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Regulation of Sarcoplasmic Reticulum Ca^{2+} Release by Serine-Threonine Phosphatases in the Heart

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Abstract

The amount and timing of Ca^{2+} release from the sarcoplasmic reticulum (SR) during cardiac cycle are the main determinants of cardiac contractility. Reversible phosphorylation of the SR Ca^{2+} release channel, ryanodine receptor type 2 (RyR2) is the central mechanism of regulation of Ca^{2+} release in cardiomyocytes. Three major serine-threonine phosphatases including PP1, PP2A and PP2B (calcineurin) have been implicated in modulation of RyR2 function. Changes in expression levels of these phosphatases, their activity and targeting to the RyR2 macromolecular complex were demonstrated in many animal models of cardiac disease and humans and are implicated in cardiac arrhythmia and heart failure. Here we review evidence in support of regulation of RyR2-mediated SR Ca^{2+} release by serine-threonine phosphatases and the role and mechanisms of dysregulation of phosphatases in various disease states.

Keywords

Sarcoplasmic reticulum calcium release; ryanodine receptor; serine threonine phosphatase; cardiac arrhythmia; heart failure

Introduction

Components of cardiomyocyte Ca^{2+} handling machinery are exquisitely well tuned with each other to ensure robust repetition of the cardiac contraction and relaxation cycle over the whole lifespan. Metabolic demands of the body are constantly changing and multiple cellular signaling cascades, regulating cardiac function that affect distinct components of sarcoplasmic reticulum (SR) Ca^{2+} release/cytosolic Ca^{2+} removal network, must do so with

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Conflict of Interest

None

highest degree of order. Phosphorylation of the plasmalemmal L-type Ca^{2+} channel that provides a trigger for SR Ca^{2+} release and phosphorylation of phospholamban, a negative regulator of SR Ca^{2+} ATPase activity, results in enhanced Ca^{2+} influx and enhanced SR Ca^{2+} uptake respectively during β -adrenergic stimulation [1]. Despite ongoing controversy, the body of evidence suggesting that activity of cardiac SR Ca^{2+} release channel, the ryanodine receptor (RyR2), is regulated by reversible phosphorylation as well is growing [2–4]; and the increase in RyR2 activity was implicated in increased rate of SR Ca^{2+} release during β -adrenergic stimulation [5]. Convergence on protein phosphorylation provides extremely efficient coordination of cellular Ca^{2+} transport pathways to increase cardiac output in response to sympathetic stimulation in health [1]. In disease states the ability to maintain appropriate levels of phosphorylation of relevant proteins and RyR2 in particular is compromised, leading to defective Ca^{2+} homeostasis, thereby contractile impairment and increased propensity to malignant stress-induced Ca^{2+} -dependent cardiac arrhythmias.

The cardiac SR Ca^{2+} release channel RyR2 consists of four ~560 kDa subunits [6] with 46 serine/threonine residues per subunit that can be phosphorylated (www.phosphosite.org). However, studies of only three residues S2031, S2808 and S2814 (human nomenclature here and below) so far confirmed their functional relevance [7–9]. An electron microscopy study showed that S2031 is located in domain 4 in the cytoplasmic assembly of the RyR2 structure [10]. The S2808 and S2814 residues are located within the flexible linker that connects repeat 3 and 4 domains within the α -helical scaffold in the central region in the turret of the RyR2 facing dyadic space [11]. Interestingly, this linker contain in total 5 sites that can be phosphorylated by protein kinase A (PKA) and/or Ca^{2+} -calmodulin protein kinase type two (CaMKII) *in vitro* making it a phosphorylation “hot spot”. Furthermore, 4 out of these 5 sites and several additional residues in close proximity have also been detected to be phosphorylated *in vivo* [12,13]. The RyR2 macromolecular complex encompasses a wide network of proteins involved in control of phosphorylation state of the channel. Protein kinase A (PKA), Ca^{2+} -Calmodulin dependent protein kinase type II (CaMKII), phosphodiesterase 4D (PDE4D), protein phosphatase type 1 (PP1), protein phosphatase type 2A (PP2A) and Ca^{2+} -dependent protein phosphatase type 2B (PP2B) also known as calcineurin can be immunoprecipitated with RyR2 (Fig. 1) [14–17]. This level of complexity underscores the critical importance of the fine-tuning of RyR2 phosphorylation and thereby its function in the heart. Altered expression profiles, localization and activities of serine-threonine phosphatases found in multiple animal models of cardiac disease and humans highlights the importance of understanding of mechanisms of phosphatase-dependent regulation of activity of target proteins including RyR2.

1) The Structure and regulation of Serine-Threonine phosphatases

PP1, PP2A and PP2B present in the RyR2 macromolecular complex account for approximately 90% of phosphatase activity in the heart [18,19] and these phosphatases were distinguished based on their enzymatic activities. The combinatorial structural nature of these enzymes allows specific subcellular targeting and substrate affinity [20]. PP1 exists as a dimer, consisting of catalytic and regulatory subunits. Studies show that there is no freely available PP1 in the cardiac cell, but rather competition of >200 regulatory subunits to form a holoenzyme complex with a catalytic subunit [21–23]. Three types of catalytic subunits

(PP1 α , PP1 γ and PP1 δ) are expressed by three different genes [24,25], with further diversification achieved by PP1 α and PP1 γ each having different splice variants (PP1 α_{1-3} and PP1 $\gamma_{1/2}$) [23,26,27]. The >200 PP1 regulatory subunits can be classified by their activity into two groups: either those that regulate PP1 activity, or those that target PP1 to specific substrates (including glycogen-targeting, plasma membrane targeting and myosin-targeting subunits) [20,21,26]. PP2A structure is more complex than the PP1 holoenzyme, typically existing as a trimer with catalytic (PP2A-C α , PP2A-C β), structural scaffolding (PP2A-A α , PP2A-A β) and regulatory subunits. Regulatory subunits are grouped into four families (PP2A-B, PP2A-B', PP2A-B'', PP2A-B''') with many of these having different splice variants and multiple isoforms (for example, B56 α of the PP2A-B family is one of the most studied isoforms). The members are coded by at least 17 distinct genes, with large sequence diversity. Calcineurin also typically exists as a dimer, consisting of calmodulin-binding catalytic (CNA α , CNA β or CNA γ) and calcium-binding regulatory subunits (CNB α or CNB β) [28]. However, the enzyme can sometimes be modulated by additional interacting proteins, such as muscle A-kinase anchor protein (mAKAP) or Cain, a calcineurin inhibitor [29–32].

Pioneering work from AR Marks' group showed that phosphatases PP1 and PP2A are tethered to RyR2 via the leucine-isoleucine zipper motif of their regulatory subunits spinophilin (PPP1R9B) and PR130 respectively [33,34]. Later studies suggest that the number of regulatory subunits that localize phosphatase activity to the RyR2 microdomain may be higher. PP2A was found to scaffold to mAKAP within the complex via regulatory subunit B56 δ , and B56 α has also been shown to tether phosphatase catalytic subunits in a similar fashion [35,36]. Furthermore, posttranslational modifications of catalytic and regulatory subunits provide an additional layer of control of local phosphatase activity via several feedback loops. For example, phosphorylation of Inhibitor 1 (I1) can potentially inhibit PP1 [37] and form a positive feedback loop, amplifying the phosphorylation of several substrates in β -adrenergic stimulation including RyR2 and phospholamban [37,38], while phosphorylation at Serine-566 and reduced methylation at Leucine-309 of catalytic PP2A subunits causes a destabilization in the interaction with the regulatory subunit, serving as a negative feedback loop on the target phosphorylation and reducing its activity [35,39]. Phosphorylation of Tyrosine 307 also contributes to regulation, determining the localization and substrate specificity of the catalytic PP2A subunit [40,41]. PP2A phosphorylation also modulates PDE4D3, the phosphodiesterase anchored on the mAKAP scaffold within the RyR2 complex. Specific PP2A inhibitors have also been identified (I1_{PP2A} and I2_{PP2A}), but the expression and consequences of phosphorylation of these proteins on PP2A is yet to be explored [41,42]. MicroRNAs, small ~22 nucleotides noncoding RNAs that control protein expression through interference with translation by annealing to target mRNAs, have recently emerged as potent regulators of expression levels of phosphatases [43]. Several subunits of serine/threonine phosphatases were validated as targets for muscle-specific microRNAs; including catalytic subunits of PP2A by miR-133[36], regulatory subunit of PP2A B56 α by miR-1[44], and catalytic subunits of calcineurin by miR-499[45].

2) The Effects of Serine-Threonine phosphatases and kinases on the RyR channel function

The pharmacological enhancement of Serine-Threonine phosphatases suppresses SR Ca^{2+} release while inhibition enhances it in cardiomyocytes (Fig. 2A) via modulation of activity of many Ca^{2+} transport complexes including SERCa-phospholamban, plasmalemmal L-type Ca^{2+} channel and RyR2 [41,46–50]. Under β -adrenergic stimulation, when SERCa activity is high and current via L-type Ca^{2+} channels is maximal, phosphatase inhibition promotes the generation of pro-arrhythmic spontaneous Ca^{2+} waves (Fig. 2B). This indicates a key role of RyR2-bound phosphatases in maintenance of stable SR Ca^{2+} release during stress.

Early works specifically focused on the role of reversible phosphorylation of RyRs demonstrated the complex nature of such regulation. Takasago et al. showed that RyR2 could be phosphorylated by multiple exogenous Serine-Threonine kinases including PKA, PKG, PKC and Ca^{2+} -Calmodulin dependent protein kinases. In parallel the authors demonstrated accelerated [H3] ryanodine binding suggestive of increased RyR activity because for ryanodine to bind the channel must be in the open state [51,52]. Interestingly, their attempt to activate endogenous CaMKII resulted in decreased ryanodine binding. Chu et al. showed that CaMKII-dependent phosphorylation of RyR from skeletal muscle can be effectively reversed by incubation of junctional SR fraction with serine-threonine phosphatases PP1, PP2A and PP2B (calcineurin, in the presence of Ca^{2+}) [53]. Experiments using single RyR channels from skeletal muscle incorporated into lipid bilayer demonstrated that activation of endogenous CaMKII causes reduction in RyR activity reversible by application of serine-threonine phosphatase [54]. Similar results were obtained by H. Valdivia's group for RyR2 [55]. In this work application of exogenous acid phosphatase increased [H3] ryanodine binding and the open probability (P_0) of RyR2s reconstituted into lipid bilayers by increasing the rate of opening and promoting the appearance of a longer open state with no effect on single channel conductance. Purified exogenous CaMKII in the presence of calmodulin produced opposite effects reversible by application of acid phosphatase, application of CaMKII inhibitory peptide or replacement of ATP with the non-hydrolysable analogue of ATP. In 1991 Witcher et al. identified S2809 (human nomenclature S2808) as a unique site for phosphorylation by CaMKII and showed that phosphorylation at this site increases channel P_0 [8]. However, later work by Hain et al. [56,57], challenged this notion. The authors demonstrated that pretreatment of RyR2 channels with acid phosphatase or PP1 promotes channel block with Mg^{2+} , which can be relieved by incubation with exogenous CaMKII. On the contrary, activation of endogenous CaMKII resulted in reduction of channel activity in the presence of Mg^{2+} reversible by application of serine-threonine phosphatases. The authors concluded that RyR2 activity is governed not by one but multiple phosphorylation sites, and phosphorylation/dephosphorylation of these sites can lead to distinct functional consequences.

The latest revisions of the role of reversible phosphorylation of RyR2 resulted in identification of three functionally relevant RyR2 Serines. Importantly S2808, besides CaMKII, has been proposed to be a substrate for PKA [58], PKG [52] and possibly PKC [59]. S2814 was identified by Wehrens et al. as a CaMKII specific site [9], while S2031 as a PKA specific site [7]. However, data from our laboratory [36] suggests that S2031 can also be phosphorylated by CaMKII. The growing body of evidence strongly supports that

maximal phosphorylation at these sites is indeed associated with increased activity of the channel and consequent dephosphorylation with PP1 reduces its activity [59–61]. In addition to being responsive to $[Ca^{2+}]$ at cytosolic activation sites, RyR2 exhibits strong sensitivity to $[Ca^{2+}]$ from luminal side [62]. Li et al. showed that simultaneous phosphorylation of RyR2 at S2808 and S2814 increased luminal Ca^{2+} activation of the channels in lipid bilayers experiments [60]. Furthermore, Xiao et al. [63] demonstrated that PKA activates single RyR2 channels reconstituted in lipid bilayers only in the presence of Ca^{2+} from the luminal side. In addition to increasing channel activity, PKA phosphorylation dissociated RyR2 activity from sensitivity to cytosolic Ca^{2+} [59].

Little is known with regard to the specificity of phosphatases towards distinct RyR2 phosphorylation sites. Huke and Bers [64] demonstrated that PP1 can dephosphorylate S2808 and S2814, while PP2A can dephosphorylate S2814 and not S2808. In support of PP1 specificity to S2814, Chiang et al [65] showed that a spinophilin KO mouse model showed enhanced RyR2 phosphorylation at this site and furthermore, overexpression of spinophilin in HEK293 cells leads to a decrease in phosphorylation at S2814, but conversely not at S2808. Our experiments with muscle-specific microRNAs miR-1/miR133 mediated suppression of PP2A activity in the RyR2 microdomain showed preferential control of RyR2 phosphorylation at S2814 and S2031 and not S2808 [36,44]. The specificity of PP2B is yet to be determined.

Importantly, we showed that dephosphorylation of RyR2 incorporated into lipid bilayers with PP1 also increases RyR2 Po [66]. Carter et al. [59,61] demonstrated that PP1-mediated increase in RyR2 activity is not associated with changes in sensitivity to cytosolic Ca^{2+} and stems from abbreviation of closed states of the channel. At the cellular level, exposure of rat ventricular myocytes with plasmamembrane permeabilized with saponin to catalytic subunits of PP1 and PP2A resulted in dramatic increase in RyR2-mediated SR Ca^{2+} leak and rapid depletion of SR Ca^{2+} stores, preventable by preincubation of myocytes with phosphatase inhibitors [66]. To prevent potential effects of depletion of the stores which confound interpretation of results, Liu et al. [67] used mouse model overexpressing skeletal type of SR Ca^{2+} ATPase SERCa1a. Application of PP1 in permeabilized myocytes from these mice produced only a transient increase in Ca^{2+} spark frequency at unchanged SR Ca^{2+} content, suggestive that partial dephosphorylation of RyR2 enhances channel activity, which nearly returns to normal when more complete dephosphorylation state is achieved.

To summarize, RyR2 has multiple phosphorylation sites that can be targeted by multiple protein kinases and phosphatases. Both maximum phosphorylation and incomplete dephosphorylation of RyR2 result in increased activity of the channel. Fully dephosphorylated and intermediate phosphorylation states correspond to low “stabilized” RyR2 activity (Fig. 3). This may help to reconcile seemingly contradicting results from different groups because reducing or enhancing effects of phosphatases and kinases on RyR2 activity are likely depend on the initial level of RyR2 phosphorylation at multiple sites.

3) The effect of genetic ablation of RyR2 phosphorylation sites on Ca^{2+} release

a) Serine 2031—Over the last fifteen years generation of phospho- and dephosphomimetic mutants, substituting Serine of interest for Aspartic Acid (D) or Alanine (A) respectively, became a routine approach for assessment of the effects of reversible phosphorylation on protein function [68,69]. The SR Chen group demonstrated that human recombinant S2031A mutant channels reconstituted into lipid bilayers behaved similar to WT [63], which is not unexpected because basal phosphorylation at this site consistently reported as very low [36,70,71]. The difference was apparent when channels were exposed to PKA, with S2031A exhibiting reduced sensitivity to luminal $[\text{Ca}^{2+}]$ in comparison to WT or S2031D. Interestingly, earlier work of Wehrens et al. [72] also using human recombinant RyR2s showed that PKA-mediated increase in S2031A Po is similar to WT, while phosphomimetic mutant S2031D displayed low level of activity. The latter data directly challenges functional relevance of S2031 site, implying that other residues confer PKA-dependent effects on RyR2 function.

b) Serine 2814—The basal level of phosphorylation of CaMKII site S2814 assessed using phospho-site specific antibodies is generally reported as intermediate. It can be increased ~2–3 fold in the presence of phosphatase inhibitors and β -adrenergic agonist isoproterenol [64,71,73] and significantly decreased in the presence of Ca^{2+} chelator EGTA [70] or phosphatases [64]. Myocytes isolated from S2814A mutant mouse generated by Van Oort et al. [74] exhibited no obvious changes in SR Ca^{2+} release. Spark frequency in permeabilized S2814A myocytes was normal and Ca^{2+} transients and SR Ca^{2+} content measured in intact cells were not different from wild type. On the contrary, S2814D phosphomimetic mutant exhibited enhanced RyR2-mediated SR Ca^{2+} leak, reduced Ca^{2+} transients and SR Ca^{2+} content. Importantly, genetic ablation of S2814 was protective against pacing-induced arrhythmias versus WT mice after transverse aortic constriction (TAC) surgery. Further works confirmed that knock-in (KI) non-phosphorylatable S2814A is protective against ischemia-reperfusion induced arrhythmias, delays development of heart failure after TAC [75] and reduces abnormalities in Ca^{2+} homeostasis attenuating ventricular arrhythmias in Duchene muscular dystrophy [76]. Furthermore, S2814A KI prevents induction of atrial fibrillation in FKBP12.6 knockout mice by reducing enhanced RyR2 activity assessed by measuring Ca^{2+} sparks [77] and reverses the increase in atrial ectopy associated with PP1 depletion from the RyR2 complex in spinophilin knock-out mice [65].

c) Serine 2808—The roles of phosphorylation and dephosphorylation of S2808 continues to be intensely debated. Depending on the animal species and specific antibodies used, the basal level of S2808 phosphorylation is reported being either intermediate or high [36,61,64,67,71,73]. Earlier work using recombinant human S2808A channels reconstituted in lipid bilayers demonstrated that ablation of this phosphorylation site significantly reduced increase in Po upon application of PKA [72], which was later challenged by Xiao et al. [63], whose study showed no changes in effectiveness of PKA-mediated facilitation of mutant channel activity. Both groups did not report an increase in S2808A mutant activity in comparison to WT at basal conditions. Experiments using KI S2808A mouse model showed very modest differences in Ca^{2+} handling [78]. Namely, Ca^{2+} transient was slightly but significantly lower in S2808A KI in the presence of isoproterenol at 3 Hz pacing - the

highest frequency studied. Also, the Ca^{2+} transient decay was slower at 2 Hz with and without isoproterenol. Experiments in permeabilized myocytes in the presence of okadaic acid to block phosphatases and KN93 to block CaMKII showed no difference in cAPM-dependent increase in Ca^{2+} spark frequency. Remarkably, Western blot analysis using site-specific anti-phospho-serine antibodies revealed that ablation of S2808 is accompanied by marked increase in phosphorylation of S2031 and S2814 in the presence of isoproterenol suggestive of adaptive remodeling. This important finding is often overlooked and phosphorylation of all three serines is usually not reported in mouse models with an S substitution on A or D especially after β -adrenergic stimulation. Taking into account works from SR Chen [7] and X Wehrens [75] groups one would expect that increased phosphorylation of S2031 and S2814 will result in enhanced RyR2 activity and increased Ca^{2+} release, which is apparently not the case in S2808A mice challenged with isoproterenol [78,79]. Single channels from WT and S2808A hearts pretreated with PP1 showed no difference in P_o at low $[\text{Ca}^{2+}]$ at the luminal side of the channel [80]. In similar conditions no effects of PKA on WT and S2808A RyR2 P_o were detected [78]. However, in the presence of physiological 1 mM luminal $[\text{Ca}^{2+}]$ activity of S2808A was significantly reduced in comparison with WT [80]. In isolated myocytes this led to diminished rate of recruitment of RyR2 clusters during depolarization, increased time to peak of Ca^{2+} transients, increased SR Ca^{2+} load and profoundly slower propagation of spontaneous Ca^{2+} waves under β -adrenergic stimulation. Stabilizing effects of the S2808A mutation on RyR2 activity and/or SR Ca^{2+} release were reported in KI models generated by AR Marks and X Wehrens [72,76,81,82]. To further explore protective potential of S2808 ablation Liu et al. [67] used mouse model where SR Ca^{2+} uptake and leak were dramatically increased by expressing skeletal SR Ca^{2+} ATPase and knocking out calsequestrin to disrupt control of RyR2 activity by luminal Ca^{2+} . Importantly, profound abnormalities in Ca^{2+} release present in double mutant (DM) model were not lessened but exacerbated by introduction of S2808A. Moreover, the most pronounced defects in Ca^{2+} release and lowest survival rate were observed in S2808A^{+/-} mice. In contrast to DM, application of PP1 failed to produce increase in Ca^{2+} spark frequency in permeabilized S2808A^{+/-} myocytes, but instead evoked significant decrease in Ca^{2+} spark frequency. In S2808A^{+/+} PP1 failed to produce any effects. To summarize, ablation of phosphorylation at Ser-2808 enhances RyR2 leakiness at high SR Ca^{2+} loads and the activating effects of incomplete dephosphorylation on RyR2 are greater than those of complete loss of phosphorylation.

4) Serine Threonine phosphatases in cardiac disease

a) Heart failure—Phosphorylation of the RyR2 macromolecular complex and the altered expression and regulation of associated phosphatases have long been implicated in contractile dysfunction in heart failure (HF). Early studies of phosphatase levels in HF suggested that expression and activity of PP1 and PP2A were increased in failing human ventricles [83], as well as in a canine model of HF [84] and rat models of myocardial infarction [85,86]. PP2B is also significantly upregulated and activated in failing hearts, with both phosphorylated and total levels of the protein found to be increased [87–90]. Briston et al. [91] reported an increase in levels of both PP1 and PP2A in a tachypacing-induced sheep model of HF, with reduced PKA activity and a significant reduction of RyR2 phosphorylation at S2808, with reduced SR Ca^{2+} release which was restored by activation of

adenylate cyclase by forskolin. Altered regulation of PP1 inhibitor I1 has been implicated in the increased PP1 activity, with phosphorylation of I1 at Threonine-35 depressed in human HF [92,93]. Furthermore, phosphorylation of I1 at Serine-67 [94] is also increased and I1 protein levels are overall decreased in the heart [93], which appears consistent with the enhanced PP1 activity and decreased inhibitory action of I1 observed in HF.

Other groups have reported enhanced RyR2 phosphorylation with increased Ca^{2+} leak. Our work in a canine model of HF suggests that destabilized activity due to CaMKII hyperphosphorylation, as well as thiol oxidation by reactive oxygen species (ROS) results in progressive alterations of RyR2 function, a common mechanism across the progression of HF that impairs Ca^{2+} cycling and cardiac function [3]. Marx et al. [58] showed that there was a significant decrease in levels of PP1 and PP2A associated with the RyR2 macromolecular complex in failing human and canine hearts, even with the rise of cellular levels of PP1. RyR2 phosphorylation by PKA increased fourfold in HF with increased single channel activity, with a decrease in phosphatase bound to the macromolecular complex, resulting in diastolic Ca^{2+} leak and SR Ca^{2+} depletion. Experiments in a rabbit model of HF by Ai et al. [95] also reported increased SR diastolic Ca^{2+} release because of increased phosphorylation at S2814 CaMKII phosphorylation sites of RyR2, which was attributed to reduced levels of PP1 and PP2A. In both canine and human HF, systemic administration of β -adrenergic receptor blockers appears to reverse PKA hyperphosphorylation of RyR2, restoring normal function and macromolecular complex composition, as well as restoring sensitivity to β -agonists [96–98]. PP1 and PP2A targeting to the RyR2 complex was also restored to normal levels by this approach [98].

To explore why there are decreased levels of phosphatase associated to RyR2 and decreased PP2A activity sometimes observed in HF, we [36], in line with others [99] found that the expression levels of PP2A catalytic subunits B56 α and B56 δ were decreased in myocytes from canine hearts with HF induced by rapid pacing, leading to excessive RyR2 phosphorylation at S2031 and S2814 and diminished SR Ca^{2+} release due to excessive RyR2 mediated diastolic SR Ca^{2+} leak [80][3]. Phosphorylation at S2808 remained unchanged, as did the levels of PP1 catalytic subunits. Another possibility is that in many models of HF, posttranslational modifications may account for the depletion of RyR2-associated phosphatases and not necessarily from a decrease in regulatory subunits. In human and canine HF, decreased PP2A catalytic subunit methylation was reported at Leucine-309, as well as decreased levels of leucine carboxymethyltransferase 1 (LCMT-1), an enzyme that regulates methylation [39]. Meanwhile levels of phosphorylation at Tyrosine-307 of catalytic subunits increased, suggesting that these posttranslational modifications lead to the possible exclusion of regulatory subunits for active and RyR2-associated holoenzymes.

However, while many report increased RyR2 phosphorylation in models of HF and attribute this to the altered Ca^{2+} handling and characteristic depressed cardiac performance of the heart, others report abnormal Ca^{2+} release with no structural or functional changes in canine HF RyR2 compared to control, including phosphorylation at S2808 [100]. Xiao et al. [7] also reported similar levels of phosphorylation at both S2808 and S2031 in canine failing and non-failing hearts. In a comparison of non-ischemic vs. ischemic human HF [75],

authors reported increased CaMKII phosphorylation of RyR2 in patients with non-ischemic HF only, and in mice found increased S2814 phosphorylation only after thoracic aortic banding (pressure overload), not after myocardial infarction (MI). AR Marks' group have found PKA hyperphosphorylation in diverse models of human, mouse and rat heart failure [58,96,101,102], attributing the discrepancies between other groups work to the continuing activity of phosphatases during the isolation of cardiomyocytes and perfusion of intact hearts and stressing the importance of phosphatase inhibitors in such experiments [102]. It is apparent that phosphorylation of RyR2 and the dysregulation of associated phosphatases is an important factor in HF, independently of the model used and the seemingly opposite results.

b) Ventricular Arrhythmia—Heart failure is accompanied with increased arrhythmic risk with ~50% of patients dying suddenly during ventricular tachycardia and fibrillation [103]. Increased levels of circulating catecholamines and enhanced β 2-adrenergic responsiveness are hallmarks of HF [104,105], with cardiomyocytes exhibiting increased SR Ca^{2+} leak and increased propensity for diastolic Ca^{2+} waves when under β -adrenergic stimulation [3,106–109]. Enhanced RyR2 activity in HF due to the loss of PP1 and PP2A from the channel complex was first demonstrated in single channel studies by Marx et al. [58]. Increased activity of RyR2 has been subsequently linked to different patterns of abnormal Ca^{2+} handling observed in HF, such as self-propagating waves [110] and triggered activity arising from proarrhythmic early after depolarizations (EADs) [111] or delayed after depolarizations (DADs) [3,106] that can lead to ventricular arrhythmia [106,107,112,113]. RyR2 hyperactivity has been implicated in the multiple pathophysiological forms of HF [108,114]: even in early, compensated stages of disease where Ca^{2+} transient amplitude remains unchanged, it appears that RyR2 activity is abnormally high [3]. Pharmacological inhibition of CaMKII with a specific CaMKII inhibitor KN93 confirmed the critical role of the kinase in arrhythmogenesis, with diastolic Ca^{2+} waves abolished in HF cardiomyocytes. We showed that the dissociation of PP2A in canine HF cardiomyocytes underlies increased RyR2 phosphorylation at S2814 and S2031 by CaMKII, an increased frequency of diastolic Ca^{2+} waves and DADs, and the increased propensity for arrhythmogenesis [36].

In failing human hearts, I1 is downregulated [37,92,93] due to increased phosphorylation of the inhibitor at Serine 67 and Threonine 75 [94,115]. In a study of I1-overexpressing mice and I1-KO mice [116], El-Armouche et al. found that the loss of I1 leads to reduced β -adrenergic sensitivity, protecting from catecholamine-induced arrhythmia. Authors also found that KO mice exhibited lower levels of RyR2 phosphorylation at S2814, while overexpression of I1 led to cardiac dysfunction and spontaneous hypertrophy, suggesting the upregulation of I1 exacerbates the detrimental effects of β -adrenergic stimulation on the failing heart.

c) Atrial fibrillation—Atrial fibrillation is the most common form of arrhythmia in humans. Increased arrhythmic risk in AF is usually attributed to an increased substrate for reentry due to altered expression patterns of sarcolemmal ion channels and tissue fibrosis [117]. However, over the last ten years substantial evidence was accumulated that RyR2 dysfunction underlies ectopic activity that is present in AF [118,119]. Expression levels and

activity of PP1 and PP2A are markedly increased in human AF [120,121], however RyR2 phosphorylation levels were shown to be increased [119,122–124]. Chelu et al. suggested that enhanced activity of CaMKII due to phosphorylation of T287 can outweigh increased phosphatase activity to shift balance toward increased RyR2 phosphorylation increasing its activity [123]. El-Armouche et al. proposed an alternative hypothesis [120]. They reasoned that phosphatase activity could be distributed unevenly between subcellular compartments. Indeed they found that phosphorylation of I-1 at the T35 site was tenfold higher in the SR, which is consistent with decreased phosphatase activity in RyR2 microdomain and can explain RyR2 hyperphosphorylation. As a proof of concept Chiang et al. [65] generated a spinophilin KO mouse model which demonstrated enhanced propensity to AF due to enhanced activity of phosphorylated RyR2, with PP1 depleted from the channel complex.

d) Hereditary Arrhythmia—The first evidence that aberrant regulation of RyR2 by phosphatases can contribute to inherited arrhythmia came from the work of DeGrande et al. [125] studying mechanisms of arrhythmia linked to mutations in ankyrin-B. Ankyrin adaptor proteins mediate the attachment of membrane proteins to the cytoskeleton [126], and the ankyrin-B isoform is found at cardiac SR junctions with the transverse tubule [127] with a role in PP2A targeting and regulation [128,129]. Ankyrin-B dysfunction has been linked to both acquired and congenital forms of arrhythmia, with both humans and mice with ankyrin B loss-of-function mutations displaying stress-induced arrhythmias [130,131]. DeGrande et al. [125] identified enhanced RyR2 S2814 phosphorylation levels in mouse models of cardiac ankyrin-B syndrome due to alterations in the CaMKII signaling pathway. In normal hearts, ankyrin-B directly associates with B56 α , the PP2A regulatory subunit, but in ankyrin-B deficient cardiomyocytes there was a lack of B56 α targeting, likely contributing to the increase in RyR2 phosphorylation.

Later our laboratory demonstrated that the mislocalization of phosphatases from RyR2 complex can contribute to cardiac arrhythmia associated with the loss of function of ether go-go (HERG) potassium channels that underlie long QT syndrome type 2 (LQT2) [73]. Rabbits that overexpress dominant-negative mutant of the human gene KCNH2 (HERG-G628S) in the heart to eliminate IKr currents exhibit prolonged QT interval and a high incidence of sudden cardiac death due to ventricular tachyarrhythmias [132]. In this model of LQT2 syndrome, PP1 and PP2A phosphatases are depleted from the RyR2 macromolecular complex [73], leading to increased RyR2 phosphorylation at S2808 and S2814 and increased RyR2-mediated SR Ca²⁺ leak. In the presence of isoproterenol, LQT2 myocytes exhibited enhanced propensity to generate EADs, which were preventable by inhibition of CaMKII. Loss of PP1 from the RyR2 complex in this model was attributable to downregulation of spinophilin that tethers PP1 activity to the channel and enhanced expression of PPP1R3A that localizes PP1c to glycogen. We did not find substantial changes in expression levels of regulatory subunits of PP2A. However, we found increase in Y307 phosphorylation of PP2Ac, which was reported to destabilize PP2Ac interaction with regulatory and anchoring subunits [73].

5) Perspective

Taken together, these findings imply that altered RyR2 phosphorylation and therefore altered RyR2 function due to the changes in local phosphatase activities is a common phenomenon in broad range of acquired and inherited cardiac diseases. Manipulations with phosphatase activities localized to RyR2 present an attractive therapeutic strategy. However, it will be challenging to achieve given the involvement of Serine-Threonine phosphatases in numerous cellular processes including hypertrophic signaling, cell death, metabolism and mitochondrial function [133–135]; multilayered control mechanisms that regulate expression and distribution of phosphatases in the cell, and presence of phosphatases in multiple tissues. Pharmacological inhibitors and activators of phosphatases that used as immunosuppressants or in anti-cancer therapy often exhibit adverse effects on the heart. For example patients treated with FTY720 (fingolimod), an enhancer of PP2A activity, exhibit bradycardia [136]. Interestingly, a recent report showed that FTY720 efficiently prevents arrhythmias associated with ischemia-reperfusion injury in rats [137]. The authors attribute beneficial effects of FTY720 to activation of the Pak1-Akt pathway, while potential effects on Ca^{2+} homeostasis were not studied. Patients treated with PP2B inhibitors for prolonged period of time develop diastolic dysfunction [138]. Furthermore, recent study using a mini pig model of compensated HF showed that administration of Cyclosporine A, a PP2B inhibitor, not only failed to normalize diminished Ca^{2+} transients, but even failed to alter enhanced PP2B activity in failing hearts [139]. Targeted expression/knockout of regulatory subunits that tether phosphatase activities to specific substrates appears to be a more straightforward approach. Nevertheless, altering levels of specific regulatory subunits often leads to unexpected results. For example, mouse models with overexpression and knockdown of the regulatory subunit of PP2A B56 α exhibit increased PP2A activity with unchanged and reduced phosphorylation levels of RyR2 respectively [140,141]. So far the most promising finding was in a KO model of Inhibitor 1 of PP1. I1 ablation promoted dephosphorylation of RyR2 at S2814 and attenuated arrhythmogenesis, mildly affecting β -adrenergic sensitivity [116,142]. Another promising therapeutic target that largely remains unexplored is microRNAs. Overexpression of muscle specific miRNA, miR-499 which regulates expression of catalytic subunits of PP2B led to reduction in cardiac dysfunction induced by ischemia-reperfusion injury [45]. Despite complexity, the ways in which RyR2-bound phosphatase activities are affected and altered locally deserve further in-depth investigation.

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Abbreviations

PP1	protein phosphatase type 1
PP2A	protein phosphatase type 2
PP2B	protein phosphatase type 2B
RyR2	ryanodine receptor type 2

SR	sarcoplasmic reticulum
SERCa	SR Ca ²⁺ ATPase
CaMKII	calcium-calmodulin dependent protein kinase type 2
PKA	protein kinase A
PKC	protein kinase C
PKG	protein kinase G
I-1	inhibitor of phosphatase PP1 type 1
HF	heart failure
AF	atrial fibrillation
Ank-B	ankyrin-B
LQT2	long QT syndrome type 2

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Highlights

- Serine-threonine phosphatases regulate RyR2 activity and thereby SR Ca^{2+} release
- Phosphatases PP1, PP2A and PP2B are associated with RyR2 macromolecular complex
- Local RyR2-bound phosphatase activities are altered in broad range of cardiac diseases
- Manipulation of RyR2-bound phosphatase activity is an attractive therapeutic strategy

Figure 1. The RyR2 macromolecular complex with associated accessory proteins that influence its phosphorylation status

The action of protein kinases CaMKII and PKA on RyR2 phosphorylation sites S2031, S2808 and S2814 are opposed by protein phosphatases PP1, PP2A and PP2B. PP1c and PP2Ac are directed to the complex via their regulatory subunits, spinophilin and PR130 and B56α respectively. In addition, PP2A scaffolds to the complex via B56δ and mAKAP, which is anchoring PP2B, PKA and PDE4D.

Figure 2. The effects of pharmacological modulators of phosphatase activity on SR Ca^{2+} release
A. Representative recording of Ca^{2+} transients recorded in rat ventricular myocytes field stimulated at 0.5 Hz under baseline conditions (black), after incubation with PP2A activator FTY720 (100 nM, 30 min., grey), incubation with PP2B inhibitor Cyclosporine A (100 nM, 30 min., blue), and after complete inhibition of PP1 and PP2A with Calyculin A (1 μM , 15 min., red). Measurements of SR Ca^{2+} content by application 10 mM caffeine demonstrate that FTY720 reduces Ca^{2+} stores, while inhibition of phosphatases increases Ca^{2+} transient without increase in SR Ca^{2+} content. **B.** Representative confocal line-scan images of Ca^{2+} transients in periodically paced rat cardiomyocytes in the presence of β -adrenergic agonist isoproterenol. Phosphatase inhibition with Calyculin A (1 μM , 15 min.) promotes generation of arrhythmic spontaneous Ca^{2+} waves.

Figure 3. Both phosphorylation and dephosphorylation increase RyR2 channel activity

A. Schematic representing the effect of kinase and phosphatase of the RyR2 tetramer and its open probability (P_o). Blue circles represent unphosphorylated sites, where phosphatase activity is high, and red circles represent phosphorylated sites, where kinase activity is high. Maximum phosphorylation and incomplete dephosphorylation of RyR2 results in increased P_o . Fully dephosphorylated and intermediate phosphorylation states correspond to low “stabilized” P_o . **B.** SR Ca^{2+} release depends upon RyR2 function at different phosphorylation states. Increased phosphatase activity in rat ventricular myocytes causes diminished Ca^{2+} transient due to enhanced RyR2 mediated loss of SR Ca^{2+} during diastole exacerbating effects of depressed SERCa-mediated SR Ca^{2+} uptake due to dephosphorylation of phospholamban, and reduced Ca^{2+} influx through dephosphorylated sarcolemmal L-type Ca^{2+} channels. In the presence of β -adrenergic agonist isoproterenol (ISO, 100 nM) increased RyR2 phosphorylation contributes to increased Ca^{2+} transient by acceleration of SR Ca^{2+} release during systole. When phosphatase activity localized to

RyR2 is decreased, RyR2 achieves maximum phosphorylation under β -adrenergic stimulation, which results in massive diastolic SR Ca^{2+} leak. In conditions when SERCa mediated SR Ca^{2+} uptake and L-type Ca^{2+} channel-mediated influx are high this leak evolves into propagating spontaneous Ca^{2+} waves promoting arrhythmogenic disturbances of membrane potential (delayed after depolarization, red arrows). Right panel demonstrates representative simultaneous line scan recording of Ca^{2+} transients and voltage in current clamped rat ventricular myocyte under 100 nM ISO with PP2A dissociated from the RyR2 macromolecular complex by adenovirus-mediated overexpression of microRNA miR-1.