Reply to 'Mechanisms of ryanodine receptor 2 dysfunction in heart failure'

Haikel Dridi, Alexander Kushnir, Ran Zalk, Qi Yuan, Zephan Melville and Andrew R. Marks

We appreciate that Francisco J. Alvarado and Héctor H. Valdivia write in their Correspondence (Mechanisms of ryanodine receptor 2 dysfunction in heart failure. *Nat. Rev. Cardiol.* https://doi.org/10.1038/s41569-020-00443-x (2020))[1] that they agree with the fundamental premise of our Review (Intracellular calcium leak in heart failure and atrial fibrillation: a unifying mechanism and therapeutic target. *Nat. Rev. Cardiol.* https://doi.org/10.1038/s41569-020-0394-8 (2020))[2] on the causal role of intracellular Ca^{2+} leak in cardiovascular arrhythmias. However, they take issue with our discussion of the mechanistic role of cAMP-dependent protein kinase (PKA) phosphorylation of Ser2808 in the ryanodine receptor 2 (RYR2) in heart failure (HF) progression[3] and claim that our findings have not been reproduced by others. We disagree. Indeed, as we show below, data from the Valdivia laboratory provide proof of the reproducibility of our results.

Our Review[2] summarizes the mechanisms that impair regulation of the Ca^{2+}-release channel, RYR2, and how they contribute to HF and atrial fibrillation (AF). We provide an overview of how inherited genetic variants and stress-induced phosphorylation and oxidation of RYR2 cause dissociation of the RYR2-stabilizing protein calstabin 2 (also known as FK506-binding protein 12.6) and a pathological diastolic Ca^{2+} leak from the sarcoplasmic reticulum (SR) that triggers arrhythmias and impairs cardiac contractility[3].

PKA and Ca^{2+}/calmodulin-dependent protein kinase type II (CAMKII) phosphorylate RYR2 at Ser2808[3] and Ser2814[4,5], respectively. The crucial role of PKA phosphorylation of RYR2 caused by chronic β-adrenergic activation during HF has been demonstrated by replacement of Ser2808 with an alanine residue in RYR2-Ser2808Ala mice, which have blunted inotropic and chronotropic responses to β-adrenergic stimulation and are protected against HF progression owing to reduced SR Ca^{2+} leak[6–8].

Moreover, despite their claims to the contrary, data published by Benkusky, Valdivia and colleagues[9] support our findings that preventing PKA phosphorylation of RYR2 by mutation of Ser2808 to Ala2808 protects against HF progression[6–8]. Indeed, in their Supplementary Table 1, Benkusky and colleagues report complete preservation of cardiac function in their own RYR2-Ser2808Ala mice at 11 weeks after aortic banding-induced HF[9] (TABLE 1). Moreover, in their Fig. 5, they report that the Ca^{2+} transient amplitude is reduced in cardiomyocytes from RYR2-Ser2808Ala mice during isoprenaline stimulation[9] (FIG. 1). Therefore, Benkusky and colleagues confirm our finding that Ser2808 is a crucial PKA phosphorylation site on RYR2 that is required for β-adrenergic stimulation of Ca^{2+} release from the SR. In vivo data showing complete protection against HF progression in RYR2-Ser2808Ala mice confirm our results and are the most important evidence for the crucial role of Ser2808. However, Alvarado and Valdivia have accused us of 'cherry picking' their data to support our findings. We simply point out that their own data[9] show significant blunting of the β-adrenergic response and protection against HF progression, supporting the reproducibility of our findings. Furthermore, these findings are clinically relevant because patients with HF who are treated with β-blockers show reduced HF progression and decreased mortality[10].

Alvarado and Valdivia also take issue with the finding that RYR2-Ser2808 is the only physiologically relevant PKA site on RYR2. They point out that many potential PKA sites exist on this massive protein (nearly 5,000 residues). However, when we replaced Ser2808 with an alanine residue, the RYR2 channel could not be phosphorylated by PKA in vivo during isoprenaline stimulation[6–8]. Xiao and colleagues[11] have proposed that RYR2-Ser2030 (and not RYR2-Ser2808) is the important PKA phosphorylation site on RYR2 and that the RYR2-Ser2030Ala substitution blunts the β-adrenergic responses in isoprenaline-treated hearts[12] and that

Table 1 | **Structural and functional echocardiographic parameters of wild-type and RYR2-Ser2808Ala mice**

Paramater	Control		Banded (4 weeks)		Banded (11 weeks)	
	Wild-type	RYR2-Ser2808Ala	Wild-type	RYR2-Ser2808Ala	Wild-type	RYR2-Ser2808Ala
Age (weeks)	12.7 ± 0.4	14.0 ± 0.4	16.7 ± 0.9	18.8 ± 0.4	23.7 ± 0.4	25.0 ± 0.4
Body weight (g)	26.3 ± 0.5	29.1 ± 1.3	28.9 ± 0.9[a]	29.0 ± 0.5	31.5 ± 1.0[b]	30.1 ± 0.7
Heart rate (bpm)	439.5 ± 27	471.3 ± 24	454.3 ± 24	508.8 ± 29	513.1 ± 22	548.3 ± 12[a]
Left ventricular mass / body weight (g)	2.98 ± 0.2	3.03 ± 0.2	5.17 ± 0.4[b]	4.87 ± 0.4[b]	5.27 ± 0.4[a]	5.05 ± 0.3[b]
Posterior wall thickness in diastole (mm)	0.77 ± 0.0	0.77 ± 0.0	1.19 ± 0.1[a]	1.05 ± 0.1[a]	1.24 ± 0.1[a]	1.13 ± 0.0[b]
Anterior wall thickness in diastole (mm)	0.73 ± 0.0	0.76 ± 0.0	1.26 ± 0.1[a]	1.13 ± 0.1[b]	1.23 ± 0.0[a]	1.12 ± 0.1[b]
Left ventricular diameter in diastole (mm)	3.3 ± 0.1	3.5 ± 0.15	3.1 ± 0.1	3.4 ± 0.14	3.3 ± 0.1	3.5 ± 0.14
Fractional shortening (%)	52.1 ± 3.2	51.0 ± 2.6	46.9 ± 1.6	48.3 ± 1.1	42.6 ± 1.2[a]	51.9 ± 5.1
Isovolumetric relaxation time (ms)	0.017 ± 0.0	0.018 ± 0.0	0.018 ± 0.0	0.016 ± 0.0	0.019 ± 0.0	0.017 ± 0.0

Data from the Valdivia laboratory are presented as the means ± s.e.m. ($n = 6–7$). [a]$P < 0.05$ versus control. [b]$P < 0.001$ versus control. In agreement with our studies, Benkusky and colleagues show that cardiac function is preserved in RYR2-Ser2808Ala mice during aortic banding. Cardiac function (measured as fractional shortening of the left ventricle) was significantly reduced in wild-type mice with aortic banding (from 52.1 ± 3.2% at baseline to 42.6 ± 1.2% at 11 weeks; $P < 0.05$), whereas cardiac function was completely preserved in their RYR2-Ser2808Ala mice with aortic banding (from 51.1 ± 2.6% at baseline to 51.9 ± 5.1% at 11 weeks), confirming our findings. Reproduced with permission from REF.[9].

RYR2-Ser2030Ala mice have a reduced β-adrenergic response[13]. By contrast, both our findings[8] and those of others, including Huke and Bers[14], did not report phosphorylation of RYR2-Ser2030 in vivo or in isolated cardiomyocytes, respectively

Some discrepancies between our results and those of Valdivia and colleagues might be explained by the following observations. The RYR2-Ser2030Ala mice used by Valdivia and colleagues have significantly decreased RYR2 levels (see Fig. 2a in the article by Potenza and colleagues[13]), which might contribute to the blunted β-adrenergic response observed by Valdivia and colleagues in RYR2-Ser2030Ala mice. The activities of phosphatases and phosphodiesterases, which are part of the RYR2 complex[3,15], were not evaluated in their study. The RYR2-Ser2030Ala mice had cardiac hypertrophy at baseline (see Figure S1 in the article by Potenza and colleagues[13]) and high basal levels of Ser2808 phosphorylation. The increase in PKA phosphorylation at Ser2808 in response to isoprenaline treatment was significantly reduced in the RYR2-Ser2030Ala mice compared with wild-type control mice (see Fig. 7 in the article by Potenza and colleagues[13]), which could also blunt the β-adrenergic response. Finally, structural images of the RYR2 channel produced using cryogenic electron microscopy show that Ser2030 is located in the bridging solenoid, which is not near to the known phosphorylation sites on the channel[16].

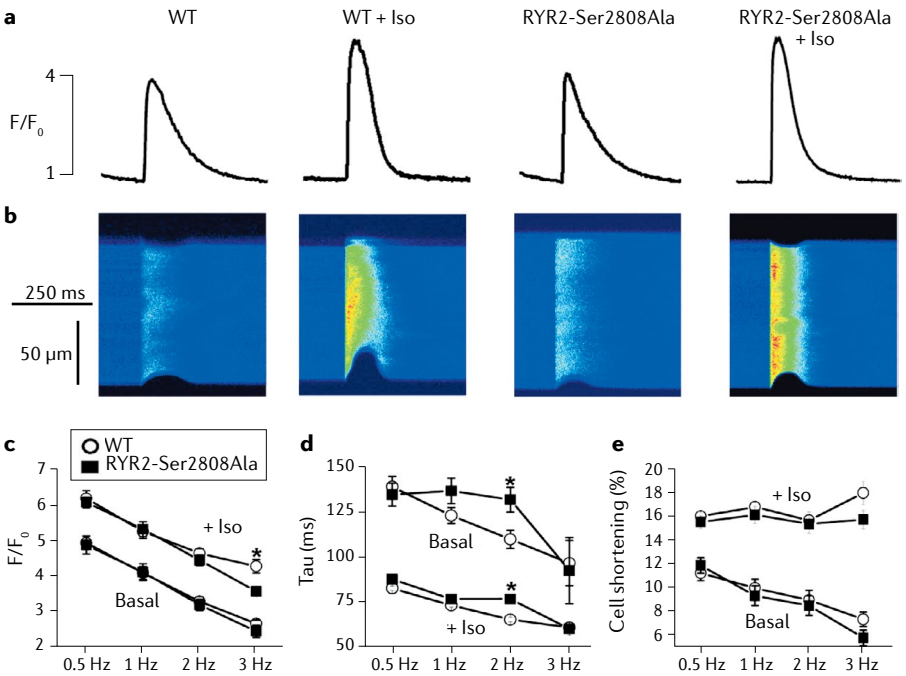

Fig. 1 | **Intracellular Ca^{2+} transients and contractions in WT and RYR2-Ser2808Ala cardiomyocytes.** These data are from the Valdivia laboratory. **a** | Representative Ca^{2+} transients from wild-type (WT) and RYR2-Ser2808Ala mouse cardiomyocytes field stimulated at 2 Hz. **b** | The associated confocal line-scan images for the Ca^{2+} transients depicted in panel **a**. **c** | Line graph depicting the relationship between stimulation frequency and the amplitude of the Ca^{2+} transient (F/F_0). This graph shows a reduced Ca^{2+} transient amplitude with isoprenaline (Iso) stimulation under field stimulation at 3 Hz in RYR2-Ser2808Ala cardiomyocytes compared with wild-type cardiomyocytes, which supports our findings that Ser2808 is crucial to the β-adrenergic response. (In mice the physiological heart rate frequency is ~10 Hz or 600 bpm, such that the blunting of the β-adrenergic response is revealed only as the stimulation frequency is increased towards the physiological level.) **d** | The mono-exponential decay of the Ca^{2+} transient (tau). **e** | The percentage of cell shortening before (basal) and after (+ Iso) β-adrenergic stimulation. *$P < 0.05$ (WT versus RYR2-Ser2808Ala). $n = 243$ and 197 transients (WT basal and with Iso, respectively); $n = 183$ and 189 transients (RYR2-Ser2808Ala basal and with Iso, respectively). Reproduced with permission from REF.[9].

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Competing interests
A.R.M. and Columbia University, USA, own shares in ARMGO Pharma, a biotechnology company developing ryanodine receptor-targeted drugs. The other authors declare no competing interests.