Calcium cycling proteins and heart failure: mechanisms and therapeutics

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Ca²⁺-dependent signaling is highly regulated in cardiomyocytes and determines the force of cardiac muscle contraction. Ca²⁺ cycling refers to the release and reuptake of intracellular Ca²⁺ that drives muscle contraction and relaxation. In failing hearts, Ca²⁺ cycling is profoundly altered, resulting in impaired contractility and fatal cardiac arrhythmias. The key defects in Ca²⁺ cycling occur at the level of the sarcoplasmic reticulum (SR), a Ca²⁺ storage organelle in muscle. Defects in the regulation of Ca²⁺ cycling proteins including the ryanodine receptor 2, cardiac (RyR2)/Ca²⁺ release channel macromolecular complexes and the sarcoplasmic/endoplasmic reticulum Ca²⁺ ATPase 2a (SERCA2a)/phospholamban complex contribute to heart failure. RyR2s are oxidized, nitrosylated, and PKA hyperphosphorylated, resulting in “leaky” channels in failing hearts. These leaky RyR2s contribute to depletion of Ca²⁺ from the SR, and the leaking Ca²⁺ depolarizes cardiomyocytes and triggers fatal arrhythmias. SERCA2a is downregulated and phospholamban is hypophosphorylated in failing hearts, resulting in impaired SR Ca²⁺ reuptake that conspires with leaky RyR2 to deplete SR Ca²⁺. Two new therapeutic strategies for heart failure (HF) are now being tested in clinical trials: (a) fixing the leak in RyR2 channels with a novel class of Ca²⁺-release channel stabilizers called Rycals and (b) increasing expression of SERCA2a to improve SR Ca²⁺ reuptake with viral-mediated gene therapy.

There are many potential opportunities for additional mechanism-based therapeutics involving the machinery that regulates Ca²⁺ cycling in the heart.

Excitation-contraction coupling

With each beat of the heart, Ca²⁺ is released from the sarcoplasmic reticulum (SR) via the ryanodine receptor 2, cardiac (RyR2), raising the cytosolic Ca²⁺ concentration about ten-fold (~1 μM) and activating cardiac muscle contraction (Figure 1). The Ca²⁺ is then pumped back into the SR by the sarcoplasmic/endoplasmic reticulum Ca²⁺ ATPase 2a (SERCA2a), lowering the cytosolic Ca²⁺ concentration to baseline levels (~100 nM) and causing relaxation. The Ca²⁺ release and reuptake cycle is initiated by the action potential, an electrical signal that depolarizes the plasma membrane and the specialized invagination called the transverse tubule (T tubule). Voltage-gated Ca²⁺ channels on the T tubule are activated by depolarization and allow a small amount of Ca²⁺ to run down its concentration gradient from mM external Ca²⁺ concentration to nM internal Ca²⁺ concentration. This entering Ca²⁺ binds to and activates RyR2 channels, which release Ca²⁺ stored at high concentration (in the millimolar range) in the SR. The Ca²⁺ binds to troponin C, allowing actin-myosin cross-bridging and the thick and thin filaments of the sarcomere to slide past each other, shortening the sarcomere and causing cardiac muscle contraction.

Heart failure

Heart failure (HF) is the leading cause of mortality and morbidity in developed countries. The incidence of HF continues to increase after age 65, affecting nearly 1 in 100 individuals (1). This is despite substantial advances in the care of patients, brought about by coronary care units and the development of devices for the treatment of HF including biventricular pacing and left ventricular assist devices (LVADs) (2). The most common cause of HF in developed countries is atherosclerosis and concomitant ischemic heart disease. Other causes include hypertension (which leads to hyper trophy that can degenerate to dilated cardiomyopathy and HF), dilated non-ischemic cardiomyopathies, and much rarer genetic causes. While HF initially involves the myocardium, resulting in decreased cardiac performance, it rapidly affects multiple organs including, most prominently, the neurohormonal, circulatory, and renal systems. Indeed, patients with HF have chronic activation of the sympathetic nervous system, which results in a maladaptive attempt to improve cardiac function. Moreover, β-adrenergic agonists or phosphodiesterase (PDE) inhibitors do increase contractility by increasing cAMP and increasing Ca²⁺ release, but they also increase mortality. In fact, blocking neurohormonal pathways is the focus of current HF therapy, and while this improves survival, it is limited by side effects and the requirement to titrate drugs to physiological parameters such as heart rate (HR) and blood pressure (3). Additional current therapies are aimed at reducing the symptoms of HF (e.g., diuretics for pulmonary and peripheral congestion), but they do not inhibit HF progression.

The search for novel therapeutics for HF has led investigators to examine the mechanisms underlying HF with the hope that this approach will uncover potential therapeutic targets to slow HF progression, improve quality of life, and reduce mortality.

Much attention has been paid to understanding the role of defects in Ca²⁺ regulation in HF (4). This is because, as noted above, Ca²⁺ is the signal that regulates cardiac muscle contraction. Cardiac contractility is determined by the amplitude and kinetics of Ca²⁺ cycling, which in turn are regulated by phosphorylation and dephosphorylation of key proteins involved in excitation-contraction (EC) coupling by kinases and phosphatases. Stress-induced activation of G protein-coupled β-adrenergic receptors (β-ARs) activates adenyl cyclase, which in turn leads to cAMP production and PKA activation. PKA phosphorylates many proteins in cardiac muscle, including those

Conflict of interest: Andrew R. Marks is a consultant for and owns shares in ARMGO Pharma Inc., a start-up company developing RyR-targeted therapeutics.

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involved in EC coupling: the L-type Ca$^{2+}$ channel, RyR2, and phospholamban (Figure 2), as well as troponin I, myosin binding protein C, and protein phosphatase inhibitor-1. PKA phosphorylation of these proteins (e.g., RyR2) results in increased HR as well as increased amplitude and velocity of Ca$^{2+}$ release and reuptake and enhanced contractility (5). Whereas PKA is the “on switch” for cardiac HR and contractility, the off switches are the protein phosphatases, including PP1 and PP2A, as well as PDE4D3, which hydrolyzes cAMP (6).

It is now generally accepted that defective SR Ca$^{2+}$ handling plays an important role in HF pathophysiology (7). This defective SR Ca$^{2+}$ handling is characterized chiefly by leaky RyR2 channels, due to stress-induced dissociation of the stabilizing RyR2 subunit calstabin2 (also known as FKBP12.6) resulting in a diastolic SR Ca$^{2+}$ leak, reduced SR Ca$^{2+}$ content, and decreased Ca$^{2+}$ transient (6–10). Compounding this problem is impaired SR Ca$^{2+}$ uptake due to reduced activity of SERCA2a, as a consequence of both reduced SERCA2a expression and increased inhibition of the pump by phospholamban (11). Thus, these two major players in cardiac EC coupling, the SR Ca$^{2+}$ uptake and SR Ca$^{2+}$ release channel (RyR2), are co-mitigators of the transient inward current (15).

Impaired SERCA2a function and enhanced Na$^{+}$/Ca$^{2+}$ exchanger (NCX) activity have been proposed as causes of reduced SR Ca$^{2+}$ load in HF (12). Moreover, aberrant diastolic release of SR Ca$^{2+}$ can generate a transient inward current that causes delayed afterdepolarizations (DADs) (10, 13).

The RyR2 leak model of HF is supported by studies demonstrating that: (a) β-adrenergic stimulation causes depletion of calstabin2 from the RyR2 complex (7); (b) HF patients have PKA-hyperphosphorylated and calstabin2-depleted RyR2 (16–23); and (c) patients whose cardiac function has been normalized by treatment with LVADs have reduced levels of circulating catecholamines (24) and reduced phosphorylation of RyR2 at Ser2809 (5). Indeed, improved cardiac function in patients treated with LVAD is associated with restoration of calstabin2 binding to RyR2 (5).

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The RyR2 leak is caused by stress-induced remodeling of the RyR2 macromolecular complex due to PKA hyperphosphorylation, nitrrosylation, and oxidation of the channel that results in depletion of calstabin2, phosphatases (25), and PDE4D3 (6) from the RyR2 channel in HF (7). Depletion of PDE4D3 and phosphatases results in elevated levels of cAMP at RyR2 (6) and a decreased rate of dephosphorylation of a hyperphosphorylated channel, promoting further PKA hyperphosphorylation (7, 25). The depletion of the channel subunit calstabin2 from the channel results in decreased systolic Ca$^{2+}$ transient amplitude secondary to reduced SR Ca$^{2+}$ stores was responsible for the decreased contractility and reduced cardiac output in HF (12). Moreover, aberrant diastolic release of SR Ca$^{2+}$ can generate a transient inward current that causes delayed afterdepolarizations (DADs) (10, 13).

Ca$^{2+}$ signaling defects in HF: leaky RyR2 channels, decreased SR Ca$^{2+}$ content, and reduced transients
Over 20 years ago, reports of reduced Ca$^{2+}$ transient amplitude, increased Ca$^{2+}$ transient duration, prolonged Ca$^{2+}$ transient decay time, and more recently reduced SR Ca$^{2+}$ load suggested that a
in destabilization of the channel closed state and the diastolic SR Ca\textsuperscript{2+} leak that reduce the SR Ca transient, resulting in impaired contractility and trigger fatal cardiac arrhythmias (5, 26). Importantly, the same stress-induced remodeling affects the skeletal muscle RyR1 complex and contributes to weakened skeletal muscle function and impaired exercise capacity in HF (27, 28).

The discovery of leaky RyR2 in failing hearts also provides a mechanism to explain the therapeutic efficacy of β-AR blockers in HF. Indeed, β-blockers inhibit PKA phosphorylation of Ser2808 in murine RyR2 (Ser2809 in human RyR2) by blocking β-ARs. The LTCC is also phosphorylated, and NCX expression is upregulated. An important contributor to impaired Ca\textsuperscript{2+} handling in HF is PKA hyperphosphorylation of RyR2. This leads to a higher sensitivity to Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release at low cytoplasmic Ca\textsuperscript{2+} concentrations, resulting in increased RyR2 open probability at low Ca\textsuperscript{2+} concentrations and a diastolic SR Ca\textsuperscript{2+} leak. The long-term effect of the diastolic Ca\textsuperscript{2+} leak is depletion of SR Ca\textsuperscript{2+} stores. SERCA2a expression and activity are decreased in HF, which is linked to phospholamban hypophosphorylation. In contrast, NCX expression and activity are upregulated in HF. Arrows indicate increased or decreased expression or activity in HF.

**Figure 2**

Defective Ca\textsuperscript{2+} handling in failing hearts due to sympathetic overactivity. Chronic activity of the sympathetic nervous system leads to phosphorylation of the β-AR, activation of β-AR kinase, and desensitization of β-ARs. The LTCC is also phosphorylated, and NCX expression is upregulated. An important contributor to impaired Ca\textsuperscript{2+} handling in HF is PKA hyperphosphorylation of RyR2. This leads to a higher sensitivity to Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release at low cytoplasmic Ca\textsuperscript{2+} concentrations, resulting in increased RyR2 open probability at low Ca\textsuperscript{2+} concentrations and a diastolic SR Ca\textsuperscript{2+} leak. The long-term effect of the diastolic Ca\textsuperscript{2+} leak is depletion of SR Ca\textsuperscript{2+} stores. SERCA2a expression and activity are decreased in HF, which is linked to phospholamban hypophosphorylation. In contrast, NCX expression and activity are upregulated in HF.

**Arrhythmias: lessons from catecholaminergic polymorphic VT**

Catecholaminergic polymorphic VT (CPVT) is a rare inherited form of exercise-induced sudden cardiac death (SCD) that occurs in individuals with structural normal hearts and normal ECGs. Mutations in RyR2 have been linked to CPVT (13, 34).

We originally reported that RyR2 CPVT mutations reduced the affinity for calstabin2, resulting in leaky channels during exercise (13). Treating mice with a Rykal (a novel class of drugs described below that prevent diastolic SR Ca\textsuperscript{2+} leak via RyR2 channels) has been shown to prevent exercise-induced depletion of calstabin2 from the RyR2 complex and reduced VT and SCD (9, 13). Calstabin2-deficient and haploinsufficient mice exhibit CPVT (13) but do not develop HF. Presumably the reason for the lack of cardiac dysfunction is that, in contrast to the post-MI model, in which calstabin2 depletion from the RyR2 complex has been shown to promote HF progression, the calstabin2-deficient mice have otherwise normal cardiac function and are able to compensate for the chronic diastolic SR Ca\textsuperscript{2+} leak in the absence of a compromised ventricle (e.g., no MI). However, PDE4D3-deficient mice that have chronic PKA hyperphosphorylation of RyR2 and RyR2-S2808D mice that mimic chronically PKA-hyperphosphorylated RyR2 both have calstabin2 depletion from the RyR2 complex, and both exhibit progressive cardiac dysfunction leading to a dilated cardiomyopathy in the absence of MI (6). This indicates that the combination of calstabin2 deficiency plus another insult (e.g., PKA hyperphosphorylation of RyR2) is sufficient to cause cardiac dysfunction in the absence of MI (7). However, the loss of calstabin2 alone, without another insult to the myocardium, can be compensated for at baseline, although this causes SCD with exercise due to leaky RyR2.

**Fixing leaky RyR2 channels**

Identification of the diastolic SR Ca\textsuperscript{2+} leak via RyR2 as a mechanism underlying HF progression and cardiac arrhythmias has led to novel therapeutic approaches. JTV-519 (K201), a 1,4-benzothiazepine, was noted to have effects on intracellular Ca\textsuperscript{2+} (35) and cardioprotective effects (36). However, the target was not known and there was no mechanism of action for JTV-519. Moreover, JTV-519 was found to inhibit Na\textsuperscript{+}, Ca\textsuperscript{2+}, and K\textsuperscript{+} currents (37, 38). Using a canine model of pacing-induced HF, Matsuhashi and colleagues reported that JTV-519 improved cardiac function (19).

Testing the drug in calstabin2-deficient mice showed that the ability of JTV-519 to prevent HF progression and fatal cardiac arrhythmias and improve skeletal muscle function requires stabili-
lization of the closed state of RyR2 by calstabin2 (28, 39). Moreover, JTV-519 had no effect on the gating properties of normal RyR channels and no effects in healthy dogs and mice (9).

My laboratory generated many derivatives of the 1,4-benzothiazepine JTV-519 and have developed a novel class of Ca\textsuperscript{2+} release channel stabilizers known as Rycals. An orally available Rycal, S107, improves skeletal muscle force generation and exercise capacity, reduces arrhythmias, and improves muscle function in mice with Duchenne muscular dystrophy by reducing pathologic SR Ca\textsuperscript{2+} leak in cardiac and skeletal muscle (7, 9, 40–43). Rycals are protective against post-MI HF progression (7, 44) and have been shown to suppress VT/ventricular fibrillation (VT/VF) and SCD in murine models of human CPVT. S107 also raises the seizure threshold in mice with leaky neuronal RyR2 channels and improves exercise capacity in mouse models of sarcopenia (age-related loss of muscle function) (6, 9, 10, 34, 41).

Dantrolene, a drug used to prevent malignant hyperthermia in patients with mutations in RyR1 who have been exposed to volatile anesthetics, has been proposed to have therapeutic potential in heart disease (45). The Na\textsuperscript{+} channel antagonist flecainide prevents ventricular arrhythmias in patients with CPVT mutations (46). Based on single-channel data, flecainide has been proposed as an open channel blocker of RyR2. However, it is unlikely that this could be the mechanism of its anti-arrhythmic activity in CPVT, since blocking the open state of RyR2 would seriously impair cardiac contractility. Moreover, the leak that triggers the fatal ventricular arrhythmias in CPVT is a diastolic SR Ca\textsuperscript{2+} leak that occurs when the channel is supposed to be tightly shut, not open (8, 13, 39). Finally, it is more likely that some other activity of flecainide, such as its well-documented sodium channel blockade (47), is the mechanism underlying its anti-arrhythmic actions in CPVT.

Focus on the pump

SERCA activity and protein levels are decreased in failing hearts (11). Mice that are haploinsufficient for the SERCA2a gene (\textit{Atp2a2}) develop accelerated HF progression compared with wild-type controls (48). However, patients with an \textit{ATP2A2} mutation and haploinsufficiency (Darier disease) do not have cardiac dysfunction (49). Nevertheless, restoration of normal levels of SERCA2a has been targeted as a novel therapeutic for HF (4). Phospholamban controls the affinity of SERCA2 for Ca\textsuperscript{2+}. Unphosphorylated phospholamban inhibits SERCA2 Ca\textsuperscript{2+} affinity, and phosphorylation by PKA and Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II (CaMKII) relieves this inhibition and results in enhanced SR Ca\textsuperscript{2+} uptake. However, a human mutation in phospholamban results in severe dilated cardiomyopathy (50).

Increasing SR Ca\textsuperscript{2+} uptake

Both SERCA overexpression and phospholamban inhibition have been targeted as therapeutic strategies for HF. Cardiac-specific expression of SERCA2a was shown to improve contractility (51). However, results regarding arrhythmias are controversial. Chen et al. reported increased arrhythmias leading to mortality.
after MI in transgenic rats (52), whereas decreases in ventricular arrhythmias after postischemic injury were reported (53). Beneficial results observed in preclinical testing have led to clinical trials in patients with HF to enhance SR Ca\(^{2+}\) uptake, including a first-in-human gene therapy trial, Calcium Uptregulation by Percutaneous administration of gene therapy In cardiac Disease (CUPID), using an adeno-associated type I vector carrying SERCA2a. In 39 patients with New York Heart Association class III/IV HF, treatment with the SERCA2a adeno-virus resulted in improvement or stabilization in the New York Heart Association class, Minnesota Living With Heart Failure Questionnaire, 6-minute walk test, peak maximum oxygen consumption, N-terminal pro-hormone brain natriuretic peptide levels, and left ventricular end-systolic volume, as well as decreased frequency of cardiovascular events and duration of hospitalizations (54). Further trials are planned.

Controversies in the field
The diastolic SR Ca\(^{2+}\) leak model for HF and arrhythmias first proposed in 2000 (5) has generated great interest and has led to clinical trials for HF and arrhythmias as noted above. Indeed, many laboratories have examined aspects of our findings. While essentially all of our findings have been reproduced by others (16, 17, 19–23, 55–57), controversy surrounding our work has emerged over the years, with reports challenging our studies as summarized recently (58). I have previously addressed this controversy and pointed out that most of the differences are based either on opposite interpretations of the same findings or on profound differences in experimental approach (59).

We originally reported that RyR2s are PKA hyperphosphorylated in human and canine failing hearts (5) and defined PKA hyperphosphorylation as phosphorylation of 3–4 of the four PKA sites (Ser2808) in the RyR2 homotetramer (60, 61). We also reported that PKA hyperphosphorylation of RyR2 was associated with depletion of the stabilizing subunit calstabin2 from the channel (there are four calstabin2 bound to each RyR2 channel, again one per monomer; ref. 5) and that this renders the channel “leaky,” meaning that at diastolic cytosolic Ca\(^{2+}\) concentration (∼100 nM) when the RyR2 channel should be tightly closed, the PKA-hyperphosphorylated, calstabin2-depleted channels are not tightly closed; that is, HF RyR2 channels have increased single-channel open probability when recorded in planar lipid bilayers (5). This results in a diastolic SR Ca\(^{2+}\) leak that depletes SR Ca\(^{2+}\), contributing to impaired contractility and HF progression, and provides signals that lead to membrane depolarization and triggering of fatal ventricular arrhythmias in failing hearts (13, 34).

Alternative mechanisms have been proposed to explain SR Ca\(^{2+}\) leak in HF. CaMKIIδ levels are elevated in human HF (62), and there is an increase in CaMKII-dependent phosphorylation of RyR2 in HF. The observation that mice expressing the CaMKII inhibitory peptide AC3-I are protected against HF led to the proposal that CaMKII inhibitors may prevent HF progression (63). It has been reported that PKA hyperphosphorylation of RyR2 is involved in ischemic (post-MI) but not in non-ischemic (aortic banding) cardiomyopathy, and that CaMKII phosphorylation of RyR2 is the mechanism for the leak (64). The aortic banding model is a hypertrophy model in which cardiac function is initially increased and later decreases. It is possible that elevated CaMKII phosphorylation of RyR2 is associated with the hypertrophy.

Moreover, we developed genetically altered mice that harbor RyR2 that cannot be phosphorylated by CaMKII (RyR2-S2814A) and showed that they were not protected against post-MI HF progression (65). Instead, RyR2 CaMKII phosphorylation is required for the rate-related increase in contractility (65). CaMKII is Ca\(^{2+}\) activated and is exposed to more Ca\(^{2+}\) at higher HRs, resulting in increased CaMKII phosphorylation of RyR2, increased SR Ca\(^{2+}\) release, and increased contractility (known as the Bowditch phenomenon) (65). HR is typically elevated in HF due to increased levels of catecholamines, and thus CaMKII activation during HF is likely a result of increased HR.

We originally reported that PKA hyperphosphorylation of RyR2 causes depletion of calstabin2 from the RyR2 complex (5). We subsequently observed that oxidation and nitrosylation of RyR2 also cause depletion of calstabin2 from the RyR2 complex, and that the combination of all three delete nearly all of the calstabin2 from the channel complex (7, 44). Some of the divergent results reported in the literature concerning the effects of PKA phosphorylation on calstabin binding to the channels could be due to variations in the state of oxidation of the channels. For example, we found that when RyR2-S2808D channels were expressed in HEK cells, there was progressive oxidation of the channel, such that the binding of calstabin2 to the channel decreased each day the transfected cells were in culture (7). This progressive oxidation is likely due to mitochondrial Ca\(^{2+}\) overload resulting from leaky RyR2 channels and ROS production, which oxidizes the channel (41).

Future directions: new and old pathways, additional therapeutic targets
It should be evident from Figure 1 that there are many potential therapeutic targets in the key pathways that regulate Ca\(^{2+}\) handling in cardiac muscle. For example, the cardiac RyR2 is a macromolecular signaling complex with multiple enzymes and targetting proteins (15), each of which should be explored as a potential cause of, and therapeutic target for, HF at the cellular, animal, and human genetic levels (8). Moreover, recent work on mechanisms of SR Ca\(^{2+}\) leak in different disease models has shown the critical role of nitrosylation of RyR1 in muscular dystrophy (43) and RyR2s (42) and of interactions between RyR1 and mitochondria in skeletal muscle, whereby oxidation of RyR1 causes Ca\(^{2+}\) overload of mitochondria and further oxidation of the channel, worsening the SR Ca\(^{2+}\) leak (41). These studies suggest that targeting molecules involved in oxidation/nitrosylation and mitochondria should be explored for future therapeutics.

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