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COMMENTARY

Excitation–Contraction Coupling

Do CPVT-linked mutations alter RYR2 regulation by cytosolic 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 Ca^{2+} release channel, known as type-2 ryanodine receptors (RYR2s), by the mechanism of cytosolic Ca^{2+} activation, thereby Ca^{2+}-induced 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 Ca^{2+} release aberrancy with these RYR2 mutations is observed as a diastole Ca^{2+} leak and spontaneous Ca^{2+} release, driving electrogenic Na^{+}–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 Ca^{2+} activation, and therefore lower the luminal Ca^{2+} threshold for spontaneous Ca^{2+} release from SR. During β-adrenergic stimulation, more Ca^{2+} are taken up into SR, which facilitates the spontaneous 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 Ca^{2+} at the diastole conditions. Thus, upon β-adrenergic stimulation, locally spilled Ca^{2+} from SR activates the mutant RYR2 from the cytosolic side, resulting in the spontaneous 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 Ca^{2+} concentration (~0.1 μM) and the occurrence of spontaneous Ca^{2+} release and oscillation, as well as a threshold of luminal Ca^{2+} concentration for spontaneous Ca^{2+} release. They tackled the controversial cytosolic Ca^{2+} versus luminal Ca^{2+} issue using two parallel experimental techniques: (1) simultaneous fluorescence measurements of cytosolic and luminal Ca^{2+} of heterologous HEK293 cells expressing recombinant RYR2 mutant proteins, and (2) in vitro RYR2 activity measurements by [^{3}H]ryanodine binding methods to determine RYR2 activity at 0.1 μM cytosolic Ca^{2+} concentration.

Authors defined A_{rest} as an index for RYR2 activity in the resting cells with 0.1 μM cytosolic Ca^{2+}. They calculated A_{rest} using parameters obtained from [^{3}H]ryanodine binding assay which determined the bell-shaped cytosolic Ca^{2+}-dependent activities of RYR2 (Fig. 2 A). They proposed that an A_{rest} value depend not only on a dissociation constant for activating Ca^{2+} (K_{ACa}) but on maximal gain of Ca^{2+} activated RYR2 channels, A_{max}, and a dissociation constant for inhibiting Ca^{2+} (K_{ICa}), which is usually >10,000 times higher than resting Ca^{2+} concentration, therefore, minimally affects A_{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 Ca^{2+} in [^{3}H]ryanodine binding studies was normalized to the peak activity at ~100 μM Ca^{2+}, and the apparent activating Ca^{2+} affinities of RYR2 mutants were compared. Under this data analysis, possible differences of A_{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_{rest} values and luminal Ca^{2+} thresholds for spontaneous Ca^{2+} release in CPVT-linked RYR2 mutants. In detail, they found that high RYR2 activity at resting cytosolic Ca^{2+} (high A_{rest} values) reduced the luminal Ca^{2+} threshold, resulting in a high rate of Ca^{2+} oscillation even with low SR Ca^{2+} load (Fig. 2 B), supporting an idea that CPVT mutations affect cytosolic

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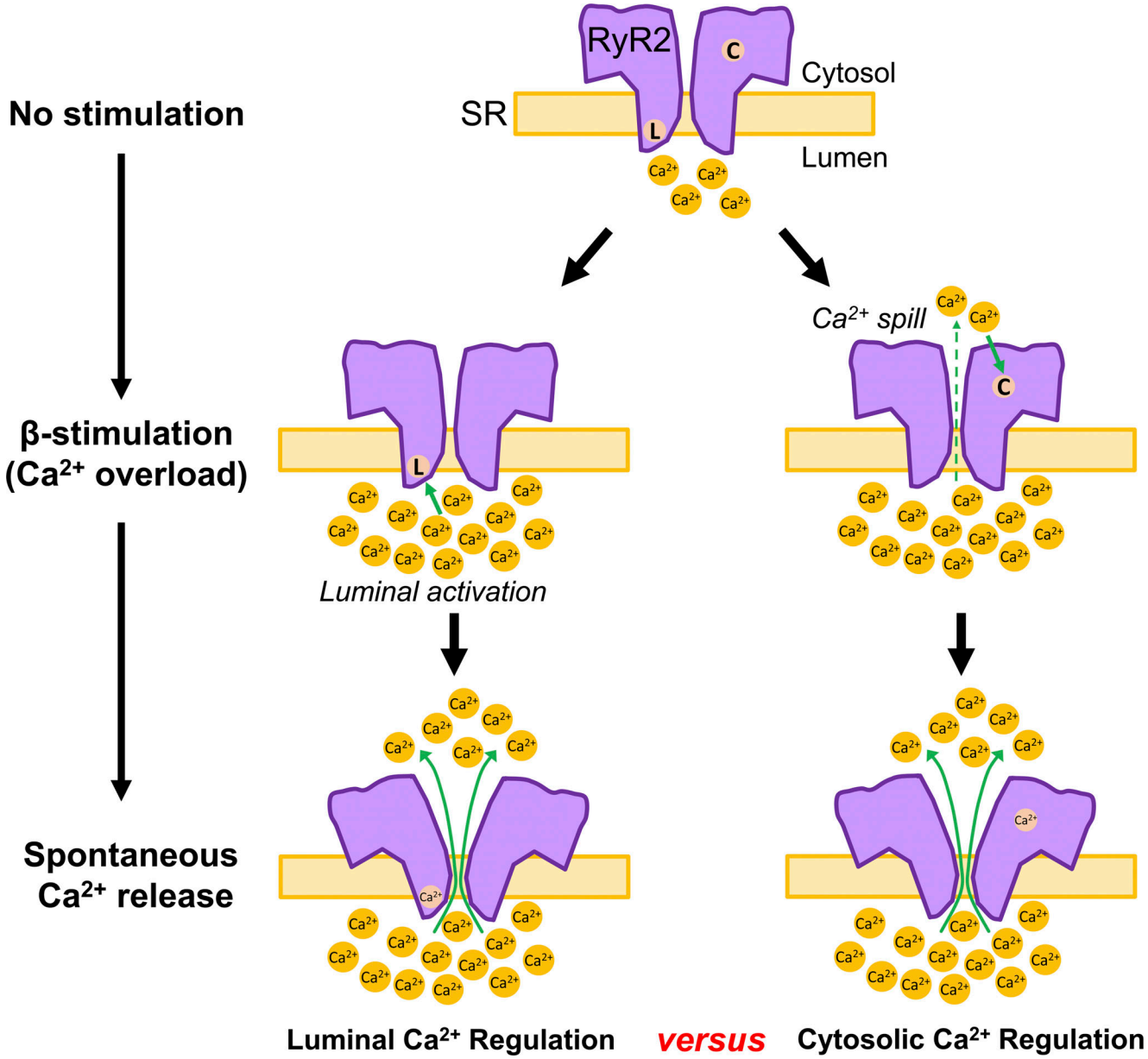

Figure 1. **Two mechanisms for spontaneous Ca^{2+} release from SR during β-adrenergic stimulations.** RYR2 have luminal (L) and cytosolic (C) Ca^{2+} regulatory sites. β-Adrenergic stimulations increase SR Ca^{2+} concentration, which activates RYR2 through luminal regulatory sites; thus, spontaneous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

Ca^{2+} regulation. On the other hand, the RYR2 mutants with low A_{rest} indexes required a high SR Ca^{2+} load, and thereby more local Ca^{2+} spills to trigger the Ca^{2+} 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^{2+} and luminal Ca^{2+} affinity but also SR store-operated Ca^{2+} 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^{2+} 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^{2+} release through CPVT RYR2 mutants was caused by their enhanced activity in cytosolic Ca^{2+} regulation. However, this does not rule out a luminal Ca^{2+} regulatory mechanism in RYR2. Ca^{2+}-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

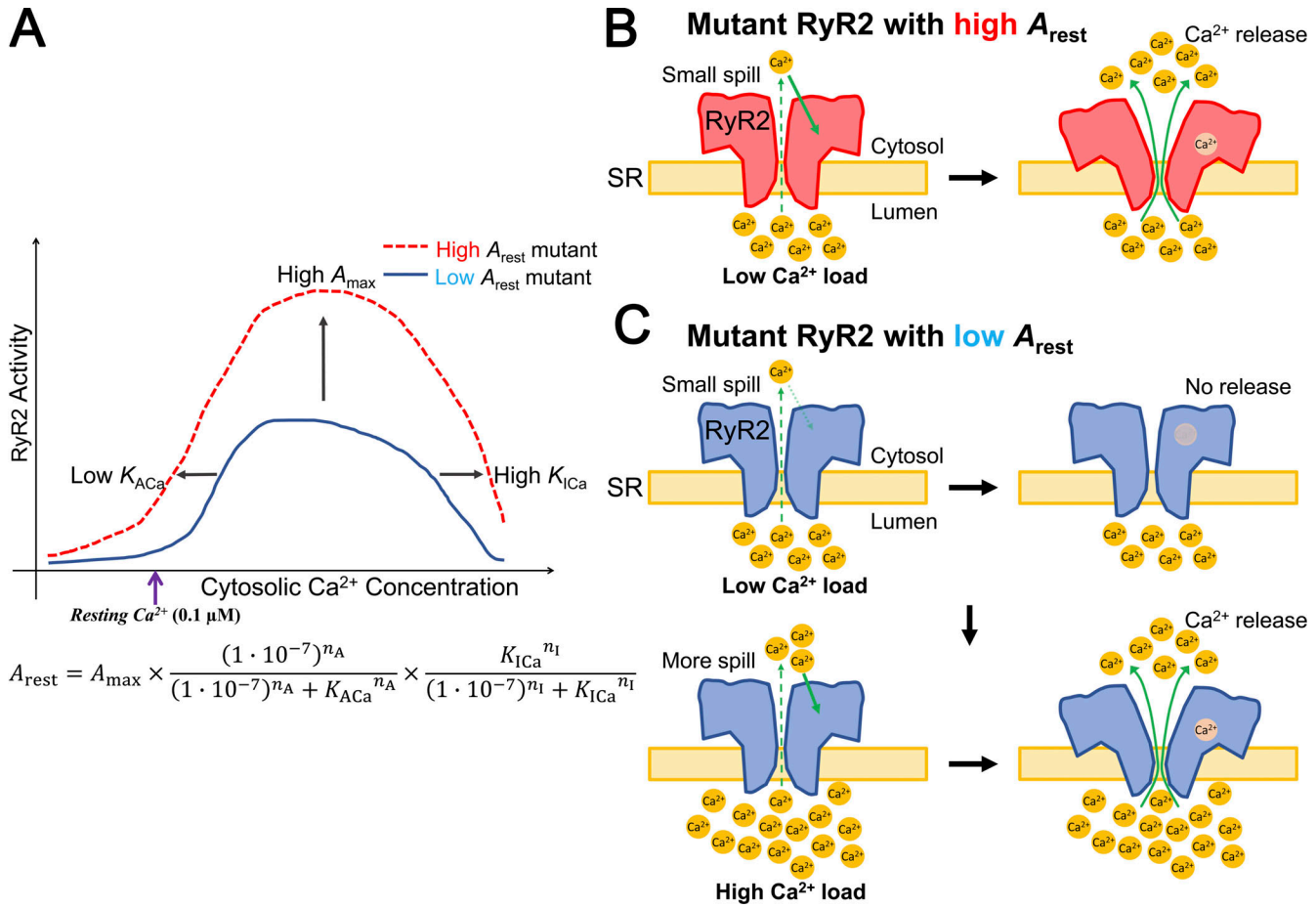

Figure 2. **Spontaneous SR Ca^{2+} releases through mutant RYR2s with high or low A_{rest} indexes. (A)** Fitting of bell-shaped cytosolic Ca^{2+}-dependent curves of RYR2 channel activity obtained from [^{3}H]ryanodine binding assay determines the three parameters, A_{max}, K_{ACa}, and K_{ICa}, which 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 ~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 μM and even the highest affinity CPVT mutant (H4762P-RYR2) is ~3 μM. 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^{2+} 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^{2+} (a Hill coefficient is 2); thus, A_{rest} values would be different in the presence of large populations of heterotetrameric mutant RYR2.

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