Editorial

A Guide for the Perplexed
Towards an Understanding of the Molecular Basis of Heart Failure
Andrew R. Marks, MD

Heart failure is a complex disorder involving maladaptive responses that result in defective regulation and function of multiple biological systems. Central to our understanding of heart failure and to the ability to design and test novel therapeutic approaches that will prolong survival and improve quality of life for the millions of individuals worldwide is the need to gain a better understanding of the molecular pathogenesis of the disorder.

In the search for molecular physiological defects in failing hearts, it is logical to examine the mechanism of excitation-contraction (EC) coupling in which cardiomyocyte membrane depolarization, because of the cardiac action potential, is translated into mechanical contraction in the heart. This system requires the normal function of 3 key elements: (1) calcium (Ca\(^{2+}\)) entry via the voltage-gated Ca\(^{2+}\) channel (VGCC) on the plasma membrane (transverse tubule); (2) Ca\(^{2+}\) release via the ryanodine receptor/Ca\(^{2+}\) release channel (RyR2); and (3) Ca\(^{2+}\) uptake via the Ca\(^{2+}\)-ATPase on the sarcoplasmic reticulum (SR) (Figure 1).

Data from our laboratory have shown that RyR2s in failing hearts are defective because of a chronic hyperadrenergic state of heart failure that results in hyperphosphorylation of the channel by cAMP-dependent protein kinase (PKA).\(^1\) PKA hyperphosphorylation makes the RyR2 channels in failing hearts leaky by depleting these macromolecular complexes of the stabilizing protein FK506 binding protein (FKBP12.6). One of the roles of FKBP12.6 is to stabilize the RyR2 in the closed state during diastole to ensure against an aberrant SR Ca\(^{2+}\) leak, which can trigger cardiac arrhythmias by initiating delayed afterdepolarizations (DADs).\(^2,3\)

We have shown that PKA phosphorylation of RyR2 at serine 2809 causes dissociation of FKBP12.6 from the channels.\(^1\) RyR2s are homotetramers comprised of 4 RyR2 monomers, each of which has a single PKA phosphorylation site (serine 2809) and binds one FKBP12.6. Transient PKA phosphorylation of 1, 2, or 3 of the 4 PKA sites on RyR2 and the concomitant dissociation of 1, 2, or 3 of the 4 FKBP12.6 bound to the channel transiently increases the activity of the channel by shifting the Ca\(^{2+}\) dependence for activation to the left. Dephosphorylation of RyR2 by phosphatases that are bound to the channel reverses this activation of the channel.\(^4\) Thus, this form of regulation of RyR2 provides a mechanism for transiently increasing systolic SR Ca\(^{2+}\) release in a physiological manner to increase cardiac contractility.

An important problem in heart failure is that RyR2s are chronically PKA hyperphosphorylated and depleted of FKBP12.6, and as a result become leaky. These chronically defective RyR2 channels contribute to depletion of SR Ca\(^{2+}\) stores that in turn explain, in part, the reduced contractility of cardiac muscle in failing hearts. An important and obvious question is why, after hundreds of millions of years of evolution, a system that is so critical to our survival is so susceptible to defective regulation (after all, heart failure is the leading cause of mortality in the developed world)? One answer is that heart failure itself is a new disease, as well as a post-reproductive age disease. People simply did not live long enough, until the last several hundred years, for heart failure to be a significant problem. This, combined with the fact that when it does occur, heart failure typically affects individuals past their reproductive age, explains the lack of evolutionary pressure to adapt to conditions in which cardiac muscle becomes dysfunctional such as myocardial infarctions and viral cardiomyopathies.

The clinical course of heart failure, therefore, represents a maladaptive activation of the classic “fight or flight” stress response in a futile attempt to increase cardiac output. In the absence of alternative signaling pathways this futile, maladaptive response results in the chronic hyperadrenergic state of heart failure. One consequence of this chronic maladaptive state is dysregulation of key EC-coupling molecules in such a way that global cardiac muscle function is depressed. This maladaptive response drives the progressive downhill clinical course of patients with heart failure. For this reason, novel therapeutic approaches that target the defective molecular signaling that causes defective contractility hold tremendous promise for the future.

Under normal physiological conditions, the classic “fight or flight” stress response involves activation of the sympathetic nervous system resulting in catecholamine release. Catecholamine-mediated activation of β-adrenergic receptors in the heart stimulates a signaling pathway that increases PKA phosphorylation of each of the 3 key components of cardiac EC coupling, resulting in increased Ca\(^{2+}\)-dependent contractility.

Thus, during normal transient sympathetic nervous system stimulation, PKA phosphorylation activates the trigger for cardiac EC coupling (by PKA phosphorylating the VGCC responsible for the Ca\(^{2+}\) influx that activates RyR2), SR Ca\(^{2+}\) release via RyR2 (by PKA phosphorylating RyR2), and SR...
Figure 1. Regulation of key molecules in cardiac EC coupling by stress activated pathways. The normal fight or flight stress response activates 3 key molecules involved in cardiac EC coupling via PKA phosphorylation: (1) the trigger for cardiac EC coupling, the voltage-gated Ca$^{2+}$ channel (VGCC); (2) the SR Ca$^{2+}$ release channel RyR2; and (3) the Ca$^{2+}$ uptake pathway (via PKA phosphorylation of phospholamban which reduces inhibition of the Ca$^{2+}$-ATPase SERCA2a). These regulatory events conspire to increase systolic SR Ca$^{2+}$ release and thereby increase contractility, providing increased cardiac output to meet metabolic demands of stressful conditions. In failing hearts, this system becomes defective because of the maladaptive chronic hyperadrenergic state of heart failure, resulting in PKA-hypophosphorylated RyR2 channels that cause a diastolic SR Ca$^{2+}$ leak$^1$ that conspires with reduced SERCA2a-mediated SR Ca$^{2+}$ uptake (due in part to PKA hypophosphorylated phospholamban which inhibits SERCA2a) to deplete SR Ca$^{2+}$ and contribute to contractile dysfunction of cardiac muscle.

Ca$^{2+}$ uptake via SERCA2a (by PKA phosphorylating phospholamban resulting in reduced inhibition of SERCA2a). It is this system, in addition to others, that becomes defective in failing hearts.

In our model of the likely molecular basis of heart failure (Figure 1), SR Ca$^{2+}$ leak via PKA hyperphosphorylated RyR2 combines with depressed SR Ca$^{2+}$ uptake (due to downregulation of SERCA2a as a result of PKA hypophosphorylation of phospholamban and reduced numbers of SERCA2a) to deplete SR Ca$^{2+}$ content.

As with most new models, especially those proposed in a field that is complex and that has proven to be extraordinarily difficult to dissect at a molecular level, our SR Ca$^{2+}$ leak model has been criticized. A recent study reported that PKA phosphorylation of RyR2 has no effect on Ca$^{2+}$ spark rate in murine cardiomyocytes at rest.$^5$ Some have interpreted this study as evidence that PKA phosphorylation of RyR2 does not modulate channel function in cells and therefore has no effect on SR Ca$^{2+}$ release. In purely evolutionary terms, it would be hard to explain why the macromolecular complex that controls PKA phosphorylation of RyR2 has been conserved over hundreds of millions of years from the worm Caenorhabditis elegans to Homo sapiens$^6$ if it has no fundamental functional role. Rather, I suggest that one ask the question of whether the experimental design needs to be reconsidered. Indeed, it is important to remember that in the heart there are stringent mechanisms designed to prevent aberrant SR Ca$^{2+}$ leak through RyR2 under physiological conditions, and that these controls only break down under extreme pathological conditions, such as heart failure and certain conditions associated with cardiac arrhythmias.

Two of the important molecular mechanisms that ensure that the SR Ca$^{2+}$ release channel stays tightly closed during diastole are FKBP12.6, which stabilizes the closed state of the channel, and Mg$^{2+}$, which is present at mmol/L concentrations in cardiac myocytes and is a potent inhibitor of RyR2. Li et al$^7$ examined the effects of isoproterenol-induced PKA phosphorylation of RyR2 in cardiomyocytes. For technical reasons (the permeabilization protocol they used resulted in a high SR Ca$^{2+}$ leak even before application of isoproterenol), they performed their experiments at extremely low cytosolic Ca$^{2+}$ levels of 10 nmol/L and 50 nmol/L, which are even below the 100 nmol/L level of Ca$^{2+}$ in resting cardiomyocytes during diastole. Thus, the study by Li et al$^7$ shows that isoproterenol-induced PKA phosphorylation of RyR2 does not cause a significant diastolic SR Ca$^{2+}$ leak. This is a very good thing, because otherwise every time an individual with a normal heart exercised (causing PKA phosphorylation of RyR2), he/she would be fire off DADs and would likely go into ventricular tachycardia. The experiments by Li et al$^7$ confirm that in normal (non-failing) cardiomyocytes, PKA phosphorylation of RyR2 under resting diastolic conditions does not cause an SR Ca$^{2+}$ leak via RyR2. What the experiments by Li et al$^7$ do not address is whether the chronic PKA hyperphosphorylation of RyR2 that occurs in failing hearts causes an increase in diastolic SR Ca$^{2+}$ leak. Moreover, the study by Li et al also does not address the question of whether PKA phosphorylation of RyR2 increases EC coupling gain in normal hearts. EC coupling gain can be defined as the ratio of SR Ca$^{2+}$ release to Ca$^{2+}$ current. The analysis of experiments designed to address the question of whether PKA phosphorylation of RyR2 increases EC coupling gain is complex because multiple variables are changing at the same time due to activation of the Ca$^{2+}$ current (the trigger), RyR2 (Ca$^{2+}$ release), and SR Ca$^{2+}$ uptake. Nevertheless, the effect of PKA phosphorylation of RyR2 on EC coupling gain should be assessed in the initial phase of EC coupling. It is during this initial phase of EC coupling when the cytosolic Ca$^{2+}$ that activates RyR2 is beginning to rise that the effects of PKA phosphorylation of RyR2 are physiologically important and are most likely to increase EC coupling gain. PKA phosphorylation increases the sensitivity of RyR2 to activating Ca$^{2+}$, if one examines the total SR Ca$^{2+}$ released divided by the total Ca$^{2+}$ current the effects of PKA phosphorylation of RyR2 on EC coupling gain will likely be obscured.

A recent study by Jiang et al$^8$ reports that they were unable to reproduce 3 of the key findings in our studies on heart failure: (1) PKA hyperphosphorylation of RyR2 in failing hearts; (2) depletion of FKBP12.6 from the RyR2 channel complex by PKA hyperphosphorylation of RyR2; and (3) increased activity of RyR2 channels at low (diastolic) Ca$^{2+}$. An accompanying editorial in *Circulation Research* suggests that on the basis of these conflicting findings, the issue of whether defects in RyR2 actually occur in failing hearts remains open.$^9$ Thus, it is important to examine whether Jiang et al$^8$ in fact have done experiments that should cause
reexamination of the findings on which the heart failure diastolic SR Ca\(^{2+}\) leak model is based.

The experiments in which Jiang et al\(^6\) examine PKA phosphorylation of RyR2 and conclude that it is not significantly increased in failing hearts are incomplete. Jiang et al used a back-phosphorylation method to measure PKA phosphorylation of RyR2. This method requires two measurements: (1) PKA phosphorylation of RyR2 with radioactive ATP to determine the extent to which the channel protein can be phosphorylated; and (2) determination of the amount of RyR2 protein in each sample. Both measurements are essential for obvious reasons; the more protein in a given sample, the greater the PKA phosphorylation signal will be, regardless of the actual phosphorylation status of the channel. Although they did not measure the amount of RyR2 in their samples, Jiang et al reported that the back-phosphorylation assay showed that RyR2 were not PKA hyperphosphorylated in failing hearts.\(^6\) They also used a commercially available phospho-specific antibody that was designed to recognize phosphorylated serine 2809 on RyR2. We have made our own phospho-specific antibody that was designed to recognize phosphorylated serine 2809 on RyR2 and have provided this antibody at no cost to all investigators who have requested it. As with the back-phosphorylation studies, Jiang et al\(^6\) did not measure the amounts of RyR2 protein in their samples. Nevertheless, their data with the phospho-epitope antibody suggest that 50% (2/4) of their heart failure samples did exhibit RyR2 PKA hyperphosphorylation compared with controls. Thus, these incomplete studies from Jiang et al do not appear to be sufficiently convincing to refute the findings of multiple controlled studies,\(^1,11,12,18-21\) which show PKA hyperphosphorylation of RyR2 in heart failure.

In our initial report on the discovery of defective RyR2 channels in failing hearts, we were careful to point out that not all RyR2 channels from failing hearts are abnormal.\(^1\) Indeed, we reported that although \(\approx70\%\) of RyR2 channels from failing hearts exhibited abnormal single channel properties, only 15% exhibited the most severe defects.\(^1\) Jiang et al\(^6\) reported normal RyR2 in failing hearts. It is not surprising that one can find a normal RyR2 channel in failing hearts, because in a population of channels, not all are abnormal. Moreover, the conditions used by Jiang et al to study RyR2 channel function are such that they would never detect the defects in channel function that we have reported in failing hearts. We clearly specified that the abnormal RyR2 channel behavior occurs at diastolic levels of cytosolic Ca\(^{2+}\) (eg, \(\approx100\) nanomolar).\(^1\) Indeed, it makes little sense to explore whether an SR Ca\(^{2+}\) “leak” occurs through RyR2 using measurements made at systolic levels (eg, micromolar) of cytosolic Ca\(^{2+}\). After all, when the channel is already open and pouring Ca\(^{2+}\) out from the SR to activate systole, a “leak” is irrelevant. In any case, we specified that the RyR2 channel defects occur at diastolic levels of cytosolic Ca\(^{2+}\), which are typically about 100 nanomolar. Jiang et al\(^6\) showed that they failed to detect defects in RyR2 channel function in channels recorded at either 5 micromolar or 100 micromolar cytosolic cytosolic Ca\(^{2+}\). Thus, Jiang et al\(^6\) used levels of Ca\(^{2+}\) that are 1000 times higher than diastolic Ca\(^{2+}\) levels, and they did not look for abnormal RyR2 activity at diastolic (nanomolar) levels of cytosolic Ca\(^{2+}\).

With regard to the third important finding of ours addressed by Jiang et al,\(^6\) they report that PKA phosphorylation of RyR2 does not dissociate FKBP12.6 from RyR2. They PKA phosphorylated ventricular homogenates, pelleted the samples, and immunoblotted for FKBP12 and FKBP12.6. FKBP12.6 should only appear in the pellet if it is bound to the much larger RyR2. However, FKBP12 is a cytosolic protein that should not be present in the pellet because it lacks high affinity binding to canine RyR2. Jiang et al\(^6\) showed a large amount of FKBP12 in the membrane pellet, proving that their membrane pellets were contaminated with cytosolic proteins as FKBP12 is quite abundant in the cytosol of muscle.\(^8\) Jiang et al also showed that FKBP12.6 was present in the membrane (pellet) fraction in both control and PKA-phosphorylated samples, and concluded that PKA phosphorylation does not dissociate FKBP12.6 from RyR2. However, because their membrane pellets were contaminated with cytosolic proteins, they cannot conclude from this experiment that PKA phosphorylation does not dissociate FKBP12.6.

This review points out the importance of careful assessment of studies to ensure that valid and useful comparisons are made. In the present case, our findings have been fully supported by independent studies from other laboratories.\(^9-12\) Moreover, our data are not at odds with those from many other laboratories that have focused more on the defects in SR Ca\(^{2+}\) uptake in heart failure. Clearly, an increased SR Ca\(^{2+}\) leak and a decreased SR Ca\(^{2+}\) uptake will conspire to achieve the same end, which is depletion of SR Ca\(^{2+}\). Both the SR Ca\(^{2+}\) leak that we have described and the defect in SR Ca\(^{2+}\) uptake proposed by multiple groups require further study, and both may need to be addressed in novel therapeutic approaches in heart failure.

One way to determine whether the diastolic SR Ca\(^{2+}\) leak or decreased SR Ca\(^{2+}\) reuptake is the dominant lesion in heart failure is to assess the effects of therapies designed to treat heart failure by fixing either the leak or the defect in uptake. Indeed, multiple studies have shown that inhibiting phospholamban as a means of increasing SR Ca\(^{2+}\) reuptake can prevent heart failure in some genetic models of cardiomyopathy. What remains to be determined is whether upregulation of SR Ca\(^{2+}\) reuptake can restore cardiac function in a long-term model of preexisting heart failure. Moreover, in pursuing such studies, it will be important to determine whether or not the increased energetic cost of up-regulating the SR Ca\(^{2+}\)-ATPase is detrimental in the setting of cardiomyopathy. In addition, because SR Ca\(^{2+}\) overload has classically been used as a model to study DADs that trigger cardiac arrhythmias, it will be important to demonstrate in long-term animal models that phospholamban inhibition is not pro-arrhythmogenic.

Recent studies based on our discovery of the diastolic SR Ca\(^{2+}\) leak via PKA hyperphosphorylated RyR2 in failing hearts\(^4\) have shown that inhibiting diastolic SR Ca\(^{2+}\) leak by stabilizing the binding of FKBP12.6 to RyR2 with the 1,4-benzothiazepine derivative, JTV519, can significantly improve cardiac hemodynamics in a canine model of heart failure.\(^1,12\) These studies provide substantial support for our model of the mechanism of heart failure that includes an important role for diastolic SR Ca\(^{2+}\) leak via RyR2 (Figure 1). Indeed, the strategy of treating heart failure based on fixing the
Figure 2. A molecular model of heart failure. The maladaptive response to cardiac muscle damage from myocardial infarction (MI), viral infection, or pressure overload due to hypertension (HTN) or valve disease results in activation of signaling pathways including adrenergic, renin angiotensin (RAS), and cytokine, designed as stress responses. The chronic activation of these pathways in heart failure results in defects in EC coupling including: (1) PKA hyperphosphorylation of the cardiac ryanodine receptor (RyR2) that results in depletion of FKBP12.6 (which helps keep the channel closed in diastole) from the channel macromolecular complex, causing a diastolic Ca2+ leak that depletes sarcoplasmic reticulum (SR) Ca2+; and (2) reduced SR Ca2+ reuptake via SERCA2a. Other defects in the maladaptive response in heart failure include alterations in the cytoskeleton15–16 and the extracellular matrix (imbalance between matrix metalloproteinases [MMPs] and tissue inhibitors of metalloproteinases [TIMPs]). 

SR Ca2+ leak through RyR2 in failing hearts is particularly attractive for several reasons: (1) stabilizing FKBP12.6 binding to RyR2 inhibits SR Ca2+ leak by stabilizing the closed state of the channel in diastole2 and should not inhibit activation of the channel in systole; therefore, this approach should not impair contractility; (2) inhibiting the SR Ca2+ leak through RyR2 should reduce, rather than increase, energy consumption because the SR Ca2+ leak itself requires increased SERCA2a activity to maintain low diastolic Ca2+; and (3) stabilizing the closed state of RyR2 in diastole should not be arrhythmogenic. The finding that inhibiting RyR2-mediated SR Ca2+ leak in failing hearts improves heart failure in a large animal model provides strong rationale for pursuing this strategy in additional animal models of heart failure with long-term follow up.

The challenge remains, as always, to objectively assess new information as it comes forth in this complex field (Figure 2) and to maintain a rigorous commitment to careful, controlled studies. Indeed, these requirements are not new in the field of medicine. The 12th century physician-philosopher Moses Maimonides (the title of this essay is borrowed and modified from one of his most famous writings13) wrote in his physician’s oath over 800 years ago, “Grant me the strength, time and opportunity always to correct what I have acquired, always to extend its domain; for knowledge is immense and the spirit of man can extend indefinitely to enrich itself daily with new requirements. Today he can discover his errors of yesterday and tomorrow he can obtain a new light on what he thinks himself sure of today.”

References
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