

**COMMENTARY**

Excitation–Contraction Coupling

# Do CPVT-linked mutations alter RYR2 regulation by cytosolic $\text{Ca}^{2+}$ in cardiomyocytes?

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The release of calcium ions from sarcoplasmic reticulum (SR) during cardiac action potentials is essential for cardiac muscle contraction. In cardiac excitation–contraction coupling, a small calcium influx through the plasmalemma activates the SR  $\text{Ca}^{2+}$  release channel, known as type-2 ryanodine receptors (RYR2s), by the mechanism of cytosolic  $\text{Ca}^{2+}$  activation, thereby  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release (Bers, 2002). Dysfunctional RYR2 regulations are known to associate with multiple cardiomyopathies, including various types of arrhythmogenesis as well as cardiac hypertrophy and failure. Over 200 mutations in human RYR2 gene have been reported to associate with catecholaminergic polymorphic ventricular tachycardia (CPVT), in which patients suffer life-threatening arrhythmias triggered by emotional or exercise stresses. One well-known SR  $\text{Ca}^{2+}$  release aberrancy with these RYR2 mutations is observed as a diastole  $\text{Ca}^{2+}$  leak and spontaneous  $\text{Ca}^{2+}$  release, driving electrogenic  $\text{Na}^+$ - $\text{Ca}^{2+}$  exchanger, thereby, delayed- or early-afterdepolarizations (Wehrens et al., 2005). Molecular mechanisms underlying such gain-of-function RYR2 mutations are, however, controversial (Fig. 1). Chen and colleagues proposed that CPVT-associated RYR2 mutations increase sensitivity of RYR2 to luminal  $\text{Ca}^{2+}$  activation, and therefore lower the luminal  $\text{Ca}^{2+}$  threshold for spontaneous  $\text{Ca}^{2+}$  release from SR. During  $\beta$ -adrenergic stimulation, more  $\text{Ca}^{2+}$  are taken up into SR, which facilitates the spontaneous  $\text{Ca}^{2+}$  release (Chen et al., 2014; Jiang et al., 2005; Jiang et al., 2004). On the other hand, it is also conceivable that CPVT mutations render RYR2 easily open by cytosolic ligands, e.g., cytosolic  $\text{Ca}^{2+}$  at the diastole conditions. Thus, upon  $\beta$ -adrenergic stimulation, locally spilled  $\text{Ca}^{2+}$  from SR activates the mutant RYR2 from the cytosolic side, resulting in the spontaneous  $\text{Ca}^{2+}$  release and oscillation. In this issue of *JGP*, Kurebayashi et al. (2022) demonstrated systematic examinations that showed tight correlation between RYR2 regulation by resting cytosolic  $\text{Ca}^{2+}$  concentration ( $\sim 0.1 \mu\text{M}$ ) and the

occurrence of spontaneous  $\text{Ca}^{2+}$  release and oscillation, as well as a threshold of luminal  $\text{Ca}^{2+}$  concentration for spontaneous  $\text{Ca}^{2+}$  release. They tackled the controversial cytosolic  $\text{Ca}^{2+}$  versus luminal  $\text{Ca}^{2+}$  issue using two parallel experimental techniques: (1) simultaneous fluorescence measurements of cytosolic and luminal  $\text{Ca}^{2+}$  of heterologous HEK293 cells expressing recombinant RYR2 mutant proteins, and (2) in vitro RYR2 activity measurements by [<sup>3</sup>H]ryanodine binding methods to determine RYR2 activity at  $0.1 \mu\text{M}$  cytosolic  $\text{Ca}^{2+}$  concentration.

Authors defined  $A_{\text{rest}}$  as an index for RYR2 activity in the resting cells with  $0.1 \mu\text{M}$  cytosolic  $\text{Ca}^{2+}$ . They calculated  $A_{\text{rest}}$  using parameters obtained from [<sup>3</sup>H]ryanodine binding assay which determined the bell-shaped cytosolic  $\text{Ca}^{2+}$ -dependent activities of RYR2 (Fig. 2 A). They proposed that an  $A_{\text{rest}}$  value depend not only on a dissociation constant for activating  $\text{Ca}^{2+}$  ( $K_{\text{ACa}}$ ) but on maximal gain of  $\text{Ca}^{2+}$  activated RYR2 channels,  $A_{\text{max}}$ , and a dissociation constant for inhibiting  $\text{Ca}^{2+}$  ( $K_{\text{ICa}}$ ), which is usually  $>10,000$  times higher than resting  $\text{Ca}^{2+}$  concentration, therefore, minimally affects  $A_{\text{rest}}$  (see Eqs. 1–3 in Kurebayashi et al., 2022). They also pointed out that, in some earlier studies (Jiang et al., 2005; Jiang et al., 2004), mutant RYR2 activation by cytosolic  $\text{Ca}^{2+}$  in [<sup>3</sup>H]ryanodine binding studies was normalized to the peak activity at  $\sim 100 \mu\text{M}$   $\text{Ca}^{2+}$ , and the apparent activating  $\text{Ca}^{2+}$  affinities of RYR2 mutants were compared. Under this data analysis, possible differences of  $A_{\text{max}}$  values among the RYR2 mutants were not considered, which may be one of the reasons for the controversy. In the present study, the authors examined wild type and 10 different CPVT-linked RYR2 mutants and found tight correlation between  $A_{\text{rest}}$  values and luminal  $\text{Ca}^{2+}$  thresholds for spontaneous  $\text{Ca}^{2+}$  release in CPVT-linked RYR2 mutants. In detail, they found that high RYR2 activity at resting cytosolic  $\text{Ca}^{2+}$  (high  $A_{\text{rest}}$  values) reduced the luminal  $\text{Ca}^{2+}$  threshold, resulting in a high rate of  $\text{Ca}^{2+}$  oscillation even with low SR  $\text{Ca}^{2+}$  load (Fig. 2 B), supporting an idea that CPVT mutations affect cytosolic

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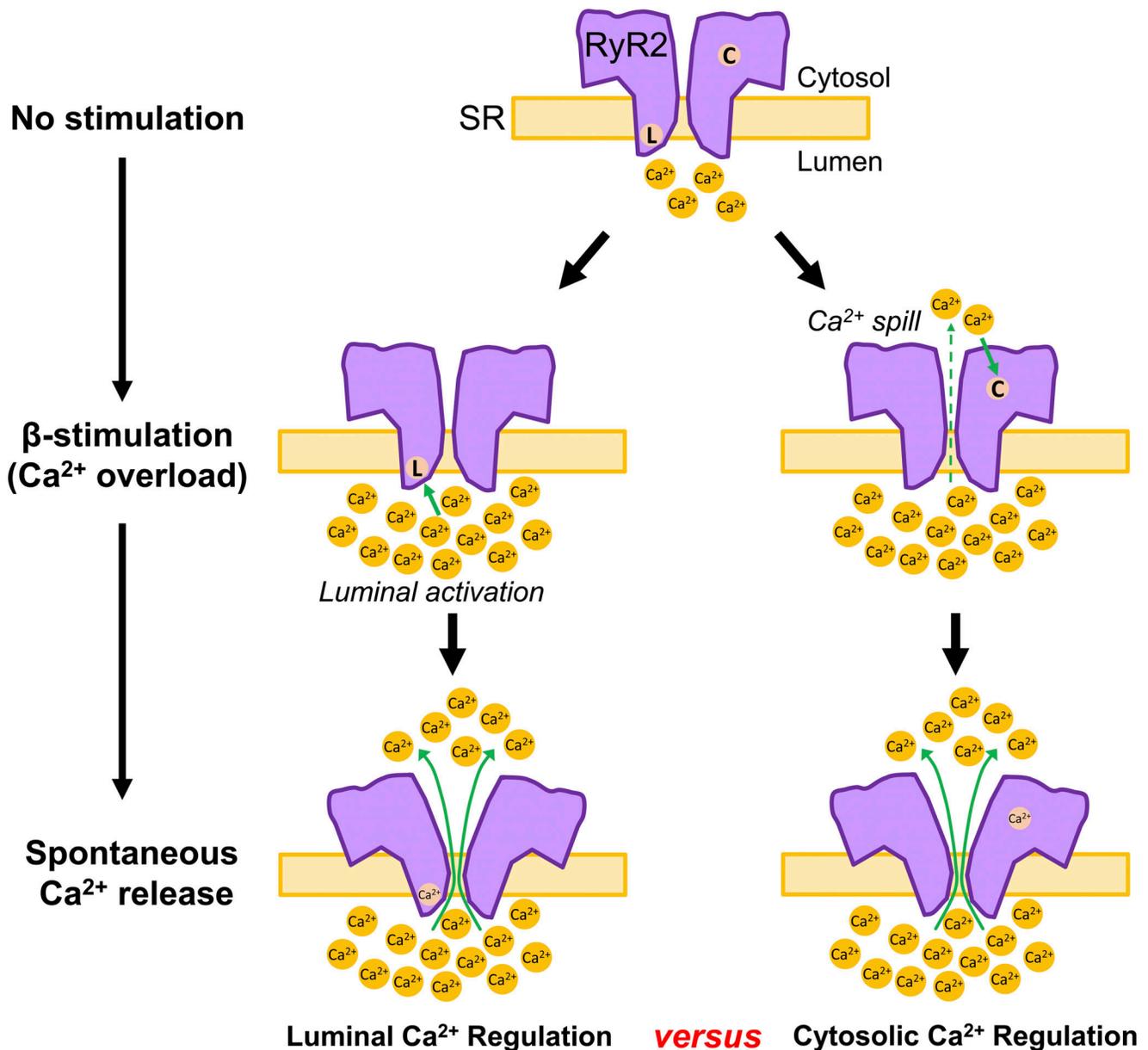


Figure 1. **Two mechanisms for spontaneous Ca<sup>2+</sup> release from SR during  $\beta$ -adrenergic stimulations.** RYR2 have luminal (L) and cytosolic (C) Ca<sup>2+</sup> regulatory sites.  $\beta$ -Adrenergic stimulations increase SR Ca<sup>2+</sup> concentration, which activates RYR2 through luminal regulatory sites; thus, spontaneous Ca<sup>2+</sup> release occurs (left). On the other hand, increased luminal Ca<sup>2+</sup> causes a spill of Ca<sup>2+</sup> to the cytosol, which activates RYR2 through cytosolic regulatory sites (right). In both cases, CPVT-linked mutations enhance the Ca<sup>2+</sup> activation. In this issue of *JGP*, Kurebayashi et al. (2022) showed the evidence for cytosolic regulation of CPVT-linked RYR2 mutants; however, a possibility for synergetic effects of the two mechanisms still remains.

Ca<sup>2+</sup> regulation. On the other hand, the RYR2 mutants with low  $A_{rest}$  indexes required a high SR Ca<sup>2+</sup> load, and thereby more local Ca<sup>2+</sup> spills to trigger the Ca<sup>2+</sup> release and oscillation (Fig. 2 C). Their wet-lab findings are also supported by mathematical simulation in which the authors took not only cytosolic Ca<sup>2+</sup> and luminal Ca<sup>2+</sup> affinity but also SR store-operated Ca<sup>2+</sup> entry to the cytosol into their considerations. In addition, they found that a human patient harboring a CPVT mutation showing high  $A_{rest}$  value in their experiments tended to exhibit onset of arrhythmogenic symptoms at a younger age, indicating pathological relevance of the experimental  $A_{rest}$  index. Overall, the induction of the  $A_{rest}$  value is a key point in this article to address controversial issues.

This article advanced our understanding of Ca<sup>2+</sup> signaling mechanisms underlying CPVT pathology; however, their intriguing findings also open a door for other challenging questions as the authors discussed and partly addressed. The article showed quantitative evidence that spontaneous Ca<sup>2+</sup> release through CPVT RYR2 mutants was caused by their enhanced activity in cytosolic Ca<sup>2+</sup> regulation. However, this does not rule out a luminal Ca<sup>2+</sup> regulatory mechanism in RYR2. Ca<sup>2+</sup>-dependent regulation from both the cytosolic and luminal side may work synergically. The authors partly addressed this question by constructing RYR2 carrying double mutations, one on the CPVT site (R2474S) and the other on the previously reported luminal

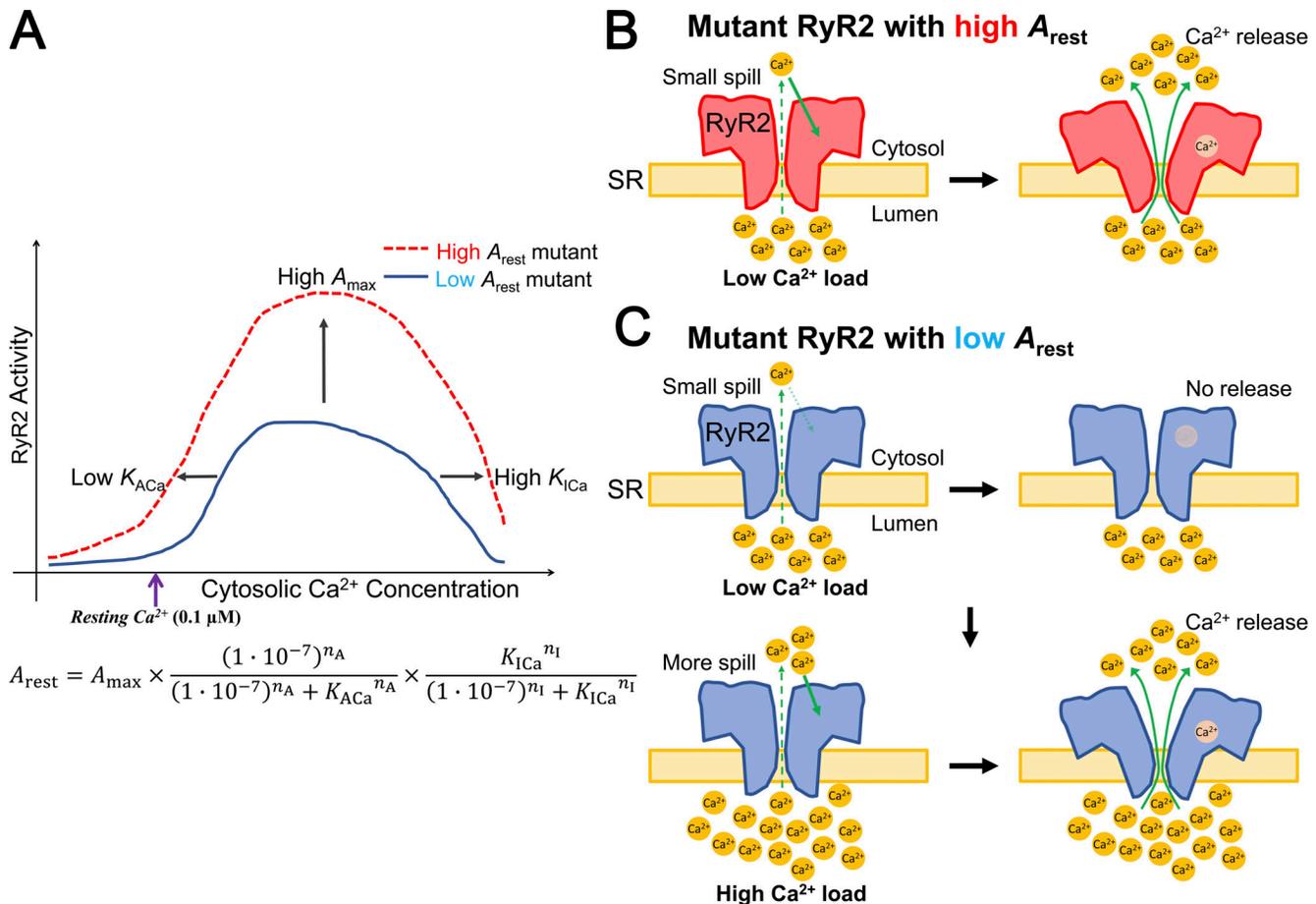


Figure 2. **Spontaneous SR  $Ca^{2+}$  releases through mutant RYR2s with high or low  $A_{rest}$  indexes.** (A) Fitting of bell-shaped cytosolic  $Ca^{2+}$ -dependent curves of RYR2 channel activity obtained from [<sup>3</sup>H]ryanodine binding assay determines the three parameters,  $A_{max}$ ,  $K_{ACa}$ , and  $K_{ICa}$ , which are used to calculate the  $A_{rest}$  index using the equation shown below the graph ( $K_{ACa}$  and  $K_{ICa}$  are in mol/liter). Constant values of Hill coefficients ( $n_A = 2.0$  and  $n_I = 1.0$ ) were used to obtain the best curve fitting. (B) Mutant RYR2s with high  $A_{rest}$  index spontaneously release  $Ca^{2+}$  at resting state, even with low SR  $Ca^{2+}$  load and only small  $Ca^{2+}$  spill to the cytoplasm. (C) Mutant RYR2s with low  $A_{rest}$  index do not release  $Ca^{2+}$  spontaneously at resting state when SR  $Ca^{2+}$  is low. Once SR  $Ca^{2+}$  loading level increases, more  $Ca^{2+}$  spills, and thus mutant RYR2s start releasing  $Ca^{2+}$  spontaneously.

$Ca^{2+}$  sensing site (E4872; Chen et al., 2014). The double mutations R2474S/E4872Q slightly increased the spontaneous  $Ca^{2+}$  oscillation frequency and cytosolic  $Ca^{2+}$ -dependent RYR2 activity compared with E4872Q mutation only, suggesting that increased spontaneous  $Ca^{2+}$  oscillation in R2474S CPVT mutant does not require the putative luminal  $Ca^{2+}$  binding site. One problem in this experiment is that the E4872Q mutation greatly reduced cytosolic  $Ca^{2+}$ -dependent RYR2 activities, thereby  $\sim 0 A_{rest}$ . With this loss-of-function property of E4872Q mutation, it is difficult to quantitatively evaluate and compare the  $A_{rest}$  values of the double mutants; thus, a synergetic role of luminal  $Ca^{2+}$  activation for the spontaneous  $Ca^{2+}$  release cannot be excluded.

Recently, the cryo-EM-based structural mapping identified a cytosolic  $Ca^{2+}$  binding site in RYRs (des Georges et al., 2016; Gong et al., 2019). Mutations on this site attenuated cytosolic  $Ca^{2+}$ -dependent activation of RYRs (Chirasani et al., 2019; Guo et al., 2020; Murayama et al., 2018). Thus, we may test inversely whether CPVT mutations together with cytosolic  $Ca^{2+}$  site mutations still increase luminal  $Ca^{2+}$  activation of the mutant RYR2. However, this will require an experimental platform where luminal  $Ca^{2+}$  is

controlled, such as single channel bilayer recording. Another interesting question is about a global structural impact by CPVT mutations. Since RYR2 mutations examined in this study spread all over the large protein complex, these mutations may allosterically alter the conformation of cytosolic or luminal  $Ca^{2+}$  sensing site (or their surrounding domains). Does the structural rearrangement of the  $Ca^{2+}$  binding site also correlate with the  $A_{rest}$  value? Investigators have started providing high-resolution structures of the recombinant mutant RYR proteins (Iyer et al., 2020); therefore, this challenging question may be answered in the near future.

The paper uses heterologous cell expression of the CPVT mutant RYR2. This is an advantage to focus solely on the intrinsic properties of RYR2 proteins, but can be a drawback because the system excludes the possibility that CPVT mutations on RYR2 alter protein interactions or post-translation modifications. The authors reported that the dissociation constant of activating  $Ca^{2+}$  in wild type RYR2 is  $>10 \mu$ M and even the highest affinity CPVT mutant (H4762P-RYR2) is  $\sim 3 \mu$ M. These values are somewhat beyond the threshold of the cytosolic  $Ca^{2+}$  concentration triggering  $Ca^{2+}$ -induced  $Ca^{2+}$  release in cardiomyocytes.

This discrepancy may have been caused by the difference between heterologous cell and cardiac cell environments. It also should be noted that one well-characterized CPVT pathology is that RYR2 mutations causes RYR2 hyperphosphorylation at Ser2809, resulting in the dissociation of FKBP12.6, thereby causing leaky Ca<sup>2+</sup> release (Wehrens et al., 2003). In this regard, it is important to evaluate the CPVT mechanism proposed by authors in the cardiac cell environment with adrenergic stimulation. Recent advances in stem cell technology and genetic techniques are helpful in creating a series of CPVT mutant cardiac muscle cell lines by introducing point mutations in RYR2 gene in the pluripotent stem cells using a gene editing technique and then differentiating them into cardiomyocytes (Wei et al., 2018).

Lastly, most of the CPVT-linked RYR2 mutations in human patients are heterozygote, expressing various types of heterotetrameric RYR2, while the authors' experiments with transfection of the mutant RYR2 cDNA mimic the homozygote situation. I noticed that the homozygous mutant RYR2 expression is likely to exhibit significant functional differences from wild type, which is valuable information as a phenotypic characterization of the gene mutations; however, one interesting experiment would be coexpression of wild type and mutant RYR2 cDNAs in the HEK293 cells. The authors assume partial cooperative regulation of tetrameric RYR2 by cytosolic-activating Ca<sup>2+</sup> (a Hill coefficient is 2); thus, A<sub>rest</sub> values would be different in the presence of large populations of heterotetrameric mutant RYR2.

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