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An invited review following *the Soujinkai Young Investigator Award*: Ryanodine Receptor Bound Calmodulin as a Novel Therapeutic Target of Arrhythmias and Heart Failure

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Abstract Life-threatening situations that can arise in patients with heart failure are widely recognized in clinical cardiology. This occasionally results from ventricular arrhythmias and hence the management of lethal arrhythmias is now one of the most important issues in the treatment of heart failure. In the failing cardiomyocytes, an abnormal regulation of intracellular calcium (Ca^{2+}) handling by a Ca^{2+} release channel of the sarcoplasmic reticulum (SR), so-called ryanodine receptor (RyR2), is known to play an important role in the mechanism underlying cardiac arrhythmias. On the other hand, in catecholaminergic polymorphic ventricular tachycardia (CPVT), mutation-linked defective channel gating of the RyR2 is also known as a major pathogenic mechanism underlying fatal arrhythmias. The knowledge gained from these studies upon the RyR2 and its modulatory proteins, such as calmodulin (CaM), may lead to the development of a new pharmacological or genetic strategy for the treatment of heart failure and cardiac arrhythmias. In this review, we focused on the role of CaM on the regulation of the channel gating of the RyR2 in the pathogenesis of heart failure and fatal arrhythmias, and discuss the possibility of developing a novel therapeutic strategy targeting the RyR2.

Key words: heart failure, lethal arrhythmia, cardiac ryanodine receptor, calmodulin

The cardiac ryanodine receptor (RyR2) plays a key role in the regulation of intracellular Ca^{2+} homeostasis, and its defectiveness leads to heart failure and lethal arrhythmias.[1] More than 150 RyR2 missense mutations so far identified are partly linked to CPVT.[2] These mutations are not randomly distributed, but cluster in 3 well-defined regions of the RyR2 that correspond to malignant hyperthermia or central core disease mutable regions, designated as the N-terminal domain (1-619 amino acid), central domain (2000-2500 amino acid), and C-terminal transmembrane domain. Such a unique distribution of missense mutations within the RyR2 suggests that the RyR2 shares a common domain mediated channel regulatory mechanism. Based on the notion that a single amino-acid mutation at different domains results in a nearly identical phenotype of the channel dysfunction such as hyperactivity of the channel to Ca^{2+} and/or agonists, Ikemoto and colleagues proposed a "domain switch hypothesis".[3,4] Namely, in the non-activated or normal state, the N-terminal and central domains make close contact at several sub domains (domain

zipping), whereas in an activated or diseased state the interaction between both domains are disturbed (domain unzipping), thereby leading to destabilized channels.

Calmodulin (CaM), one of the accessory proteins of RyR2, has recently attracted attention in the context of the defective channel gating of the RyR2 in various heart diseases. CaM inhibits the RyR2 opening probability at a physiological [Ca^{2+}] by binding to CaMBD, suggesting that the RyR2-bound CaM (CaM-RyR2) stabilizes the closed state of RyR2 channels in the normal resting state. In this regard, the CaM binds to the restricted region {CaM binding domain (CaMBD): 3583-3603} within the RyR2 midway between the so-called "clamp" region and transmembrane channel region of the RyR2. On the other hand, there is a domain (4026-4172 for RyR2 and 4064-4210 for RyR1), which has EF hand motifs with a similar amino-acid sequence to CaM; so-called CaM-like domain (CaMLD).[5] Gangopadhyay and Ikemoto proposed a diseased RyR2 model in which CaMBD forms an abnormal activation link with CaMLD, competing with CaM, leading to hypersensitized channel gating to Ca^{2+}, where in a normal state, CaM serves as a natural molecular wedge for the CaMBD-CaMLD link and hence stabilizes the channel.[6] They further demonstrated that endothelin-induced cardiomyocyte hypertrophy was prevented by interfering with the CaMBD-CaMLD link using an anti-CaMBD antibody,[7] suggesting that cardiac hypertrophy is partly caused by destabilized RyR2 and the subsequent Ca^{2+} leak mediated through the production of the abnormal activation link between the CaMBD and CaMLD. In support of that idea, Huang and colleagues showed that Arg3595 in CaMBD and Lys4269 in CaMLD are both located in a mutually interactive distance, by a methods using cryo-electron microscopy and a fluorescence resonance energy transfer.[8]

The critical role of the RyR2-bound CaM in the development of cardiac disorders was shown in a recent report by Meissner and colleagues.[9] They generated a knock-in (KI) mouse model in which RyR2 was unable to bind to CaM and found that mutant mice developed hypertrophic cardiomyopathy with a severely impaired contractile function and died early.[9] We also previously found that the CaM binding affnity to RyR2 was reduced in pacing-induced heart failure,[10,11] CPVT-associated RyR2 mutations (S2246L,[12] R2474S,[13-15] and N4103K[16]), or under oxidative stress conditions.[17]

In the present study, we evaluated the role of the RyR2-bound CaM in the pathogenesis of cardiac arrhythmias observed in transverse aortic constriction (TAC)-induced failing hearts, and especially examined whether GSH-CaM (Gly-Ser His-CaM, by which the binding affinity to the RyR2 was markedly enhanced) would correct the aberrant spontaneous Ca^{2+} release and arrhythmias in TAC mice. Indeed, here we found that GSH-CaM inhibited spontaneous Ca^{2+} sparks/transients as arrhythmogenic substrates.[18]

RyR2 bound endogenous CaM is decreased in TAC cardiomyocytes

Eight weeks after the TAC or sham operation, the frequency of premature ventricular contractions during a resting condition was significantly higher in TAC mice. Intraperitoneal administration of caffeine and epinephrine revealed a high inducibility of bidirectional or polymorphic ventricular tachyarrhythmias in TAC mice but not in sham mice. Then, we isolated the cardiomyocytes from TAC mice. The endogenous CaM co-localized with the RyR2 along the Z-line was measured by an immunofluorescence analysis. The amount of the endogenous CaM significantly decreased in the TAC cardiomyocytes as compared with the sham cardiomyocytes. Next, we measured the binding affinity of exogenous CaM to the RyR2 in SR vesicles, using the cross-linker, sulfo-SANPAH. The binding affinity of CaM to the RyR2 indeed decreased in the TAC SR vesicles.

Restoration of a normal CaM binding to the RyR2 reduced spontaneous Ca^{2+} release events in failing cardiomyocytes

To examine whether a correction of the defective interaction between CaM and RyR2 inhibits spontaneous Ca^{2+} release events, we measured the frequency of Ca^{2+} sparks (SpF) in saponin-permeabilized cardiomyocytes. The

baseline Ca^{2+} SpF significantly increased in TAC cardiomyocytes. The addition of GSH-CaM, but not WT-CaM, inhibited the increase in the SpF. Both the full width at half-maximum and full duration at half-maximum increased in TAC, and GSH-CaM restored both parameters to a greater extent than WT-CaM. These results suggest that the enhancement of the CaM binding affinity to the RyR2 stabilizes the channel, thereby inhibiting Ca^{2+} leaks in failing cardiomyocytes. We also examined whether the spontaneous Ca^{2+} transients increased in intact failing cardiomyocytes. The spontaneous Ca^{2+} transients were virtually absent in sham cardiomyocytes. However, TAC cardiomyocytes exhibited frequent spontaneous Ca^{2+} transients when the pacing rate increased from 1 to 5 Hz. Importantly, the spontaneous Ca^{2+} transients were inhibited by the incorporation of the GSH-CaM (with the use of a protein delivery kit), but not by that of WT-CaM.

The mechanisms underlying triggered activity and arrhythmias

Heart failure is associated with structural and electrophysiological remodeling, leading to a tissue heterogeneity that enhances arrhythmogenesis and the propensity for sudden cardiac death.[19] Early afterdepolarizations (EADs) are typically observed in cardiac tissues exposed to injury, altered electrolytes, hypoxia, acidosis, catecholamines, and pharmacological agents, including antiarrhythmic drugs. Ventricular hypertrophy and heart failure also predispose to the development of EADs.[20] EADs are commonly associated with the prolongation of the repolarization and are promoted by bradycardia or pauses.[21] In contrast, the appearance of DADs is commonly associated with rapid heart rates.[21] Burashnikov and Antzelevitch proposed a novel mechanism of triggered activity, termed a "late phase 3 EAD," which combines the properties of both EADs and DADs, but has its own unique character.[22,23] Late phase 3 EAD-induced triggered extrasystoles represent a new concept of arrhythmogenesis in which abbreviated repolarization permits a "normal SR calcium release" to induce an EAD-mediated closely coupled triggered response, particularly under conditions permitting intracellular calcium loading. This late phase 3 EAD is assumed to be tachycardia-pause-dependent. EAD, DAD, and late phase 3 EAD induced triggered activity are all capable of initiating and maintaining cardiac arrhythmias. Therefore, reducing the triggered activity by restoring the binding affinity of CaM to RyR2 and hence stabilizing the RyR2 may be helpful in preventing arrhythmias in failing hearts.

A postulated role of the RyR-bound CaM in the development of cardiac hypertrophy

The CaM binding to the amino terminal of GRK5 has been shown to be required for its nuclear translocation in hypertrophic transcriptional signaling mediated by angiotensin II (AngII) and phenylephrine (PE).[24] However, it was unclear where the CaM required for the translocation of GRK to the nucleus comes from. With regard to this, we recently reported that in the hypertrophic signaling the CaM conveyed by GRK5 was supplied by the RyR2 as a macromolecular complex.[25] Namely, various hypertrophic triggers like angiotensin II and pressure-overloads commonly displace CaM from the RyR2, subsequently accelerating the binding of the displaced CaM to the GRK, thereby driving a nuclear translocation of CaM with GRK5 to the nucleus and kinase-dependent nuclear export of the hypertrophy/HF-related transcriptional regulators HDAC4 and 5.[25]

Leaky RyR2 channels as a therapeutic target for arrhythmias and heart failure

We have shown that a treatment with dantrolene, a specific drug for the treatment of malignant hyperthermia, which is known to be an inherited disease with (skeletal) an RyR1 mutation-linked disorder, may be a new therapeutic strategy against heart failure and arrhythmias.[18] Dantrolene was found to inhibit a Ca^{2+} leak through RyR1 and 2 by correcting the defective inter-domain interaction (unzipping) between the N-terminal domain and central domains of RyRs. The binding site of dantrolene is located at the 600-610 amino acid residue or RyR2 (N-terminal

domain). Hartmann and colleagues also reported that dantrolene significantly suppressed the arrhythmogenicity in human diseased cardiomyocytes.[26]

Now, our clinical research is in progress to evaluate the efficacy and safety of dantrolene against heart failure and arrhythmias in patients with chronic heart failure (NYHA II-III) and a reduced ejection fraction in a multicenter, randomized double-blind, and controlled study (SHO-IN TRIAL, UMIN000028766). The primary outcomes of the study are a composite of death (cardiovascular and non-cardiovascular death), lethal arrhythmias (ventricular tachycardia [VT] storm, sustained VT, and ventricular fibrillation) or a first hospitalization for an exacerbation of heart failure 2 years after starting the administration of dantrolene or a placebo.

Conclusion

In failing cardiomyocytes, the binding affinity of CaM to RyR2 was significantly impaired, leading to the abnormal Ca^{2+} handling in the intracellular homeostasis. Restoration of the binding affinity of CaM to the RyR2 improved the RyR2 dysfunction. Targeting the binding affinity of CaM to the RyR2 may be a novel therapeutic strategy against cardiac arrhythmias and heart failure.

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Conflict of Interest

The authors declare no conflict of interest.

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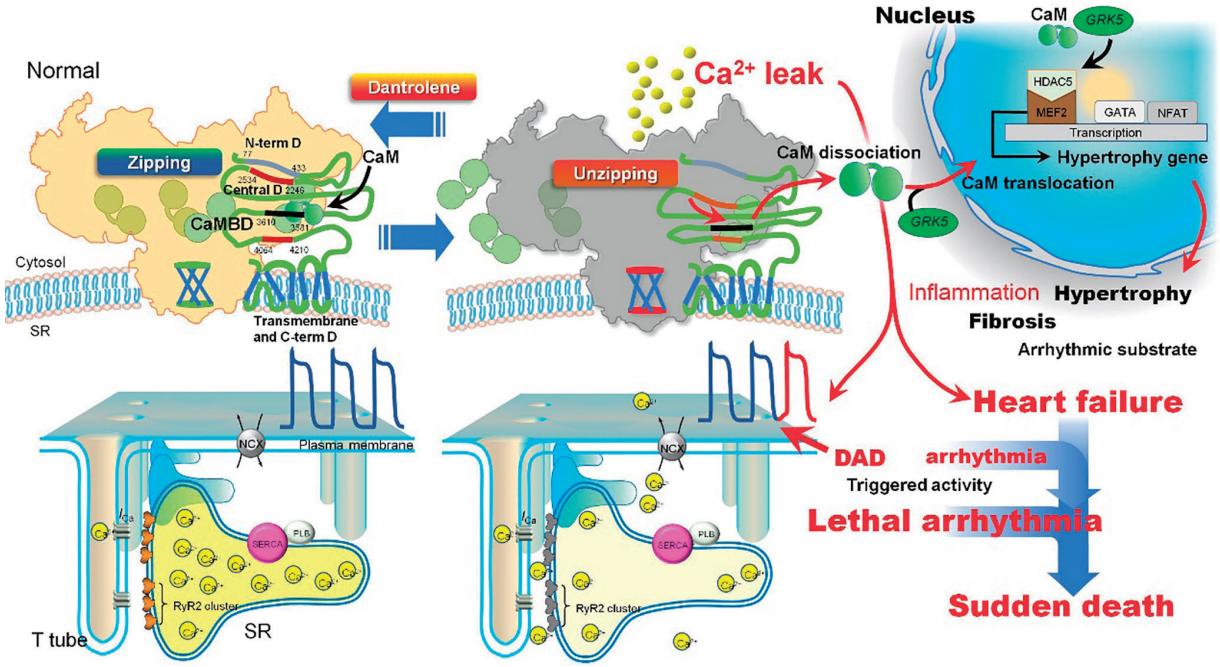

Fig. 1
A postulated molecular mechanism of heart failure and lethal arrhythmias.
Unzipping of an interacting pair of N-terminal D and Central D may allosterically displace CaM from the RyR2, thereby making the channel leaky. This may increase the diastolic intracellular Ca^{2+} level, in turn activating the Na^{+}-Ca^{2+} exchanger and hence accelerating the influx of Ca^{2+}, causing DADs and triggered activity. On the other hand, a translocation of the displaced CaM together with GRK5 to the nucleus may activate the genes related to hypertrophic signaling, causing cardiac hypertrophy and inflammation with arrhythmias (originating from the atrial and ventricular myocardium). In end stage heart failure, an arrhythmogenic substrate ultimately leads to lethal arrhythmias and sudden death. Dantrolene, by restoring a normal zipped state and allosterically restoring the CaM binding to the RyR2 in the diseased hearts, may be a novel therapeutic strategy for heart failure and lethal arrhythmias. N-term D, N-terminal domain; Central D, Central domain; CaM, Calmodulin; CaMBD, Calmodulin binding domain; C-term D, C terminal domain; GRK5, G protein-coupled receptors kinase 5; HDAC5, histone deacetylase 5; HDAC4, histone deacetylase 4; MEF2, myocyte enhancer factor-2; NAFT, nuclear factor of activated T cells; SERCA, sarcoplasmic reticulum Ca^{2+}-ATPase; PLB, phospholamban; NCX, Na^{+}-Ca^{2+} exchange; SR, sarcoplasmic reticulum; RyR2, cardiac ryanodine receptor; DAD, delayed afterdepolarization.