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Review

Understanding How Phosphorylation and Redox Modifications Regulate Cardiac Ryanodine Receptor Type 2 Activity to Produce an Arrhythmogenic Phenotype in Advanced Heart Failure

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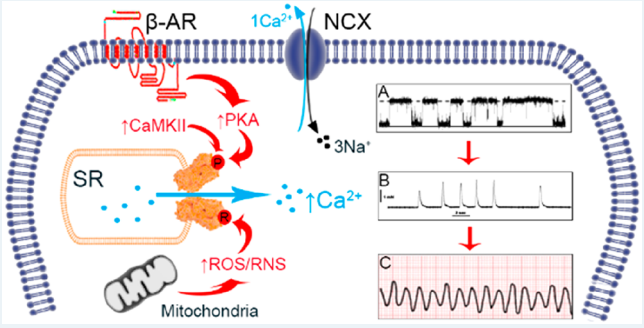
ABSTRACT: Heart failure (HF) is a global pandemic with significant mortality and morbidity. Despite current medications, 50% of individuals die within 5 years of diagnosis. Of these deaths, 30−50% will be a result of sudden cardiac death from ventricular arrhythmias. This review discusses two stress-induced mechanisms, phosphorylation from chronic β-adrenoceptor (β-AR) stimulation and thiol modifications from oxidative stress, and how they modulate the cardiac ryanodine receptor type 2 (RyR2) and foster an arrhythmogenic phenotype. Calcium (Ca^{2+}) is the ubiquitous secondary messenger of excitation−contraction coupling and provides a common pathway for contractile dysfunction and arrhythmia genesis. In a healthy heart, Ca^{2+} is released from the sarcoplasmic reticulum (SR) by RyR2. The open probability of RyR2 is under the dynamic influence of co-proteins, ions, and kinases that are in strict balance to ensure normal physiological functioning. In HF, chronic β-AR activity and production of reactive oxygen species and reactive nitrogen species provide two stress-induced mechanisms uncoupling RyR2 control, resulting in pathological diastolic SR Ca^{2+} leak. This increased cytosolic [Ca^{2+}] promotes Ca^{2+} extrusion via the local Na^+/Ca^{2+} exchanger, resulting in net sarcolemmal depolarization, delayed after depolarization and ventricular arrhythmia. Experimental models researching oxidative stress and phosphorylation have aimed to identify how post-translational modifications to the RyR2 macromolecular complex, and the associated Na^+/Ca^{2+} cycling proteins, result in pathological Ca^{2+} handling and diastolic leak. However, the causative molecular changes remain controversial and undefined. Through understanding the molecular mechanisms that produce an arrhythmic phenotype, novel therapeutic targets to treat HF and prevent its malignant course can be identified.



KEYWORDS: *heart failure, arrhythmia, ryanodine receptor, phosphorylation, redox, beta-adrenoceptors*

BURDEN OF HEART FAILURE AND ARRHYTHMIA

Heart failure (HF) with reduced ejection fraction (HFrEF) is a global pandemic, affecting ∼26 million people worldwide.[1] In Australia, the prevalence of HF is 1.0−2.0% of the population, consistent with North America and Europe.[2] HF is more prevalent in the elderly, with the mean age of patients admitted with a primary diagnosis of HF ranging from 70 to 75 years of age.[1] Approximately 11% of the world's population is over the age of 60 years, and this is predicted to reach 22% by 2050.[3] Consequently, the prevalence of HF will increase.

HFrEF is a chronic disease with a malignant prognosis. Impaired contractility is the central pathophysiological feature, which determines quality of life and the risk of sudden cardiac death (SCD). In 1993, the reported median survival following HF diagnosis was 1.7 years for men and 3.2 years for women.[4] Corresponding 1 and 5 year survival rates were 57 and 25% in men and 64 and 38% in women, respectively. Of these deaths, 30−50% were the result of SCD from ventricular arrhythmias.[4] Patients at greatest risk of SCD are those with a left ventricular function (LVF) ≤ 35% despite optimal guideline-directed medical therapy (GDMT). There is consensus within global guidelines that these individuals derive benefit from an implantable cardioverter−defibrillator (ICD). These devices can abort malignant ventricular arrhythmias by delivering high-voltage shocks. Despite the development of disease modifying medications and utilization of ICDs, present registries report an all-cause 1 year mortality of 23.6%.[5] However, controversy

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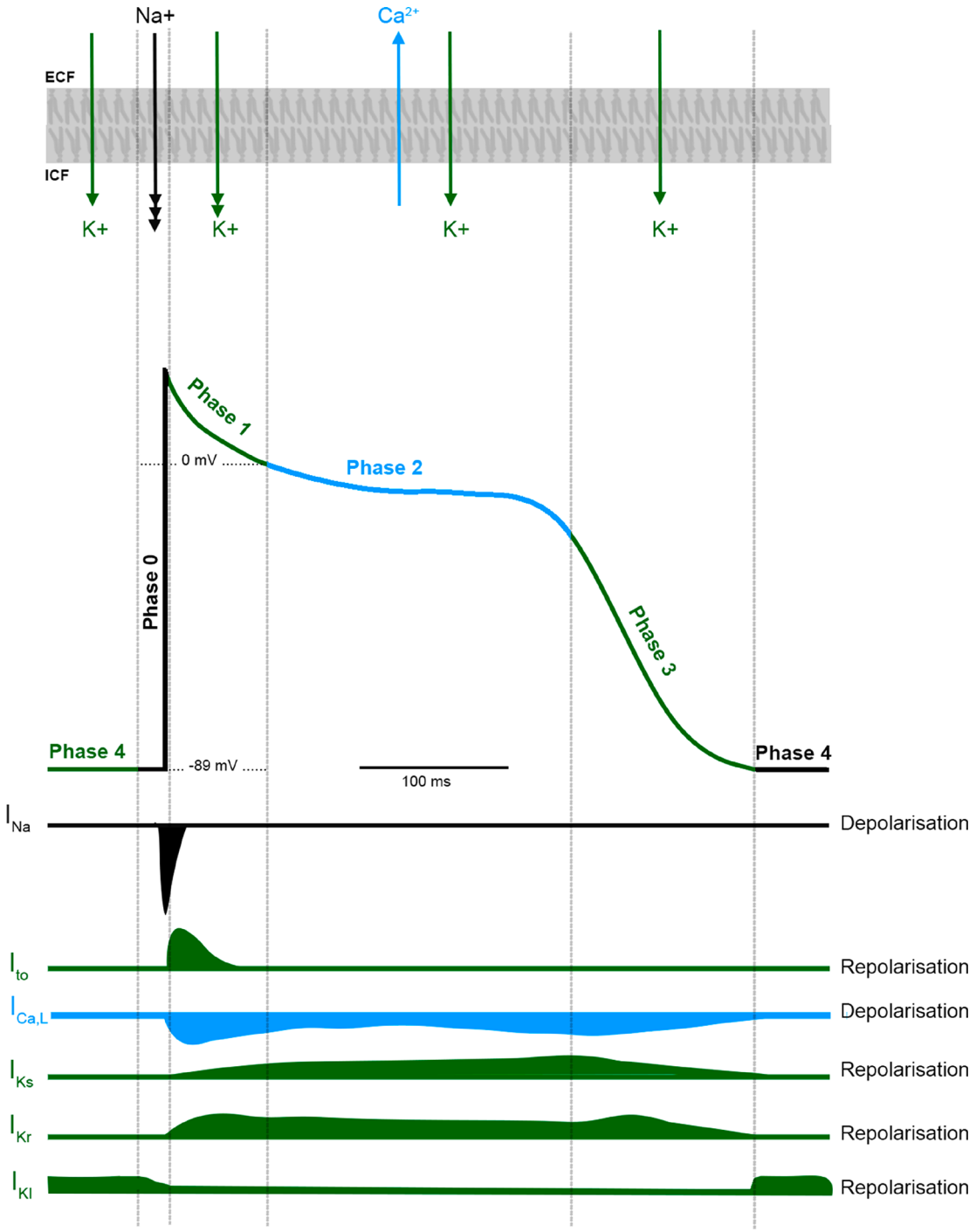

Figure 1. Action potential of ventricular myocardium. Illustration of the different phases of a ventricular myocyte action potential and the relationship of the different ionic currents involved in its generation.

exists within the published literature. Contemporary studies recruiting nonischemic HF patients with LVF ≤ 35% revealed no significant benefit in total mortality at 5.6 years with ICD therapy.[6] However, prespecified subgroup analysis showed evidence of a prognostic benefit in patients younger than 68 years. Thus, the potential utility of ICDs depend on the patient's risk of sudden arrhythmic death relative to nonsudden death. Older patients are twice as likely to die of causes other than SCD.[7] Taken together, young patients with nonischemic cardiomyopathy remain at high risk for SCD despite GDMT. Identifying cellular mechanisms implicated in the development of contractile dysfunction and generation of arrhythmia may provide novel medication targets with the potential to benefit millions of people suffering from HF.

CARDIAC EXCITATION–CONTRACTION COUPLING

In order to understand the complex interplay of cardiac contraction and development of ventricular arrhythmia, it is important to have an appreciation of cardiac excitation–contraction coupling (ECC): the process of translating an electrical action potential (AP) into mechanical output.

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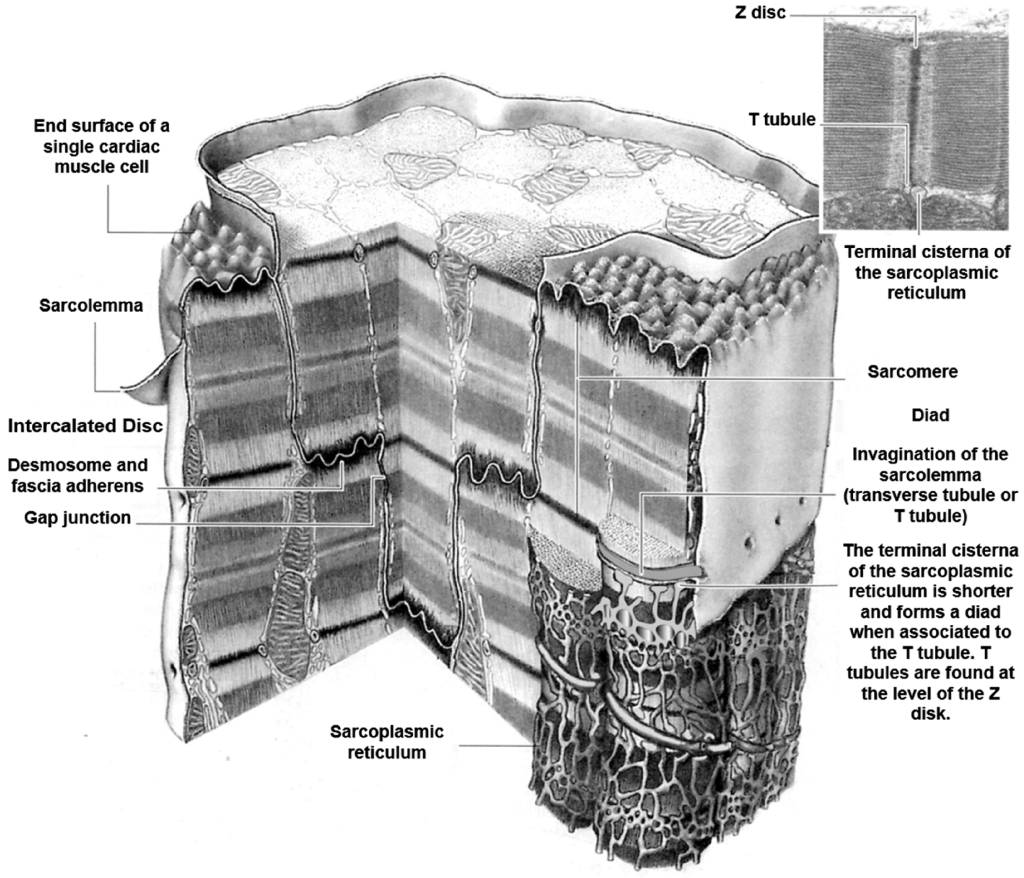

Figure 2. Schematic diagram of a cardiomyocyte. Each cardiomyocyte is surrounded by the meshwork of the sarcolemma which forms invaginations called T tubules. At the intercalated disc, desmosomes and gap junctions can be found. Reproduced with permission from ref 15. Copyright 1984 Williams & Wilkins.

Action Potential. The ventricular AP is a consequence of sequential activation and inactivation of ion channels conducting inward sodium (Na^+) and potassium (K^+) depolarizing currents and outward K^+ repolarizing currents (Figure 1).[8] Depolarization increases intracellular calcium concentration $[Ca^{2+}]_i$, which is central to regulating troponin C (TnC), exposing actin to the myosin heads, resulting in force generation.[9,10]

As an AP propagates, it evokes depolarization of adjacent cells by increasing the resting membrane potential (E_m) to a threshold that activates voltage-gated Na^+ (Nav) channels (INa-fast, Nav1.5). The inward sodium current (I_{Na}) sets up a positive feedback loop creating depolarization and the rapid upstroke of an AP, characteristic of phase 0. Phase 1, or early repolarization, results from inactivation of Nav channels and activation of the fast transient voltage-gated outward K^+ current (I_{to}). The AP then enters a long plateau with a steady E_m, phase 2, a balance between outward flow of K^+ and inward voltage-gated Ca^{2+} current ($I_{Ca,L}$) through L-type calcium channels (LTCC).[8] This influx of Ca^{2+} is the primary trigger for ECC. Eventually, the LTCC is inactivated, and the outward flow of K^+ predominates, facilitating repolarization (phase 3). Multiple types of voltage-gated K^+ currents and non-voltage-gated inwardly rectifying K^+ currents contribute to repolarization, including I_{Kr} [$I_{K(rapid)}$], I_{Ks} [$I_{K(slow)}$], and I_{Ku} [$I_{K(ultrarapid)}$].[8] Phase 4 reflects the cell at rest. Here, the membrane is most permeable to K^+ and the flow is driven by an ionic concentration gradient, generated by Na^+/K^+-ATPase pumps. Non-voltage-gated inward rectifying K^+ channels (I_{Ki}) primarily contribute to the maintenance of a resting diastolic potential of −89 mV.[8,11] The stability of the resting membrane potential affects cardiac excitability and AP morphology. The ion channels discussed are composed of many pore-forming subunits and channel accessories which underlie the channels' contribution to the myocardial AP. Mutations in genes encoding these subunits have been found to underpin several genetic cardiac arrhythmia syndromes.[8,12,13]

Sarcolemma. The sarcolemma is a highly specialized plasma membrane (Figure 2) which includes ion channels, transporters and pumps that are responsible for ionic fluxes involved in ECC and calcium homeostasis. The sarcolemma exhibits both regional specialization, important for spatial and temporal summation of local Ca^{2+} transients, and global orchestration of cell-wide Ca^{2+} transients.[9] First, it forms transverse tubules (T tubules), invaginations that extend deep into the interior of the cardiomyocyte, which couple with the terminal cisternae of the sarcoplasmic reticulum (SR), the intracellular Ca^{2+} reservoir.[14] Second, cardiomyocytes interdigitate and are closely opposed at intercalated discs. Here, cells form mechanical connections and gap junctions to provide a low-resistance electrical pathway that allows the AP to propagate, coordinating the heart to function as an electrical syncytium.[9,14]

Calcium-Induced Calcium Release. The efflux of Ca^{2+} from the SR is crucial for the activation of contraction as independently Ca^{2+} influx via LTCC is too small and

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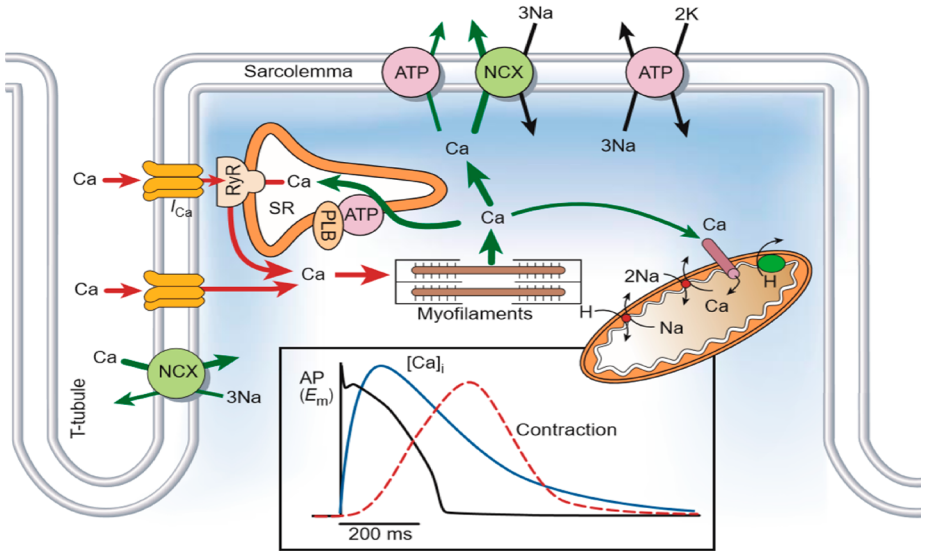

Figure 3. Excitation−contraction coupling and control of Ca^{2+} transients in ventricular myocytes. Action potential stimulates intracellular Ca^{2+} influx ($I_{Ca,L}$) via voltage-gated L-type calcium channels. Ryanodine receptors (RyR) in the sarcoplasmic reticulum (SR) are activated by the local increases in cytosolic Ca^{2+} releasing the SR's stored Ca^{2+}, a process called calcium-induced calcium release. Ca^{2+} binds to troponin C, and the myofilaments contract. Ca^{2+} is sequestered into the SR via the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA), and to the extracellular space via the Na^+/Ca^{2+} exchanger (NCX). Reproduced with permission ref 17. Copyright 2002 Springer Nature Limited.

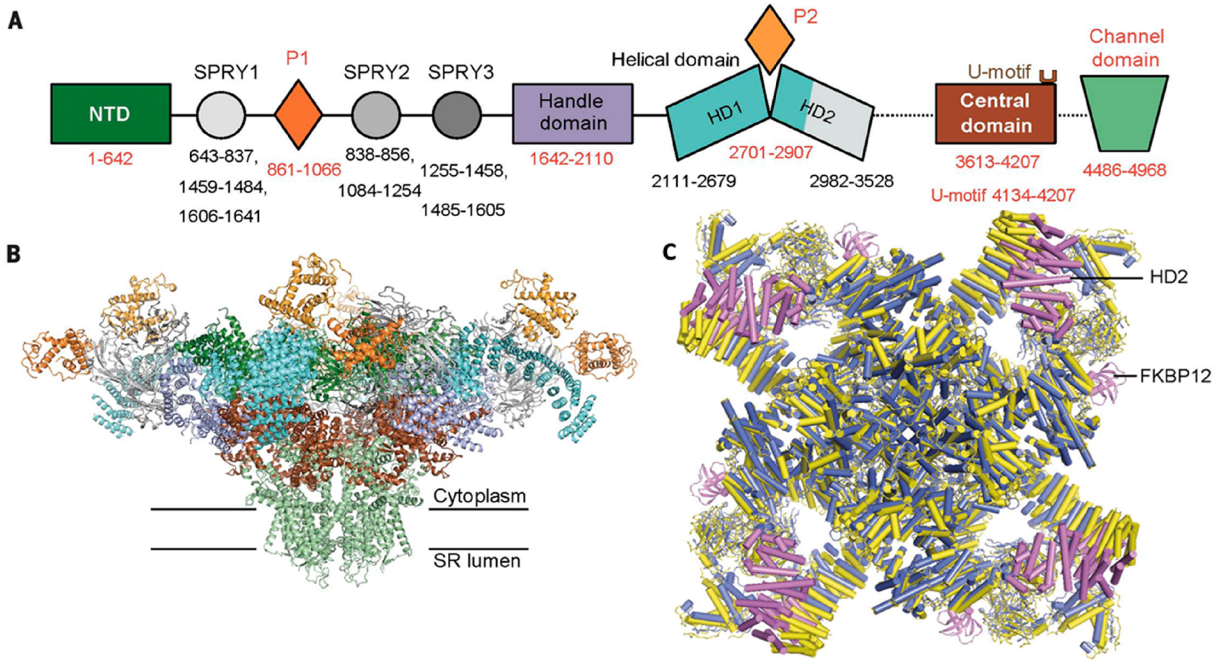

Figure 4. Cryo-EM structure of RyR2. (A) Domain organization of a RyR2 depicting 8 of the cytoplasmic structural domains attached to the channel domain−the N-terminal domain (NTD), the P1 domain, the three SPRY domains (SPRY 1, 2, and 3), the Jsol/handle (handle), the helical/Bsol domain (helical), and the central (central) domain. (B) Structure of the RyR2 in a closed formation at a resolution of 4.2 Å. The tetrameric structure reveals the large cytoplasmic region and domain colors are the same scheme as in panel A. (C) Structural comparison of RyR2 (yellow) with rRyR1 and the position of FKBP12 binding. Reproduced with permission from ref 25. Copyright 2016 American Association for the Advancement of Science.

insufficient to engage the contractile machinery. Calcium-induced calcium release (CICR) is the most widely accepted mechanism of coordinated SR Ca^{2+} release[16−18] (Figure 3). The voltage-dependent LTCCs are found clustered on membrane microdomains on T tubules, which are closely opposed to a RyR2 found on the membrane of the SR. Groupings of LTCCs and RyR2s in so-called Ca^{2+} release units (CRU) or couplons are the initiation sites for where each AP may cause transient flows of Ca^{2+} from the SR to occur. This spatial arrangement of Ca^{2+} cycling proteins is important to the efficiency of ECC.[19] LTCCs are activated when the transmembrane potential increases to −55 mV allowing an inward $I_{Ca,L}$.[9] RyR2 within the corresponding couplon releases Ca^{2+} from the SR lumen to the cytosol. This local increase in concentration of intracellular Ca^{2+} from a single RyR2 is termed a calcium spark.[20] Subsequently, neighboring RyR2 are

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activated via the local influx of Ca^{2+} (>10 μM), and the spatial temporal summation of these sparks forms a Ca^{2+} wave which propagates through the cardiomyocyte. Once the cytosolic [Ca^{2+}] reaches a threshold, it acts as a switch, binding troponin C and allowing the contractile machinery to engage.[10,17] The amount of Ca^{2+} released from the SR determines the Ca^{2+} transient amplitude which correlates well with strength of contraction.[21]

INTRACELLULAR CALCIUM HANDLING IN HEALTHY CARDIOMYOCYTES

ECC is a delicate balance of highly orchestrated intracellular signaling pathways converting electrical activation into mechanical force. Ca^{2+} is the ubiquitous second messenger pertaining to electrophysiology and mediating contraction. It is becoming clear that abnormalities in Ca^{2+} homeostasis play a key role in the *sine qua non* of systolic HF, reduced contraction, and the generation of arrhythmias from pathological diastolic SR Ca^{2+} release.[17,21,22]

In normal physiology, cellular Ca^{2+} must remain in a steady state between contraction and relaxation. This is attained by cycling between the cytoplasm and SR.[18] In systole, intracellular Ca^{2+} must rise significantly from resting level, achieved by the RyR2 and LTCC. In relaxation, prior to the next AP, cytosolic [Ca^{2+}] must return to baseline and SR Ca^{2+} content must be replenished, achieved by a combination of (i) sequestration into the SR via sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA2a) and (ii) removal from the cytosol by sarcolemmal Na^+/Ca^{2+} exchanger (NCX) which is equivalent to the small amount of Ca^{2+} that entered through LTCC.[17,18] The co-localization of these Ca^{2+} extrusion pathways allows them to interact and compete for Ca^{2+} within a specialized microenvironment.[23]

Cardiac Ryanodine Receptor Type 2. RyR2 is a 2.2 MDa homotetramer which comprises four 560 kDa subunits extending from the SR lumen to the cytosol.[24,25] It is the principle cardiac SR Ca^{2+} release channel. Three isoforms of RyR are expressed in striated muscle: RyR1 predominates in skeletal muscle, RyR2 predominates in cardiomyocytes, and RyR3 predominates in developing skeletal muscle. Peng et al.[25] has illustrated the domain organization at a resolution of 3.8 Å (Figure 4). The primary structure includes a large cytoplasmic region, six transmembrane regions, and a small SR luminal region. Under physiological conditions, RyR2 activation is primarily regulated by Ca^{2+}. However, RyR2 also acts as a scaffold for regulatory subunits to assemble upon, forming the SR Ca^{2+} release complex. Consequently, RyR2 regulation is dynamic, influenced by the integrated effects of functional ligand binding.[25,26]

Calcium. Regulation of RyR2 gating by intracellular Ca^{2+} is central to the controlled release of SR Ca^{2+}. The RyR2 open probability (P_o) has a steep dependence on cytoplasmic [Ca^{2+}], with Hill coefficients between 2 and 4.[27] At low levels of cytoplasmic [Ca^{2+}] (~100−200 nM), RyR2 channels are inactive, with a low P_o. Ca^{2+} influx, through LTCC, increase cytoplasmic [Ca^{2+}] within the diadic cleft to submicromolar concentrations.[9] Ca^{2+} binds RyR2 at high-affinity activation sites (A-site).[28] This facilitates SR Ca^{2+} release, resulting in local positive feedback, CICR. The P_o reaches a nadir as cytoplasmic [Ca^{2+}] reaches 10 μM. Any further increase in [Ca^{2+}] will result in a decreasing P_o, suggesting that Ca^{2+} binds to low-affinity in-activation sites.[29]

In total, four Ca^{2+} regulation sites have been proposed: two activation sites located on cytoplasmic (A-site) and luminal (L-site) domains and two cytoplasmic inhibitory sites (I1 and I2).[27] Des George et al. used cryo-EM reconstruction to localize the A-site Ca^{2+}-binding site at the interdomain interfaces of the RyR1 central domain and C-terminal domains.[30] Evidence for luminal activation has proved challenging. While it has been recognized that increased SR [Ca^{2+}] can trigger Ca^{2+} release, it was not until 1994 that Sitsapesan and Williams observed direct luminal activation.[31] Laver et al. have proposed both direct luminal activation and a secondary but stabilizing feed-through mechanism.[32] As SR Ca^{2+} content increases, Ca^{2+} binds specific luminal activation sites (L-site). The subsequent passage of Ca^{2+} accesses and binds the cytoplasmic A-site, stabilizing the open state. RyR2 can be inactivated by the I1 (low-affinity) I2 (high-affinity) sites. The I1 inactivation site helps produce the bell-shaped curve of activation and inactivation to cytoplasmic [Ca^{2+}] at micro- and millimolar concentrations, respectively.[28,32] Magnesium (Mg^{2+}) can act as a competitive antagonist of Ca^{2+} at both the A- and I1-sites, directly inhibiting RyR2 activation and increasing the required Ca^{2+} activation concentration.[33]

Adenine Nucleotides. Adenosine-derived nucleosides, especially ATP, are physiological regulators of RyR2. They enable greater increases in channel activity relative to smaller changes in cytosolic Ca^{2+}. The degree to which they increase P_o is dependent on the number of phosphates present on the ribose ring. ATP produces the greatest increase in P_o, and AMP produces the smallest increase.[30] Ca^{2+} and ATP act synergistically, with maximal P_o occurring when both are present.[24] Mg^{2+} may also form a complex with ATP: MgATP, the predominate form of biologically active ATP. As previously described, increasing [Mg^{2+}] inhibits RyR2 but it may also alter the ratio ATP/MgATP in favor of MgATP, decreasing RyR2 activity.[24] What remains unclear is whether ATP or MgATP regulates activity.

FKBP12 and FKBP12.6. Calstabin 1 and 2, also known as FKBP12 and FKBP12.6, are two Ca^{2+}-channel-binding and stabilizing proteins. FKBP12 and FKBP12.6 preferentially bind RyR1 and RyR2, respectively, interacting with a hydrophobic cluster in the SPRY1 domain and junctional solenoid, stabilizing the link between the cytoplasmic region and the pore of RyR2 in a closed conformation.[16,30] This prevents Ca^{2+} leak and regulates the dynamic interaction of RyRs, promoting coupled gating and improving the efficiency of ECC.[34,35] Genetic deletion of FKBP12.6 or pharmacological removal with either FK506 or rapamycin alters the biophysical properties of RyR2 and induces significant subconductance openings.[35] In these models the resultant increase in SR Ca^{2+} release has been associated with ventricular arrhythmias.[34]

Calmodulin (CaM). CaM is a 16.7 kDa cytosolic Ca^{2+}-binding protein that regulates RyR2 by direct binding. CaM binds RyR2 stoichiometrically, 4 CaMs per tetrameric RyR2, at high affinity subunits (RyR2 amino acids 3581−3610).[36] CaM inhibits RyR2 opening at all [Ca^{2+}]. In diastole, it is reported that CaM stabilizes and maintains RyR2 in the closed state, helping terminate Ca^{2+} release.[37] Mutations in the highly conserved CaM-binding region severely reduce and prevent CaM−RyR2 binding.[36] In animal models this has been associated with severe hypertrophic cardiomyopathy and catecholaminergic polymorphic ventricular tachycardia (CPVT).[38] In nonischemic HF, CaM−RyR2 binding affinity is reduced, contributing to the increased diastolic Ca^{2+} leak,

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like mice carrying the human CPVT mutations (R2474S). Oxidative stress, which will be discussed in detail later, has been the proposed mechanism for reducing the binding affinity of CaM.[39] LTCCs are another important binding partner for CaM. Ca^{2+} that enters via LTCCs binds CaM which in turn, allows it to interact with the LTCC to increase Ca^{2+} entry to initiate ECC.[21]

Triadin, Junctin, and Calsequestrin. A complex consisting of triadin, junctin, and calsequestrin (CSQ) act as a Ca^{2+} sensor on the RyR2 luminal side. They participate in intra-SR Ca^{2+} buffering and modulate release. Triadin and junctin are membrane-bound proteins that interact directly with RyRs, but also serve as connecting proteins, associating with CSQ.[24] CSQ is a low affinity, high-capacity Ca^{2+} storage protein which dissociates from RyR2 as luminal Ca^{2+} increases, increasing RyR2 P_o and normalizing SR Ca^{2+} content. Cardiomyopathies and arrhythmic pathologies associated with an imbalance in Ca^{2+} handling have been linked to a deficiency in SR luminal CSQ and a lack of, or overexpression of triadin and junctin.[24,40]

L-Type Ca^{2+} Channel. LTCCs are responsible for the voltage dependent inward $I_{Ca,L}$ transient responsible for the plateau phase of an AP and triggering CICR. The maximal amplitude of the $I_{Ca,L}$ is delayed due to the channels time dependent nature, and product of high channel conductance with low driving force ($E_m - E_{Ca}$).[41] LTCC termination is primarily mediated by cytosolic Ca^{2+}-dependent inactivation via CaM. As local $[Ca^{2+}]$ increases from $I_{Ca,L}$ and SR release, Ca^{2+} binds CaM which binds to the carboxy terminus of the LTCC.[42] This local negative feedback highlights the importance of the co-location of key calcium handling proteins and how local $[Ca^{2+}]$ can derive an effect over global cellular Ca^{2+} content. LTCC are also regulated by protein kinase A (PKA) and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII). They increase $I_{Ca,L}$ amplitude and sensitivity, with activation occurring at more negative E_m.[41]

Sarcoplasmic Reticulum Ca^{2+}-ATPase. Removal of cytosolic Ca^{2+} and restoration of the SR Ca^{2+} reservoir is primarily achieved by SERCA2a. SERCA2a is crucial for diastolic Ca^{2+} cycling, promoting efficient muscle relaxation and, by restoring the SR Ca^{2+}reservoir, it has an important inotropic contribution for the following contraction. Schultz et al. developed SERCA2a knockout mice to understand its role in Ca^{2+} homeostasis and cardiac contractility.[43] Homozygous null ($SERCA2a^{-/-}$) mice died early in development, while heterozygous ($SERCA2a^{-/+}$) mice, unlike the wild-type (WT) mice, rapidly developed cardiomyopathy when the heart was stressed by pressure overload. In contrast, overexpression was well-tolerated, leading to increased SR Ca^{2+} restoration, increasing contractile strength and improving lusitropy (relaxation). After myosin ATPase, SERCA2a is the largest consumer of ATP, and studies suggest it is the ATPase most sensitive to a reduction in supply of ATP.[44] HF induces a state of increased ATP demand from reduced pump function and increased sympathetic activity which may affect ECC coupling and contractile function.[45] SERCA2a activity is regulated by two molecules: phospholamban (PLN) and sarcolipin (SLN). PLN is a phosphoprotein that regulates SERCA2a pump activity by changing its affinity for Ca^{2+} depending on cytosolic $[Ca^{2+}]$.[45] PLN can be phosphorylated by PKA and CaMKII at Ser-16 and Thr-17, respectively, after activation of either β_1-AR or β_2-ARs,[46,47] thus releasing SERCA2a from PLN inhibition. Preventing phosphorylation at Ser-16 increases PLN affinity, suppressing SERCA2a function and is correlated with reduced Ca^{2+} uptake and cardiac contractility.[48] Sarcolipin, when coexpressed with SERCA2a, decreases Ca^{2+} affinity, inhibiting the SERCA2a pump.[45]

Na^+/Ca^{2+} Exchanger. Cardiac NCX is located primarily at the T tubule with recent high-resolution imaging suggesting partial co-localization with RyR2.[49] NCX is the main effector of cardiac myocyte Ca^{2+} extrusion. It exchanges Ca^{2+} with a Na^+/Ca^{2+} stoichiometry of 3:1 producing an ionic charge from a gain of one net charge. Under the control of local E_m and intracellular and extracellular $[Na^+]$ and $[Ca^{2+}]$, NCX can both extrude Ca^{2+} and work in reverse for Ca^{2+} influx. The initial upstroke of the AP increases the E_m such that the NCX favors Ca^{2+} influx. As the RyR2 is activated to release Ca^{2+} there is a local rise in intracellular $[Ca^{2+}]$ versus global intracellular $[Ca^{2+}]$, causing NCX Ca^{2+} efflux. As repolarization proceeds, Ca^{2+} extrusion continues to be thermodynamically favored. The intracellular and SR Ca^{2+} content can be raised by modest increases in $[Na^+]_i$, inhibiting Ca^{2+} extrusion. While an increased SR Ca^{2+} content can increase contractile strength, conditions of Ca^{2+} overload may promote spontaneous Ca^{2+} release and formation of Ca^{2+} waves. This Ca^{2+} current may activate NCX in its forward-mode, extruding Ca^{2+}. This generates a depolarizing current termed a delayed after depolarization (DAD), which if large enough can produce a triggered beat by activating the rapid Na^+ inward current.[50,51]

POST-TRANSLATIONAL MODIFICATIONS

At basal resting conditions when Ca^{2+} influx remains constant, efflux must remain unchanged within cardiomyocytes to ensure a steady state.[18] The channels, pumps, and transporters discussed are regulated by multiple ligands and complex signaling cascades. They modulate the activity and amplitude of Ca^{2+} transients in response to changing demands for cardiac output. A further level of control is provided by post-translational modifications which target vulnerable residues on both ion channels and associated ligands. Alterations in sympathetic tone and redox balance drive phosphorylation and thiol modifications, two post-translational modifications which are highly regulated in the normal heart, fine-tuning Ca^{2+} handling. Unfortunately, in pathological states, such as HF, this balance can be lost resulting in deleterious Ca^{2+} handling.

β-Adrenoceptor Signaling. Sympathomimetic stimulation of the β-AR is the primary neurohormonal mechanism which augments the force and frequency of cardiac contraction. In exercise, cardiac output is increased nearly 5-fold within seconds.[52] At least two cardio-stimulatory β-ARs are expressed in cardiomyocytes and the sino atrioventricular conducting system of human heart.[53−55] Activation of β_1-AR and β_2-AR causes positive inotropic, chronotropic, dromotropic and lusitropic effects.[54−57] Both β_1-AR (Ser49Gly, Arg389Gly),[58] and β_2AR (Arg16Gly, Gln27Glu, and Thr164Ile)[59] exist as polymorphisms. Inotropic and lusitropic effects of (−)-noradrenaline through β_1-AR and (−)-adrenaline through β_2-AR are conserved across all polymorphisms in human right atrium from nonfailing hearts[55] despite enhanced β_1AR Gsα-protein cyclic AMP−PKA signaling through Arg389-β_1AR compared to Gly389-β_1AR.[58] There is some evidence for a third β-AR, β_3-AR, which has been reported to mediate cardio-depressant effects in human ventricle[60] and cardiostimulant effects in human atrium,[61] but questions remain about its functional significance in human heart.[62−64]

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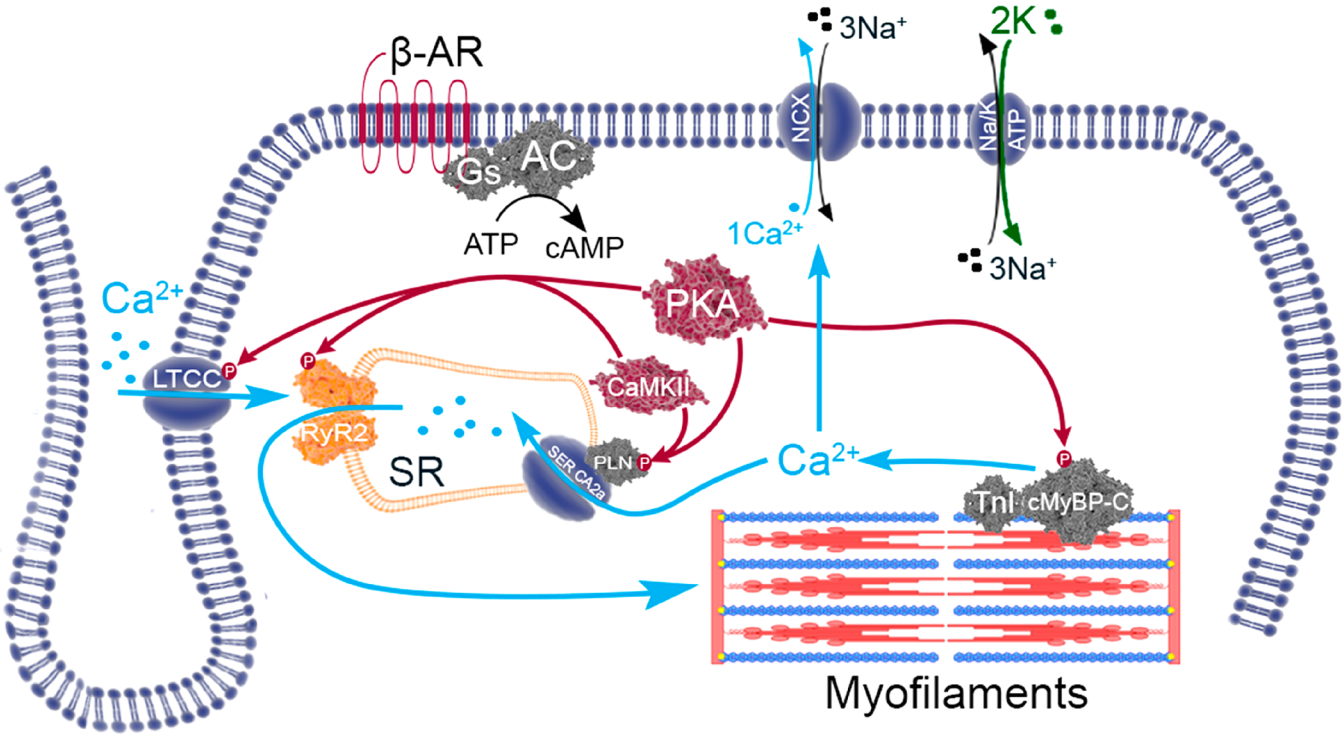

Figure 5. Post-translational phosphorylative signaling. PKA is the main downstream effector of β-AR signaling. CaMKII responds primarily to the local Ca^{2+} environment. Phosphorylation of RyR2 by PKA and CAMKII increases the P_o. Current evidence suggests serine 2808 is the primary target of PKA although evidence also exists for CaMKII. Serine 2814 is a CaMKII phosphor-specific site. PLN is phosphorylated by both PKA and CaMKII, releasing SERCA2a from PLN inhibition, increasing Ca^{2+} uptake and contractile strength. Both PKA and CaMKII can phosphorylate the LTCC increasing the inward Ca^{2+} current. PKA-dependent phosphorylation of both TnI and cardiac myosin-binding protein-C (cMyBP-C) exert lusitropic effects, accelerating relaxation.

In the human heart, activation of β_1-AR and β_2-AR by endogenous agonists (−)-noradrenaline and (−)-adrenaline results in coupling to the $G_{\alpha s}$-protein−adenylyl cyclase−cAMP−protein kinase pathway.[46,47,55] The intracellular elevation of cAMP activates cAMP-dependent protein kinase (PKA). PKA-catalyzed phosphorylation regulates pivotal proteins responsible for ECC. β_1-AR- and β_2-AR-mediated lusitropic effects result from phosphorylation of PLN and TnI.[46,47,55] Phosphorylation of PLN relieves its inhibition of SERCA2a, increasing the rate of Ca^{2+} uptake into the SR, accelerating the decline in cytosolic [Ca^{2+}]. Phosphorylation of TnI reduces the affinity of Ca^{2+} for TnC accelerating myofilament relaxation.[55] Together these two mechanisms improve diastolic performance. The inotropic effect is achieved from greater availability of Ca^{2+}. By enhancing SERCA2a activity (via PLN phosphorylation), there is improved diastolic restoration of SR Ca^{2+}, improving its availability for ECC. Furthermore, phosphorylation of LTCCs increases inward Ca^{2+} influx.[17,18,52] A new steady state is achieved from increased cellular Ca^{2+} and greater amplitude of Ca^{2+} transients, increasing contraction and reducing time to peak force[55] (Figure 5).

β_1-AR- and β_2-AR-mediated inotropic and lusitropic effects in human heart are regulated by chronic administration of β-blockers to patients with coronary artery disease or HF.[55,57] Increased inotropic and lusitropic effects through activation of β_1-AR and β_2-AR were observed following atenolol or metoprolol. However, chronic administration of carvedilol was notable for causing reduced potency of (−)-adrenaline at β_2-ARs with smaller reductions for (−)-noradrenaline at β_1-ARs.[65]

The β_1-AR can be activated by β-blockers such as (−)-pindolol and (−)-CGP 12177 at higher concentrations (~2−3 log units) than those required to block β_1-AR and β_2-ARs.[66] (−)-Pindolol and (−)-CGP 12177 cause positive inotropic and lusitropic effects in the human heart which are resistant to the blockade of β_1- and β_2-ARs by (−)-propranolol at concentrations (200 nM)[67,68] that effectively block the inotropic effects of (−)-noradrenaline through β_1-AR and (−)-adrenaline through β_2-AR.[69] These studies indicate β_1-AR has two binding sites, one that is sensitive to blockade by (−)-propranolol (activated by (−)-noradrenaline) and another which is relatively insensitive (activated by (−)-pindolol and (−)-CGP 12177). Activation of both sites in the human heart results in β_1-AR coupling to the Gsα−cAMP−PKA pathway.[68]

Redox Signaling. ROS and RNS are highly reactive oxygen-/nitrogen-containing molecules. They are kept in strict balance, as low levels are required for site-specific protein modifications and normal intracellular signaling. ROS are generated by xanthine oxidase (XO) and nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOX) and are byproducts of mitochondrial oxidative phosphorylation.[70] The electron transport chain leaks electrons which combine with oxygens forming superoxide anions ($O_2^{-}\bullet$). Superoxide dismutase (SOD) converts $O_2^{-}\bullet$ into hydrogen peroxide which may react with transition metals to form hydroxyl radicals (OH·). XO is involved in the purine degradation pathway and produces electrons which transfer to oxygen generating hydrogen peroxide. The enzyme NOX was originally described in neutrophils producing superoxide as a byproduct. In cardiac cells they produce lower levels of ROS. Nitric oxide synthase (NOS) generates nitric oxide (NO·) and

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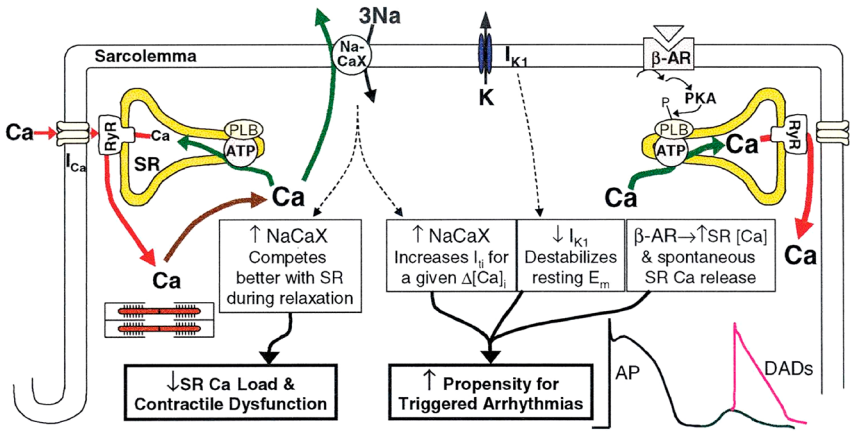

Figure 6. Altered calcium handling in HF, a cause of contractile dysfunction and arrhythmogenesis. Increased NCX and reduced SERCA2a expression alters cytosolic Ca^{2+} cycling reducing SR Ca^{2+} content, made worse by the increased SR Ca^{2+} leak. Decreased Ca^{2+} transients reduce myofilament activation and impair contraction. Enhanced NCX, reduced I_{K1}, and residual β-AR responsiveness contribute to arrhythmogenesis through triggered activity (DADs). Source: Reproduced with permission from ref 22. Copyright 2001 American Heart Association.

RNS. Uncoupled NOS can also produce $O_2^{-}\bullet$ which combines with NO· to produce the powerful oxidant peroxynitrate (ONOO·).[70] Preventing the uncontrolled production of ROS/RNS are glutathione peroxidase and SOD. Reduced glutathione (GSH) is the major cytosolic ROS scavenger transforming to its oxidized state, GSSG. Glutathione reductase reduces GSSG to GSH and is crucial to maintaining the redox buffer system protecting the cell from ROS-induced damage. NADPH is required for thioredoxin, another ROS scavenger, and helps maintain the ratio of reduced to oxidized glutathione. Oxidative stress occurs when there is excess ROS produced relative to the antioxidant buffer systems. Nitrosative stress results from impaired NO· signaling, increasing RNS. ROS and RNS are tightly coupled within biological systems, working together to modulate signaling, known as the nitro-redox balance. This relationship is complex as both species can modify the same thiols, and there is cross talk between the enzymes that produce them. NO· deficiency can result in increased ROS production. Perturbation of this system can result in impaired contractility and the modification of proteins involved in ECC, a hallmark of HF.[71]

HEART FAILURE AND CALCIUM BALANCE

Failing myocytes display dysfunctional intracellular Ca^{2+} homeostasis evidenced by fragmentation of the normally synchronized Ca^{2+} release with spontaneous diastolic Ca^{2+} leak, associated with contractile dysfunction and arrhythmogenesis (Figure 6). HF studies have consistently shown reduced SERCA2a expression and upregulation of NCX resulting in reduced SR Ca^{2+} content.[22,72] Pogwizd et al. induced HF in rabbits by aortic insufficiency.[22] They observed enhanced inward NCX current and a 2-fold increase in NCX mRNA. SERCA2a expression was unchanged but function was reduced by ∼24%. They concluded NCX must compete better with SERCA2a in diastole, opposing restoration of the SR Ca^{2+} content. This is consistent with findings in human failing hearts which revealed ∼25% reduction in SERCA2a expression and ∼2-fold increase in NCX contributing to systolic dysfunction.[72]

Physiologically, CICR controls the release of Ca^{2+} via the RyR2. The magnitude and frequency of the Ca^{2+} transient is affected by the P_o of RyR2 and SR Ca^{2+} content, with low levels of Ca^{2+} release occurring when content is reduced. Under conditions of SR Ca^{2+} overload the RyR2 is activated independent of cytosolic $[Ca^{2+}]$. This spontaneous release is known as store-overload-induced Ca^{2+} release (SOICR) and is one of the proposed mechanisms behind the initiation of DADs leading to arrhythmia.[73] Fatal ventricular arrhythmias in nonischemic HF patients result from triggered activity, termed early after depolarizations (EADs) and DADs.[74] EADs occur in the late plateau or repolarization phase (phase 3) from decreased outward K^+ currents. DADs predominate in the HF population and are oscillations of the transmembrane potential in phase 4 of a preceding AP from Ca^{2+}.[9] An initial understanding of the role of Ca^{2+} came from arrhythmogenic studies of digitalis glycosides. Dogs perfused with an increasing concentration of ouabain produced ventricular ectopic beats. These correlated with DADs, isolated from individual Purkinje fibers, and voltage clamp techniques linked a "transient inward current" in repolarization to a rise of intracellular $[Ca^{2+}]$.[75] SOICR occurs when the SR Ca^{2+} content exceeds a threshold level either in conditions that favor cytosolic and cellular Ca^{2+} overload or a reduction in the threshold value required to trigger spontaneous release, largely dependent on the properties of the RyR2. However, in HF one would expect less spontaneous Ca^{2+} release as the SR Ca^{2+} content is reduced. How can this paradox be resolved?

Stress-Induced Mechanisms Underlying Arrhythmia. Failing myocytes display a greater amount of SR Ca^{2+} leak for any given SR Ca^{2+} content. This suggests the threshold content for SOICR to be reduced, with the RyR2 being more sensitive to cytosolic and SR $[Ca^{2+}]$. However, it may be that RyR2 is dysfunctional, remaining open independent of SR $[Ca^{2+}]$ content. Advanced HF fosters an environment of chronic oxidative stress and elevated sympathetic tone altering the delicate balance of post-translational modifications fine-tuning Ca^{2+} handling. Although HF demonstrates reduction in β_1-AR density[53] and reduced β_1-AR responses,[53,55] there remains residual β-AR responsiveness[46] which activates PKA and neighboring components of Ca^{2+} signaling, elevating local SR Ca^{2+} content and increasing RyR2 P_o. Oxidative stress has been implicated in abnormal RyR2 activity and increased SR Ca^{2+} leak in many cardiac pathologies including myocardial infarction and HF.[39,70] Under normal physiological conditions the RyR2 channel exists in a stable closed, zipped conformation, preventing deleterious Ca^{2+} leak. The stressed

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environment of HF can uncouple the delicately balanced post-translational modifications, increasing diastolic RyR2 P_o and creating a "leaky" phenotype. The HF environment is one of arrhythmia generation and the dysfunctional RyR2, its catalyst.[76]

Phosphorylation. Phosphorylation can be performed by several kinases, but pertinent to RyR2 and Ca^{2+} signaling are CaMKII and PKA. CaMKII responds primarily to the local Ca^{2+} environment, while PKA provides a systemic effect, being the main downstream effector of β-AR signaling. Present on the cytoplasmic domain of RyR2 are several residues which may undergo phosphorylation. Although it is still under debate, three main functional sites have been identified. Two are PKA-specific sites, serine (S) 2030 and S2808, and one is a CaMKII-specific site, S2814.[70]

Serine 2808. Controversy exists as to whether S2808 is the primary substrate of PKA, CaMKII, or both, as experiments of phosphospecific antibodies produced mixed results. In 2000, Marx et al. reported PKA hyperphosphorylation was associated with dissociation of FKBP12.6 from the channel complex, increasing RyR2 Ca^{2+} sensitivity for activation, elevating the P_o.[77] Their study had access to human failing hearts, which similarly exhibited PKA hyperphosphorylation and reduced FKBP12.6 binding with subsequent increased channel P_o. Extensive investigations by Wehrens et al., first utilizing immunoblotting with phopho-epitope-specific antibodies,[78,79] and then genetically altered mice in which Ser2808 was replaced by alanine, identified Ser2808 as the functionally important PKA site.[80] In RyR2-S2808A mice, β-AR activation was unable to induce PKA phosphorylation of RyR2 and deplete FKBP12.6 from the channel complex. Their mouse models used an ischemic mechanism for deleterious remodeling and induction of HF. Notably, disruption of RyR2 hyperphosphorylation protected against progressive cardiac dysfunction following ischemia. However, this hypothesis is not universally accepted as a number of groups have been unable to confirm the fundamental aspects of this hypothesis. MacDonnell and colleagues, using the same RyR2-S2808A mouse model, were unable to detect PKA-mediated S2808 phosphorylation or an increase in SR Ca^{2+} leak following activation of β-AR with isoprenaline compared to WT.[81] The reasons for these fundamentally different results remain to be understood. While it seems indisputable that S2808 is indeed phosphorylated, its functional effect remains to be agreed upon. This is made more complex by "how much" phosphorylation is required. Under basal conditions, without β-AR stimulation or PKA activation, S2808 has been shown to be constitutively phosphorylated at high levels (>50% of maximum).[82] This raises the question of whether the observed PKA phosphorylation in HF is pathological. Indeed some groups have also failed to detect S2808 phosphorylation in HF,[83] and another group observed that both minimal and maximal S2808 phosphorylation achieved RyR2 activation, suggesting a "V" shaped curve of dependence.[84] Despite the extensive body of work by Marks' group,[77,79,80] it remains a concern that groups using similar models cannot replicate and validate their results. Further studies are required to understand the role of PKA S2808 phosphorylation and its functional effect on RyR2 P_o.

Serine 2814. Wehrens et al. demonstrated that CaMKII specifically phosphorylates S2814.[79] The implications of this were investigated with knock-in mice, RyR2-2814 (S2814D), mimicking constitutive phosphorylation. Increases in Ca^{2+} transients were similar between S2814D mice and WT mice with CaMKII activation, suggesting S2814 was the principle site of CaMKII-mediated phosphorylation. This was confirmed by the inhibition of CaMKII with KN-93, preventing phosphorylation in WT and reducing Ca^{2+} transients, but this had no effect in S2814D mice. The alterations in Ca^{2+} signaling were associated with increased Ca^{2+} diastolic leak, ventricular arrhythmias, and the development of HF.[85] Uchinoumi et al. sought to uncover the mechanism for this increased Ca^{2+} leak.[86] They used fluorescence resonance energy transfer (FRET) to measure RyR2-bound CaM and binding of fluoroescent-DPc10 (F-DPc10) peptide to prove conformational change. DPc10 is in the RyR2 CPVT mutational hotspot. It is linked to domain unzipping and pathological Ca^{2+} leak observed in CPVT and HF, which dantrolene can reverse.[87] WT mice activated by CaMKII and phophomimetic RyR2-S2814D knock-in mice displayed increased diastolic Ca^{2+} leak, reduced CaM−RyR2 affinity, and an unzipped (pathological) RyR2 conformational change as evidenced by increased F-DPc10 access. These same observations were revealed in myocytes of rabbits with HF, induced by a nonischemic model, which had elevated CaMKII activity and S2814 phosphorylation. This suggests HF can activate CaMKII to phosphorylate and induce a common pathological (unzipped) conformational change in RyR2, characterized by reduced CaM−RyR2 affinity and increased diastolic Ca^{2+} leak.[86] This conformational change was overcome by dantrolene which binds amino acids 601−620 of RyR2, stabilizing the channel gating in a closed state. The effect of β-AR on CaMKII requires further understanding, but in transgenic mice, with ablation of S2814, spontaneous Ca^{2+} waves were reduced, and they were protected against catecholaminergic-induced arrhythmias.[82] The effect of CaMKII phosphorylation of S2814 has produced more consistent and reproducible observations than S2808, but it remains debated as to which one is more functionally important.

Serine 2030. Serine 2030 has been championed by Xiao et al.[88] Using site-phosphor-specific antibodies, they observed S2030, not S2808, was the major PKA site responding to β-AR stimulation. At basal conditions, before β-AR stimulation, S2030 was not phosphorylated, while S2808 was. They also observed increased spontaneous Ca^{2+} waves suggesting phosphorylation of S2030 was the critical event for stress-induced arrhythmia. In resting conditions, S2030 appears to be unphosphorylated, however we have not defined its role in Ca^{2+} signaling in healthy cardiomyocytes, let alone HF.[26]

Multi-Phosphosite Model. Our current models on phosphorylation are simplistic with most evidence supporting activation of RyR2 from PKA phosphorylation of S2808, dissociating FKBP12.6 from the channel complex and CaMKII by phosphorylation of S2814. This model does not consider the effect of S2030, nor does it recognize other undiscovered RyR2 phosphorylation sites.[82] They have also been discussed as mutually exclusive events, but it is perfectly conceivable that this is not absolute. Structural models reveal S2808 and S2814 to be co-located on the "phosphorylation hotspot", a prominent and exposed epitope of the helical domain.[82] Phosphorylation maintains a more flexible configuration, favoring an open state conducive with diastolic Ca^{2+} leak. This provides an interesting question about the current models. Are the effects observed as simple as one kinase phosphorylating one site producing one effect? The sites in the

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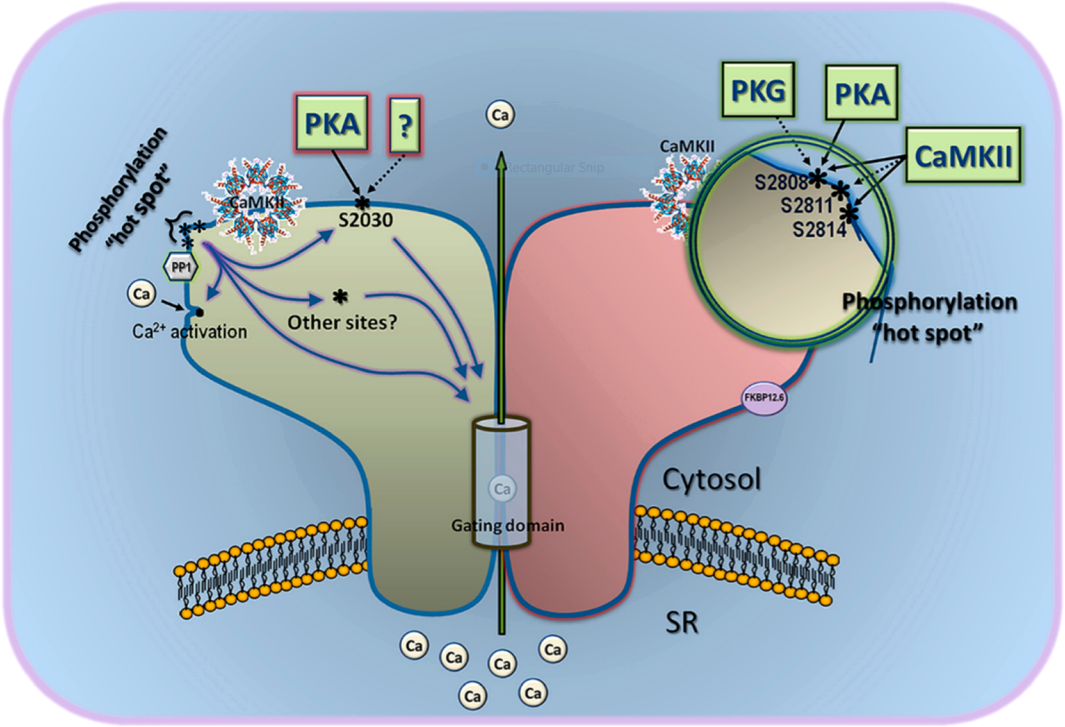

Figure 7. RyR2 phosphorylation sites. Depiction of the three phosphorylation sites targeted by several kinases on RyR2. S2808 and S2814 are located in the "phosphorylation hotspot", an exposed protruding epitope on RyR2. There may still be sites to be discovered here (i.e., S2811). S2030 is shown as separate from the hotspot. These sites may work independently or together by differential regulation of PKA and CaMKII. The effect on channel gating may be direct or indirect via interaction with classical Ca^{2+} activation sites. Reproduced with permission from ref 82. Copyright 2014 Camors et al.

phosphorylation hotspot may indeed have the same end effect, due to their location, but they may also provide redundancy and a graded response dependent on sequential phosphorylation. The integrated effect of S2030 may explain the distinct effects of CaMKII and PKA on the phosphorylation hotspot, providing a "multiphosphosite model" (Figure 7). Of course, there may be other undiscovered sites which may affect these mechanisms. While recognizing these weaknesses in the models, there are also differences in experimental conditions which may explain divergent results. RyR2 function is dependent on SR Ca^{2+} content and local Ca^{2+} transients. No one unified method has been utilized to control this, which complicates the interpretation of phosphorylation effects. Second, in intact organelle systems, there are competing intracellular signaling cascades and protective mechanisms reversing phosphorylation to mitigate their effects. β-AR stimulation affects multiple signaling cascades confounding the interpretation of specific β-AR activated PKA events. LTCC can undergo phosphorylation by both CaMKII and PKA, increasing the $I_{Ca,L}$ which may activate RyR2, and as previously illustrated, SERCA2a activity is amplified by PLN phosphorylation which increases following β-AR stimulation.

This review focuses on cardiac stress induced by phosphorylation from chronic β-AR stimulation, thiol modifications from oxidative stress, and how they modulate the RyR2 to produce an arrhythmogenic phenotype. We note that there is evidence that RyR2 phosphorylation is modulated by PKG[89] and through activation of muscarinic (M2 and M3) receptors.[90] In murine ventricular myocytes and canine failing ventricular myocytes, the nonselective muscarinic receptor agonist carbachol increased phosphorylation of S2808 but reduced phosphorylation of S2814. These effects were attributed to to M2-PKG phosphorylation of S2808 and M3-mediated reduction in reactive oxygen species which in turn reduced CaMKII phosphorylation of S2814.[90] The net effect was that S2808 phosphorylation increased fractional Ca^{2+} release from SR and that S2814 dephosphorylation mediated decrease in diastolic Ca^{2+} leak.[90]

Redox Balance. Oxidative stress has been observed in the myocardium and plasma of patients with HF. Oxidative stress describes a pathologically mediated redox imbalance, consequential to a diminished antioxidant pool caused by consumption and reduced generation.[76] There are several sources of ROS/RNS in HF (mitochondria, NADPH oxidase, xanthine oxidase, and uncoupled NOS), but their relative contributions remain unknown. Kohlhass et al. demonstrated how disturbed Ca^{2+} and Na^{+} handling affects mitochondrial ROS generation. Failing myocytes consume increasing amounts of ATP. There is an increase in electron leak from the electron transport chain of mitochondria which either gets oxidized by NADPH or forms $O_2^{-}\bullet$. In order to maintain homeostasis, an increase in NADPH production is required. However, reduced cytosolic Ca^{2+} transients and increased intracellular $[Na^{+}]$ in HF results in mitochondrial Ca^{2+} efflux via mitochondrial NCX. Reduced mitochondrial Ca^{2+} hampers the activation of Kreb cycle dehydrogenases, hampering regeneration of NADPH. Consequently, failing myocytes display pronounced oxidation of NADPH to $NADP^{+}$ and increased mitochondrial H_2O_2, worsening oxidative stress.[91] As HF progresses, oxidative stress worsens, perpetuating a deleterious cycle. Ca^{2+} handling is redox-sensitive. In failing myocytes, ROS have been implicated in pathological SR Ca^{2+} leak from both direct changes to ion channel activity and

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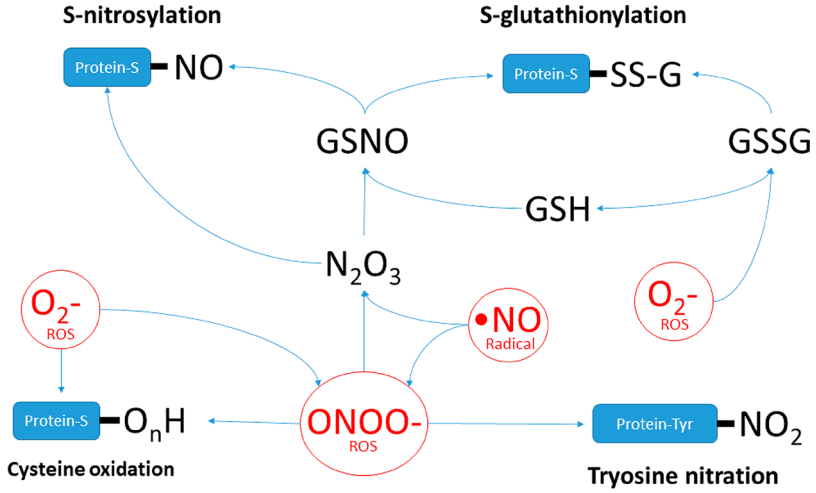

Figure 8. ROS/RNS induced post-translational modifications. Diagram depicts the chemical relationships between different ROS and RNS and how they impact post-translational modifications. GSNO: *S*-nitrosoglutathione. GSSG: oxidized glutathione.

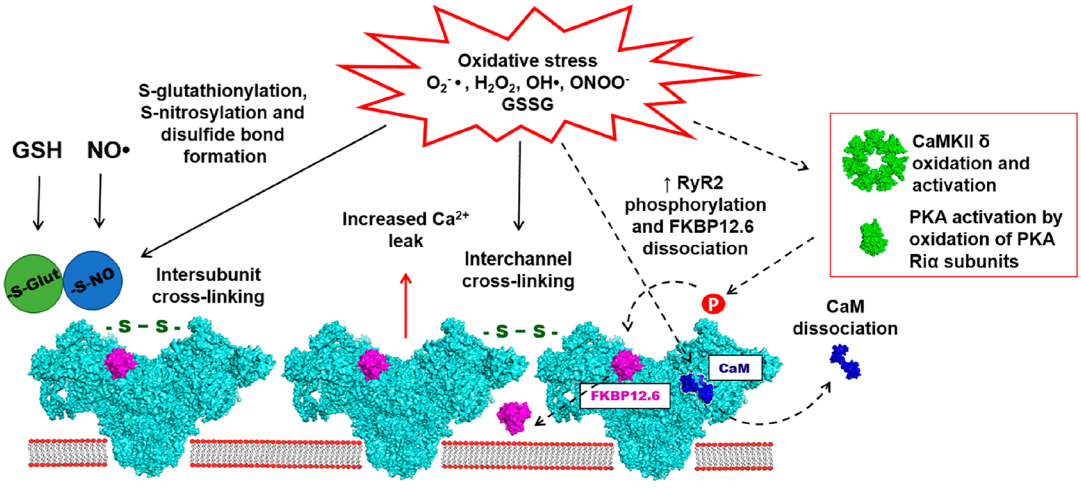

Figure 9. Direct and indirect effects of redox dependent post translation modifications of cardiac RyR2.ROS/RNS induce direct modifications by *S*-glutathionylation, *S*-nitrosylation, and disulfide bond formation on RyR2 (solid arrows). Indirect effects are communicated by the activation of PKA and CaMKII and dissociation of the regulatory ligand CaM (dashed arrows). The principal effect is increased RyR2 P_o causing pathological SR diastolic Ca^{2+} leak. Reproduced with permission from ref 70. Copyright 2018 Nikolaienko et al.

indirect by amplifying redox-regulated protein kinase activity.[70,76]

Redox Regulation of RyR2. The RyR2 is highly redox sensitive, due to the presence of 89 cysteine residues (with thiol side chains) per monomer, with 21 of these found "free" of thiol modification.[92] ROS/RNS oxidize vulnerable cysteine residue thiol side chains, producing sulfenic, sulfinic, and sulfonic acids. These groups can then react to produce *S*-nitrosylation, *S*-glutathionylation, and disulfide bond formation altering the channels P_o (Figure 8).[70] Generally, the evidence supports activation of RyR2 following cysteine oxidation, but results have been heterogeneous relating to differences in conditions and experimental models used.

Initial models with rat cardiomyocytes revealed concentrations of 1 μM H_2O_2 had no effect on cytosolic Ca^{2+}, while 100 μM H_2O_2 caused a marked and sustained increase in cytosolic Ca^{2+}.[93] Guinea pig models showed a biphasic response with an initial increase followed by suppression of cellular Ca^{2+} at 5 min to 1 mM H_2O_2.[94] Jones et al. explored this relationship, observing that oxidation caused a concentration and time-dependent effect on the threshold for SOICR.[95] Following an initial reduction in threshold for SOICR, prolonged exposure increased the threshold, represented by an abrupt loss in SOICR events. They exposed HEK293 cells expressing RyR2 to supraphysiological amounts of H_2O_2 (0.01−1 mM). Molecular models suggest thiol groups present in the channel pore and transmembrane domains have different threshold levels for oxidation, with less reactive thiols inducing a delayed inhibition of RyR2, explaining this biphasic response.[95] Importantly, the supraphysiological H_2O_2 levels in these experimental models are unlikely to represent the lower levels of pathological oxidative stress induced by HF. This would account for the decrease in release threshold observed in HF models, representing milder oxidizing conditions. In canine models of tachycardia-paced-mediated HF, single RyR2 channel activity was significantly higher than control. They observed a rightward shift in the ratio of reduced and oxidized glutathione which correlated with a reduction in free thiols on RyR2, consistent with redox imbalance. The increased P_o resulted from an increase in sensitivity of RyR2 to SR luminal Ca^{2+}, linking redox modifications to reduced threshold for SOICR to occur. This effect was partially reversed in the

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presence of reducing agents, while oxidation of controls reproduced the HF phenotype and luminal Ca^{2+} sensitivity.[96] This incomplete reversal could be explained by permanent oxidative modifications or synergistic post-translational effects like phosphorylation. Lastly, RyR2 exists in a basal oxidized state, the degree of which is unknown. Using an artificial bilayer model, Hanna et al. noticed that RyR2 requires mild oxidizing conditions to mimic the healthy in situ state.[97] They observed that in reduced conditions, the P_o of RyR2 did not increase with rising luminal $[Ca^{2+}]$, as would be expected in healthy myocardium, but was corrected in mildly oxidizing states. Compared to lipid bilayer models, the junctional microdomain of intact organelles may create an enclosed microenvironment, where an oxidizing redox potential may be maintained by NADPH oxidase NOS2 and not reflect the reduced global environment of the cytoplasm. Unfortunately, it has proven challenging to characterize the sites of modifications. In RyR1, cysteine 3635 is found in the CaM binding site and can undergo functionally significant *S*-nitrosylation. Despite RyR1 and RyR2 displaying ~70% homology, the corresponding hyper-reactive cysteines found in RyR2 (cysteine 3602) have revealed inconsistent results with Ca^{2+} handling. While it did not affect CaM regulation of RyR2, it did suppress SOICR and was an important determinant of the activation and termination of Ca^{2+} release.[98]

Taken altogether, redox modifications by ROS consistently cause RyR2 Ca^{2+} mishandling which are time- and concentration-dependent.[70] The cysteine residues that confer significant functional change have yet to be defined. HF induces chronic increases in ROS which may account for the observed increased RyR2 activity. These ex vivo models, using isolated myocytes, have inherent limitations. Exogenous models cause a global increase in ROS; however, free radicals are highly active diffusing only nanometers. This suggests endogenous systems compartmentalize, creating microenvironments where ROS have local effects on closely apposed targets. Furthermore, these models do not fully reflect the dynamic redox environment present in intact organelles, nor do they measure the integrative effect of competing intracellular signaling cascades and modulating proteins that regulate RyR2 activity which may also undergo redox modulation (Figure 9).

Redox Regulation of the RyR2 Junctional Complex. The altered association of accessory proteins within the RyR2 junctional complex may contribute and underpin redox-mediated changes in HF. The binding affinity of CaM to RyR2 is reduced when oxidized by H_2O_2, increasing diastolic Ca^{2+} leak. Oda et al. performed an elegant study of permeabilized rat ventricular myocytes.[39] H_2O_2 (50 μM) reduced the content of free thiols on RyR2, decreased CaM−RyR2 binding but did not alter FKBP12.6−RyR2 association. They confirmed increased DPc10 association indicating domain unzipping. When evaluating the relative contributions, 60% was from H_2O_2 changes on RyR2, and 40% was from effects on CaM, suggesting a combined mechanism for RyR2 domain unzipping. The conformational change was reversed with dantrolene and restored CaM−RyR2 binding. The study controlled for the effects of CaMKII with a direct inhibitor, KN-93. Redox reactions activate CaMKII in a fashion similar to phosphorylation, by oxidation of methionine residues 281/282 found in the regulatory domain, preserving kinase activity. While models of ROS and CaMKII produce phenotypically similar effects of RyR2 activation, the contribution of redox-activated CaMKII is still not understood. H_2O_2-activated CaMKII can contribute to arrhythmogenesis which is reversed by KN-93 inhibition. Rabbit ventricular myocytes had greater potential for EADs from impairment of late Na+ current inactivation reducing repolarization reserve.[99] Finally, PKA is activated independent of cAMP by oxidation. Cysteines found within the regulatory subunits of PKA are oxidized leading to their dissociation.[70] In a study examining RyR2 dissociation from FKBP12.6, oxidation was not shown to directly effect RyR2-FKBP12.6 binding; instead, its effect was indirect via phosphorylation.[77] In SR vesicles, H_2O_2 alone or in combination with CaMKII had no effect on FKBP12.6 dissociation from RyR2. However, RyR2 oxidation with PKA caused almost complete FKBP12.6 dissociation. It is important to realize that concurrent hyper-phosphorylation is present in studies examining RyR2 redox modifications and effects were not always evaluated.

These studies highlight the indirect effects of redox modifications on the RyR2 activity. Lipid bilayer experiments using purified RyR2 do not mimic the dynamic and competing signaling cascades present in cellular systems, nor do they recreate the microenvironment of the couplon. Key Ca^{2+} and Na^+ channels that are sensitive to redox regulation contribute to the local ionic environment affecting RyR2 P_o. Direct ROS effects on RyR2 are most likely a local effect, a consequence of low diffusion capacity, while protein kinases provide a mechanism by which ROS can translate their effect across the cell. These inherent limitations may contribute to the disappointing results obtained when translating findings to an intact organelle system.

Redox Regulation of SERCA2a and NCX. SERCA2a contains 25 cysteine residues, although only 1 or 2 have shown functional importance. By exposing permeabilized myocytes to NADH, Zima et al. found SR Ca^{2+} was reduced by >50% with associated reduction in SERCA2a activity. This was attenuated by the addition of superoxide dismutase, a reducing agent.[100] The mechanism is unclear, but oxidation denatures the SERCA2a ATP-binding site, reducing its activity.[101] This inhibitory effect is offset, indirectly, by the phosphorylation of PLN by redox-activated PKA and CaMKII leading to stimulation of SERCA2a activity. The predominant effect of ROS/RNS on SERCA2a is unknown, but likely to be negative, and may depend on the time and concentration of exposure and the predominant kinase that mediate indirect ROS/RNS effects.[76] NCX contains cysteine residues in functional significant domains. H_2O_2 and $O_2^{-}\bullet$ have been shown to activate NCX but results have been inconsistent, relating to concentration and source.[102] The α1 subunit of the LTCC contains over 10 cysteines which can be the subject of redox modifications. Like SERCA2a, direct oxidation appears to inhibit inward $I_{Ca,L}$, while CaMKII and PKA increase it.[76,102]

S-Nitrosylation of RyR2. NO· exerts its effects via two mechanisms. First, NO· binds guanylate cyclase stimulating cGMP production which allosterically activates phosphodiesterase-2 which decreases the levels of cAMP reducing PKA activity. Second, NO· may transfer to a nucleophilic thiol group, a process called *S*-nitrosylation. For this to occur, thiol groups must first be oxidized. The cystine residues involved in NO-mediated RyR2 modulation are not well understood. In skeletal muscle RyR1, *S*-nitrosylation of Cys3635 activates the channel, and the effect was abolished with the expression of a C3635A mutant.[103] The corresponding RyR2 Cys3602 was not required for activation.[102] The effect of *S*-nitrosylation on

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gating properties is controversial and likely relates to experimental conditions: using endogenous or exogenous NO·, the concentration of NO·, and cytosolic environment. Purified canine RyR2 incorporated into proteoliposomes revealed increased Ca^{2+} transients with SIN-1, a NO·/$ONOO^{-}$● donor. As the number of nitrosylated thiol groups increased, progressive activation was noted. The use of dithiothreitol, a reducing agent, reversed the number of NO groups and activation.[92] Studies using an endogenous source have utilized NOS1, found in the SR near RyR2, which induces *S*-nitrosylation. RyR2 of $NOS1^{-/-}$ mice had reduced nitrosylation, but there was also a measurable increase in ROS and concomitant thiol oxidation. Cardiomyocytes displayed reduced SR Ca^{2+} content and marked diastolic Ca^{2+} leak, generating proarrhythmic Ca^{2+} waves.[104] The isolated cardiomyocytes were paced at 4 Hz and displayed a HF phenotype with reduced contractility. Wang et al. challenged this view, observing hyponitrosylation and reduced SR Ca^{2+} content with NOS1 deficiency.[105] The RyR2 P_o was reduced as was the frequency of SR Ca^{2+} sparks. Here cardiomyocytes were placed in lipid bilayers and observed under basal conditions. The difference in physiological state (paced vs quiescence) and the additional oxidative effects recorded in the first experiment may explain the discrepancy in results. Elevated ROS are a hallmark of HF, and in normal physiology, the balance in activity of nitric oxide and ROS is tightly coupled, coined the nitroso-redox balance. Cutler et al. set out to clarify this relationship using paced whole-heart studies of Langendorff perfused guinea pigs.[106] Reduced NOS1 activity was associated with significant nitroso-redox imbalance. There was reduced *S*-nitrosylation and increased oxidation of RyR2 with a decrease in RyR2 thiol content. Isolated myocytes exhibited an increase in spontaneous Ca^{2+} release which, unlike control, were enough to mediate triggered arrhythmic beats. The nitroso-redox balance is tightly controlled in healthy hearts. Physiological levels of *S*-nitrosylation may be required for normal RyR2 functioning, reducing RyR2 P_o. In an environment of oxidative stress, *S*-nitrosylation may be protective, preventing oxidation of reactive thiols and overactivation.[70] However, the experiments by Cutler et al. revealed further insights. First, the arrhythmic events only occurred under conditions of elevated intracellular Ca^{2+} and were not appreciated under normal intracellular Ca^{2+} conditions.[106] Second, the arrhythmic phenotype was compounded by the addition of H_2O_2, producing an increase in sustained ventricular arrhythmias of ∼150%, compared to NOS1 inhibition or H_2O_2 alone. Initially, greater nitroso-redox imbalance was thought to underlie this, but no significant difference in RyR2 oxidative state was appreciated. Instead, there was an increase in peak LTCC current which can further increase intracellular Ca^{2+}. Together this suggests that nitroredox imbalance may not, on its own, account for arrhythmogenesis but increase the potential, and it is the integrated effects of multiple pathways that together, result in arrhythmogenesis.

COMBINING THE MODELS

In its simplest form, HF creates a stressed environment promoting pathological diastolic Ca^{2+} leak and reducing SR lumen $[Ca^{2+}]$ and systolic Ca^{2+} release, causing reduced contractility. The increased cytosolic $[Ca^{2+}]$ promotes NCX, which HF upregulates, to extrude Ca^{2+} causing a net sarcolemmal depolarization and DAD generation (Figure 10). One of the fundamental challenges scientists face is translating how an experimental model, usually in its purest form (single cell), performs within the complex dynamics of competing cascade signaling of an intact organism. Unfortunately, many models fail to mimic the combined effects of these post-translational modifications in human cardiomyocytes with HF.

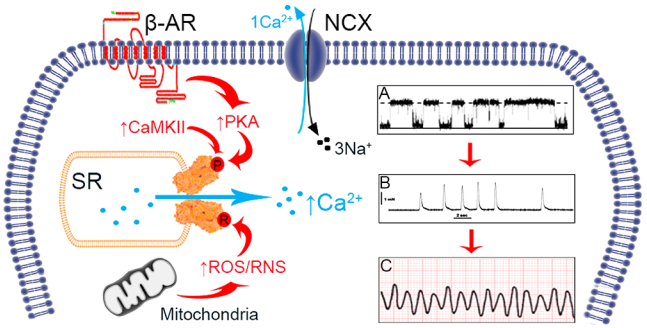

Figure 10. Stress-induced mechanisms of arrythmia. In HF, there is chronic *β*-AR activity and increased ROS/RNS generation. Vulnerable residues on RyR2 are susceptible to phosphorylation and redox modifications, which increase RyR2 P_o (diagram A). In diastole there is pathological SR Ca^{2+} leak which can activate neighboring NCX, extruding Ca^{2+} causing sarcolemma depolarization; the generation of DADs and spontaneous contractions (diagram B). Ultimately this may degenerate into ventricular arrythmia (diagram C) and SCD.

HF leads to a progressive destabilization of RyR2 function. Initial phospho-modulating effects predominate, partially driven by *β*-AR activity, with late thiol oxidative effects in advanced HF. RyR2 exhibits a progressive loss in ability to become refractory to luminal SR $[Ca^{2+}]$, and the potential for spontaneous release occurs at lower luminal Ca^{2+} content.[107] The progression of HF parallels the worsening potential for diastolic Ca^{2+} leak, Ca^{2+} waves, and DADs. Walweel et al. investigated these combined effects in an elegant study using healthy and failing human hearts.[108] S2808 and S2814 were hyperphosphorylated implicating both CaMKII and PKA pathways, and increased thiol modification was associated with reduced FBP12.6 affinity for RyR2 which, as discussed, may increase channel activity. Two further observations were made. First, phosphorylation and thiol oxidation were synergistic, significantly increasing the P_o. Second, within individual hearts there was heterogeneity in RyR2 activity. While normal hearts displayed 95% low-activity RyR2, failing hearts displayed 30%. Approximately 20% of RyR2 were not regulated by intracellular Ca^{2+}; consequently, in diastolic conditions they would be near maximally activated.[108] These differences may result from compartmentalization, leading to pockets of cells with higher degrees of post-translational modifications and chronic activation of regulatory proteins PKA and CaMKII. Graded sequential modifications may provide a unifying mechanism for gradual deterioration in contractile function and worsening arrhythmogenic potential.

Another issue scientists face is translating the observed single-cell events to the coordinated and sustained activity of myocytes at a ventricular level. Cutler et al. observed that the arrhythmic events produced by NOS1 inhibition only occurred with increased intracellular Ca^{2+}.[106] We now appreciate the negative cardiac remodeling of HF may predispose to abnormal electro-mechanical coupling and electrophysiological dysfunction due to changes in spatial temporal relationships. In

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rodents, HF disrupted the T tubule architecture, separating it from the SR luminal surface where RyR2 locates.[19,109] This was associated with a temporal delay in ECC and, in a large number of CRUs, prevented RyR2 activation via CICR.[110] The ultrastructure of the T tubule interface is important as it contains the extracellular space within the T tubule and both the cytosolic and junctional SR subspaces which exert local effects on the constituent ion channels.[23] RyR2 molecules that are uncoupled from sarcolemmal Ca^{2+} channels may amplify pathological Ca^{2+} release. By having a large population of available CRUs after phase 2 of an AP, a mechanism is provided whereby spontaneous Ca^{2+} release can produce a Ca^{2+} wave.[110,111] Cardiac resynchronization has been shown to restore T tubule architecture and reduce the burden of arrythmia in HF.[112]

HEART FAILURE MEDICATIONS

Inotropes. A 1986 review article on novel positive inotropic agents predicted the future of therapy for systolic HF.[113] They altered cardiac performance by targeting a common principle, altering cardiomyocyte handling of Ca^{2+}. After three decades, only digoxin remains in HF guidelines as a long-term agent, although it is a weak recommendation from evidence suggesting reduction in hospitalizations.[114] Digoxin, a cardiac glycoside, inhibits Na^+/K^+ ATPase increasing cytosolic Na^+. This influences the NCX driving force, thermodynamically favoring cytosolic Ca^{2+} accumulation improving.[115] In patients presenting in cardiogenic shock, with impaired end organ perfusion, conventional inotropic agents are recommended.[114] Catecholamines are direct β-AR agonists exploiting the $G_{\alpha s}$-protein−adenylyl cyclase−cAMP−protein kinase pathway. Milrinone, a phosphodiesterase-3 inhibitor, prevents cAMP hydrolysis increasing PKA activity. Because β-AR density is reduced in HF and many patients are prescribed long-term β-blockers, they were assumed to be more effective, but trials have been neutral with increased arrhythmic burden. Their unifying mechanism is the exploitation of Ca^{2+} handling, which is a dual-edged sword. Although improving contractile dynamics, catecholamines put further stress on failing cardiomyocytes with detrimental alterations to myocardial energetics and arrhythmic potential by mechanisms discussed. Clinical trials are at best neutral and at worst negative from increased arrhythmic death opposing their use in long-term management.[116] Novel agents are now being developed. Omecamtiv mecarbil is a first-in-class direct myosin activator targeting the myocardial machinery independent of Ca^{2+}.[117] Alternatively, we are discovering additional properties of current GDMT. The β_2-AR-selective (13-fold selective for β_2-AR vs β_1-AR) inverse antagonist/β-arrestin-biased agonist carvedilol[65,118,119] has proven positive effects over other β-blockers for the treatment of HF. Unlike other β-blockers, carvedilol displays an additional antioxidant effect.[120]

Carvedilol. The additional ROS-scavenging properties of carvedilol may explain its beneficial effect on HF and the prevention of ventricular arrhythmias over other β-blockers. In a canine model of pacing-induced HF, cells incubated with carvedilol had reduced ROS levels and restoration of defective interdomain interactions, promoting a stable zipped state, improved Ca^{2+} transients, and cell shortening.[120] They also exposed SR from normal hearts to SIN-1 leading to reduction of reactive thiols and defective interdomain interaction (unzipping) of the RyR2 with increased SR Ca^{2+} leakage. Once again, carvedilol restored normal interdomain interaction preventing defective RyR2 Ca^{2+} handling, thus improving myocyte function. This antioxidant effect occurred at low concentrations (30 nmol/L). Nonetheless, chronic administration of carvedilol was associated with reduced S2808 phosphorylation suggesting β-AR modulation, but the predominant effect was attributed to its antioxidant ability. A similar dose of metoprolol, eliciting an equivalent β-blocking effect, had no beneficial effect on cardiomyocyte function. To explain this, DPc10 was administered, abolishing carvedilols beneficial effects. As DPc10 induces domain unzipping without affecting PKA and FKBP12.6 phosphorylation, this added further weight that the primary action was via reducing oxidative stress. However, the poor translation of clinical effect with antioxidants suggest another mechanism may explain its positive effects in clinical trials. Zhou et al. compared 14 different β-blockers.[121] They found that only carvedilol suppressed SOICR by reducing the duration and increasing the frequency of RyR2 channel opening. SOICR can generate DADs, and this attribute may contribute to its favorable antiarrhythmic effects, independent of its antioxidant and β-AR antagonistic effects. However, supraphysiological concentrations (0.3−1 μM) were required to suppress SOICR, while the concentration required for β-blockade is only ∼1 nM. These studies used HEK293 cells, not human failing cardiomyocytes. It is therefore important to systematically assess the efficacy of carvedilol and establish whether the pleotropic effects (β-AR antagonism, ROS scavenging, and direct RyR2 modulation) act alone or in concert, protecting against the generation of malignant arrhythmias in human failing cardiomyocytes. Notably, the pharmacological properties of carvedilol also extend to β-arrestin-biased agonism through occupation of the orthosteric ligand-binding pocket of β_2-ARs[118,119] to cause G-protein-independent β_2-AR phosphorylation, internalization, EGFR transactivation, and ERK1/2 phosphorylation. In mouse cardiomyocytes, despite carvedilol exhibiting β-arrestin-biased agonism through the β_2-AR, it does not increase contractility, unlike the selective β_2-AR pepducin ICL1-9 which does through a Ca^{2+}-independent mechanism.[119] Gαi-protein stabilizes β_1-AR orthosteric bound carvedilol in a conformation that favors β-arrestin 1/2 signaling.[122] Interestingly, carvedilol does not recruit Gαi-protein to β_2-ARs, nor do other tested β-blockers (acebutolol, alprenolol, propranolol, metoprolol, and carazolol) recruit Gαi-protein to β_1-AR.[122] It is important to understand the significance of carvedilol-induced biased agonism in human heart and whether it confers beneficial outcomes in disease settings of HF and arrhythmogenesis.

Future Medicines Directly Targeting the Ryanodine Receptor. Chronic oxidative stress and phosphorylation have been identified to impart deleterious post-translational modifications of cardiac RyR2. They converge to produce excessive spontaneous diastolic SR Ca^{2+} leak facilitating arrhythmogenesis via DADs. Intuitively, medicines that target RyR2 to selectively inhibit diastolic SR Ca^{2+} leak could provide benefit to patients with HF at risk of ventricular arrhythmias.

Some drugs have already been shown to reduce diastolic RyR2 Ca^{2+} leak. Notably, dantrolene reduces diastolic Ca^{2+} leak in failing canine,[123] rabbit,[124] and human heart[125] but not corresponding species (canine, rabbit, and human) nonfailing heart.[124,125] Despite these promising results, the implementation of dantrolene into clinical practice is limited by adverse effects including hepatotoxicity. More recently, it was shown that another hydantoin compound, phenytoin, inhibits diastolic Ca^{2+} leak from human failing heart but not nonfailing

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heart.[125] Phenytoin (100 μM) caused 50% reduction in P_o of RyR2 channels from ventricle of HF patients with IHD but had no effect on RyR2 channels from nonfailing hearts under diastolic (cytoplasmic Ca^{2+} 100 nM) conditions.[125] In complementary experiments in sheep ventricle, it was shown that both phenytoin and dantrolene reduce mean RyR2 channel open time, increase RyR2 closed time, but have no effect on RyR2 P_o at systolic Ca^{2+} concentrations (Ca^{2+} 100 μM).[125] It is important that therapies do not depress systolic function, especially in the compromised failing heart. Interestingly, phenytoin and dantrolene do not compete for the same binding site on RyR2.[125] Furthermore, inhibition by dantrolene but not phenytoin was dependent on the presence of calmodulin.[125] These studies suggest phenytoin and dantrolene can stabilize the remodeled RyR2 by restoring the "zipped" configuration of the N-terminal and central domains to maintain the closed state of RyR2 during diastole. At least two separate sites for phenytoin and dantrolene may be targeted with the same effect. Dantrolene binds to domain 601−620 of RyR2,[123,126] but the specific phenytoin-binding site is not yet known.[125] Phenytoin is currently indicated for epilepsy and status epilepticus. It is estimated the concentrations of phenytoin required to stabilize RyR2 are approximately 1/2 log unit less than those used clinically.[125] The repurposing of phenytoin may provide an opportunity to cautiously test RyR2 inhibition in HF patients. Interestingly, phenytoin also has class IB antiarrhythmic properties but has been replaced for clinical use by other medicines with lower toxicity profiles.[127] The development of high-throughput screens may expedite the discovery of high-affinity (nM), selective RyR2 diastolic Ca^{2+} inhibitors[128] for testing in the human failing heart.

Gene Therapy. The novel mechanism of gene transfer is being studied by the Calcium Upregulation by Percutaneous Administration of Gene Therapy in Cardiac Disease (CUPID) investigators.[129,130] Adeno-associated viruses (AAV) are utilized as vectors to deliver and transfer known genes of interest to cardiomyocytes. SERCA2a is perhaps the first of many to be tested in the clinical realm. In animals, SERCA2a gene transfer resulted in reverse left ventricular remodeling, improved survival, and normalized Ca^{2+} handling.[131] The antiarrhythmic properties may originate from a reduction in Ca^{2+} alternans which protects from re-entrant excitation by regional AP duration gradients.[132] The CUPID 1 trial performed a single intracoronary infusion of recombinant AAV serotype 1 in patients with advanced HF. At 12 months, there were no safety concerns, and gene therapy reduced HF event rates.[130] Unfortunately, the phase 2b CUPID 2 trial of 250 HF patients revealed no evidence of improved outcomes.[129] This was despite the entry requirements and treatment algorithms being similar. The mechanism of delivery optimizes vector uptake. Intracoronary delivery is practical, while other forms, such as myocardial injection, would be invasive and negatively affect the risk/benefit ratio. Perhaps counterintuitive, an increased ratio of empty capsid particles to AAV viral capsid particles may increase gene transfer as empty capsid particles may act to neutralize antibodies.[133] Is affecting one gene enough, especially in the context of advanced HF, whereby, irrespective of the success in gene therapy delivery, it will remain insufficient to alter the trajectory of disease? Importantly, the two CUPID trials have shown SERCA2a gene therapy and its delivery to be safe. Gene therapy remains a promising field for development, and other proteins could be targeted in the future to reduce SCD in patients with HF.

CONCLUSIONS

HF induces a state of increased sympathetic tone and redox imbalance. The pathognomonic feature of HFrEF, impaired contractility, and the generation of fatal arrhythmias share a common mechanism, deleterious intracellular Ca^{2+} signaling. Experimental models researching redox modifications and phosphorylation have thrown light on how post-translational modifications affect Ca^{2+} and Na^{+} cycling proteins within the T tubule microdomain and terminal cisternae of the SR. Much interest has surrounded the RyR2 macromolecular complex. Increased phosphorylation and oxidation are associated with increased diastolic activity and pathological RyR2 Ca^{2+} handling which is recognized as a key component of the arrhythmic phenotype in HF. Currently, the causative molecular changes remain controversial and undefined. Current experimental models have been limited in dissecting the individual and synergistic contributions of RyR2 stress-induced modifications and how single-cell studies translate to ventricular arrythmias in the clinical realm. We are beginning to appreciate the complex interplay of competing cascade signaling and the importance of spatial temporal relationship, within microdomains, which compromise increased sympathetic tone, redox imbalance, and negative remodeling of HF. It follows that antiarrhythmic treatment will likely require the modification of multiple targets. First, the reversal of HF and promotion of positive remodeling is paramount to reduce both oxidative and phosphorylative stress. Second, by defining the molecular sites involved in arrhythmia genesis on RyR2 and other Na^{+} and Ca^{2+} cyclic proteins associated within the calcium release units, novel medication targets can be developed to work synergistically and reduce the rate of sudden cardiac death in heart failure.

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Notes

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LIST OF ABBREVIATIONS

AAV = Adeno-associated viruses
AP = Action potential
Ca^{2+} = Calcium
CRU = Ca^{2+} release units
CaM = Calmodulin
CaMKII = Ca^{2+}/calmodulin-dependent protein kinase II
CASQ2 = Calsequestrin 2
CICR = Calcium-induced calcium release
CPVT = Catecholaminergic polymorphic ventricular tachycardia
DAD = Delayed after depolarization
ECC = Excitation−contraction coupling
E_m = Membrane potential
F-DPc10 = Fluoroescent-DPc10
FRET = Fluorescence resonance energy transfer
GDMT = Guideline-directed medical therapy
GSH = Reduced glutathione
GSNO = *S*-Nitrosoglutathione
GSSG = Oxidized glutathione
HF = Heart failure
HFrEF = Heart failure with reduced ejection fraction
$I_{Ca,L}$ = Inward Ca^{2+} current (LTCC)
ICD = Intracardiac defibrillators
IHD = Ischemeic heart disease
I_{Na} = Inward sodium current
K^+ = Potassium
FKBP12.6 = 12.6 kDa FK506-binding protein
LTCC = L-Type calcium channels
LVF = Left ventricular function
Na^+ = Sodium
Nav = Voltage-gated Na^+
NCX = Na^+/Ca^{2+} exchanger
NO· = Nitric oxide
NOS = Nitric oxide synthase
O_2· = Superoxide anions
OH· = Hydroxyl radicals
ONOO· = Peroxynitrate
PKA = Protein kinase A
PLN = Phospholamban
P_o = Open probability
ROS/RNS = Reactive oxygen species/reactive nitrogen species
RyR2 = Ryanodine receptor
SCD = Sudden cardiac death
SERCA2a = Sarcoplasmic reticulum Ca^{2+}-ATPase
SLN = Sarcolipin
SOD = Superoxide dismutase
SOICR = Store-overload-induced Ca^{2+} release
SR = Sarcoplasmic reticulum
Tm = Tropomyosin
TnC = Troponin C
TnI = Troponin I
T tubules = Transverse tubules
WT = Wild-type
β-AR = β-Adrenoceptor

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