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Original article

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.

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1. 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 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+}-calmodulin-dependent

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; I1, 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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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.

2. 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 (PP1α, PP1γ and PP1δ) are expressed by three different genes [24,25], with further diversification achieved by PP1α and PP1γ each having different splice variants ($PP1\alpha_{1-3}$ 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-Cα, PP2A-Cβ), structural scaffolding (PP2A-Aα, PP2A-Aβ) 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, B56α 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 (CNAα, CNAβ or CNAγ) and calcium-binding regulatory subunits (CNBα or CNBβ) [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 B56δ, and B56α has also been shown to

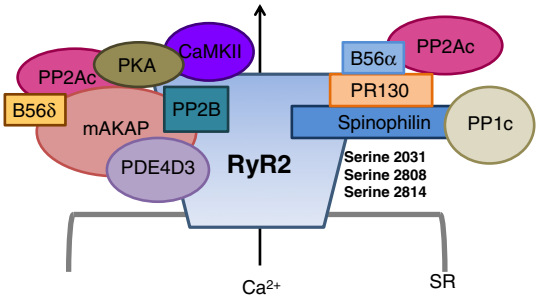

Fig. 1. The RyR2 macromolecular complex with associated accessory proteins that influence its phosphorylation status. The action of protein kinases CaMKII and PKA on RyR2 phosphorylation sites S2031, S2808 and S2814 are opposed by protein phosphatases PP1, PP2A and PP2B. Catalytic subunits PP1c and PP2Ac 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.

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 potently 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 B56α by miR-1 [44], and catalytic subunits of calcineurin by miR-499 [45].

3. 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 (Po) 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

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