

## Posttranslational modifications of cardiac ryanodine receptors: Ca<sup>2+</sup> signaling and EC-coupling

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## **Abstract**

In cardiac muscle, a number of posttranslational protein modifications can alter the function of the  $\text{Ca}^{2+}$  release channel of the sarcoplasmic reticulum (SR), also known as the ryanodine receptor (RyR). During every heartbeat RyRs are activated by the  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release mechanism and contribute a large fraction of the  $\text{Ca}^{2+}$  required for contraction. Some of the posttranslational modifications of the RyR are known to affect its gating and  $\text{Ca}^{2+}$  sensitivity. Presently, research in a number of laboratories is focussed on RyR phosphorylation, both by PKA and CaMKII, or on RyR modifications caused by reactive oxygen and nitrogen species (ROS / RNS). Both classes of posttranslational modifications are thought to play important roles in the physiological regulation of channel activity, but are also known to provoke abnormal alterations during various diseases. Only recently it was realized that several types of posttranslational modifications are tightly connected and form synergistic (or antagonistic) feed-back loops resulting in additive and potentially detrimental downstream effects. This review summarizes recent findings on such posttranslational modifications, attempts to bridge molecular with cellular findings, and opens a perspective for future work trying to understand the ramifications of crosstalk in these multiple signaling pathways. Clarifying these complex interactions will be important in the development of novel therapeutic approaches, since this may form the foundation for the implementation of multi-pronged treatment regimes in the future.

## **Keywords**

cardiac muscle; ryanodine receptor; calcium signaling; oxidation; nitrosylation; cardiomyopathy; heart failure

## 1. Introduction

In cardiac muscle, ryanodine receptors (RyRs) serve as  $\text{Ca}^{2+}$  release channels of the intracellular  $\text{Ca}^{2+}$  store, the sarcoplasmic reticulum (SR). Thereby, they provide a large fraction of the  $\text{Ca}^{2+}$  required to initiate muscle contraction from beat to beat. They are normally activated by a small amount of  $\text{Ca}^{2+}$  entering into cardiac muscle cells from the extracellular space, via voltage-dependent  $\text{Ca}^{2+}$  channels. This  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release (CICR) from the SR is the mechanism which amplifies the  $\text{Ca}^{2+}$  signal and governs excitation-contraction (EC) coupling by activation of RyRs (for review see [1]).

Research on the RyR, both on its structure and function, has been carried out over the last decades using multiple experimental approaches and techniques to overcome the difficulty of examining a channel that is located intracellularly and therefore not easily accessible. This includes assays using isolated SR vesicles (e.g. [2,3]), single RyR channels reconstituted into lipid bilayers (e.g. [4-9]), permeabilized cardiomyocytes [10,11], but also various biochemical techniques (e.g. see [12,13]). Cellular ultrastructural and co-localization information has been obtained with immunocytochemistry and electron tomography [14,15] and structure on the molecular level has been assessed with cryo-electron microscopy [16,17].

Many of these studies have confirmed the potential of RyRs to undergo several of the numerous known posttranslational modifications and a number of reports have provided evidence for functional consequences resulting from some of these modifications. These data were frequently obtained in very artificial experimental systems and under conditions far away from the natural environment of the RyRs. Therefore, it often remained unclear whether and how these observations on or near the molecular level would translate into intact and living cardiomyocytes and into the entire organ or organism [18].

Some time ago it became practical to closely examine RyR function *in-situ* and within its native environment, which means inside living cells. This has become possible because of groundbreaking developments of technologies to faithfully image subcellular and microdomain  $\text{Ca}^{2+}$  signals with appropriate spatial and temporal resolution. These developments were significantly driven by the chemical synthesis of bright and kinetically fast fluorescent  $\text{Ca}^{2+}$  indicators [19,20] and the simultaneous advancements of laser-scanning confocal microscopy combined with digital image acquisition and processing [21].

Since several excellent reviews cover many aspects of RyR posttranslational modifications on the biochemical and molecular level [22-30], here we will concentrate on recent findings that have been obtained by examining RyR activity and cardiac  $\text{Ca}^{2+}$  signaling on the cellular level, where the channels can be examined in their native environment. In particular, we will focus on the

consequences of a combined impact of several mechanisms and their mutual interactions during physiological regulation of RyRs and during the development of cardiac diseases affecting RyR function.

## 2. The ryanodine receptor

### 2.1. *The RyR macromolecular complex*

In mammals three RyR isoforms are known: the skeletal muscle form RyR1, the cardiac RyR2 and the more broadly expressed brain form RyR3. The cardiac RyR2 is a large macromolecular complex consisting of a homo-tetramer with 4 subunits comprising a molecular mass of 565 kDa each, totaling 2.2 MDa (for review see [31]). This complex is regulated and modulated in numerous ways by ions (e.g.  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{H}^+$ ), by small molecules (e.g. ATP, cADPR) and by proteins (e.g. sorcin, calstabin2, junctin, triadin). Important for this review, the macromolecular complex is also associated with protein kinases (e.g. protein kinase A (PKA),  $\text{Ca}^{2+}$ /caldmodulin dependent kinase II (CaMKII)) and phosphatases (e.g. phosphatase 1 and 2A). Some of the kinases and phosphatases are tethered to the channel and held near their target sites by means of anchoring proteins. On the RyR itself, several phosphorylation sites have been identified (see chapter 3). Furthermore, the RyR complex comprises several free cysteines that can be subject to reversible oxidative modification (see chapter 4).

### 2.2. *The $\text{Ca}^{2+}$ signaling microdomain in the vicinity of the RyRs*

In cardiac muscle, a large fraction of the RyRs are organized in dyads, where the SR membrane contains a cluster of 30-250 RyRs [32] and comes in close contact (gap of ~15 nm) with the T-tubular membrane, which harbors the voltage-dependent L-type  $\text{Ca}^{2+}$  channels. Opening of one or more L-type channels can activate CICR via several RyRs within a cluster. The tiny SR  $\text{Ca}^{2+}$  release generated by these few opening channels gives rise to a  $\text{Ca}^{2+}$  spark, an elementary  $\text{Ca}^{2+}$  signaling event, which can be detected and analyzed using confocal imaging of  $\text{Ca}^{2+}$  sensitive fluorescence indicators (for reviews see [33,34]). During each heart beat, a large number of  $\text{Ca}^{2+}$  sparks is activated simultaneously, summing up to form the cardiac  $\text{Ca}^{2+}$  transient for the activation of contraction.  $\text{Ca}^{2+}$  sparks and even smaller  $\text{Ca}^{2+}$  release events,  $\text{Ca}^{2+}$  quarks, can also occur spontaneously, for example during diastole [35,36]. Spontaneous  $\text{Ca}^{2+}$  sparks and  $\text{Ca}^{2+}$  quarks are considered to occur accidentally and underlie the SR  $\text{Ca}^{2+}$  leak, but they usually cause no harm. However, under conditions of SR  $\text{Ca}^{2+}$  overload and in circumstances which sensitize the RyRs, single  $\text{Ca}^{2+}$  sparks can initiate larger  $\text{Ca}^{2+}$  signals, such as  $\text{Ca}^{2+}$  waves, traveling along the myocytes in a saltatory fashion from sarcomere to sarcomere [37-40]. These  $\text{Ca}^{2+}$  waves have a substantial arrhythmogenic potential, since they are able to initiate  $\text{Ca}^{2+}$  activated currents, such as

the  $\text{Na}^+ \text{-Ca}^{2+}$  exchange current ( $I_{\text{NCX}}$ ), which in turn may depolarize the cardiomyocyte and even trigger premature action potentials.

### 2.3. $\text{Ca}^{2+}$ dependent activation and inactivation of the RyRs

The open probability of RyRs depends steeply on the cytosolic  $\text{Ca}^{2+}$  concentration. The increase of the RyR open probability subsequent to openings of L-type  $\text{Ca}^{2+}$  channels and entry of  $\text{Ca}^{2+}$  into the dyadic cleft is the main mechanism for activation of CICR during physiological activity. The  $\text{Ca}^{2+}$  sensitivity of the RyRs *in-situ* is low enough to ensure independent activation of adjacent  $\text{Ca}^{2+}$  spark sites, to allow for the regulation of cardiac  $\text{Ca}^{2+}$  signals by virtue of local control and recruitment of  $\text{Ca}^{2+}$  sparks. The  $\text{Ca}^{2+}$  sensitivity of the channels for this type of activation is known to depend on several modulators as mentioned above (e.g. pH,  $\text{Mg}^{2+}$ , sorcin etc.), but also on several regulatory or disease-associated posttranslational protein modifications (see chapters 3 and 4). Inactivation of the RyRs and termination of the  $\text{Ca}^{2+}$  sparks *in-situ* is less well understood and is the focus of significant ongoing research efforts. The presently favored mechanism is based on regulation of the RyR by the  $\text{Ca}^{2+}$  concentration inside the SR. Thereby, lowering the SR  $\text{Ca}^{2+}$  concentration during a spark would make the RyRs insensitive for  $\text{Ca}^{2+}$  on the cytosolic side of the channels, which causes their deactivation. This is proposed to occur via a retrograde signal mediated by allosteric interactions between calsequestrin (acting as the  $\text{Ca}^{2+}$  sensor) and junctin and/or triadin and the RyR [2,8,41]. This mode of spark termination could be stabilized by a reinforcing mechanism that has been proposed recently based on model predictions. The local SR depletion and subsequent decay in  $\text{Ca}^{2+}$  release flux from the SR during a  $\text{Ca}^{2+}$  spark may contribute to the self-termination, because of the resulting decline of the dyadic  $\text{Ca}^{2+}$  concentration [42]. Other proposed mechanisms for spark termination include  $\text{Ca}^{2+}$  dependent inactivation of the RyRs [43], but this alternative seems to be of rather minor impact since up to  $100 \mu\text{M}$  cytosolic  $\text{Ca}^{2+}$  no RyR inactivation was observed in permeabilized cardiomyocytes [44]. Another possibility is stochastic attrition, where the probabilistic simultaneous closure of all RyRs in one cluster would interrupt their mutual activation by CICR within the dyadic cleft [45]. When all channels close, the very high dyadic  $\text{Ca}^{2+}$  concentration drops to low cytosolic levels within a few milliseconds [46]. However, the probability of all channels to be closed simultaneously is very low given the known number of RyRs in a cluster, unless their gating is partly coupled [47].

The  $\text{Ca}^{2+}$  release termination mechanism mediated by lowering of the SR luminal  $\text{Ca}^{2+}$  concentration and deactivation of the RyRs could also be important under conditions of SR  $\text{Ca}^{2+}$  overload, the opposite of the depletion during a  $\text{Ca}^{2+}$  spark and CICR. By sensitizing the RyRs for  $\text{Ca}^{2+}$  on the cytosolic side, elevations of intra SR  $\text{Ca}^{2+}$  could initiate or facilitate store-overload induced  $\text{Ca}^{2+}$  release (SOICR) and arrhythmogenic  $\text{Ca}^{2+}$  waves [40,48].

## 2.4. RyR pharmacology

Several pharmacological compounds change the function of the channel (for review see [49]). Here we only briefly summarize some pharmacological approaches which have been used in studies of posttranslational RyR modifications. Experimentally, a millimolar concentration of caffeine is often applied as an activator, for example to rapidly empty the SR of  $\text{Ca}^{2+}$  and to estimate SR  $\text{Ca}^{2+}$  content. Tetracaine is utilized as a blocker of the RyRs, for example to eliminate the diastolic SR  $\text{Ca}^{2+}$  leak and estimate its magnitude from the resulting drop of the cytosolic  $\text{Ca}^{2+}$  concentration [50]. Ryanodine in low concentrations ( $\text{nM}$  to  $\mu\text{M}$ ) locks the channel in a very long-lasting subconductance state, thereby depleting the SR of  $\text{Ca}^{2+}$  and eliminating the SR buffer function. For this purpose, it is often combined with thapsigargin, an inhibitor of the SR  $\text{Ca}^{2+}$  pump (SERCA). At high concentrations ryanodine may block the RyR channel. Recently, few drugs already accepted as pharmacotherapeutics in humans have been discovered to have beneficial off-target effects on the RyRs. These drugs are presently considered for re-purposing, other compounds targeting the RyR macromolecular complex are under development [51,52].

Pharmacological tools are also available to alter mechanisms of posttranslational modifications. This includes activators and inhibitors for protein kinases [53], phosphatases and NO synthases [54]. Donors and scavengers for NO, reactive nitrogen and oxygen species (RNS and ROS) have been applied with some success (e.g. [55]. In general, the specificity of these tools is quite limited, with the exception of inhibitory peptides for some of the protein kinases (e.g. PKI for PKA, AIP for CaMKII).

## 3. RyR phosphorylation

As mentioned above, the mechanism and functional consequences of RyR phosphorylation has attracted much recent attention. Interest in this issue was inspired by a report suggesting that PKA dependent “hyperphosphorylation” of RyRs could occur during heart failure (HF) thereby aggravating this condition. Hyperphosphorylation was proposed to promote the  $\text{Ca}^{2+}$  sensitivity of RyRs resulting in elevated open probability. This in turn would cause a substantial diastolic SR  $\text{Ca}^{2+}$  leak, which could contribute to low SR  $\text{Ca}^{2+}$  content, smaller  $\text{Ca}^{2+}$  transients and hence weak heart beat [12]. Using mainly biochemical and molecular biology approaches, serine 2808 and 2030 on the RyR have been identified as possible phosphorylation sites for protein kinase A (PKA), and serine 2814 for CaMKII. However, the specificity of these sites for the mentioned kinases remains a disputed issue [12,56-58] and additional sites are likely to exist [59]. Moreover, a fierce controversy revolves around the functional consequence and pathophysiological relevance of the phosphorylation at these sites [60,61]. This debate may result from differences in experimental approaches, methods and tools, but also from variations of the particular animal and disease models.

### *3.1. Phosphorylation by CaMKII*

The pictures which emerges from the literature seems to be more clear for the consequences of CaMKII activation which leads to phosphorylation of serine 2814 on the RyR and possibly other sites [59,61], among many collateral targets. CaMKII activity seems to produce quite consistent functional changes of the RyRs that are reconcilable with the general prediction over a wide range of experimental settings and approaches, extending from single channel experiments to cellular  $\text{Ca}^{2+}$  signaling and a variety of transgenic animals. In single channel experiments the open probability of the RyRs was generally found to be increased upon phosphorylation by CaMKII [62] (but see [63]). In isolated cardiomyocytes activation of CaMKII was associated with an increase of the  $\text{Ca}^{2+}$  spark frequency [64]. Transgenic mice overexpressing the cardiac isoform of CaMKII also showed elevated  $\text{Ca}^{2+}$  spark frequencies, leading to pronounced SR  $\text{Ca}^{2+}$  leak, lower SR  $\text{Ca}^{2+}$  content and a susceptibility for arrhythmias [65,66]. Ablation of CaMKII resulted in a protection of the animals from cardiac hypertrophy, possibly mediated by the unavailability of CaMKII signaling in the pathways of excitation-transcription coupling [67,68]. To obtain further insight into the functional role of serine 2814 on the RyR several mouse models were engineered to specifically scrutinize this site. In one animal the serine 2814 was replaced by an alanine, which removes its capability to become phosphorylated by CaMKII (S2814A mouse). Hearts of these animals and cardiomyocytes isolated from them showed blunted force-frequency relationships [69] and the mice were protected from arrhythmias induced by tachypacing after being subjected to transverse aortic constriction (TAC) to induce hypertrophy and failure [70]. Conversely, the S2814D RyR, where serine is replaced by aspartic acid, mimics constitutive CaMKII dependent RyR phosphorylation and increases the open probability of the channels in bilayer experiments. These cells showed elevated  $\text{Ca}^{2+}$  spark frequencies that could not be further increased by CaMKII activation [70] and the mice developed a propensity for arrhythmias and sudden cardiac death when stressed with catecholaminergic challenges or tachypacing subsequent to TAC.

Taken together, these and numerous other studies draw a picture whereby in the short-term CaMKII dependent phosphorylation substantially modifies RyR function, cardiac  $\text{Ca}^{2+}$  signaling and EC-coupling. Overall, these signaling systems seem to become boosted, more active and  $\text{Ca}^{2+}$  sensitive but less well controlled, from the molecular to the cellular and organ level. Thus, CaMKII has been considered as a treatment target for multiple short term and long-term cardiac conditions that are associated with disturbances of  $\text{Ca}^{2+}$  signaling and CaMKII activation [71-74].

### *3.1. Phosphorylation by PKA*

PKA dependent phosphorylation and “hyperphosphorylation” of the RyRs at serine 2808 has been proposed to dissociate the stabilizing protein calstabin 2 (a.k.a FKPB-12.6) from the macromolecular complex, a sequence of events that is suggested to be followed by major functional changes of the channels resulting in diastolic  $\text{Ca}^{2+}$  leak, SR  $\text{Ca}^{2+}$  depletion and weak heart beat [12]. Obviously, this mechanism could be very important both for the physiological regulation of the channels during stress as well as for their pathophysiological malfunctioning. Therefore, it has attracted substantial research efforts from several laboratories. While in general phosphorylation of the S2808 site has been confirmed by various labs, specificity for PKA of this site, the conditions under which phosphorylation would occur and whether or not this leads to calstabin 2 dissociation have remained equivocal [56]. An additional PKA site has been identified at serine 2030 [57,75]. On the single channel level, functional changes after PKA-dependent phosphorylation have been described some time ago [76,77]. On the cellular level, the consequences of PKA-dependent RyR phosphorylation have been more difficult to pinpoint, partly because of the complex adjustments of multiple signaling networks downstream the activation of PKA in intact or permeabilized cells. Changes of  $\text{Ca}^{2+}$  spark parameters indeed were observed upon application of cAMP in permeabilized mouse cardiomyocytes, but were entirely attributable to the concomitant SERCA stimulation resulting from PLB phosphorylation, as they were not present in cells isolated from PLB ablated mice, where SERCA is already maximally stimulated [11]. Two-photon photolysis of caged  $\text{Ca}^{2+}$  to artificially trigger  $\text{Ca}^{2+}$  sparks suggested changes of RyR gating after  $\beta$ -adrenergic stimulation, since in resting Guinea pig myocytes larger  $\text{Ca}^{2+}$  release events were observed despite a decline of SR content [78]. However, when analyzing the frequency of spontaneous  $\text{Ca}^{2+}$  sparks at rest, this was later found to most likely depend on CaMKII activation [79].

Because of these difficulties to dissect the consequences of  $\beta$ -adrenergic stimulation on RyR function, transgenic animals have been engineered specifically targeting the serine 2808 site. Several animal models have been created where this serine is replaced by alanine, resulting in S2808A channels which can no longer be phosphorylated at this site [58]. Another model are the S2808D mice, which have a modification which corresponds to constitutively phosphorylated RyRs. Unfortunately, the generation of these animals has not fulfilled the expectation to clarify the open issues, as the results published in several reports have again been controversial. Initial studies with the S2808A mice showed that the modification was very subtle, did not disturb normal cardiac function and the animals had no overt phenotype. However, after myocardial infarction (MI) these mice were protected from developing heart failure and from arrhythmias induced by phosphodiesterase inhibition [58]. Reconstituted S2808A channels did not show elevated open probability after MI, in contrast to those from WT mice. This difference on the molecular level was proposed to be the underlying mechanism preventing SR  $\text{Ca}^{2+}$  leak, weak heartbeat and the susceptibility to arrhythmias in S2808A mice.

In a different laboratory, a further S2808A mouse was engineered and these animals were subjected to a pressure overload heart failure model after TAC [80]. In this study, no obvious cardioprotection was conferred to the animals by ablating the 2808 phosphorylation site. Furthermore, no differences between WT and S2808A RyRs were present in the open probability and gating kinetics of reconstituted channels. This study then examined  $\text{Ca}^{2+}$  signaling and EC-coupling on the cellular level, including an analysis of  $\text{Ca}^{2+}$  sparks and waves. Again, no significant differences were observed between the two groups of animals. These observations led the authors to conclude that the serine 2808 site only has a limited role in the pathogenesis of heart failure.

At present it remains unsettled why these apparently similar studies led to essentially opposite conclusions. One may speculate that the disease models (i.g. pressure overload versus ischemia / inflammatory disease) could result in different outcomes, or that the RyRs of the two engineered animals do not operate in a perfectly identical way. Alternatively, some of the resulting functional modifications may be rather subtle, and can be compensated by auto-regulatory features of the cardiac EC-coupling machinery [81] and are therefore difficult to detect.

Starting from the latter possibility, a detailed study was carried out to examine SR  $\text{Ca}^{2+}$  release kinetics, their intersarcomeric synchronization, and the improvement of this parameter by  $\beta$ -adrenergic stimulation when the communication between L-type  $\text{Ca}^{2+}$  channels and RyRs was challenged [82]. The reasoning for this approach was the notion that these events occur at the very interface between the L-type  $\text{Ca}^{2+}$  channels and the RyRs and might therefore reveal even subtle changes. When this communication was tested by using very small  $\text{Ca}^{2+}$  currents as triggers, substantial intersarcomeric desynchronization was observed. This was resynchronized upon  $\beta$ -adrenergic stimulation in the WT [83] but not in the S2808A cells. Furthermore, unlike WT cells,  $\text{Ca}^{2+}$  wave propagation was not accelerated upon  $\beta$ -adrenergic stimulation in S2808A cells. Together with the long delays observed in the release synchronization, this suggested the possibility of an intra-SR mechanism [40,48], whereby SR  $\text{Ca}^{2+}$  loading via SERCA would lead to sensitization of the RyR from the luminal side, thereby pushing the channel over the trigger threshold. The possibility of an intra-SR mechanism was then confirmed in reconstituted single RyR channels. At high SR  $\text{Ca}^{2+}$  concentrations, and only under this condition, WT channels indeed responded with a significantly larger increase in open probability upon PKA dependent phosphorylation than S2808A channels. Regarding the ongoing controversy, the main conclusion from these studies is that the effects of serine 2808 phosphorylation are present but delicate and may be difficult to detect when SR  $\text{Ca}^{2+}$  content is not controlled experimentally (e.g. *in-vivo*, when the auto-regulatory adjustments of SR  $\text{Ca}^{2+}$  content mentioned above may compensate for small changes of RyR open probability).

Taken together it appears that the mechanisms and consequences of PKA dependent RyR phosphorylation are less clear and potentially more subtle than those mediated by CaMKII. Whether and how these delicate changes translate into the *in-vivo* situation is difficult to

extrapolate and will require more research. In support for this expectation, a recent cross-breeding experiment between dystrophic *mdx* and S2808A mice indicated that the RyR mutation confers significant protection for cardiac disease manifestations and progression of the dystrophic cardiomyopathy in these animals (see below) [84].

#### 4. Redox modification of RyR.

##### 4.1. RyR oxidation by ROS

Changes of the cellular redox state give rise to another category of posttranslational RyR modifications, which do not only have a modulatory function but also play an important role in the development of various cardiac diseases. The term “intracellular redox potential” broadly describes the balance between reduced and oxidized proteins within cells, which in turn is determined by the level of generation and buffering of cellular reactive oxygen and reactive nitrogen species (ROS/RNS). There are multiple sources of ROS/RNS within the cell. They include but are not limited to NADPH oxidase (NOX), xanthine oxidase (XO), mitochondria and nitric oxide synthase (NOS). On the other end there are various cellular antioxidant defense components such as catalase, superoxide dismutase, thio- and glutaredoxins, glutathione peroxidase, glutathione, vitamins A, C and E, etc. Under physiological conditions, the extent of ROS/RNS accumulation is finely controlled by these scavenging and reducing mechanisms, and at low concentrations ROS/RNS serve as important intracellular messengers. An imbalance between generation of ROS/RNS and the efficiency of cellular defense systems can lead to a transient or persistent oxidative/nitrosative stress resulting in redox modifications of various cellular proteins, including those involved in  $\text{Ca}^{2+}$  homeostasis. The RyR is an important example, since it is known to be very susceptible to redox modifications. Each cardiac RyR tetramer contains a total of 364 cysteines [85]. In the presence of a physiological concentration of one of the major cellular “redox buffers” glutathione (5 mM) about 84 of these cysteines are free. The sulfhydryl groups of these cysteines are subject to reversible cross-linking, S-nitrosylation and S-glutathionylation. Numerous studies of RyRs incorporated in lipid bilayers convincingly showed that reversible redox modifications significantly affect the activity of RyR channels. Oxidative conditions generally increase the RyR open probability, while reducing agents do the opposite (e.g. [85-88]). Therefore, the functional consequence of a moderate cellular oxidative/nitrosative stress could be immediate enhancement of  $\text{Ca}^{2+}$  release from the SR in response to a given physiological trigger. This possibility has been supported by experiments with isolated SR vesicles (e.g. [85,89]). The increased  $\text{Ca}^{2+}$  sensitivity of RyRs and subsequently larger  $\text{Ca}^{2+}$  transients could have a positive inotropic effect on the cardiac function [90]. However, severe oxidative stress can cause irreversible and sustained activation of RyRs [85], increased  $\text{Ca}^{2+}$  leak from the SR, decreased SR  $\text{Ca}^{2+}$  load and finally a decline of beat-

to-beat cellular  $\text{Ca}^{2+}$  transients with contractile dysfunction. Such conditions are usually associated with or even caused by the development of various cardiac abnormalities. Therefore, the role of RyR redox modifications in cardiac pathophysiology is currently under intensive investigation in multiple laboratories around the world.

When the experimental gear was shifted from molecular and vesicles towards studies of cells, organs and organisms, it became obvious that the findings obtained from isolated RyR channels cannot be translated to more complex biological systems without a critical reevaluation. Besides the presence of various cellular sources for ROS, redox modification targets multiple intracellular sites including major proteins involved in EC coupling and all of them need to be considered in order to identify the link between each modification and the resulting changes of RyR function [91]. To discriminate between correlative, adaptive and causal posttranslational RyR modifications is often a daunting task.

Cardiac muscle has a substantial NOX activity (for reviews see [92,93]). It has been reported that NOX2 is the predominant isoform expressed in T-tubular and SR membranes of mature cardiomyocytes. Therefore, it is strategically positioned to modulate the activity of the RyRs. NOX is an enzyme that utilizes NADPH to produce superoxide anion. NOX2 was found to be overexpressed and/or its activity increased in dystrophic hearts [55,94], in hearts of patients with a history of atrial fibrillation [95], and in hearts subjected to tachycardic preconditioning [96]. Although the exact mechanisms of NOX activation under these pathological conditions remain unclear, it was shown that ROS produced by NOX stimulates SR  $\text{Ca}^{2+}$  release via at least two pathways: 1) direct oxidation or S-glutathionylation of RyRs or 2) indirectly through CaMKII activation [97] followed by phosphorylation of the RyRs. Reducing or ROS scavenging compounds could generally mitigate or prevent the consequences of oxidative stress in these experimental models. Another widely recognized source of ROS production in cardiac myocytes are mitochondria [98,99]. Mitochondria always generate a small amount of ROS through leakage in the electron transport chain during respiration. Under some pathophysiological conditions, such as ischemia/reperfusion, ROS produced by mitochondria become the main contributors to cellular oxidative stress. In this situation mitochondrial  $\text{Ca}^{2+}$  overload and subsequently ROS overproduction may trigger mitochondrial permeability transition, which in turn boosts ROS production via ROS-induced ROS release mechanisms [100,101]. There are also several reports indicating upregulation of XO activity in experimental models of heart failure [102]. Furthermore, contractile function and myocardial efficiency in HF could be improved by the treatment the animals with the xanthine oxidase inhibitor allopurinol [103,104]. Overall, regardless of the source of their generation, ROS and subsequent oxidative modifications of RyRs have been directly held accountable for augmented stretch-induced  $\text{Ca}^{2+}$  responses and hypersensitive EC-coupling in dystrophic cardiomyocytes [55,105-108] as well as in impaired  $\text{Ca}^{2+}$  signaling in failing [109,110] and diabetic hearts [111,112].

#### *4.2. RyR modifications by RNS*

The two major isoforms of NO synthase (NOS) in cardiac myocytes are eNOS and nNOS. They have a specific sub-cellular localization and are possibly aimed at different targets in their microdomains, due to the short range of NO diffusion. The eNOS isoform is localized in the plasma membrane in caveolae through interaction with caveolin-3. In healthy cardiac muscle nNOS is mainly located in the SR membrane, linked to the RyRs. In failing or diseased hearts nNOS may partly redistribute to the sarcolemma. Normally, the iNOS isoform is not present in significant amounts, but this may be different during the development of cardiac diseases. NO produced by these enzymes can bind to free thiol groups on various proteins, including RyR, causing S-nitrosylation and conformational changes. Alternatively, NO can act via the cGMP dependent pathway and activated PKG, a protein kinase which is thought to phosphorlyate the RyR at the S2814 CaMKII site [59]. This and the presence of many other targets for NO and PKG, most notably the L-type  $\text{Ca}^{2+}$  channels, make it very challenging to attribute any functional change to a specific RyR posttranslational modification in the setting of experiments with intact cells.

An important role for RyR nitrosylation in cardiac EC-coupling and  $\text{Ca}^{2+}$  signaling was suspected already some time ago (for review see [90]), when it was found that the stretch-induced enhancement of cardiac  $\text{Ca}^{2+}$  signals and elevation of  $\text{Ca}^{2+}$  spark frequency was blunted in the presence of L-NAME, an unspecific inhibitor of all NOS isoforms [113]. The effect of stretch could be mimicked by adding the NO donor SNAP, which nearly doubled the  $\text{Ca}^{2+}$  spark frequency. Additional studies reported that NO could have diverse actions, depending on the preexisting extent of  $\beta$ -adrenergic stimulation. NO donors increased the  $\text{Ca}^{2+}$  spark frequency in a cGMP independent way at low (10 nM) concentrations of ISO, presumably by RyR nitrosylation [114]. At 1  $\mu\text{M}$  ISO a decrease of the spark frequency was observed, however this was accompanied (or caused) by a reduction of the SR  $\text{Ca}^{2+}$  content. In nNOS-/ mice, but not in eNOS-/ mice, hyponitrosylation of the RyRs was observed, confirming that the structural proximity between nNOS and RyR is functionally relevant. Interestingly, these RyRs exhibited more extensive oxidative modifications, leading to elevated SR  $\text{Ca}^{2+}$  leak [115]. Thus, constitutive RyR S-nitrosylation in WT animals appears to confer some protection of the channels against more severe oxidative modifications. This may be important in various diseases, where changes of the nitrosylation have been implied in their pathology, but also in conferring some cardioprotection [116].

In one study the extent of RyR nitrosylation was quantified in mice with dystrophic cardiomyopathy and found to be increased around 4-5 fold [117], while PKA dependent RyR phosphorylation was not significantly elevated. This was accompanied by a doubling of the frequency of spontaneous  $\text{Ca}^{2+}$  sparks and a propensity for arrhythmias. The extent of RyR oxidation and CaMKII-dependent

RyR phosphorylation was not assessed directly, but the protective effect of N-acetyl cysteine (NAC) suggests an important role of oxidative stress in dystrophic cardiomyopathy, as reported earlier ([55,94]).

The general concept which emerges from these partly controversial studies, although rather diffuse, suggests that the reciprocal interactions between RyR modifications resulting from ROS and RNS and their functional outcome are very complex and not yet fully understood. While some observations suggest quite synergistic actions, in other experimental settings more competitive effects between ROS and RNS modifications become apparent. Interactions between ROS and RNS are possible in various ways, for example through their tightly connected chemistries (e.g. superoxide and NO can combine to form peroxynitrite [118] or by competing for the same thiols on the RyR. A further complication in the interpretation of the findings may arise from the finding that RyR2 is nitrosylated via S-nitrosoglutathione (GSNO) and not by NO directly [88]. Further, most experiments were carried out at ambient oxygen pressure (~150 mmHg), but in the tissue there is much less oxygen (~10 mmHg). The degree of oxidation and the function of the cardiac RyR is modified by ambient O<sub>2</sub>. In any case, it seems that the precise balance between ROS and RNS is important, and that a NO/ROS disequilibrium can lead to abnormal RyR channel behavior [119].

## 5. Cross-talk between redox modifications and phosphorylation in disease

Recently, a number of studies have been carried out in a variety of cardiac disease models, focusing on modifications of RyR function and the conceivably underlying posttranslational modifications. A common finding in many of these studies was a sequential (i.e. during disease development) or simultaneous presence of several posttranslational RyR modifications. While such a pattern could result from parallel but unrelated changes of the involved pathways, it seems more plausible that these modifications are not independent from each other. There are numerous possibilities for significant cross-talk and synergisms among these signaling pathways, for example between ROS, phosphorylation and Ca<sup>2+</sup> signals (for reviews see [120,121]. In one scenario, boosting the Ca<sup>2+</sup> transient by phosphorylating various Ca<sup>2+</sup> signaling proteins may elevate mitochondrial Ca<sup>2+</sup> content, followed by an increased mitochondrial metabolism and ROS production [98]. Mitochondrial ROS can further augment the Ca<sup>2+</sup> signals by oxidizing multiple Ca<sup>2+</sup> signaling proteins, as described above, but also by activating CaMKII via redox modification. This occurs in addition to the stimulation by the larger Ca<sup>2+</sup> transients themselves [97], thereby establishing multiple and coupled positive feed-back loops [122]. A multitude of cardiac diseases are accompanied by oxidative stress which can initiate similar vicious cycles, just starting from another pathomechanism. Examples are congestive heart failure [109], dystrophic cardiomyopathy [55], diabetes [112] and ischemia/reperfusion [123]. Since phosphorylation, as well as oxidation and elevated Ca<sup>2+</sup> concentration will increase the RyR open probability, the CICR mechanism will

become very sensitive and unstable. To what extent these multiple changes and the concomitant posttranslational RyR modifications exert additive effects and whether they develop rapidly or slowly during the development of a disease is not yet established and needs to be investigated in the future. However, a number of recent studies are in line with this possibility, which would be relevant for future therapeutic strategies. In the presence of synergistic pathomechanisms, multi-pronged therapies could be designed to target several of these vicious cycles simultaneously.

Interactions between RyR oxidation and phosphorylation have been studied in dystrophic cardiomyopathy, a disease that combines a high degree of oxidative stress and excessive  $\text{Ca}^{2+}$  signals after mechanical stress, resulting from the lack of the protein dystrophin [55]. In one example, a cross-breeding approach has been applied to test for rescue from this disease by eliminating not the main pathomechanism, but another step in the vicious cycle [84]. Dystrophic *mdx* mice were crossed with RyR-S2808A mice, which carry RyRs that cannot be phosphorylated at this site. Ablation of this phosphorylation site protected these animals, even though not the main pathomechanism was targeted, but rather one of the other steps in the positive feed-back loop. Unlike *mdx* mice, these animals did not develop cardiac hypertrophy with fibrosis and showed improved cardiac function. Further, they were protected from isoproterenol-induced arrhythmias and SR  $\text{Ca}^{2+}$  leak. These findings suggest that PKA dependent RyR phosphorylation contributes to the abnormal  $\text{Ca}^{2+}$  homeostasis in dystrophic cardiomyopathy. Interestingly, and in apparent contrast to these findings, another study was not able to detect significant PKA dependent RyR phosphorylation in *mdx* mice [117]. However, more recent studies suggest that the disease phenotype and the pattern of RyR posttranslational modifications change in the course of dystrophic cardiomyopathy, and other diseases as well. PKA activation seems to become important only at later stages, and is associated with elevated SR  $\text{Ca}^{2+}$  leak and reduced  $\text{Ca}^{2+}$  content [108].

## 6. RyR mutations and posttranslational modifications

Another layer of complexity is added to the intricate mutual interactions of all the posttranslational modifications discussed above in patients carrying a mutation of the RyR2 [124,125]. Many of these patients are prone to stress-induced arrhythmias, manifesting themselves as catecholaminergic polymorphic ventricular tachycardias (CPVTs), potentially leading to sudden cardiac death. Cell lines and transgenic animals have been engineered expressing RyRs harboring mutations that were identified in families with CPVT patients [126-129]. These animals replicate the human disease phenotype and serve as disease models to investigate pathomechanisms arising from RyR mutations and to develop therapeutical approaches. In these disease models, arrhythmias could be provoked by tachypacing and/or by  $\beta$ -adrenergic stimulation. In cellular experiments, these cardiomyocytes exhibited elevated  $\text{Ca}^{2+}$  spark frequencies, a propensity for

diastolic  $\text{Ca}^{2+}$  waves with delayed after-depolarizations and reduced wave thresholds. These are all signs for disturbed channel gating with a predisposition towards abnormal  $\text{Ca}^{2+}$  sensitivity of mutated channels (cytosolic or SR luminal). In summary, the phenotypes resulting from RyR mutations share many features with the functional consequences of the posttranslational modifications discussed above.

## 7. Conclusion and outlook

While we start to understand the consequences of various posttranslational RyR modifications on the molecular level, we also develop the awareness for the extraordinary complexity of this issue on the cellular and organ level. This partly results from the multiple crosstalks and interactions of the various signaling pathways and their intertwined positive and negative feed-back loops. A large amount of research will thus be required to address the question whether all the regulatory and / or pathophysiologically important mechanisms changing RyR function behave in additive, competitive or mutually exclusive ways. One example should be mentioned here: It is well established, that physical or emotional stress can trigger CPVTs in patients harboring cardiac RyR mutations. However, it is so far unknown what exactly triggers these arrhythmias during the stress. Is it provoked by additional RyR sensitization originating from PKA or CaMKII dependent RyR phosphorylation? Or is it the result of the concomitant stimulation of the SERCA after PLB phosphorylation? Or is it the combination of the two possibilities which is particularly detrimental? To answer these and many similar questions relevant for other cardiac conditions, it needs to be understood in more detail, on the molecular level, how these modifications interact to bring about functional change. These findings then need to be integrated into the more complex situation of intact cells, organs and organisms. Based on the presently available literature, only partly discussed above, one is inclined to predict that such interactions exist and are very important. Understanding these interactions will lay the foundation for the development of mechanism based therapies, potentially targeting several synergistically acting mechanisms simultaneously.

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## Figure legends:

**Fig. 1.** Modulation of the ryanodine receptor (RyR) by  $\text{Ca}^{2+}$  and phosphorylation.  $\text{Ca}^{2+}$  influx via the L-type Ca channel (LTCC) activates the RyR and triggers  $\text{Ca}^{2+}$  release from the sarcoplasmic reticulum (SR), a process referred to as  $\text{Ca}^{2+}$  induced  $\text{Ca}^{2+}$  release or CICR, leading to myocyte contraction. The levels of free cytosolic  $\text{Ca}^{2+}$  are tightly regulated by the SR  $\text{Ca}^{2+}$  ATPase (SERCA) and the sarcolemmal  $\text{Na}^+ \text{-Ca}^{2+}$  exchanger (not indicated). After CICR and contraction, the  $\text{Ca}^{2+}$  store is refilled by pumping  $\text{Ca}^{2+}$  back into the SR thereby re-establishing diastolic  $\text{Ca}^{2+}$  levels. The sensitivity of RyR toward activating  $\text{Ca}^{2+}$  is modulated by phosphorylation. Stimulation of the  $\beta_1$ -adrenoreceptor ( $\beta$ -AR) leads to Gs-protein-mediated activation of adenylyl cyclase (AC) and further cAMP-dependent activation of PKA. PKA can directly phosphorylate RyR at several phosphorylation sites, presumably at S2808, possibly inducing dissociation of calstabin 2, and at S2030, but also modulates the LTCC and SERCA function, the latter by phosphorylation of phospholamban (PLB). Increased cytosolic  $\text{Ca}^{2+}$  levels activate CaMKII, which directly phosphorylates RyR at S2814. Similar to PKA, CaMKII also phosphorylates PLB and the LTCC leading to global changes in myocyte  $\text{Ca}^{2+}$  homeostasis.

**Fig. 2.** Redox-modifications of RyRs. Changes in the redox potential of the myocyte have been shown to have a serious influence on protein function, especially at the level of the RyR. The main sources for the production of reactive oxygen species (ROS) in cardiomyocytes are the sarcolemmal NADPH oxidase (NOX), the xanthine oxidase (XO) and the mitochondrial electron transport chain (complex I through IV). ROS can glutathionylate free cysteine residues on the RyR and also act in an indirect way via CaMKII activation and subsequent RyR phosphorylation. Nitric oxide synthases (NOS) are mainly responsible for the production of nitric oxide (NO) and reactive nitrogen species (RNS). In cardiomyocytes, sarcolemmal endothelial NOS (eNOS), which co-localizes with caveolin-3 (Cav3) in caveolae, and RyR-associated neuronal nNOS are primarily responsible for the production of NO, causing S-nitrosylation at free thiol groups of the RyR and many other proteins. RNS also lead to PKG activation and RyR phosphorylation presumably at S2814. Most likely, these mechanisms work synergistically and induce parallel modifications of RyR function.

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Figure 1 tiff

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## Ca<sup>2+</sup> dependence and RyR phosphorylation

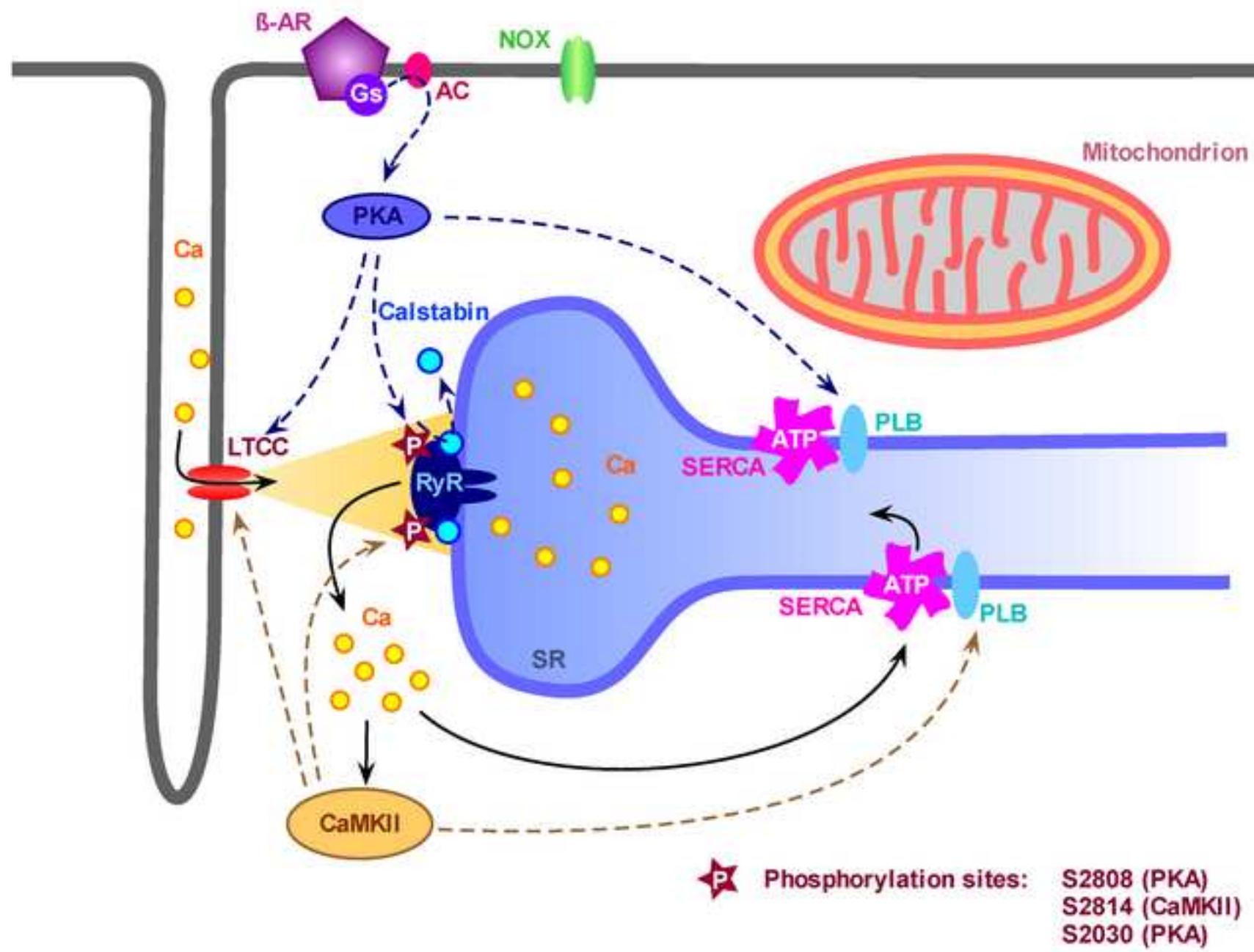


Figure 2 tiff

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## Redox modifications of RyR

