Targeting Cardiomyocyte Ca\textsuperscript{2+} Homeostasis in Heart Failure

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Abstract: Improved treatments for heart failure patients will require the development of novel therapeutic strategies that target basal disease mechanisms. Disrupted cardiomyocyte Ca\textsuperscript{2+} homeostasis is recognized as a major contributor to the heart failure phenotype, as it plays a key role in systolic and diastolic dysfunction, arrhythmogenesis, and hypertrophy and apoptosis signaling. In this review, we outline existing knowledge of the involvement of Ca\textsuperscript{2+} homeostasis in these deficits, and identify four promising targets for therapeutic intervention: the sarcoplasmic reticulum Ca\textsuperscript{2+} ATPase, the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger, the ryanodine receptor, and t-tubule structure. We discuss experimental data indicating the applicability of these targets that has led to recent and ongoing clinical trials, and suggest future therapeutic approaches.

Keywords: Cardiac myocytes, calcium homeostasis, heart failure, SR Ca\textsuperscript{2+} ATPase, Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger, ryanodine receptor, t-tubules.

I) INTRODUCTION

Heart failure treatment has historically undergone several paradigm shifts. Early pharmacological approaches included cardiac glycosides, which may be effective in relieving patients' symptoms, but carry a notoriously high risk of toxicity. In the 1980s, adrenergic agonists and phosphodiesterase inhibitors were introduced with great expectations, as it seemed intuitive to treat impaired contractility with positive inotropes. However, these agents were observed to increase mortality as they induced maladaptive cardiac responses and exhausted the failing heart. Consequently, the mainstay of today’s treatment is based on inhibitors of the chronic neurohumoral activation in heart failure, such as \beta-adrenergic blockers and angiotensin converting enzyme (ACE) inhibitors. Unfortunately, these treatments often only provide heart failure patients with symptomatic relief and temporarily impede disease progression. Therefore, new treatment strategies are needed that target the basal pathogenic mechanisms of this disease. The two main causes of death in heart failure patients, declining cardiac pump function and arrhythmias, have both been linked to disrupted Ca\textsuperscript{2+} homeostasis in cardiac muscle cells (cardiomyocytes) [1]. While the details of these deficiencies continue to be unraveled, existing data suggest that targeting deficient Ca\textsuperscript{2+} handling in failing cardiomyocytes has enormous therapeutic potential. In this review, we will examine the potential benefits of modulating several key players in Ca\textsuperscript{2+} homeostasis, with discussion of recent and ongoing clinical trials. However, to place these proposed therapies in their proper context, we will first briefly outline the involvement of Ca\textsuperscript{2+} in disrupted contraction and relaxation, arrhythmogenesis, and signaling in failing cardiomyocytes.

Impaired Cardiomyocyte Contraction and Relaxation

There is general agreement that smaller and slower contraction of individual cardiomyocytes contributes to reduced left ventricular contraction (systole) in heart failure [1, 2]. Contractility is regulated by a process known as excitation-contraction (EC) coupling [3]. This process is initiated as the action potential propagates over the surface membrane and into invaginations called t-tubules, triggering the opening of L-type Ca\textsuperscript{2+} channels (LTCCs). The resulting Ca\textsuperscript{2+} influx in turn triggers additional Ca\textsuperscript{2+} release from the sarcoplasmic reticulum (SR) via Ca\textsuperscript{2+} release channels called ryanodine receptors (RyRs) (Fig. 1A). This process, known as Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release (CICR), is made possible by close proximity between LTCCs and RyRs in functional units known as dyads. Ca\textsuperscript{2+} release from a dyad is called a Ca\textsuperscript{2+} spark, and the spatiotemporal summation of Ca\textsuperscript{2+} sparks constitutes the Ca\textsuperscript{2+} transient. Contraction is triggered as Ca\textsuperscript{2+} binds to the myofilaments.

Fundamental to the reduction in contractility in heart failure is reduced EC-coupling gain, meaning that less Ca\textsuperscript{2+} is released from the SR upon LTCC activation, resulting in decreased magnitude of Ca\textsuperscript{2+} transients and contractions [4, 5]. This reduction in Ca\textsuperscript{2+} release in failing cells largely results from decreased SR Ca\textsuperscript{2+} content which, in turn, results from a) reduced SERCA2a function, b) increased RyR leak and/or c) increased NCX function, which competes with SERCA2a for Ca\textsuperscript{2+} [1] (Fig. 1B). Reduced EC-coupling gain additionally results from impaired communication between LTCCs and RyRs. In heart failure, t-tubule organization is disrupted, leading to “orphaned” RyRs which no longer have opposed LTCCs (Fig. 1B). Thus, Ca\textsuperscript{2+} release is de-synchronized across the cell, which slows the rising phase of the Ca\textsuperscript{2+} transient (Fig. 2A), and may contribute to reduced contractile power in this condition [2]. Ca\textsuperscript{2+} release is also de-synchronized in failing cells due to a sub-population of Ca\textsuperscript{2+} sparks with slowed kinetics, which we have hypothesized result from dispersion of RyRs in the dyad [6]. Finally, alterations in action potential configuration may de-synchronize Ca\textsuperscript{2+} release [7-9], as a prolonged action potential reduces the magnitude and kinetics of the Ca\textsuperscript{2+} current, making triggering of SR Ca\textsuperscript{2+} release less efficient. Thus, the nature of reduced EC coupling gain in heart failure is complex.

Relaxation of cardiomyocytes is reported to be slowed and/or less extensive in failing cells, which impairs the ability of the ventricle to fill with blood before the next heartbeat. This phase of the cardiac cycle, known as diastole, has gained appreciable attention in recent years with the realization that a significant proportion of heart failure patients exhibit impaired ventricular filling but normal systole, a condition known as heart failure with preserved ejection fraction (HFpEF) or diastolic heart failure [10]. At the cardiomyocyte level, for efficient relaxation to occur, Ca\textsuperscript{2+} needs to be efficiently removed from the cytosol following release. The main pathways for removal are Ca\textsuperscript{2+} recycling into SR by the SR Ca\textsuperscript{2+} ATPase 2a (SERCA2a), and Ca\textsuperscript{2+} extrusion mediated by the plasma membrane Na\textsuperscript{+}/Ca\textsuperscript{2+}-exchanger (NCX). Depending on the specific
heart failure phenotype, both routes for Ca\textsuperscript{2+} removal may be impaired (Fig. 1B) [11]. Thus, targeting cardiomyocyte Ca\textsuperscript{2+} homeostasis has the potential to improve both systolic and diastolic function in heart failure patients.

**Ca\textsuperscript{2+}-Dependent Arrhythmogenesis**

30-50% of heart failure patients are reported to die from sudden cardiac death, and the majority of these deaths are linked to ventricular tachycardia [12]. Ventricular tachycardia can result from spontaneous electrical activity of cardiomyocytes. In some cases, a spontaneous action potential may be triggered by a phasic depolarization during the downstroke of the action potential, called an early afterdepolarization (EAD). While the precise mechanisms underlying EAD generation remain debated and may vary in different settings, there is general agreement that many EADs result from inappropriate re-opening of LTCCs or other depolarizing currents [13].

**Fig. (1). Excitation-contraction (EC) contraction coupling in the normal and failing heart.** In healthy cardiomyocytes (A), the action potential propagates into the t-tubules, opening voltage-gated L-type Ca\textsuperscript{2+} channels (LTCCs). The resulting Ca\textsuperscript{2+} influx triggers the opening of Ca\textsuperscript{2+} release channels (ryanodine receptors, RyRs) in the membrane of the sarcoplasmic reticulum (SR). Released Ca\textsuperscript{2+} initiates contraction as it binds to the contractile apparatus, and relaxation occurs as Ca\textsuperscript{2+} is recycled into the SR by the SR Ca\textsuperscript{2+} ATPase 2a (SERCA2a), and removed from the cell via the Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger (NCX). During heart failure (B), Ca\textsuperscript{2+} release is impaired, leading to slower and smaller contractions. Reduced SR Ca\textsuperscript{2+} content results from decreased SERCA2a activity, increased RyR leak and, in some cases, increased NCX activity. Systolic dysfunction also results from disruption of T-tubule structure, which functionally “orphans” some RyRs from LTCCs. Slowed and incomplete Ca\textsuperscript{2+} removal from the cytosol impairs cardiomyocyte relaxation, and promotes hypertrophy and apoptosis signaling. Triggered cardiac arrhythmia has been linked to RyR leak, and removal of released Ca\textsuperscript{2+} by NCX, causing DADs. EADs may result from inappropriate re-opening of Ca\textsuperscript{2+} channels. Deficient Ca\textsuperscript{2+} cycling is also linked to altered Na\textsuperscript{+} homeostasis, following downregulation of the Na\textsuperscript{+}-K\textsuperscript{+} ATPase (NKA) and increased late Na\textsuperscript{+} current.
Abnormal Ca\(^{2+}\) homeostasis can also promote arrhythmogenesis via delayed afterdepolarizations (DADs). These events are triggered by spontaneous Ca\(^{2+}\) release from the SR, resulting in depolarization as the released Ca\(^{2+}\) is removed from the cell by NCX [13]. Thus, targeting cardiomyocyte Ca\(^{2+}\) homeostasis has great potential for the prevention of triggered arrhythmias in heart failure patients as it is involved in the generation of both EADs and DADs.

Ca\(^{2+}\)-Dependent Signaling

Beyond its role in triggering contraction/relaxation and contributing to the electrical stability of the cardiomyocyte, intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) is also critically involved in signaling. For example, the Ca\(^{2+}\)-dependent calcineurin-NFAT pathway is centrally involved in mediating pathologic hypertrophy [14] and inflammation [15]. While the precise nature of the Ca\(^{2+}\) signal which triggers this pathway remains unclear, accumulating evidence suggests that Ca\(^{2+}\) levels in the dyadic cleft may be critically involved [16, 17]. (Fig. 1B). Ca\(^{2+}\)-dependent signaling is also involved in cardiomyocyte stress-responses and apoptosis, which additionally contribute to impaired myocardial function. Indeed, reduced SR Ca\(^{2+}\) content, as is known to occur in failing myocytes, has recently been linked to activation of the unfolded protein response, apoptosis, and altered SR structure [18]. Finally, disrupted Ca\(^{2+}\) homeostasis is known to offset the finely tuned balance between energy demand and availability in the heart. Energy wasting during heart failure results from futile Ca\(^{2+}\) cycling, and ATP production is reduced following Ca\(^{2+}\)-mediated mitochondrial dysfunction [19]. As will be described in the following sections, several proposed therapies aimed at improving contraction/relaxation in cardiomyocytes or arrhythmia suppression may simultaneously inhibit detrimental Ca\(^{2+}\) signaling.

Based on the above discussion, several key regulators of Ca\(^{2+}\) homeostasis emerge as potential therapeutic targets in failing cardiomyocytes, namely i) SERCA2a, ii) NCX, iii) RyR and iv) t-tubule structure. In the remainder of this review we will discuss the functional control of these targets, their dysregulation in heart failure, and ongoing efforts to reverse these impairments. This discussion will highlight opportunities to specifically target different heart failure phenotypes on a patient-to-patient basis.

II) SERCA

SERCA2a Function

SERCA2a contributes to Ca\(^{2+}\) removal from the cytosol by recycling Ca\(^{2+}\) into the SR. SERCA2a is thus an important regulator of diastolic function since, together with NCX, it sets the rate of Ca\(^{2+}\) transient decline and resting Ca\(^{2+}\) levels, and thereby the rate and extent of cardiomyocyte relaxation [11]. SERCA2a function also contributes to control of systolic function, by regulating the SR Ca\(^{2+}\) load available for release. SERCA2a activity is primarily determined by cytosolic Ca\(^{2+}\) levels and its endogenous inhibitor phospholamban (PLB). Since Ca\(^{2+}\) is usually the rate limiting factor for SERCA2a activity, increased [Ca\(^{2+}\)]\(_i\) directly enhances SR Ca\(^{2+}\) pumping [20]. PLB is a reversible inhibitor of SERCA2a, which acts by decreasing the Ca\(^{2+}\) affinity, but not the maximum pumping capacity (V\(_{\text{max}}\)) of SERCA2a. Phosphorylation of PLB by protein kinase A (PKA), a cAMP-dependent kinase, relieves this inhibition (Fig. 2B). PKA is activated by intracellular cAMP, which is amplifi ed via the β-adrenergic pathway. As PLB is one of the major substrates for PKA, PLB phosphorylation is accordingly one of the primary mechanisms for increasing SR Ca\(^{2+}\) content and relaxation rate following β-adrenergic stimulation [11, 21]. The main mechanism that terminates the β-adrenergic cascade is cAMP degradation by a class of enzymes called phosphodiesterases (PDEs), which importantly modulate SERCA2a function. The inhibitory effects of PLB are also relieved via phosphorylation by Ca\(^{2+}\)/calmodulin-dependent kinase type II (CaMKII). As CaMKII is activated by increased [Ca\(^{2+}\)]\(_i\), this mechanism enables SERCA2a stimulation at high pacing rates [11]. It is currently unclear whether the Ca\(^{2+}\) signal for activation of CaMKII is Ca\(^{2+}\) levels near the dyad, and/or integrated [Ca\(^{2+}\)]\(_i\) during the Ca\(^{2+}\) transient. Dephosphorylation of PLB is mediated mainly by protein phosphatase 1 [22] (Fig. 2B).
contribute to this discrepancy. Regional differences in SERCA expression have also recently been suggested to underlie these conflicting results [29].

SERCA2a activity may also be reduced in heart failure due to altered protein regulation, as a number of studies have reported augmented PLB inhibition. Although most studies have failed to demonstrate alterations in PLB levels in heart failure [27, 28, 30]. SERCA2a downregulation gives a relative increase in PLB compared to SERCA2a, and this may increase inhibition of the remaining pumps. Most important, however, seems to be a reduced phosphorylation state of PLB observed in human heart failure [27, 31] and experimental models of heart failure [28]. In the failing human heart, phosphorylation at threonine 17 has been suggested to be reduced despite increased CaMKII activity, due to increased activity of the phosphatase calcineurin [32]. Furthermore, reduced ser16 phosphorylation has been attributed to increased activity of protein phosphatase 1 in both human heart failure [33, 34] and animal models [28].

As expected based on above discussions, downregulation of SERCA2a levels, and thus activity, have been correlated with both systolic and diastolic dysfunction in human heart failure [26, 35]. Similar findings have been made in mouse models with SERCA2a ablation. Mice with heterozygous SERCA2 knockout (+/-) showed systolic and diastolic dysfunction in human heart failure [26, 35]. As expected based on above discussions, downregulation of SERCA2a activity may also be reduced in heart failure due to conflicting results [29].

Expression have also recently been suggested to underlie these changes. Transgenic mice overexpressing SERCA2a exhibit enhanced cardiac contractility and relaxation [36]. Furthermore, conditional cardiomyocyte SERCA2a gene knockout caused decreased rates of cytosolic Ca2+ removal, reduced SR Ca2+ content and Ca2+ transient magnitude (Fig. 3A), and development of end-stage heart failure at several weeks following gene deletion [37-39]. These experiments illustrate the direct relationship between SERCA2a levels, Ca2+ handling, and cardiac pathology.

Reduced SERCA2a function also has important implications for other aspects of the failing phenotype, including arrhythmogenesis, mechanoenergetics and hypertrophic and apoptotic signaling [40]. SERCA2a activity has been implicated in both early and late afterdepolarizations (EADs and DADs). Reduced SERCA2a activity decreases the magnitude of Ca2+ release, resulting in decreased inactivation of L-type Ca2+ channels and predisposition for EAD generation. Effects on DADs however, may depend on the interaction between SERCA2a activity and that of other Ca2+ handling proteins. Data from the SERCA2 knockout mouse have shown that reduced SERCA2a function is associated with a decreased threshold for RYR opening [41], which appears to be due to CaMKII-dependent phosphorylation of the channel. At baseline, these cells exhibited fewer Ca2+ waves and DADs, however beta-adrenergic stimulation increases SR Ca2+ content above the threshold for RYR opening, and may explain the increased incidence of arrhythmias in this setting [42].

**Targeting Reduced SERCA2a Activity in Heart Failure**

As the detrimental effects of reduced SERCA2a function have become evident, its potential as a target in heart failure has emerged. Transgenic mice overexpressing SERCA2a exhibit enhanced cardiac function at baseline in parallel to improved cardiac output [42]. The group of Hajjar has demonstrated that mechanoenergetic wasting in aortic banded rats was reduced by SERCA2a gene transfer, and survival improved, probably due to reduction in RYR Ca2+ leak (futile Ca2+ cycling) and improved mitochondrial function [49, 50]. SERCA2a overexpression also decreases diastolic [Ca2+]i, and a resulting attenuation of calcineurin/NFAT activation may be responsible for attenuated hypertrophic and apoptotic signaling [40]. SERCA2a overexpression in rat heart failure also reduced pathologic expression of miR-1, a micro RNA involved in regulation of transcription factors, receptor ligands, apoptosis regulators and ion channels [51].

Following these positive experimental results, human trials with SERCA2a gene therapy have been initiated. A phase I trial with an adenovirus-associated viral-1 vector (AAV1) was used to deliver SERCA2a gene by intracoronary infusion. The results were published in 2009, establishing safety and feasibility [52]. A phase 2 trial, the Percutaneous Administration of Gene Therapy in Cardiac Disease (CUPID) study, was then conducted to further evaluate clinical benefits [53]. The CUPID study was a randomized, double-blind, placebo controlled trial that included 39 patients with advanced heart failure. The effects of either low, mid or high dose AAV1-SERCA2a gene infusion were compared with placebo. The study confirmed safety, and the high dose group demonstrated therapeutic response with decreased symptoms of heart failure, improved functional status, decreased levels of natriuretic peptides and attenuated adverse remodeling of the left ventricle. A three-year follow-up study of these patients showed reduced mortality in those that received high dose gene therapy [54]. High titers of adenoviral neutralizing antibodies against the AAV1 are, however, a problem in some patients that needs to be addressed.

As an alternative to gene therapy, pharmacologic agents may directly act to restore SERCA2a function. Istaroxime produces both inotropic and lusitropic effects due to its dual mechanism of action [55]. First, it directly stimulates SERCA2a, increasing both SR Ca2+ content and cytosolic Ca2+ removal (Fig. 2B). Second, it is a non-glycoside inhibitor of the Na/K ATPase, and thereby increases intracellular Na+ levels and indirectly inhibits NCX function. The resulting rise in [Ca2+]i makes more Ca2+ available for SERCA2a and increases the rate of Ca2+ removal and SR Ca2+ content. In vivo animal experiments have shown that both acute and chronic istaroxime treatment restore cardiac function with no increase in arrhythmic events or cardiac energetics [56-58]. This is contrary to the harmful profile of established inotropic drugs. The HORIZON-HF trial was a phase-2, randomized, double-blind, placebo-controlled clinical trial that evaluated hemodynamic, echocardiographic and neurohumoral effects of acute administration of istaroxime on heart failure deterioration [59, 60]. The results were promising, and included improved systolic and diastolic function, lower pulmonary capillary wedge pressure, increased blood pressure and decreased heart rate.

Another potential method of restoring SERCA2a activity is to use the inhibitory effects of PLB. Several studies of PLB-deficient mice [61-64] have demonstrated enhanced Ca2+ cycling, improved lusitropy and inotropy, no increase in mortality and abrogated progression to heart failure. Furthermore, inhibition of wild-type PLB by gene transfer of pseudophosphorylated PLB prevented heart failure development in several animal models [65-67] (Fig. 2B). Inhibition of wild-type PLB has also been demonstrated with...
cardiomyocytes were transduced with adenoviral vectors encoding loss-of-function PLB mutants [68]. In human heart failure, isolated cardiomyocytes were transduced with adenoviral vectors encoding PLB antisense RNA, with a resulting improvement of Ca\(^{2+}\) handling and contraction and relaxation velocities [69]. Somewhat worrying, however, is the link between human PLB mutations and cardiac disease. In fact, PLB null mutations cause lethal forms of hereditary dilated cardiomyopathy [70]. This suggests that knockdown or competitive inhibition of PLB inhibition should be approached with caution in humans.

Alternatively, relieved SERCA2a inhibition can also be accomplished by therapeutically increasing PLB phosphorylation. Protein phosphatase 1 (PP1), which dephosphorylates PLB, exhibits increased activity in heart failure due to i) increased expression [28, 33] and ii) reduced activity of inhibitor-1 [71] (Fig. 2B). Inhibiting the activity of PP1 is therefore a putative strategy to increase PLB phosphorylation. Indeed, gene delivery of inhibitor-1 or inhibitor-2 has been associated with reduced incidence of arrhythmia, hyper trophy, heart failure and death in animal models of heart failure [72, 73]. Delivery of short hairpin RNA targeting PP1 by adenoadsiocated viral vectors was also observed to prevent ventilricular remodeling [74]. Recently, a decoy peptide mimicking phosphorylated PLB was shown to competitively inhibit PP1 from interacting with wild-type PLB, and improved cardiac contractility resulted [75]. However, since protein phosphatase 1, together with protein phosphatase 2a, exert the majority of total phosphatase activity in the heart, inhibition of its function will affect many phosphorylated cardiac proteins and may produce undesirable side-effects. The ryanodine receptor is such a target that poses a specific concern, as hyperphosphorylation of this receptor has been hypothesized by some to be arrhythmogenic [76] (see discussion in the RyR section).

PLB phosphorylation may also be increased by enhancing kinase activity. PKA activity is dependent on cAMP levels which could be raised by either increased synthesis (via adenylyl cyclase), or reduced degradation (via phosphodiesterases) (Fig. 2B). One can envision several possible approaches to increase adenylyl cyclase activity. Although the enzyme is activated by traditional inotropic \(\beta\)-agents such as dobutamine, the \(\beta\)-receptor signaling pathway is associated with increased mortality. Gene therapy aiming at increasing adenylyl cyclase expression could bypass the detrimental \(\beta\)-receptor cascade (Fig. 2B). Adenylyl cyclase isoform 6 is activated by the \(\beta\)-receptor, and overexpression of this enzyme is reported to improve contractility and survival in animal models of heart failure [77, 78]. A human trial has been initiated to evaluate the potential of adenoviral delivery of adenylyl cyclase 6 by intracoronary infusion in human heart failure (ClinicalTrials.gov Identifier: NCT00787059). Another method to increase cAMP bioavailability, but avoid the \(\beta\)-receptor cascade, is to inhibit cAMP degradation by phosphodiesterases (PDEs). Most cardiac PDE activity has been attributed to PDE3, PDE4 and, more recently, PDE1 [79] (Fig. 2B). PDE3 inhibition is known to have inotropic effects in heart [80, 81]. However, the benefits of PDE3 inhibition have only been sustained in short-term therapy, and long-term mortality is increased predominantly due to arrhythmic events [80, 81]. It is likely that the underlying mechanisms are the same as for \(\beta\)-receptor stimulation, as increased Ca\(^{2+}\) cycling results in the generation of arrhythmogenic currents and unfavorable metabolic effects. Interestingly, PDE3 inhibition is also reported to contribute to the inotropic effects of the myofilament sensitizer Levosimendan [82].

II) NCX

NCX Function

While SERCA2a recycles Ca\(^{2+}\) into the SR, NCX is the main route for Ca\(^{2+}\) extrusion from the cardiomyocyte. NCX plays an important role in setting resting Ca\(^{2+}\) levels, and thus the extent of cardiomyocyte relaxation. However, since resting Ca\(^{2+}\) regulates SERCA2a activity, SR content, and Ca\(^{2+}\) transient magnitude, the net effect of altered NCX activity on the rate of Ca\(^{2+}\) decline can be difficult to predict, and may vary between species [11]. This functional interaction between NCX and SERCA2a is illustrated in Fig. 3A.

Although NCX functions predominantly to extrude Ca\(^{2+}\), it may also work in reverse mode resulting in Ca\(^{2+}\) influx. It is generally agreed upon that the NCX exchanges one molecule of Ca\(^{2+}\) for three molecules of Na\(^{+}\) [83], making it electrogenic. Therefore, forward-mode NCX operation results in a depolarizing current, while reverse mode carries a repolarizing current. The driving force which determines NCX direction and function are the electrochemical gradients, namely membrane potential and transmembrane gradients of Ca\(^{2+}\) and Na\(^{+}\) [84]. Negative membrane potential and low intracellular Na\(^{+}\) concentration ([Na\(^{+}\)]\(_{i}\)) promote forward operation mode. Since low cytosolic Na\(^{+}\) levels are actively maintained by the Na\(^{+}\)/K\(^{-}\)-ATPase (NKA), NCX function and NKA function are closely coupled (Fig. 1A). Indeed, experimental data suggest that NCX and the \(\alpha_{1}\)-isoform of NKA are closely localized in the t-tubules, which allows their function to be linked by [Na\(^{+}\)]\(_{i}\), in a restricted microdomain [85-87]. For example, reducing NKA\(_{\alpha_{1}}\) activity can locally elevate [Na\(^{+}\)]\(_{i}\), which inhibits Ca\(^{2+}\) removal by NCX, while making reverse-mode NCX function more likely [88].

Another mechanism of NCX regulation is the small inhibitory protein phospholemman. This is a member of the FXDY family of ion transport regulators and is found to co-localize with both NKA [89] and NCX [90] in heart (Fig. 3B). PKA and PKC phosphorylation modulate its inhibitory effect, but in a different manner in NCX compared to NKA; phospholemman phosphorylation relieves NKA inhibition but increases NCX inhibition [91]. During phosphorylation, phospholemman therefore increases contractility, by inhibiting NCX and increasing Ca\(^{2+}\) [92], but also protects against Na\(^{+}\) overload by relieved inhibition of NKA [93].

NCX in Heart Failure

NCX upregulation is a common feature of both human heart failure [94-96] and animal models of heart failure [97, 98]. As such changes often occur simultaneously with SERCA2a downregulation, a marked increase in NCX/SERCA2a ratio is commonly reported, and has been implicated in both systolic dysfunction and arrhythmogenesis [99]. Reduced contractility is caused by increased transsarcomemmal Ca\(^{2+}\) extrusion relative to SR Ca\(^{2+}\) recycling, which depletes SR Ca\(^{2+}\) stores. Accordingly, experimental overexpression of NCX in cardiomyocytes is associated with reduced SR Ca\(^{2+}\) load and contractile dysfunction [100]. Thus, in many heart failure models, NCX upregulation potentiates the loss of SR Ca\(^{2+}\) caused by reduced SERCA2a activity (Fig. 3A). On the other hand, increased NCX function may actually be beneficial for diastolic function, by correcting for defective Ca\(^{2+}\) removal due to reduced SERCA2a activity (Fig. 3A). In support of this concept, Hasenfuss et al showed that NCX levels predicted diastolic function in human heart failure, with preserved relaxation in subjects with upregulated NCX [101].

However, NCX function is dependent not only on its expression, but also by local Na\(^{+}\) and Ca\(^{2+}\) levels and action potential configuration – all of which are altered in heart failure [102]. [Na\(^{+}\)] levels, is reported to be increased in both human heart failure and animal models [103], due to either decreased Na\(^{+}\) extrusion or increased Na\(^{+}\) influx. NKA is the major Na\(^{+}\) extrusion pathway in cardiomyocytes in several, but not all models of heart failure. NKA expression is found to be decreased [103] (Fig. 1B). Downregulation of the \(\alpha_{1}\) NKA isoform is of particular significance, due to its functional coupling with NCX [104, 105]. Important Na\(^{+}\) influx mechanisms believed to contribute to Na\(^{+}\) loading are NCX [102], Na\(^{+}/H\(^{+}\) exchange (NHE) [106] and increased late Na\(^{+}\) current [107, 108]. With sufficient Na\(^{+}\) loading in end-stage heart failure, NCX-mediated Ca\(^{2+}\) extrusion may be impaired despite increased NCX expression [37, 39]. Prolongation of action potential duration in
failing myocytes can exacerbate this deficit as positive membrane potentials reduce the driving force for Ca$^{2+}$ extrusion by NCX [9, 39]. With respect to arrhythmogenesis, NCX activity may be implicated in several ways [13]. As described previously, spontaneous Ca$^{2+}$ release during diastole is a feature of heart failure, and extrusion of released Ca$^{2+}$ elicits DADs. When NCX expression is increased, the depolarizing current will be larger and DADs are more likely to trigger action potentials, ectopic beats and arrhythmias. In addition, inward NCX current associated with Ca$^{2+}$ extrusion during the downstroke of the action potential may prolong action potential duration, leading to EADs and spontaneous beats [13].

Through its regulation of resting Ca$^{2+}$ levels, NCX is also an important control point for hypertrophy signaling. In this regard, marked upregulation of Ca$^{2+}$ extrusion may effectively compensate for SERCA2a loss and prevent hypertrophy, as we recently reported in SERCA2 KO mice [36, 109]. Without such compensation, increased diastolic [Ca$^{2+}$], and hypertrophic remodeling are expected.

Fig. (3). Altered NCX activity as a therapeutic target in heart failure. A: Experimental Ca$^{2+}$ transients from SERCA2 knockout mice are dramatically reduced (left panel). Modeling data predict that simultaneous NCX ablation increases Ca$^{2+}$ transient magnitude (right). This indicates that NCX competes with SERCA2a for the same pool of Ca$^{2+}$ and reduces SR Ca$^{2+}$ content and release. Data are adapted from [227], with permission. B: NCX activity could be therapeutically modulated by direct targeting or altering electrochemical gradients. NCX inhibitors attenuate cellular Ca$^{2+}$ extrusion and thereby increase cellular Ca$^{2+}$ load and ultimately contractility. Inhibition of NKA similarly inhibits NCX-mediated Ca$^{2+}$ extrusion by increasing cellular Na$^+$ levels. However, prevention of Ca$^{2+}$ overload is desirable in patients at risk for arrhythmia, and this may be attained by inhibition of Na$^+$ influx pathways, which augments Ca$^{2+}$ extrusion.
Targeting NCX in Heart Failure

The above discussion illustrates that there is great potential for targeting NCX in heart failure, to prevent arrhythmia and improve pump function. Current pharmacological inhibitors of NCX are the drugs KB-R7943 [112], SEA-0400 [113] and SN-6 [114] (Fig. 3B). While KB-R7943 also blocks Na⁺, K⁺ and Ca²⁺ channels, SEA-0400 and SN-06 have more selective profiles. Still, regardless of pharmacological profile, NCX inhibitors indirectly inhibit LTCCs by increasing [Ca²⁺] [115]. Even so, NCX inhibition is expected to have inotropic effects by shifting the balance from Ca²⁺ extrusion to SR Ca²⁺ recycling. Furthermore, NCX blockade is expected to block generation of the arrhythmogenic inward jNCX.

NCX inhibition has been investigated in a range of different cardiac disease models, including heart failure. In a rabbit rapid-pacing model of heart failure, in vivo SEA-0400 administration was observed to reduce action potential duration, repolarization dispersion and the number of EADs and ventricular tachycardias [116]. In canine heart failure, DADs were also attenuated by SEA-0400 [117]. Inotropic effects of reduced NCX function have been experimentally demonstrated by intracellular application of the exchange inhibitory peptide (XIP) in a canine model of heart failure, with restoration of SR Ca²⁺ reuptake and release [118]. Also, pharmacological NCX blockade by SEA-0400 showed positive inotropic effects in mice with heart failure following transverse aortic constriction [115], although relaxation was slowed. This is an expected drawback of NCX inhibition for heart failure etiologies where increased NCX function compensates for impaired Ca²⁺ removal by SERCA2a. Thus, NCX inhibition may be best suited for patients which predominantly exhibit systolic dysfunction. However, based on presently available data, Antoons et al recently concluded that the effects of NCX inhibition have thus far been predominantly beneficial in reducing arrhythmias and restoring contractility [119]. An added benefit of NCX inhibition may be attenuation of myocardial fibrosis and stiffening, as reported by Kamimura et al in a rat model of heart failure with preserved ejection fraction. They hypothesized that the underlying mechanism was reduced Ca²⁺ entry into fibroblasts which attenuates collagen production [120].

Rather than directly modulating NCX function, an alternative approach is to indirectly modify NCX activity via changes in [Na⁺]. Cardiac glycosides function, at least in part, by inhibiting NKA and increasing [Na⁺], which attenuates NCX-mediated Ca²⁺ removal and augments SR Ca²⁺ load (Fig. 3B). However, such action also increases the propensity for triggered arrhythmias, and Ca²⁺ overload is implicated in myocardial dysfunction, remodeling and failure. Istaroxime, which is a non-glycoside NKA inhibitor, bypasses these side effects of Ca²⁺ overload by also stimulating SERCA2a (as discussed in the SERCA section). Alternatively, the opposite strategy of targeting pathological increases in [Na⁺] may reduce NCX-mediated Ca²⁺ overload and improve survival. As discussed above, increased NHE-1 activity has been implicated in Na⁺ gain in failing cells, and NHE-1 inhibition has been shown to experimentally reverse cardiac hypertrophy [110, 121]. However, several clinical trials have not been able to reproduce the cardioprotective effects of the NHE-1 inhibitors cariporide or eniporide in patients with acute coronary syndromes [122-124]. In fact, the most recent of these trials demonstrated cariporide-induced toxicity [124]. Targeting the late Na⁺ current is another possible approach (Fig. 3B). Ranolazine, an inhibitor of this current, has been shown to reduce [Na⁺], and reverse diastolic dysfunction in tissue preparations from failing hearts [107]. Unfortunately, the recent RALI-DHF study failed to demonstrate improved left ventricular relaxation following Ranolazine treatment in patients with heart failure with preserved ejection fraction [125].

The final putative strategy for targeting NCX function that we will consider is modulation of phospholemman. Both up- and downregulation of phospholemman expression and phosphorylation are reported in different heart failure models [91]. Further complicating interpretation of the suitability of phospholemman as a drug target is its roving inhibition of NCX and NKA. Overexpression of phosphomimetic phospholemman in mice resulted in premature death due to heart failure and arrhythmias. Ca²⁺ removal was slower and diastolic Ca²⁺ increased [126], indicative of NCX inhibition. One might expect that the opposite intervention, that is reducing phospholemman phosphorylation to activate NCX, might be a suitable therapeutic approach in heart failure. However, expected reductions in diastolic [Ca²⁺], and Ca²⁺-dependent signaling would likely come at the price of reduced contractility, as has been reported in studies examining NCX overexpression [100].

RyR Function

RyR is a large tetrameric protein localized to the SR membrane. RyR2, which is the major cardiac RyR isoform, acts as a scaffolding protein that associates with a number of proteins to form a macromolecular complex [127, 128]. This complex, which is important for RyR regulation and integrity, includes regulatory proteins such as protein kinase A, protein phosphatase 1 and 2a, calmodulin, calmodulin kinase II and phosphodiesterase 4D (PDE4D) (Fig. 4B). This structure allows tight control of RyR function via several phosphorylation sites, as well as Ca²⁺ activation and inactivation sites. The RyR2 binding protein FKB12.6 (calstabin 2) stabilizes the tetrameric conformation.

RyR in the Failing Heart

There is general agreement that RyRs become leaky during heart failure, resulting in increased frequency of Ca²⁺ sparks (Fig. 4A) and waves, which promote arrhythmogenesis as released Ca²⁺ is removed by NCX, causing DADs [13]. Leak-induced elevation of [Ca²⁺] in or near the dyad is also believed to activate pathologic signaling leading to hypertrophy and myocardial dysfunction [17, 129]. However, the effect of RyR leakage on mechanical function remains a point of discussion. Many have reported that leak-induced reduction of SR Ca²⁺ content may decrease Ca²⁺ transient amplitude and contractility. However, the concept of autoregulation put forward by the Eisner group predicts that the decrease in SR Ca²⁺ content will be counterbalanced by a larger fractional SR Ca²⁺ release due to increased RyR sensitivity, leaving Ca²⁺ transients unaltered [130]. This concept is in accordance with the phenotype of human catecholaminergic polymorph ventricular tachycardia (CPVT), where RyR leak is increased, but contractile force is preserved. However, in heart failure RyR leak is also accompanied by disturbed SERCA2a and NCX function, which is likely critical for mediating depressed contractile function. The Eisner group has also shown that increased leak does not alter global diastolic [Ca²⁺] [131], although even local increases in resting Ca²⁺ levels might inhibit relaxation.

Increased RyR open probability in heart failure has been attributed to PKA or CaMKII phosphorylation. Marx et al reported that increased β-adrenergic signaling caused PKA-mediated “hyperphosphorylation” at Ser2808 of RyR, resulting in FKB12.6 disso-
Association from the channel, destabilization of the closed conformation of RyR, and increased Ca\(^{2+}\) leak [132]. Follow-up work by this group proposed that stress-induced phosphorylation, oxidation, and nitrosylation of RyR [133] also result in depletion of protein phosphatases and PDE4D3 [134], which further potentiates RyR hyperphosphorylation and FKBP12.6 dissociation. Recently, Lundby et al mapped downstream phosphorylation sites of \(\beta\)-receptor signaling, and RyR Ser2808 was indeed identified as one phosphorylation target [135]. However, PKA phosphorylation of RyR and its relevance to heart failure pathogenesis are highly controversial [136]. Of particular note, the groups of Houser and Valdivia have been unable to reproduce PKA phosphorylation at Ser2808 as a relevant mechanism in the cardiac fight-flight response and heart failure development [137-140].

Work done in the Bers group showed that CaMKII phosphorylation of RyR in mice altered Ca\(^{2+}\) sparks [141], whereas PKA phosphorylation did not [142]. In agreement with this finding, increased RyR leak during heart failure has been linked to CaMKII...
phosphorylation at Ser2814 [143]. Recent evidence indicates that CaMKII-mediated S2814 phosphorylation actually promotes heart failure development [129, 144, 145]. Importantly, CaMKII activity is enhanced during chronic β-adrenergic and angiotensin receptor II stimulation, as seen in heart failure, and expression of CaMKII is also increased in this disease [146].

While there has clearly been much study of RyR phosphorylation in heart failure, there is also some evidence to suggest that RyR expression may be altered, at least in some models [6, 147]. In mice with heart failure following myocardial infarction, we recently observed reduced RyR expression and Ca²⁺ sparks with slow kinetics [6] (Fig. 4A). Slow spark regions also exhibited slowed Ca²⁺ release during the action potential, which contributed to de-synchronized SR Ca²⁺ release in failing cells. We have hypothesized that reduced RyR density or spadic RyR distribution may underlie these abnormal sparks.

**Targeting RyR Leak to Restore Cardiac Function**

Despite the controversies described above, there is clearly great potential for drugs that might act to reduce RyR-mediated Ca²⁺ leak, and thus prevent arrhythmias, hypertrophic signaling and possibly contractile dysfunction. Therapeutic agents may either directly interact with RyR, or indirectly reduce RyR phosphorylation by targeting upstream signaling pathways.

Several compounds, collectively called rycals, are known to bind and modulate RyR directly (Fig. 4B). One of the first drugs shown to restore abnormal RyR function was JTV519 (K201). This agent stabilizes the closed RyR conformation, but whether this is mediated by FKBP12.6 binding is still controversial. An early study in a canine model of heart failure showed that JTV519-treated hearts exhibited attenuated PKA-mediated phosphorylation of RyR, restored FKBP12.6 binding, inhibited SR Ca²⁺ leak, and ultimately rescued left ventricular function [148]. Further evidence that FKBP12.6 binding to RyR underlies these drug actions came from the observation that JTV519 effects were observed in transgenic FKBP12.6–/– but not FKBP12.6+/- mice [149, 150]. However, later studies [151, 152] have questioned such a requirement for FKBP12.6, as Yamamoto et al. proposed that JTV519 acts to stabilize RyR by restoring normal RyR interdomain interactions [152]. There are early indications that JTV519 may have therapeutic promise, as experiments in human myocardium indicated that it improved both diastolic and systolic function under Ca²⁺ overload conditions [153]. However, it is not clear that these effects stem solely from RyR stabilization, as the drug also inhibits LɔCa, LɔKr and Lk [154]. A new derivative of JTV519, S107, has a more selective profile [155], and has been reported to increase the binding of FKBP12.6 to RyR, and to reduce abnormal diastolic Ca²⁺ release, arrhythmias [155], and heart failure progression in mice [133]. In a current phase 2, placebo-controlled randomized trial, the anti-arrhythmic properties of another RyR modulating drug, S44121, are being evaluated in patients with congestive heart failure (ISCRTRN reg nr 14227980).

Flecainide and tetracaine have also been identified as possible RyR inhibitors. Flecainide is a class Ic antiarrhythmic clinically used to treat atrial fibrillation. The drug has additionally been shown to inhibit the open-state of the RyR, and thus experimentally prevent Ca²⁺ waves and arrhythmias in both mouse and human CPVT [156, 157]. However, this finding is controversial as Liu et al. [158] reported that flecainide prevented triggered activity in CPVT mice primarily by Na⁺ channel block, while Ca²⁺ homeostasis was minimally affected [158]. Tetracaine, on the other hand, is a closed-state blocker of RyR [159], and could thereby reduce diastolic Ca²⁺ leak without interfering with systolic Ca²⁺ release. Finally, there is great therapeutic potential in targeting signaling pathways that control RyR phosphorylation via PKA or CaMKII. Indeed, several existing heart failure therapies may ultimately target RyR phosphorylation via actions on these kinases. For instance, one important mechanism behind the beneficial effects of β1-antagonism may be reduced PKA- and/or CaMKII-dependent RyR phosphorylation [133] [160] (Fig. 4B). In addition, ACE inhibitors have been shown to reverse both elevated CaMKII expression and the hypertrophic phenotype in spontaneously hypertensive rats [161]. Angiotensin receptor blockers may similarly reduce CaMKII-derived leak. Specific inhibition of CaMKII by agents such as KN-93 or AIP also reduce RyR leak [143] (Fig. 4B), and have been shown to improve the force frequency-relationship in trabeculae isolated from failing human hearts [162]. KN-93 was additionally shown to prevent arrhythmia in CaMKII overexpressing mice [163]. Finally, interventions such as SERCA2a overexpression which reduce diastolic Ca²⁺ levels can inhibit RyR opening directly or via reduced CaMKII activation [48]. The benefits of CaMKII inhibition may extend beyond effects on RyRs, as CaMKII targets a range of dysfunctional processes in heart failure, including gene transcription, inflammatory signaling and fibroblast activation (reviewed in [164]).

**IV) T-TUBULE STRUCTURE**

**T-Tubule Structure and Function**

As described in the introduction, the transverse tubules (t-tubules) are invaginations of the cellular membrane which form an organized, primarily transverse, network that enables the formation of dyadic junctions between the cellular membrane and the SR [165]. T-tubules have been identified in ventricular cardiomyocytes in a wide variety of mammalian species (for a thorough review see [166]) and recent studies on larger mammals such as pig, sheep, cow, horse, and human [167-170] also found t-tubules in atrial myocytes (for review see [171]). The majority of t-tubules lie in close proximity to the Z-lines of healthy ventricular cells [172-174]. However, in addition to the transverse elements of the t-tubule system, a smaller proportion of longitudinal elements extend between Z-lines [6, 175, 176]. T-tubule geometry and distribution vary between species, and it has been suggested that ventricular cells from smaller species (with higher heart rates) have thinner t-tubules but higher overall t-tubule densities [5, 174, 177, 178].

T-tubule density and organization play an important role in determining the homogeneity of SR Ca²⁺ release. The high degree of t-tubule organization in rodent ventricle cells facilitates very synchronous Ca²⁺ release [5, 6, 179]. In cells with lower t-tubule density, such as pig ventricular myocytes, Ca²⁺ release is less synchronous [180], and in de-tubulated cells a wave-like inward propagation from the periphery to the center of the myocyte is observed [181]. In addition, cultured ventricular cells lose t-tubules progressively over time resulting in dysynchronous release of Ca²⁺ [182]. Thus, in healthy cardiomyocytes, a well-organized t-tubule network ensures close localization between LTCCs and RyRs, tight control of CICR, and uniform Ca²⁺ release across the cell.

**T-Tubules in the Failing Heart**

We and others have shown that T-tubule structure is altered in pathological states such as heart failure (for review see [2]). Depending on species, the specific heart failure etiology, and the time-point during disease progression, these alterations may include: i) disorganization of transversely-oriented t-tubules (Fig. 5A) [6, 105, 179, 183-193], ii) loss of t-tubules [5, 105, 185, 187, 188, 191, 194-200], iii) increase in the longitudinal fraction of tubules [6, 109, 179, 183, 189, 190, 193, 201, 202], and iv) dilation of t-tubules [173, 192, 199-201]. T-tubule loss or drift of transverse elements causes spatial dissociation between LTCCs in the t-tubules and RyRs in the SR, leading to the formation of orphaned RyRs (Fig. 1B). Such changes impair the ability of LTCCs to trigger SR Ca²⁺ release [6, 183], but also de-synchronize the Ca²⁺ transient, as Ca²⁺ release from orphaned RyRs can be triggered only after diffusion of Ca²⁺ from intact dyads [6, 159, 178, 188, 196, 203-205]. In the majority of studies, the relationship between T-tubule disruption...
and Ca\textsuperscript{2+} release dysynchrony has been merely correlative. However, we recently examined this issue quantitatively in a mathematical model describing spatiotemporal dynamics of Ca\textsuperscript{2+} in the cytosol and SR. With progressive disorganization of T-tubule structure during heart failure development, the model confirmed greater Ca\textsuperscript{2+} release dysynchrony (Fig. 5A). However, the model and experiments additionally showed that the magnitude of Ca\textsuperscript{2+} release and RyR Ca\textsuperscript{2+} sensitivity also affect release synchrony [193]. Of note, reduced SR content in failing cells exacerbates dysynchrony of Ca\textsuperscript{2+} release resulting from T-tubule disruption. The less homogeneous Ca\textsuperscript{2+} transient is slower and of lower amplitude in failing cells, which has been linked to slower and smaller contraction [2].

In addition to enabling dyad formation, the integrity of t-tubules plays a crucial role for action potential propagation through the myocyte. T-tubule detachment from the surface membrane prevents those tubules from receiving action potentials, and effectively orphans RyRs in their dyadic junctions. However, interesting new data indicate that detached t-tubules also impede action potential propagation into t-tubules which remain in contact with the cell surface, worsening dysynchrony of Ca\textsuperscript{2+} release [206]. Furthermore, important ion channels and transporters present in the t-tubules are expected to exhibit dramatically altered distribution following t-tubule re-organization. Such changes likely contribute to altered action potential configuration, which exacerbates impairments in CICR in failing cells [9, 207, 208]. Since the LTCC and NCX are more prominently expressed in t-tubules compared to the sarcosomal membrane [175, 209], t-tubule disruption has been linked to deficient trans-sarcosomal Ca\textsuperscript{2+} fluxes [179, 182, 183]. We have additionally linked impaired NCX-mediated Ca\textsuperscript{2+} removal in failing cells to loss of the alpha-2 NKA isoform during t-tubule disorganization, and disruption of a Na\textsuperscript{+} microdomain shared by these proteins [105].

While there are clearly a number of detrimental consequences of altered t-tubule organization in failing cells, our recent data indicate that the increased longitudinal fraction of t-tubules frequently observed during heart failure may be compensatory. We observed that in SERCA2 knockout mice, newly grown longitudinal elements form dyadic junctions with the SR [109]. Although LTCCs are not present in these tubules, they do contain NCX in close apposition to RyRs. Our mathematical modeling data suggest that such changes facilitate a greater reliance on trans-sarcosomal Ca\textsuperscript{2+} cycling, by facilitating NCX-mediated Ca\textsuperscript{2+} entry and removal, and that Ca\textsuperscript{2+} entry via NCX can elicit CICR. This hypothesis has not yet been confirmed by functional data, and it also remains to be determined whether a similar adaptive role of newly grown tubules occurs in other heart failure models.

It is currently unclear whether t-tubule disarray during heart failure is pro-arrhythmic. Data from the Wehrens group have indicated that orphaned RyRs exhibit increased activity [186], which is supported by previous reports from failing cells [6]. This finding is supported by recent data from [210, 211] and in studies of Rubatalated cardiomyocytes. Possible Therapeutic Targets to Recover the T-Tubule Network

To date, t-tubule structure has not been deliberately targeted in clinical practice. However, Sacher et al. [202] recently showed that resynchronization therapy of failing canine hearts improved the organization of t-tubules, suggesting that similar therapies in patients may also promote reverse remodeling of t-tubules. A suggested explanation for this action is that re-synchronization relieves the mechanical stress present in the late-activated lateral wall, and attenuates stress- or strain-dependent signaling which may lead to t-tubule disruption. Support for this hypothesis comes from the observation that Sildenafil treatment during pulmonary artery hypertension was observed to improve t-tubule structure by reducing afterload on the right ventricle [188]. Similar effects have been reported following pressure unloading by administration of β-receptor blockers [212] and mechanical unloading by heterotopic transplantation of post-infarction failing hearts [195]. Thus, it appears that the load and/or stretch placed on the myocardium are key factors in determining the organization/disorganization of the t-tubule network. The t-tubules themselves may transduce these signals as they contain stretch-sensitive transmembrane proteins and channels, and exhibit altered geometry during stretch and contraction of the myocyte [213, 214].

Several molecules have been described to influence t-tubule structure and function [2]. One of the more extensively studied molecules is Junctophilin-2 (JP2) which plays an important role in anchoring t-tubules to the SR (Fig. 5B). Knockout (KO) models have shown that JP2 is critical for normal cardiomyocyte function [215]. This led to several investigations of JP2 in heart failure, and it is now well documented that JP2 is markedly down-regulated in failing mice [212], rats [185, 188, 216], and humans [217]. JP2 down-regulation was observed to correlate well with t-tubule loss [185], and recent studies have shown that JP2 knockdown or transgenic expression of JP2 shRNA causes t-tubule remodeling [194]. Thus, although the molecular mechanisms underlying JP2 down-regulation in heart failure remain unclear, existing data indicate that stabilization of JP2 levels may have therapeutic potential. Interestingly, caveolin-3, a muscle-specific caveolae-related protein which associates with JP2, may be important for anchoring JP2 in the t-tubule, and is down-regulated along with JP2 in pathological states including heart failure [218] (Fig. 5B). Furthermore, caveolin-3 KO mice exhibit structural remodeling of the t-tubule network in skeletal muscles [219]. Caveolin-3 over-expression may therefore serve as a therapeutic approach to combat JP2-dependent t-tubule remodeling.

Based on the growing consensus that mechanical stress regulates t-tubule structure and function, it is perhaps not surprising that recent work has implicated a role for calcineurin-NFAT signaling. It is well-established that this pathway transduces mechanical stimuli during hypertrophy [220] through a number of effectors, including microRNAs. MicroRNA-24 (miR-24), a member of the miR-23a-27a-24-2 cluster which is up-regulated in heart failure [221], has been identified as a prominent regulator of JP2 expression in cardiac tissue [221] (Fig. 5B). Overexpression of miR-24 in cultured cardiomyocytes led to JP2 down-regulation, disrupted dyadic structure, and dysfunctional CICR. The same group has since shown that miR-24 suppression protects against heart failure progression, and associated disruption of t-tubules and Ca\textsuperscript{2+} homeostasis [222]. Thus, therapeutic inhibition of miR-24 or other components of the NFAT signaling pathway holds great potential for maintaining dyadic integrity in heart failure.

Another protein of interest is telethonin (Tcap), a stretch-sensitive protein located in the Z-disc of cardiomyocytes. Tcap KO mice were observed to exhibit progressive disruption of the t-tubule network during development [191]. These defects were especially apparent in hearts working against increased mechanical load (thoracic aortic constriction). In addition, increased Tcap expression was associated with recovery of t-tubules during reverse remodeling induced by SERCA2a gene therapy [192]. Further studies are, however, needed to elucidate the precise interplay between myocardial load, expression of Tcap, and t-tubule organization.

Amplyphinis-2 (BIN1) is a protein involved in the formation of t-tubule invaginations and the trafficking of LTCCs to the t-tubules [223, 224]. Hong et al. [225] found that BIN1 was downregulated in failing human cardiomyocytes and that this was linked to decreased expression of LTCCs in the cell membrane. However, it is important to point out that in many heart failure models t-tubule density is unaltered or even increased, including a larger fraction of longitudinally-oriented tubules [109, 179, 183, 189, 201, 202]. Whether BIN1 is involved in the growth of these new tubules re-
mains unknown, but raises the possibility that BIN1 overexpression might have therapeutic potential.

FUTURE PERSPECTIVES

As is evident from the above discussion, a growing body of preclinical data suggest that targeting abnormal Ca\(^{2+}\) handling in heart failure may be beneficial. However, much work remains to be done. Our current understanding of Ca\(^{2+}\) homeostasis during heart failure is largely focused on the end-stage of the disease, with surprisingly little known about changes in Ca\(^{2+}\) homeostasis during disease development. Existing data indicate that Ca\(^{2+}\) cycling is initially increased at early stages [226], but it is unclear if such alterations may be beneficial if maintained over longer periods, or if these apparent “compensations” in fact drive disease progression. Investigating the time course of changes in Ca\(^{2+}\) handling will generate complex data sets, and it will be a considerable challenge to integrate and interpret these data effectively. Therefore, we believe that the emerging fields of systems biology and mathematical mod-
CONCLUSION

The past several decades of investigation have revealed that altered Ca\(^{2+}\) handling is as important a pathophysiological mechanism in heart failure, and that SERCA2a, NCX, RyR, and t-tubule structure are key players. Such insight has enabled the recent therapeutic targeting of these systems, resulting in attenuation or reversal of important facets of the heart failure phenotype, including mechanical dysfunction, arrhythmogenesis, and pathological signaling. Although the majority of these data are from experimental studies, recent and ongoing clinical trials have shown promise. Since heart failure is a diverse disease entity, with variations in etiology, phenotype, stage and molecular basis, it is anticipated that future therapies based on cardiomyocyte mechanisms will have the potential to individualize treatment, and improve patient outcomes.

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CONFlict OF INTEREST

The authors confirm that this article content has no conflict of interest.

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