Defective Ryanodine Receptor Interdomain Interactions May Contribute to Intracellular Ca\(^{2+}\) Leak

A Novel Therapeutic Target in Heart Failure

Stephan E. Lehnart, MD; Xander H.T. Wehrens, MD, PhD; Andrew R. Marks, MD

Heart failure (HF) is a leading cause of morbidity and mortality, and despite optimal medical treatment, patients with NYHA class III–IV symptoms have a 2-year mortality rate approaching 50%. About half of the deaths from HF occur suddenly, and ventricular arrhythmias account for a large percentage of these sudden cardiac deaths (SCDs). There is a strong correlation between chronic increased activity of the sympathetic nervous system (SNS) and poor prognosis in HF. Interestingly, \(\beta\)-adrenergic receptor (BAR) blockers that block the target receptors for catecholamines released during SNS activation reduce HF progression and arrhythmia vulnerability in patients with HF, leading to improved survival. Activation of the BAR signaling cascade driven by the SNS caused by HF initially results in a compensatory response aimed at enhancing cardiac contractility, but when SNS activation is sustained, maladaptive changes occur. Chronic SNS activation desensitizes cardiac BAR signaling, which further contributes to reduced inotropic reserve and worsens remodeling in HF, as part of a maladaptive response that ultimately fails to protect the heart or preserve cardiac function.

Defective intracellular Ca\(^{2+}\) homeostasis has been consistently reported in HF. Because intracellular Ca\(^{2+}\) concentrations ([Ca\(^{2+}\)]\(_i\)) directly regulate contractility of cardiomyocytes, a reduced [Ca\(^{2+}\)]\(_i\) transient amplitude in HF results in decreased force development. A prolonged decay of the [Ca\(^{2+}\)]\(_i\) transient and increased diastolic [Ca\(^{2+}\)]\(_i\) may contribute to slowed relaxation of the failing heart. Several defects in the intracellular [Ca\(^{2+}\)]\(_i\) metabolism have been reported, including depressed Ca\(^{2+}\) uptake, storage, and/or release of Ca\(^{2+}\) from the sarcoplasmic reticulum (SR) storage organelle. Depressed SR Ca\(^{2+}\) release may result from decreased SR Ca\(^{2+}\) uptake via the SR Ca\(^{2+}\) ATPase (SERCA2a), as well as a competitive increase of Ca\(^{2+}\) extrusion to the extracellular space via upregulated function of the plasma membrane Na\(^+\)/Ca\(^{2+}\) exchanger.

Increased diastolic SR Ca\(^{2+}\) leak may also result in decreased SR Ca\(^{2+}\) loading and depressed contractility in HF. Diastolic SR Ca\(^{2+}\) leak is caused by “leaky” ryanodine receptor (RyR2)/SR Ca\(^{2+}\) release channels, which display an increased sensitivity to Ca\(^{2+}\)-induced Ca\(^{2+}\) release and incomplete channel closure during diastole in HF. We and others have previously demonstrated that chronic SNS hyperactivity in HF leads to increased protein kinase A (PKA) phosphorylation of RyR2 in the heart, which results in decreased binding of the channel-stabilizing subunit calstabin2 (FKBP12.6). These findings highlight the important role of defects in RyR2 in HF as previously discussed in a Circulation editorial. The role of leaky RyR2 and SNS activation in triggering ventricular arrhythmias is supported by the observation that inherited mutations in the RyR2 gene cause sudden cardiac death. Patients with catecholaminergic polymorphic ventricular tachycardia (CPVT) are heterozygous for the mutant RyR2 allele and are highly susceptible to exercise-induced cardiac arrhythmias. The new study by Oda and colleagues in this issue of Circulation expands the insights into the fundamental mechanisms of defective RyR2 function in HF and cardiac arrhythmias and suggests a role for defective interdomain interactions within RyR2 as a contributing factor in the development of an abnormal diastolic SR Ca\(^{2+}\) leak.

Concept of Intramolecular Domain Unzipping

Missense mutations in the homologous RyR1 channel gene that have been linked to diseases of the skeletal muscle, including malignant hyperthermia (MH) and central core disease (CCD), cluster in regions of the RyR1 channel that correspond to the regions of the RyR2 channel that contain the CPVT mutations. We previously noted that similarity between the distribution of CPVT and MH/CCD mutations in RyR2 and RyR1, respectively, raises the possibility that similar biophysical defects in the cardiac and skeletal RyR channels account for CPVT and MH/CCD. Indeed, the majority of MH mutations in RyR1 are located in well-defined domains of the RyR1 channel, also referred to as MH clusters or hot-spot regions. Under experimental conditions, MH mutant RyR1 channels display a phenotype similar to CPVT mutations in RyR2, hyperactivity under resting conditions, and Ca\(^{2+}\) leak from the SR into the cytoplasm. Ikemoto and colleagues have proposed that N-terminal and central domains in RyR1 contribute to the conformational stabilization of the closed state of the channel via domain-zipping interactions. More simply put, they predict that...
sequences of the channel that are not adjacent to one another in the linear peptide sequence can interact once the channel is folded into its 3-dimensional structure. Furthermore, Ikemoto and colleagues have proposed that channel opening during excitation-contraction coupling requires domain unzipping to reduce the conformational energy constraints necessary to induce the open state.\textsuperscript{34, 35} According to the domain-zipping model, MH-associated mutations occurring in either the N-terminal or central domains of RyR1 reduce the affinity of the zipper domains, resulting in leaky channels.

To test this hypothesis, a domain-specific peptide (DP4) corresponding to the putative RyR1 central zipper domain was applied to inhibit binding of the central to the N-terminal zipper domain. The resulting channel defects in RyR1 are similar to those observed in MH-associated RyR1 mutations (eg, increased channel open probability, abnormal SR Ca\textsuperscript{2+} release, and hypersensitivity to activation).\textsuperscript{33, 36, 37} A note of caution should be introduced, however, because experiments with synthetic peptides are susceptible to artifacts from unspecific peptide binding resulting from excess peptide concentrations. It was proposed that defective interdomain interactions within MH-mutant RyR1 channels contribute to leaky RyR1 channels that cause MH.\textsuperscript{38}

### Defective Intermolecular Domain Interactions in the Cardiac RyR2 Isoform and Correlation With Structural Studies

Because skeletal RyR1 and cardiac RyR2 are 66% homologous at the protein level, it was proposed that domain unzipping may also occur in the cardiac Ca\textsuperscript{2+}-release channel RyR2. Oda and colleagues have designed a synthetic peptide (DPc10), corresponding to Gly2460-Pro2495 of RyR2. Binding of the peptide DPc10 to the hypothetical N-terminal zipper domain of RyR2 was found to increase sensitivity to Ca\textsuperscript{2+}-dependent channel activation and diastolic SR Ca\textsuperscript{2+} leak to levels observed in failing hearts. In contrast, introduction of the CPVT-associated RyR2 mutation Arg2474Ser into DPc10 (DPc10-mut) abolished these effects indirectly, suggesting that the DPc10-mut peptide, which was not able to interfere with the interdomain interaction or to produce domain unzipping effects, may mimic effects of the identical CPVT mutation in the RyR2 channel, as we have shown earlier.\textsuperscript{28}

To further test the hypothesis that DPc10 produced unzipping of interacting N-terminal and central domains in RyR2, the fluorescent label MCA was cross-linked to RyR2 with DPc10 peptide targeting. Site-specific antibody labeling of calpain-digested RyR2 fragments revealed that MCA bound to the N-terminal 120 kDa of RyR2. These data support the idea that the N-terminal and central domains of RyR2 interact with each other. Interestingly, data from cryo-electron microscopy studies of RyR2 support this observation as well.\textsuperscript{39}

Serysheva and coworkers recently published the 3-dimensional RyR1 structure at 14 Å resolution, and projected residues 216 to 572 of the N-terminus within a group of structural surface domains that form the 4 corners of the cytoplasmic aspect of the channel tetramer, also referred to as the clamp regions (Figure, A and B). These findings are consistent with previous localization studies of the RyR1 and RyR3 N-termini at significantly lower resolutions.\textsuperscript{40, 41} Interestingly, the clamp structure containing the N-terminus projection was shown to undergo conformational changes corresponding to channel opening and closing\textsuperscript{12, 42} and appears to be localized near the calstabin1 (FKBP12) binding region.\textsuperscript{44} Because we and others have shown that calstabin1 (FKBP12) binds to the central domain of the full-length RyR1 involving the critical amino acid Val\textsuperscript{2461} corresponding to Ile\textsuperscript{2427} in the cardiac RyR2,\textsuperscript{45, 46} the structural and experimental data provide further support for the concept of N-terminal and central domain interaction. Moreover, Wagenknecht and colleagues have demonstrated that Thr\textsuperscript{1366} within divergent region 2 (DR2) localizes to clamp domain 6 in RyR1,\textsuperscript{47} whereas...
Thr\textsuperscript{2374} within DR3 localizes to clamp domain 9 near the calstabin binding region in RyR2 (Figure, B).\textsuperscript{48} Because of the high degree of sequence identity, it is likely that RyR1 intradomain mechanisms and interactions with calstabin1 similarly apply to the cardiac RyR2 and calstabin2, as summarized in part C of the Figure. It should be noted, however, that no direct structural evidence of interdomain interaction is available, although a casual observer of the RyR channel 3-dimensional structures will readily appreciate extensive domain interactions in the huge cytoplasmic domain of this largest of all ion channels (Figure, B).

Further support for the hypothesis of interacting N-terminal and central domains within RyR2 was obtained with the bulky fluorescence quencher QSY-BSA. The rationale of peptide targeting of the MCA fluorescent probe to the N-terminus of RyR2 was used in combination with the large fluorescence quencher to monitor accessibility of unzipped domain configurations. Measurement of QSY-BSA fluorescence quenching was considerably increased after treatment with DPC10 or cAMP (which increases PKA phosphorylation of RyR2 at Ser\textsuperscript{2809}).\textsuperscript{49} Confirming previous results, accessibility of the QSY-BSA quencher was also significantly increased in SR preparations from failing hearts, which is consistent with domain unzipping resulting from PKA hyper-phosphorylation of RyR2.\textsuperscript{21}

Under physiological conditions, stress or exercise result in transiently increased PKA phosphorylation of RyR2 at Ser\textsuperscript{2809}, which transiently decreases the binding affinity of calstabin2 to RyR2 and increases Ca\textsuperscript{2+}-dependent activation of the channel.\textsuperscript{28,49,50} Thus, after PKA phosphorylation of RyR2, there is increased SR Ca\textsuperscript{2+} release for any given trigger [Ca\textsuperscript{2+}]\textsubscript{j}.\textsuperscript{28,50} On the other hand, chronic PKA hyperphosphorylation of RyR2 in HF results in incomplete channel closure during diastole, which causes depletion of the SR Ca\textsuperscript{2+} store and reduced systolic Ca\textsuperscript{2+} release.\textsuperscript{19,21,51} Experiments with the QSY-BSA fluorescence quencher revealed that DPC10-induced domain unzipping does not induce calstabin2 dissociation by itself. Rather, the observations by Oda et al suggest that unzipping may (1) facilitate PKA phosphorylation of RyR2 and (2) enhance calstabin2 dissociation in response to PKA phosphorylation of RyR2. These data suggest that peptide-induced domain unzipping would increase the accessibility of the PKA catalytic subunit (which is bound to RyR2 via the targeting protein mAKAP) to the PKA phosphorylation site Ser\textsuperscript{2809} on RyR2.\textsuperscript{52} Alternatively, under physiological conditions PKA phosphorylation may be expected to induce conformational changes in RyR2 consistent with domain unzipping. Determination of the exact role of domain unzipping in the gating mechanism and regulation of RyR2 will require further studies. Such studies may also reveal how conformational changes in RyR2 resulting from PKA phosphorylation and domain unzipping reduce calstabin2 (FKBP12.6) binding affinity to the channel complex.

**Leaky RyR2 Channels as a Therapeutic Target for Heart Failure**

The study by Oda and colleagues provides additional confirmation that abnormal RyR2 channel function in failing hearts is caused by chronically increased levels of PKA phosphorylation of the channel resulting in dissociation of the calstabin2 (FKBP12.6) subunit, which is consistent with previous studies.\textsuperscript{20,21,23–26,53–55} Moreover, it suggests that domain unzipping may contribute to RyR2 channel dysfunction in HF. Together, these findings provide further support for the concept that normalizing RyR2 channel function may represent a novel therapeutic approach for treating HF.

Initial studies have associated normalized RyR2 function with therapeutic benefits in a canine model of HF and in patients with HF treated with \(\beta\)-blockers.\textsuperscript{22–24} It was shown that blockade of \(\beta\)AR reduced intracellular cAMP levels and decreased PKA hyperphosphorylation of RyR2, leading to normalization of RyR2 macromolecular complex stoichiometry (eg, increasing binding of calstabin2 to RyR2) and inhibition of the diastolic SR Ca\textsuperscript{2+} leak via RyR2 in failing hearts. It is well known, however, that \(\beta\)-blocker treatment offers only partial protection from HF progression and SCD and may come with unacceptable side effects, including lethargy, impotence, depression, and failure to improve exercise tolerance.\textsuperscript{56}

In proof-of-principle studies we have shown that a mutant calstabin2 with a neutralized charge that is able to bind to PKA phosphorylated RyR2 rescues channel function in CPVT mutant and PKA hyperphosphorylated RyR2, providing direct evidence for the ability of enhanced calstabin2 binding to normalize defective RyR2 function.\textsuperscript{28} Therefore, we have postulated that interventions that increase calstabin2 binding to RyR2 may fix the diastolic SR Ca\textsuperscript{2+} leak and prevent HF progression and triggered cardiac arrhythmias.\textsuperscript{21} On the basis of our initial studies that demonstrated leaky RyR2 in HF, Matsuizaki and colleagues showed that JTV-519, a 1,4-benzothiazepine derivative, slows the progression of canine HF by inhibiting RyR2 hyperactivity and intracellular Ca\textsuperscript{2+} leak.\textsuperscript{55} We have demonstrated that JTV-519 can effectively suppress ventricular arrhythmias in a genetic mouse model by rebinding calstabin2 to RyR2\textsuperscript{20} and normalize CPVT mutant RyR2 channel function,\textsuperscript{27} indicating that JTV-519 and its derivatives could constitute a novel class of drugs known as “calcium release channel stabilizers” for the treatment of heart failure and cardiac arrhythmias.

In the study by Oda et al, the DPC10 peptide, cAMP, or calstabin2 dissociation from RyR2 by FK506 increased QSY-BSA quenching, which could be inhibited by JTV-519. Moreover, SR Ca\textsuperscript{2+} store depletion in DPC10-treated or failing cardiomyocytes could be reversed by JTV-519 treatment. These data clearly establish the beneficial effects of JTV-519 at the cellular level, which extends previous in vivo findings. The experiments suggest that JTV-519 may stabilize domain interactions in the zipped state, which could be used to lead to ventricular arrhythmias. Caution must be applied to proposals to use JTV-519 for patients because it is known to have potent human K\textsuperscript{+} channel–blocking activity, which can cause QT prolongation as well as L-type Ca\textsuperscript{2+} channel-blocking activity (shown to be detrimental in HF patients). However, recently we have synthesized novel derivatives of JTV-519 that have reduced human ether-a-go-go– and L-type channel-blocking activity. These new compounds, known as calcium release channel stabilizers, have enhanced efficacy in terms of fixing the leak.
in RyR2 channel in HF and preventing exercise-induced arrhythmias in mice, raising the possibility that such compounds could be developed into therapeutics for patients.

### References


49. Wehrens XH, Lehnhart SE, Reiken SR, Marks AR. Ca\(^{2+}\)/calmodulin-dependent protein kinase II phosphorylation regulates the cardiac ryanodine receptor. *Circ Res*. 2004;94:e61–e70.


**Key Words:** Editorials calcium death, sudden heart failure sarcoplasmic reticulum
Defective Ryanodine Receptor Interdomain Interactions May Contribute to Intracellular Ca\(^{2+}\) Leak: A Novel Therapeutic Target in Heart Failure

Stephan E. Lehnart, Xander H.T. Wehrens and Andrew R. Marks

_Circulation_. 2005;111:3342-3346
doi: 10.1161/CIRCULATIONAHA.105.551861

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2005 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/111/25/3342

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation_ is online at:
http://circ.ahajournals.org/subscriptions/