII interaction may be a potential pharmacotherapeutic site, and thus more investigation is required to effectively blockade this interaction.

Involvement of CaMKII depends on heart failure model

Kirchhefer *et al.* (Kirchhefer et al. 1999) were among the first to describe increased CaMKII activity in failing hearts and differentiate its activity from protein kinase A (PKA). Subsequent analysis in a transgenic mouse model that overexpresses the cytoplasmic splice variant, CaMKII δ_C , showed a phenotypic progression towards dilated cardiomyopathy, which was associated with decreased SR Ca^{2+} content and increased fractional SR Ca^{2+}

release, consistent with abnormal RyR2 activity (Maier et al. 2003). Acute inhibition of CaMKII by drug KN-93 reduced calcium spark frequency, and suggested that in these failing mouse hearts, CaMKII is responsible for increased release of Ca^{2+} via RyR2. However, other studies have suggested that CaMKII might not play an equal role in all types of heart failure. For example, Toischer *et al.* (Toischer et al. 2010) have found that molecular signaling in mice with elevated afterload due to transverse aortic constriction (TAC) is different than mice with elevated preload with aortocaval shunt. Development of heart failure following TAC depends on activation of CaMKII, enhanced SR Ca^{2+} leak, and in late stages, fibrosis and apoptosis. In contrast, increased preload as a result of an aortocaval shunt is associated with Akt but not CaMKII phosphorylation (Toischer et al. 2010). This work highlights the importance of CaMKII as an internal sensor of intracellular $[\text{Ca}^{2+}]$ and also its importance as a transducer of aberrant Ca^{2+} signaling in preload-dependent heart failure.

Additional Mechanisms of CaMKII Activation That Impact RyR2 Function

In addition to CaMKII phosphorylation of RyR2, a variety of other post-translational modifications of RyR2 have been shown to increase susceptibility to SR Ca^{2+} leak and lethal arrhythmias. The effects of PKA phosphorylation of RyR2 have been explored by our laboratory (Sarma et al. 2010) and others (Wehrens et al. 2003; Wehrens et al. 2004a; Wehrens et al. 2004b), and several excellent reviews highlight the importance of PKA in the β -adrenergic signaling pathway (Currie 2009; Lehnart et al. 2004). Of relevance to CaMKII are the emerging concepts that CaMKII may be activated by the β -adrenergic receptor (Gao et al. 2011), and that there may be molecular cross-talk between PKA and CaMKII affecting RyR2 (Valverde et al. 2005), though further studies are required to better delineate how this may result in pathological phenotypes.

Another emerging concept is the importance of CaMKII oxidation to the progression of heart failure and arrhythmias during cardiac ischemia. CaMKII is activated by acidic and pro-oxidant signals, as is common in ischemic hearts, and thus was thought to potentially be involved in aberrant Ca^{2+} signaling in ischemia-reperfusion injury (Bell et al. 2011). Erickson *et al.* (Erickson et al. 2008) showed that oxidation of critical methionines on CaMKII were responsible for CaMKII activation to a level similar to that of autophosphorylation of the catalytic domain (Erickson et al. 2008). Utilizing angiotensin II, this group showed that an excess of reactive oxygen species (ROS) caused apoptosis via a CaMKII-dependent mechanism. Using lentiviral shRNA-silencing of CaMKII, the angiotensin II-mediated apoptosis was essentially blocked, underlining the importance of CaMKII-mediated activation in ischemic cardiomyopathy (Erickson et al. 2008). This work was further strengthened by studies by He *et al.* (He et al. 2011) who showed that aldosterone directly causes CaMKII oxidation through the recruitment of NADPH oxidase. Oxidized CaMKII was shown in rabbit cardiomyocytes to contribute to SR Ca^{2+} leak and cellular extrasystoles via a NCX-mediated increased late I_{Na} current (Wagner et al. 2011), and may contribute to sinus node dysfunction as well (Swaminathan et al. 2011). Thus, oxidation of CaMKII is an emerging mechanism that may explain arrhythmias in ischemic hearts.

Conclusions

CaMKII is a central signaling molecule, which senses cytoplasmic $[\text{Ca}^{2+}]$ and regulates Ca^{2+} homeostasis, and also is involved in chronic electrical remodeling and gene transcription. CaMKII phosphorylation of RyR2 has been shown to be an important regulatory switch modifying SR Ca^{2+} release. When hyperactive in diseased hearts, CaMKII may cause excessive diastolic Ca^{2+} “leak” leading to excess Ca^{2+} sparks, increased

incidence of plasma membrane afterdepolarizations, and lethal ventricular arrhythmias. Genetic or pharmacological inhibition of CaMKII may be a viable treatment for the prevention of cardiac arrhythmias, but given the large number of ion channels and gene targets affected by CaMKII modulation, more selective inhibitors of the RyR2-CaMKII interaction may be necessary for a more targeted approach to anti-arrhythmic therapy.

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