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Controversies in Cardiovascular Research:

Role of Ryanodine Receptor Phosphorylation in Heart Failure and Arrhythmias

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Abstract

Cardiac ryanodine receptor type-2 (RyR2) play a key role in excitation-contraction coupling. The RyR2-channel protein is modulated by various posttranslational modifications, including phosphorylation by protein kinase-A and Ca^{2+}/calmodulin protein kinase-II (CaMKII). Despite extensive research in this area, the functional effects of RyR2-phosphorylation remain disputed. In particular, the potential involvement of increased RyR2-phosphorylation in the pathogenesis of heart failure and arrhythmias remains a controversial area, which is discussed in this review article.

Keywords

Atrial fibrillation; calcium handling; heart failure; phosphorylation; ryanodine receptor

INTRODUCTION

This review focuses on recent controversies surrounding studies addressing the consequences of ryanodine receptor type-2 (RyR2) phosphorylation in heart. Ryanodine receptors serve as Ca^{2+}-release channels on endo/sarcoplasmic reticulum (ER/SR) of excitable tissues, including neurons, skeletal and cardiac muscle.[1] During the past 20 years, over 900 publications have focused on the role of RyR-phosphorylation (Fig. 1). *Circulation Research* has published the largest share of these papers, over 80 in total, including some of the most controversial work on RyR2-phosphorylation in the pathogenesis of cardiac disease. Several recent reviews have summarized major studies in the field, and already described some of the inconsistent and partly opposing conclusions thereof.[2–5] Here, we will try to delineate which concepts can be considered as mostly accepted, which require further in-depth investigations, and will discuss major unresolved controversies in the field.

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DISCLOSURES

Dr. Wehrens is a founding partner of Elex Biotech, a company that develops drugs that modulate ryanodine receptors.

RyR2-phosphorylation and excitation-contraction coupling in normal hearts

RyR2 is the major SR Ca^{2+}-release channel involved in excitation-contraction coupling, the process by which an electrical depolarizing impulse is transduced into a cardiac contraction. The amount of Ca^{2+} released from the SR via RyR2 largely determines the Ca^{2+}-transient amplitude, which correlates with the strength of systolic contraction.[6] The RyR2-channel consists of four pore-forming subunits, which associate with numerous accessory proteins including FK506-binding protein-12.6 (FKBP12.6), calmodulin (CaM), calsequestrin-2 (CSQ2), junctin, triadin, and junctophilin-2 (JPH2), all of which can regulate channel gating.[7] In addition, RyR2 is regulated at the posttranslational level by S-nitrosylation, oxidation, and protein phosphorylation.[8]

RyR2-channels contain several phosphorylation sites. The degree of steady-state phosphorylation of each site depends on a dynamic balance between multiple protein kinases and phosphatases,[9] allowing precise control of RyR2-phosphorylation and, consequently, channel activity.[10] Alterations in RyR2-phosphorylation play a critical role in various cardiac diseases, including heart failure (HF) as well as atrial and ventricular arrhythmias. Most studies to date have only focused on the effects of protein kinase-A (PKA) and Ca^{2+}/calmodulin-dependent protein kinase-II (CaMKII)-mediated RyR2-phosphorylation, although it is likely that other serine/threonine protein kinases can also phosphorylate RyR2.[10–12]

Serine-2808 (S2808, or S2809 in some species) was the first RyR2-residue identified as a phosphorylation site,[13] and is believed to be the primary target of PKA-phosphorylation.[14, 15] Addition of purified PKA to a single RyR2-channel studied in the planar lipid-bilayer system increased RyR2 open probability (Po).[14] The increased RyR2-Po was prevented by mutating S2808 to alanine (S2808A), rendering the channel unphosphorylatable.[10] Conversely, the phosphomimic mutation S2808D increased RyR2-Po in some[10, 15], but not all studies.[16] Similarly, studies in ventricular cardiomyocytes from S2808A and S2808D mice showed that the RyR2-phosphorylation level alters channel activity in intact cardiomyocytes, as evidenced by changes in Ca^{2+}-transient amplitudes and Ca^{2+}-spark frequencies, in some,[17] but not other studies.[18] Additional studies were performed by treating wild-type ventricular cardiomyocytes with PKA-inhibitors.[19] However, it is impossible to attribute the observed changes (or lack thereof) to phosphorylation of a specific RyR2-residue, because all PKA-phosphorylation sites and other PKA-target proteins will be simultaneously modified. Therefore, studies using knock-in mouse-models of missense mutations of specific phosphorylation-sites remain invaluable to study specific sites, although they also have intrinsic limitations (see below). Even using similar S2808A knock-in mouse-lines, opposing findings have been reported for the functional effects of S2808-phosphorylation..[20] For example, Marks *et al.*[20] reported blunted heart rate and contractile responses to isoproterenol (Iso), and a blunted enhancement of Ca^{2+}-transient amplitude and fractional shortening in S2808A-mice. In contrast, Houser *et al.*[21] showed unaltered chronotropic and inotropic responses to Iso *in vivo* and *in vitro*.[22] It is possible that there is a threshold below which PKA-phosphorylation

of RyR2 (at S2808) does not cause a physiological effect, but that does not appear a sufficient explanation for these clear differences. Nevertheless, it is intriguing why well-established and leading labs in the field using similar mutant recombinant channels and knock-in mouse-models arrive at opposite conclusions regarding the physiological effects of PKA-dependent RyR2-phosphorylation, particularly at the S2808-site.

Several factors might contribute to these discrepancies, including subtle differences in the animal models, reagents, experimental-procedures, and data interpretation (summarized in Table 1). Furthermore, new findings continue to refine our understanding of signaling pathways and RyR2 regulation.[5] For example, initially PKA-phosphorylation of S2808 was reported to reduce FKBP12.6-binding.[14] However, S2808D knock-in mice with constitutively-phosphorylated RyR2-channels exhibited normal FKBP12.6-binding, at least at a young age.[17] We will not discuss the controversies related to FKBP12.6 in relation to RyR2-regulation and phosphorylation due to space limitations, but refer to prior excellent review articles.[4, 5, 23] Recent studies revealed that oxidation and/or S-nitrosylation, together with RyR2-phosphorylation at S2808, are required to dissociate FKBP12.6 from RyR2, and to increase RyR2-Po.[17, 24] At present, the exact mechanisms underlying the synergy between these different posttranslational RyR2-modifications remain unknown and require follow-up studies.

Serine-2814 (S2814, in some species S2815) was the second RyR2-residue identified as a primary CaMKII-target.[10] Careful stoichiometric analysis revealed that *in vitro* CaMKII-phosphorylation could partially phosphorylate a second still-unidentified residue.[10] One study claimed that CaMKII can phosphorylate up to 3–4 sites, but these conclusions were not based on stoichiometric studies using quantifications of both total and phosphorylated RyR2.[25] Claims in the literature including some review articles, that there are 3–4 or even more phosphorylation residues per RyR2-monomer, are not based on credible data and will not help to resolve this controversy.[26] However, there is a broad consensus that CaMKII-mediated RyR2-phosphorylation increases RyR2-Po, both at the single-channel level in bilayers,[4, 10] and in ventricular-cardiomyocytes as evidenced by increased Ca^{2+}-spark frequency.[27] Mutation S2814A prevents most CaMKII-effects on RyR2, suggesting that S2814 is the major, but probably not the exclusive, RyR2-residue subject to CaMKII-phosphorylation.[10, 28] These findings are consistent with studies in CaMKII-knockout and transgenic-animals revealing altered RyR2-phosphorylation at S2814 and altered Ca^{2+}-spark frequency depending on global CaMKII-levels.[29, 30]

Some data suggest a crosstalk between the S2808 and S2814 phosphorylation-sites. For example, enhanced S2814-phosphorylation was observed in S2808A knock-in mice, suggesting that the phosphorylation-state of one residue might affect the likelihood that neighboring residues are phosphorylated, particularly following adrenergic stimulation.[18] Alternatively, this may be an artifact due to changes in the epitope for the phospho-specific antibodies. Therefore, it is important that all known RyR2-phosphorylation sites are monitored when studying (patho)physiological mechanisms using RyR2 knock-in mouse models.

Some years ago Serine-2030 (S2030, or S2031 in some species) was described as a third functional RyR2-phosphorylation site.[31] PKA-phosphorylation of this site might enhance RyR2-sensitivity to luminal (intra-SR) Ca^{2+}.[31] Other studies, however, have failed to demonstrate a measurable functional effect of this phosphorylation-site, and its physiological significance remains to be determined *in vivo*.[15, 32] Overall, it would be of great interest to identify other RyR2-phosphorylation sites that are regulated under physiological conditions or perhaps altered in cardiac diseases, to determine the exact basal-level of RyR2-phosphorylation at steady-state for each individual residue, to identify the specific kinases and their counterbalancing phosphatases that dynamically phosphorylate/dephosphorylate each respective residue, and to delineate the precise allosteric mechanisms underlying the synergetic effects of concomitant phosphorylation at multiple sites, along with other posttranslational modifications of RyR2-subunits.

Dephosphorylation of RyR2 is mediated by PP1, which is targeted to the complex by the regulatory-subunit spinophilin, and PP2A, targeted through its regulatory-subunit PR130.[33, 34] PP2A may be also targeted to RyR2 through B56α binding to ankyrin-B, which also interacts with RyR2,[35] or through the muscle-specific A-kinase-anchoring protein (mAKAP), which binds both RyR2 and PP2A-B56α.[36] Ssteady-state phosphorylation of individual RyR2 phosphorylation sites is regulated in a highly complex and dynamic manner, which should be seriously considered when quantifying the degree of phosphorylation and studying its consequences for cardiac (dys)function. Several studies have demonstrated that PP-mediated dephosphorylation of RyR2 decreased channel open probability, but other studies have reported the opposite.[9, 37–39] Moreover, it was shown that PP increases RyR2 leakiness in cells expressing WT, but not S2808A mutant RyR2 with the disabled PKA phosphorylation site.[40] Thus, the PP-mediated regulation of RyR2 remains controversial at this time.

Role of altered RyR2-phosphorylation in heart failure

Enhanced RyR2-mediated SR Ca^{2+}-leak has been observed in HF-patients and various animal models.[4, 41] It is likely that diastolic SR Ca^{2+}-leak, together with reduced SERCA2a function and enhanced NCX1 function, contributes to the depletion of SR Ca^{2+}-content in HF.[5] The causal role of RyR2-phosphorylation in HF pathogenesis, however, remains highly controversial because different groups have reported contradictory findings using similar approaches and animal models.[15, 18, 42] In addition, RyR2 is regulated by various additional posttranslational modifications, and the potential synergistic or antithetic effects with phosphorylation remain largely unexplored.[43–45]

The first paper demonstrating altered RyR2-phosphorylation in HF-patients was published in 2000 by Dr. Marks' group.[14, 15, 17] These authors[14] postulated that the hyperadrenergic state in HF increases PKA-mediated S2808-phosphorylation, reduces FKBP12.6-binding to RyR2, and enhances RyR2-Po. Several (but not all) subsequent papers validated the increased S2808-phosphorylation in HF in various species, as previously summarized.[3–5] In some cases, different groups used similar mutant mice (S2808A knock-in) and seemingly similar experimental models, yet obtained opposite results.[4] For example, the Marks lab demonstrated in S2808A knock-in mice that S2808-phosphorylation is a critical mediator of

progressive cardiac failure following experimental myocardial infarction (MI).[15] In contrast, the Houser lab showed no protective effects in S2808A-mice (generated independently by their group), despite a significant increase in S2808-phosphorylation after MI.[22] Once again, numerous factors might contribute to the discrepant results (see Table 1). Complementary studies in knock-in mice of the constitutively hyperphosphorylated S2808-site (S2808D-mice) revealed the spontaneous development of an age-dependent cardiomyopathy, which might support a role for this site in some types of HF development.[17]

It is important to consider several general concepts when comparing studies in this field. For example, since cardiac remodeling is likely heterogeneous throughout the heart, RyR2-phosphorylation levels might vary among different regions.[46] Furthermore, some studies suggest that the etiology of HF might affect the relative phosphorylation-level of different RyR2-sites.[10, 27, 28] It is also unclear whether RyR2-phosphorylation sites are differentially phosphorylated during different stages of HF or in the context of different etiologies of the disease.[47] Recent studies on human hearts revealed that both S2808 and S2814 are hyperphosphorylated during compensated cardiac hypertrophy, whereas only S2814 remained phosphorylated in patients with end-stage HF.[47] At present, it is unclear how common risk factors such as metabolic syndrome, diabetes, and ischemia (i.e., oxidative stress) might impact RyR2-phosphorylation. Additional studies in larger patient populations and large-animal models with well-controlled conditions are required to resolve such important questions.

There is now strong evidence that abnormal CaMKII-phosphorylation of RyR2 can contribute to contractile dysfunction in HF.[28, 47, 48] First, several studies showed that CaMKII hyperactivity can cause HF, for example in CaMKII-transgenic mice,[49] whereas mice overexpressing a CaMKII-inhibitor or deficient in CaMKII-δ are protected from developing HF.[50, 51] Second, RyR2 has been identified as a major downstream target of CaMKII involved in abnormal SR Ca^{2+}-leak and contractile dysfunction in HF.[48, 52] Our studies of a limited number of human failing hearts revealed increased RyR2-phosphorylation at S2814 in nonischemic dilated cardiomyopathy (DCM) but not in ischemic cardiomyopathy (ICM).[28] These findings suggest that CaMKII activation might depend on the type of HF.

Mouse HF-models demonstrated that S2814-phosphorylation was increased in mice subjected to transverse-aortic constriction (TAC) but not after MI.[28] S2814 ablation in S2814A-mice prevented progression of cardiomyopathy to severe HF in these mice, whereas no beneficial effect was noted in S2814A mice subjected to MI.[28] Moreover, constitutive S2814-phosphorylation in S2814D mice caused spontaneous late-onset HF.[27] These data suggest that S2814-phosphorylation might be involved in adverse cardiac remodeling in DCM, although additional studies are required to confirm this. These studies do not exclude the possibility of additional CaMKII-phosphorylation sites on RyR2 playing a role in DCM or other types of HF.

Some studies have demonstrated CaMKII-activation in failing hearts following MI.[50, 53] However, consistent with the lack of S2814-hyperphosphorylation following MI in mice,[28] it was not too surprising that S2814A knock-in mice were not protected from HF-

progression following MI. These findings are also in agreement with a study from another lab showing a lack of protection from MI-induced HF in S2814A-mice.[54]

Role of RyR2-phosphorylation in ventricular arrhythmogenesis

Many patients with HF die suddenly due to ventricular arrhythmias,[55] many of which are thought to be initiated by focal triggered activity, involving spontaneous diastolic SR Ca^{2+}-release events (SCaEs) via RyR2.[56] Sudden increases in SR Ca^{2+}-leak can activate a potentially-arrhythmogenic depolarizing inward Na^{+}/Ca^{2+}-exchange (NCX)-current, which can cause delayed afterdepolarizations (DADs) and trigger ventricular arrhythmias. CaMKII, which is upregulated and more active in HF, has been shown to promote SR Ca^{2+}-leak associated with triggered arrhythmias.[48, 57, 58]

Studies in S2814D-mice with constitutively-phosphorylated RyR2 revealed an increased risk for ventricular arrhythmias, even in the absence of structural heart disease.[27] Interestingly, although S2814D-mice show more spontaneous Ca^{2+}-sparks, ectopic activity was not observed under resting conditions, suggesting that a combination of increased SR Ca^{2+}-load (during fast pacing/beta-adrenergic stimulation) is required to initiate arrhythmias.[27] It remains to be studied whether concomitant S2808- and S2814-hyperphosphorylation promotes severe arrhythmogenic SR Ca^{2+}-leak, even in the absence of β-adrenergic stimulation. In addition, S2814-phosphorylation might be a key mechanism for triggered arrhythmias in HF, at least following TAC in mice.[27] Thus, it is mostly accepted that CaMKII-mediated RyR2-phosphorylation leads to SR Ca^{2+}-leak associated with arrhythmias,[7] but the potential role of S2808-phosphorylation in arrhythmogenic SCaEs remains uncertain. Finally, in HF-patients, several mechanisms likely conspire to promote triggered activity and ventricular tachycardia, including enhanced phosphorylation, oxidation and S-nitrosylation of RyR2.[5, 7] The mechanisms underlying such synergistic effects are mostly understudied and should be addressed in future studies.

RyR2-phosphorylation is also believed to participate in the pathogenesis of catecholaminergic polymorphic ventricular tachycardia (CPVT), an inherited condition characterized by exercise or stress-induced arrhythmias, syncope and sudden cardiac death.[59, 60] Over half the CPVT cases are caused by autosomal-dominant missense RyR2-mutations.[59, 61] Although over 150 different RyR2-mutations have been reported, none appear to occur within known phosphorylation-motifs.[62] Clinically, potentially-lethal arrhythmias occur predominantly following strenuous exercise or emotional stress, presumably in association with increased adrenergic activity.[59] At the cellular level, enhanced adrenergic activity activates PKA and/or CaMKII, leading to enhanced RyR2-phosphorylation.[58, 63] Most experimental studies agree that RyR2-phosphorylation can potentiate SR Ca^{2+}-leak, which is associated with ectopic activity and arrhythmias in CPVT. However, some issues remain controversial, as discussed below. We will focus only on the role of RyR2-phosphorylation in CPVT, and refer the reader to prior reviews about other aspects of CPVT including the potential role of reduced FKBP12.6-binding and domain-unzipping as mechanisms of RyR2-destabilization.[62, 64]

It is somewhat controversial whether CPVT-associated RyR2-mutations cause channel dysfunction in the absence of adrenergic stimulation and channel-phosphorylation.[64] Several studies have shown that RyR2-phosphorylation uncovers latent single-channel dysfunction of mutant RyR2.[65–67] Other studies have shown that only SR Ca2+ overload, without activation of PKA, can unmask the 'gain-of-function' phenotype of CPVT-mutant RyR2 variants.[68] However, other studies demonstrate a baseline gain-of-function,[69, 70] or even a rare loss-of-function phenotype of CPVT-mutant RyR2-channels.[71] These differences might result from different experimental approaches used or may reflect differences in phenotype-severity depending on the specific affected residue. For example, mutation V2475F appears to cause a particularly severe phenotype since homozygous knock-in mice are not viable,[72] unlike other mutant gain-of-function RyR2-strains.[27, 73–75] Interestingly, the neighboring mutation R2474S also causes a rather severe arrhythmia phenotype, compared to other RyR2-mutants (L433P, N2386I) in side-by-side studies.[75] Most studies show that the CPVT-linked mutations do not alter baseline RyR2-phosphorylation at S2808 and S2814.[67, 70, 74, 75] However, recombinant RyR2-channels with the V2475F-mutation exhibited significantly increased S2808- and S2030-phosphorylation levels at baseline, that were even more pronounced following exposure to the PKA-catalytic subunit.[72] However, the composition of recombinant RyR2 is likely different from native heart channels, so future experiments will need to confirm whether CPVT-mutations alter basal RyR2-phosphorylation levels.

Role of RyR2-phosphorylation in atrial arrhythmogenesis

Atrial fibrillation (AF) is partially characterized by Ca^{2+}-handling abnormalities.[76] Initial work showed that the incidence of spontaneous Ca^{2+}-release events (SCaEs, which include Ca^{2+}-sparks and Ca^{2+}-waves) is increased in cardiomyocytes from patients with chronic (persistent) AF (cAF), pointing primarily to RyR2-dysfunction.[77] Emerging evidence indicates that abnormal RyR2-phosphorylation indeed plays a prominent role in AF-pathogenesis. RyR2 is hyperphosphorylated at S2808 in both cAF-patients and dogs with sustained AF due to atrial tachycardia remodeling.[78] Increased S2808-phosphorylation of RyR2 in cAF-patients was surprising because cytosolic PP1 and PP2A activities are increased, which would be expected to reduce RyR2-phosphorylation levels.[79] Despite the larger SR Ca^{2+}-leak, SR Ca^{2+}-load is unaltered in cAF,[80] possibly due to increased phospholamban (PLB) phosphorylation.[79]

In dogs the increase in S2808-phosphorylation of RyR2 was associated with a stronger dissociation of FKBP12.6-subunit from RyR2-channels.[78] Mice lacking FKBP12.6 showed larger SR Ca^{2+}-leak and more SCaEs and triggered activity, along with an increased susceptibility to burst pacing-induced AF, validating the causal role of reduced FKBP12.6-levels for RyR2-dysfunction.[74, 81, 82] Similar results were obtained in mice with the E169K-mutation in JPH2, which reduced its interaction with RyR2, suggesting an important RyR2-stabilizing role also for JPH2.[83] Gain-of-function mutations in RyR2 predispose patients to CPVT and AF, and mice with these CPVT-mutations show RyR2-dysregulation and burst pacing-induced AF.[74, 75, 84] The RyR2-FKBP12.6 binding stabilizing compound S107 prevents AF-initiation in CPVT-mice, pointing to a role of FKBP12.6 for RyR2-dysfunction in CPVT-related AF.[75]

The individual contribution of S2808-phosphorylation to RyR2-dysregulation and SR Ca^{2+}-leak in cAF-patients is uncertain. While RyR2-hyperphosphorylation at S2808 appears a consistent finding in cAF-patients,[74, 78, 80, 85] pharmacological PKA-inhibition does not affect the increased RyR2-Po and SR Ca^{2+}-leak in atrial myocytes from cAF patients.[80] Although these data intuitively question an important role of S2808-phosphorylation, the apparent lack of S2808 contribution might be due to the atrial tachycardia-induced permanent increase in CaMKII-activity and S2814-hyperphosphorylation, which may mask the impact of S2808-phosphorylation for atrial function. In addition, the S2814-hyperphosphorylation might cause a stronger conformational change of RyR2 resulting in a larger increase in RyR2-Po. Extensive additional work is needed to address these interesting possibilities.

Several papers have demonstrated that increased CaMKII-dependent RyR2-phosphorylation at 2814 is the major cause of SR Ca^{2+}-leak and SCaEs in cAF-patients.[74, 80, 85, 86] CaMKII-activity may increase as a result of faster atrial rate, which promotes its auto-phosphorylation[10] or from oxidation of methionines 281/282, coupling AF-related oxidative stress to proarrhythmic Ca^{2+}-handling.[86] In addition, Thr35-hyperphosphorylation of I-1 in cAF is expected to reduce PP1-activity within the RyR2-complex,[79, 87] also increasing S2814-phosphorylation. In goats with sustained AF, CaMKII-dependent RyR2-phosphorylation is increased, likely causing SR Ca^{2+}-leak, potentially contributing to the reduced SR Ca^{2+}-load and decreased atrial contractility associated with AF.[88]

Several mouse models have validated the causal role of CaMKII-and S2814-mediated RyR2-dysfunction, increased SR Ca^{2+}-leak, and SCaEs in AF-initiation.[74, 80, 85, 86] Pharmacological CaMKII-inhibition and genetic inhibition of CaMKII-dependent S2814-phosphorylation prevent AF initiation in FKBP12.6 knock-out mice, further supporting a critical role for S2814-mediated RyR2-dysfunction in AF.[74, 82] Mice with cardiac-restricted overexpression of a repressor form of the cAMP-response element modulator (CREM) develop a complex cardiac phenotype including spontaneous-onset AF.[89, 90] CREM-Tg mice exhibit changed atrial structure and hypertrophy, along with altered conduction and Ca^{2+}-handling abnormalities including increased incidence of SCaEs and augmented SR Ca^{2+}-leak.[90] This mouse model supports a critical role for S2814-phosphorylation dependent RyR2-dysfunction in spontaneous AF.[90] CaMKII-dependent RyR2-hyperphosphorylation is likely an early event in atrial CREM-TG-mice dysfunction, since CREM-TG-mice crossed with RyR2-S2814A-mice resistant to CaMKII-dependent RyR2-hyperphosphorylation, are protected from spontaneous AF.[90] Overall, these studies support a major role for S2814-phosphorylation of RyR2 in atrial dysfunction and arrhythmogenesis in persistent AF.

Interestingly, patients with paroxysmal AF (pAF) do not exhibit changes in RyR2-phosphorylation at S2808 and S2814, despite increased SCaEs incidence and triggered activity.[91] The underlying molecular substrate involves increased SR Ca^{2+}-load and RyR2-dysregulation independent of RyR2-phosphorylation. The increased SR Ca^{2+}-load is due to PKA-dependent PLB-hyperphosphorylation, relieving PLB-inhibition of SERCA2a and increasing SR Ca^{2+}-uptake.[91] RyR2-dysregulation involves increased protein expression and higher RyR2-Po, resulting in larger likelihood and amplitude of SCaEs. A relative

deficiency of JPH2, resulting from increased RyR2 but unaltered JPH2-expression, might explain RyR2-dysfunction in pAF-patients.[83, 91]

Conclusions

Many studies have demonstrated that phosphorylation is an important mechanism by which RyR2-mediated SR Ca^{2+}-release is fine-tuned within cardiomyocytes.[5] Although our knowledge in this field is still evolving and certain concepts remain controversial due to disagreements among certain studies, it is evident that abnormal PKA- and particularly CaMKII-phosphorylation of RyR2 may contribute to the pathogenesis of HF, atrial and ventricular arrhythmias. The fact that not all studies agree is not uncommon in science, and actually helps to advance the field as a whole. It is important that there will be unrestricted exchange of reagents such as plasmids, antibodies, and knockin mouse models among labs in order to enable external confirmation of published results. As new scientific insights and technologies become available, some of the outstanding questions will undoubtedly be resolved. In the meantime, it is important that we all focus on the generation of new insights rather the perpetuation of (perceived) controversies, especially if our conclusions are based on imperfect experimental approaches. Finally, it needs to be emphasized that advancing our understanding of RyR2 regulation and dysfunction in the context of cardiac diseases is of the utmost importance, as RyR2 represents a unique and very promising therapeutic target for HF and arrhythmias.[92, 93]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

AF	Atrial fibrillation
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase-II
CPVT	Catecholaminergic polymorphic ventricular tachycardia
DCM	Dilated cardiomyopathy
HF	Heart failure
JPH2	Junctophilin-2

MI	Myocardial infarction
PKA	Protein kinase-A
PP1	Protein phosphatase type-1
PP2A	Protein phosphatase type-2A
RyR2	Ryanodine receptor type-2
CSQ2	Calsequestrin-2
SR	Sarcoplasmic-reticulum
TAC	Transverse-aortic constriction
VT	Ventricular tachycardia
WT	Wild-type

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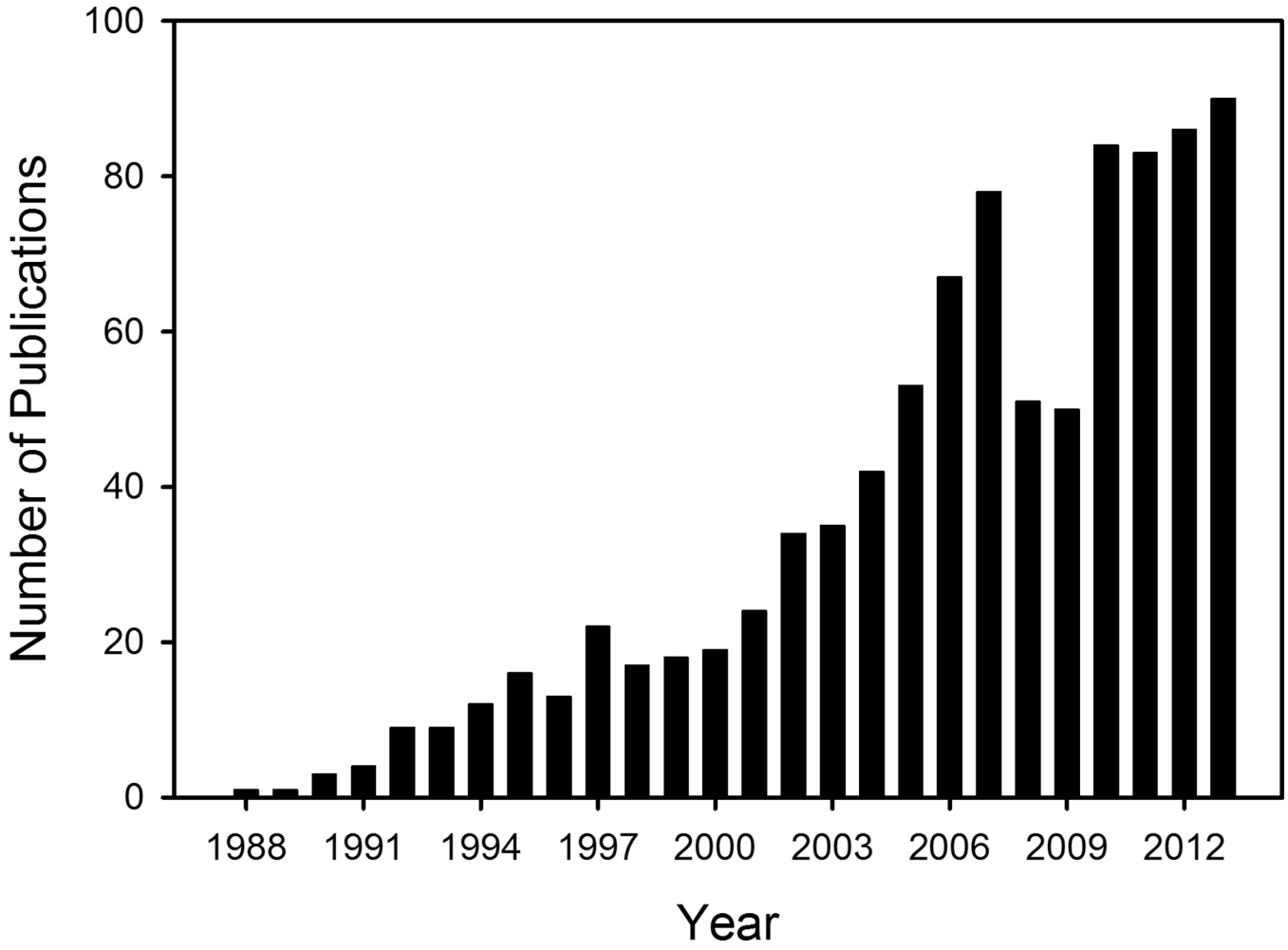

Figure 1.

Number of peer-reviewed papers published between 1988–2013, containing key words ‘ryanodine receptors’ and ‘phosphorylation’ in key bibliographic fields, according to Scopus.

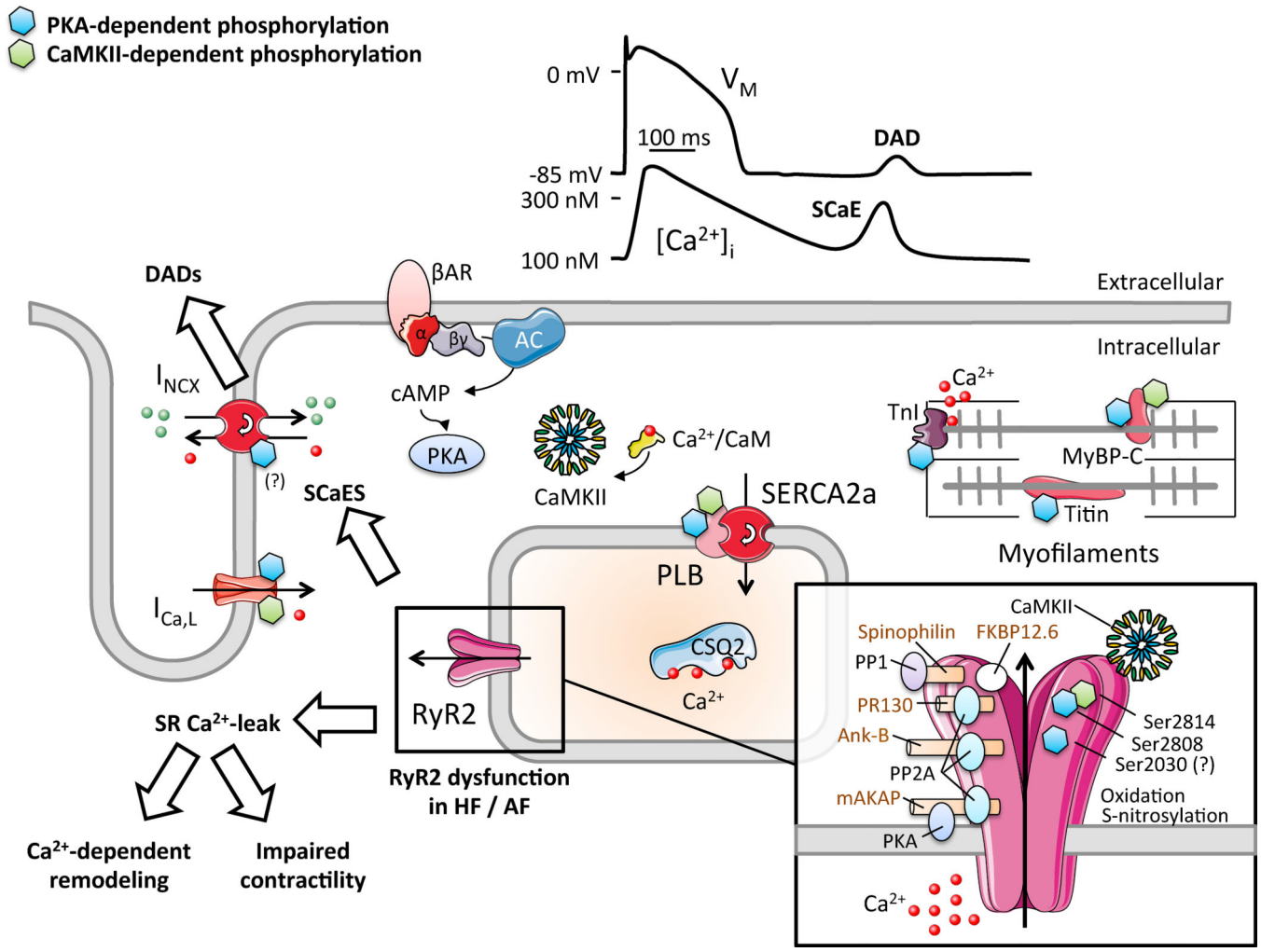

Figure 2.

Schematic representation of the key determinants of excitation-contraction coupling in cardiomyocytes. Physiologicallly, Ca^{2+}-entry through L-type Ca^{2+}-channels ($I_{Ca,L}$) triggers SR Ca^{2+}-release through RyR2. The systolic Ca^{2+}-transient activates myofilaments, initiating contraction. Diastolic relaxation occurs when Ca^{2+} is transported into SR via SERCA2a and out of the myocyte via NCX1. In heart failure (HF) and atrial fibrillation (AF), altered RyR2-phosphorylation increases SR Ca^{2+}-leak, promotes Ca^{2+}-dependent remodeling and impairs contractility. Spontaneous SR Ca^{2+}-release events (SCaEs) promote delayed afterdepolarizations (DADs) and triggered activity. Inset shows RyR2-macromolecular complex with accessory proteins, protein kinases and phosphatases (and their respective anchoring proteins) that control phosphorylation levels. Protein kinase-A (PKA) and Ca^{2+}-calmodulin-dependent protein kinase-II (CaMKII)-dependent phosphorylation sites are indicated with blue and green “P” symbols, respectively.

TABLE 1

Potential reasons for divergent experimental results and interpretations

Item	Variability	References
Animal models		
Species	Results might vary depending on species studied, i.e., RyR2 phosphorylation level at baseline or in heart failure	10, 14, 15, 38
Genetic background	Mouse strain might contribute to variable responses to β-adrenergic or pathological stress	15, 18, 20, 21, 40, 94, 95
Environmental conditions	Diet (chow) and lifestyle can affect SR Ca^{2+} handling	96–98
Circadian rhythm	Circadian rhythm can modulate pathological phenotypes as well as RyR2 properties itself	99, 100
Experimental models		
Animal model of disease	Involvement of specific RyR2 phosphorylation sites might depend on the type of experimental heart failure (i.e., LAD ligation vs. transverse aortic banding) and the time points at which phenotypes are evaluated	15, 18, 20, 21, 27, 28, 40, 54, 94, 95, 101, 102
Anesthesia / surgical techniques	Anesthesia type and surgical techniques might alter study outcomes	103, 104
Sample analysis methods	Results may dependent on analysis methods, i.e., Western Blotting vs. back-phosphorylation might reveal different results, and SR Ca^{2+} leak (tetracaine) protocol vs. Ca^{2+} spark measurements	10, 25, 105
Scale	Results might vary at the whole animal (*in vivo* vs. *ex vivo*), isolated cell (intact vs. permeabilized), and single channel level	74, 81, 86, 91, 106
Reagents		
Antibodies	Results might vary depending on antibody (i.e., epitope, purification, species). Antibodies are often not well characterized.	25, 27, 28, 32, 38, 74
Buffers, detergents, fluorophores	Buffer conditions, detergents (for solubilization of RyR2), fluorophores (for Ca^{2+} imaging) could affects results	107, 108
Redox levels	S- and Cys-nitrosylation, oxidation, tyrosine nitration levels can alter RyR phosphorylation or Ca^{2+} handling	24, 109
Data processing		
Data analysis procedures	Results might vary depending on data analysis procedures (i.e., quantification of western blot signals, processing of confocal Ca^{2+} imaging data)	110
Data quality	Proper use of positive and negative controls, etc.	
Fitting of data within conceptual models	Different groups might interpret the same data differentially based on the null hypothesis	15, 18