JBC ARTICLE

The cardiac ryanodine receptor, but not sarcoplasmic reticulum Ca^{2+}-ATPase, is a major determinant of Ca^{2+} alternans in intact mouse hearts

Received for publication, April 29, 2018, and in revised form, July 6, 2018 Published, Papers in Press, July 9, 2018, DOI 10.1074/jbc.RA118.003760

Bo Sun[‡1], **Jinhong Wei**[‡2], **Xiaowei Zhong**[‡3], **Wenting Guo**[‡3], **Jinjing Yao**[‡1], **Ruiwu Wang**[‡], **Alexander Vallmitjana**[§], **Raul Benitez**[§], **Leif Hove-Madsen**[¶], and **S. R. Wayne Chen**[‡4]

From the [‡]*Libin Cardiovascular Institute of Alberta, Department of Physiology and Pharmacology, University of Calgary, Calgary, Alberta T2N 4N1, Canada, the* [§]*Department of Automatic Control, Universitat Politècnica de Catalunya, Barcelona 08034, Spain, and the* [¶]*Biomedical Research Institute of Barcelona (IIBB), CSIC, Sant Pau, Hospital de Sant Pau, Barcelona 08025, Spain*

Edited by Roger J. Colbran

Sarcoplasmic reticulum (SR) Ca^{2+} cycling is governed by the cardiac ryanodine receptor (RyR2) and SR Ca^{2+}-ATPase (SERCA2a). Abnormal SR Ca^{2+} cycling is thought to be the primary cause of Ca^{2+} alternans that can elicit ventricular arrhythmias and sudden cardiac arrest. Although alterations in either RyR2 or SERCA2a function are expected to affect SR Ca^{2+} cycling, whether and to what extent altered RyR2 or SERCA2a function affects Ca^{2+} alternans is unclear. Here, we employed a gain-of-function RyR2 variant (R4496C) and the phospholamban-knockout (PLB-KO) mouse model to assess the effect of genetically enhanced RyR2 or SERCA2a function on Ca^{2+} alternans. Confocal Ca^{2+} imaging revealed that RyR2-R4496C shortened SR Ca^{2+} release refractoriness and markedly suppressed rapid pacing–induced Ca^{2+} alternans. Interestingly, despite enhancing RyR2 function, intact RyR2-R4496C hearts exhibited no detectable spontaneous SR Ca^{2+} release events during pacing. Unlike for RyR2, enhancing SERCA2a function by ablating PLB exerted a relatively minor effect on Ca^{2+} alternans in intact hearts expressing RyR2 WT or a loss-of-function RyR2 variant, E4872Q, that promotes Ca^{2+} alternans. Furthermore, partial SERCA2a inhibition with 3 μM 2,5-di-*tert*-butylhydroquinone (tBHQ) also had little impact on Ca^{2+} alternans, whereas strong SERCA2a inhibition with 10 μM tBHQ markedly reduced the amplitude of Ca^{2+} transients and suppressed Ca^{2+} alternans in intact hearts. Our results demonstrate that enhanced RyR2 function suppresses Ca^{2+} alternans in the absence of spontaneous Ca^{2+} release and that RyR2, but not SERCA2a, is a key determinant of Ca^{2+} alternans in intact working hearts, making RyR2 an important therapeutic target for cardiac alternans.

Intracellular Ca^{2+} alternans, one of the many forms of cardiac alternans, is a beat-to-beat alternation in the amplitude of the cytosolic Ca^{2+} transient. An increasing body of evidence indicates that Ca^{2+} alternans can occur in the absence of other forms of cardiac alternans, supporting the notion that Ca^{2+} alternans plays a primary role in the genesis of cardiac alternans (1–11). Despite the well-recognized risk of cardiac alternans in ventricular fibrillation and sudden cardiac arrest (12–19), the molecular mechanisms underlying cardiac alternans are not well understood.

Given its crucial role in cardiac alternans, understanding how Ca^{2+} alternans occurs would be key to the understanding of cardiac alternans. Over the past decades, major advances in the understanding of the mechanisms of Ca^{2+} alternans have been made. It has become clear that Ca^{2+} alternans results from altered SR[5] Ca^{2+} cycling, which is governed by SR Ca^{2+} release and reuptake (9, 11, 20–25).

Inhibiting RyR2 function either by tetracaine, intracellular acidification, or metabolic inhibition has been shown to prolong SR Ca^{2+} release refractoriness and promote Ca^{2+} alternans in isolated cardiomyocytes (26–30). On the other hand, increasing RyR2 function by caffeine shortens SR Ca^{2+} release refractoriness and suppresses Ca^{2+} alternans (31, 32). These observations suggest that the activity of RyR2 is a major determinant of SR Ca^{2+} release refractoriness and Ca^{2+} alternans. Consistent with this view, we showed that genetically suppressing RyR2 function prolongs SR Ca^{2+} release refractoriness and promotes Ca^{2+} alternans in intact hearts (32). On the other hand, shortened refractoriness of SR Ca^{2+} release as a result of CASQ2 (cardiac calsequestrin) ablation suppresses Ca^{2+} alter-

This work was supported by research grants from the Canadian Institutes of Health Research; the Heart and Stroke Foundation of Alberta, Northwest Territories, and Nunavut; the Canada Foundation for Innovation (CFI); the Heart and Stroke Foundation Chair in Cardiovascular Research; the Alberta Innovates-Health Solutions (to S. R. W. C.); Spanish Ministry of Economy and Competitiveness (MINECO) Grants DPI2013-44584-R (to R. B.) and SAF2014-58286-C2-1R (to L. H. M.), and Generalitat de Catalunya Grant SGR2014-1465 (to L. H. M. and R. B.). The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

This article contains Figs. S1–S6.

[1] Recipient of the Alberta Innovates-Health Solutions (AIHS) Fellowship Award.

[2] Recipient of the Libin Cardiovascular Institute of Alberta and Cumming School of Medicine Postdoctoral Fellowship Award.

[3] Recipient of the AIHS Studentship Award.

[4] AIHS Scientist. To whom correspondence should be addressed: 3330 Hospital Dr. N.W., Calgary, Alberta T2N 4N1, Canada. Tel.: 403-220-4235; E-mail: swchen@ucalgary.ca.

[5] The abbreviations used are: SR, sarcoplasmic reticulum; RyR2, cardiac ryanodine receptor; VT, ventricular tachycardia; CPVT, catecholaminergic polymorphic ventricular tachycardia; PLB, phospholamban; SCR, spontaneous Ca^{2+} release; SERCA2a, cardiac sarco/endoplasmic reticulum Ca^{2+}-ATPase; tBHQ, 2,5-di-tert-butylhydroquinone; GOF, gain-of-function; KO, knockout; ANOVA, analysis of variance.

nans in intact hearts (33). These findings support a general notion that suppressing the activity of RyR2 prolongs the refractoriness of SR Ca^{2+} release and promotes Ca^{2+} alternans, whereas enhancing RyR2 activity shortens the refractoriness of SR Ca^{2+} release and suppresses Ca^{2+} alternans (10, 31, 32, 34, 35). However, and contrary to this expectation, enhanced RyR2 function as a result of some genetic mutations or abnormal redox modifications has been shown to promote Ca^{2+} alternans in isolated cardiomyocytes (36–38). For instance, the CPVT-causing, gain-of-function (GOF) RyR2 mutation R4496C has been shown to reduce the refractoriness of SR Ca^{2+} release in the mouse trabecular muscle (39). However, the same GOF RyR2 R4496C mutation was found to promote Ca^{2+} alternans in isolated cardiac cells (40). Therefore, it remains unclear whether enhanced RyR2 function suppresses or promotes Ca^{2+} alternans.

These seemingly conflicting observations also raise an important question of why enhanced RyR2 activity and shortened refractoriness of SR Ca^{2+} release are unable to suppress Ca^{2+} alternans in the isolated RyR2 R4496C mutant cells. One possible explanation is that enhanced RyR2 activity would increase the propensity for spontaneous Ca^{2+} release (SCR), such as Ca^{2+} sparks and Ca^{2+} waves, which in turn would promote Ca^{2+} alternans (25, 28, 38, 41–46). Hence, it would be of interest and importance to determine whether enhanced RyR2 function promotes Ca^{2+} alternans in intact working hearts that exhibit little or no SCR during stimulation (47).

SR Ca^{2+} reuptake, another important aspect of SR Ca^{2+} cycling, is also believed to play an important role in Ca^{2+} alternans. Overexpression of the cardiac sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA2a) suppresses Ca^{2+} alternans, whereas reducing SERCA2a expression or activity promotes Ca^{2+} alternans (5, 9, 48–51). However, severely reducing the activity of SERCA2a may suppress, rather than promote, Ca^{2+} alternans, probably due to reduced SR Ca^{2+} content (25, 46, 52). Interestingly, atrial overexpression of SERCA2a has little effect on cardiac alternans (53). Hence, the effect of altered SERCA2a activity on Ca^{2+} alternans is complex and variable, and the relative contribution of altered RyR2 and SERCA2a activity to the genesis of Ca^{2+} alternans is also unclear.

In the present study, we carried out laser-scanning confocal Ca^{2+} imaging of cardiomyocytes in intact WT and RyR2 mutant hearts that exhibited little spontaneous Ca^{2+} release. We assessed the impact of the GOF RyR2 R4496C mutation on SR Ca^{2+} release refractoriness and Ca^{2+} alternans and the effects of inhibiting or enhancing SERCA2a activity on Ca^{2+} alternans in intact hearts. We demonstrate that genetically enhancing RyR2 function shortens Ca^{2+} release refractoriness and suppresses Ca^{2+} alternans in intact hearts without producing spontaneous SR Ca^{2+} release. We also demonstrate that genetically enhancing SERCA2a function by phospholamban (PLB) ablation had a relatively minor impact on Ca^{2+} alternans in intact hearts. Furthermore, we found that modest inhibition of SERCA2a function also had little impact on Ca^{2+} alternans in intact hearts. Collectively, our data demonstrate that the activity of RyR2, but not SERCA2a, is a major determinant of Ca^{2+} alternans in intact working mouse hearts.

Results

Genetically enhancing RyR2 function reduces the refractoriness of SR Ca^{2+} release in intact hearts

We have recently shown that genetically suppressing RyR2 function lengthens the refractoriness of SR Ca^{2+} release in intact hearts (32). It is unclear whether genetically enhancing RyR2 function would shorten the refractoriness of SR Ca^{2+} release in intact hearts. To address this question, we employed the RyR2 R4496C mutation, a CPVT-linked GOF RyR2 mutation that has been shown to significantly enhance the sensitivity of the channel to Ca^{2+} activation (54–56). We determined the refractoriness of SR Ca^{2+} release in isolated Langendorff-perfused intact RyR2 WT and heterozygous RyR2 R4496C mutant hearts using the S1S2 stimulation protocol (10). As shown in Fig. 1, the amplitude of Ca^{2+} transients in both the WT and RyR2 R4496C hearts decreased when the S1S2 interval was progressively reduced (from 200 to 40 ms) (Fig. 1, *A* and *B*). However, the WT and RyR2 R4496C hearts showed significantly different relationships between the Ca^{2+} transient amplitude and S1S2 interval (Fig. 1*C*) ($p < 0.05$). The Ca^{2+} transient amplitude of the RyR2 R4496C hearts recovered faster than that of the WT hearts at S1S2 intervals between 75 and 160 ms. Therefore, these data demonstrate that, contrary to the effect of suppressing RyR2 function (32), genetically enhancing RyR2 function shortens the refractoriness of SR Ca^{2+} release.

Enhancing RyR2 function markedly suppresses rapid stimulation–induced Ca^{2+} alternans in intact hearts

Prolonged refractoriness of SR Ca^{2+} release is known to promote Ca^{2+} alternans (10, 31, 32, 34, 35). Thus, a shortened refractoriness of SR Ca^{2+} release as a result of the RyR2 R4496C mutation would be expected to suppress Ca^{2+} alternans. To test this possibility, we determined the propensity for Ca^{2+} alternans in isolated Langendorff-perfused intact RyR2 WT and heterozygous RyR2 R4496C mutant hearts. As shown in Fig. 2, RyR2 WT hearts exhibited significant beat-to-beat alternations in the amplitude of Ca^{2+} transients at the stimulation frequency of 12 Hz (Fig. 2*A*). On the other hand, RyR2 R4496C mutant hearts displayed little or no beat-to-beat variations in the amplitude of Ca^{2+} transients at the same stimulation frequency (12 Hz) (Fig. 2*B*).

The frequency dependence of Ca^{2+} alternans in intact WT and RyR2 R4496C hearts is shown in Fig. 2 (*C* and *D*). Substantial Ca^{2+} alternans could be readily detected in RyR2 WT hearts stimulated at 10–11 Hz, whereas higher stimulation frequencies (13–14 Hz) were required to induce considerable Ca^{2+} alternans in RyR2 R4496C mutant hearts (Fig. 2, *C* and *D*). Furthermore, RyR2 R4496C hearts showed a significantly lower alternans ratio and alternans duration at each stimulation frequency between 10 and 14 Hz (Fig. 2, *C* and *D*) ($p < 0.01$). Taken together, these data indicate that, contrary to the effect of suppressing RyR2 function (32), genetically enhancing RyR2 function markedly suppresses rapid stimulation–induced Ca^{2+} alternans in intact hearts.

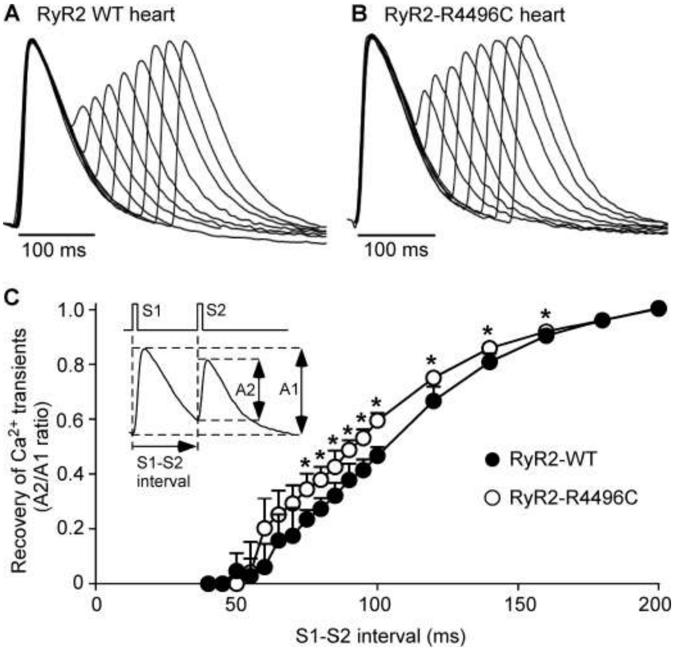

Figure 1. RyR2-R4496C mutation shortens the refractoriness of SR Ca^{2+} release. Langendorff-perfused intact RyR2 WT (*A*) and R4496C mutant (*B*) hearts were loaded with Rhod-2 AM. Hearts were first stimulated at 5 Hz for 30 beats (S1), followed by a single S2 stimulation. A series of S1S2 stimulations were repeatedly applied with progressively reduced S1S2 intervals from 200 to 40 ms. Ca^{2+} transients were recorded using line-scanning confocal imaging. *C*, the relationship between A2/A1 ratio of the Ca^{2+} transient amplitude and S1S2 interval is shown. Data shown are mean ± S.D. (*error bars*) ($n = 5$ hearts for WT, $n = 7$ hearts for R4496C) (*, $p < 0.05$).

No spontaneous Ca^{2+} sparks or Ca^{2+} waves were detected in intact RyR2-R4496C mutant hearts during Ca^{2+} alternans

It has been shown that the RyR2 R4496C mutation increases the propensity for spontaneous Ca^{2+} release (SCR; Ca^{2+} sparks and Ca^{2+} waves) (40, 55, 57). It is thought that SCR necessitates and facilitates the occurrence of Ca^{2+} alternans (25, 28, 38, 41–46). Hence, it is of interest to determine whether SCR is involved in the occurrence of Ca^{2+} alternans in intact working RyR2 R4496C mutant hearts. Fig. 3 shows Ca^{2+} transients in intact RyR2-R4496C hearts continuously stimulated with increasing frequencies from 6 to 14 Hz (Fig. 3 and Fig. S1). Surprisingly, despite the enhanced RyR2 function, intact working RyR2 R4496C mutant hearts exhibited no detectable spontaneous Ca^{2+} sparks or Ca^{2+} waves before or after the occurrence of Ca^{2+} alternans. The continued high-frequency stimulations probably override SCR in the intact working RyR2-R4496C hearts. Consistent with this view, spontaneous Ca^{2+} waves were readily observed in intact RyR2-R4496C mutant hearts, but not in WT hearts, after the cessation of pacing in the presence of high adrenergic stress (1 μM epinephrine plus 0.6 mM caffeine) (Fig. 4). It is important to note that the same condition (1 μM epinephrine plus 0.6 mM caffeine) also induced VTs in intact working RyR2-R4496C hearts as reported previously (47). Hence, the Ca^{2+} alternans observed in rapidly stimulated intact RyR2 R4496C mutant hearts are unrelated to spontaneous Ca^{2+} sparks or Ca^{2+} waves.

Enhancing SERCA2a function by phospholamban knockout (PLB-KO) has a relatively small impact on Ca^{2+} alternans in intact RyR2 WT or E4872Q mutant hearts

It is clear that modulating the activity of RyR2 has a major impact on Ca^{2+} alternans (26–30, 32). However, the relative impact of modulating the activity of SERCA2a, another key component of SR Ca^{2+} cycling, on Ca^{2+} alternans is unclear. To this end, we assessed the impact of PLB-KO on Ca^{2+} alternans in intact RyR2 WT or E4872Q mutant hearts. The RyR2 E4872Q mutation has been shown to suppress Ca^{2+} activation of RyR2 and the occurrence of spontaneous Ca^{2+} waves (32, 58, 59). As expected, PLB-KO markedly increased the amplitude of Ca^{2+} transients and reduced the transient decay time (T_{50}) as compared with WT hearts (Fig. S2), consistent with its stimulatory action on SERCA2a and SR Ca^{2+} reuptake (60). Surprisingly, PLB-KO did not significantly alter the average alternans ratios at stimulation frequencies from 5 to 14 Hz (Fig. 5). PLB-KO significantly reduced the average alternans durations only at stimulation frequencies of 10, 11, and 12 Hz (Fig. 5*D*). Thus, compared with enhancing RyR2 function, enhancing SERCA2a function as a result of PLB-KO has relatively small impact on Ca^{2+} alternans in intact WT hearts.

We also assessed whether PLB-KO could rescue the enhanced Ca^{2+} alternans in RyR2 E4872Q mutant hearts with suppressed RyR2 function. Similar to what was observed in RyR2 WT hearts (Fig. S2), PLB-KO significantly increased the amplitude of Ca^{2+} transients and reduced the T_{50} in intact RyR2 E4872Q mutant hearts (Fig. S3). PLB-KO also significantly increased the SR Ca^{2+} content in WT ventricular myocytes, as expected, and dramatically increased the SR Ca^{2+} content in RyR2 E4872Q mutant cells (Fig. S4). However, PLB-KO had no significant impact on the average alternans ratio of RyR2 E4872Q hearts at stimulation frequencies from 5 to 14 Hz (Fig. 6). PLB-KO significantly reduced the average alternans duration only at the stimulation frequency of 9 Hz (Fig. 6*D*). Thus, enhancing SERCA2a function by PLB-KO does not suppress the enhanced Ca^{2+} alternans in RyR2 E4872Q mutant hearts. These observations also suggest that, although the SR Ca^{2+} content is an important regulator of SR Ca^{2+} handling, it does not seem to play a critical role in Ca^{2+} alternans.

Effect of SERCA2a inhibition on Ca^{2+} alternans in intact hearts

We next assessed the effect of 2,5-di-*tert*-butylhydroquinone (tBHQ), an inhibitor of SERCA2a, on Ca^{2+} alternans in intact RyR2 WT and E4872Q mutant hearts. As expected, tBHQ at 3 and 10 μM significantly reduced the amplitude of Ca^{2+} transients. It also prolonged the T_{50} in RyR2 WT hearts by 13% (at 3 μM) and 16% (at 10 μM) (Fig. S5). This is consistent with the inhibitory action of tBHQ on SERCA2a and SR Ca^{2+} reuptake. However, despite its significant impact on SERCA2a, tBHQ at 3 μM did not significantly affect the average alternans ratio or duration in intact RyR2 WT hearts stimulated at a wide range of frequencies (from 5 to 14 Hz) (Fig. 7). Surprisingly, tBHQ at 10 μM markedly reduced both the average alternans ratio and duration in intact RyR2 WT hearts at stimulation frequencies of 11–14 Hz (Fig. 7). These data indicate that, depending on the extent of SERCA2a inhibition, reducing

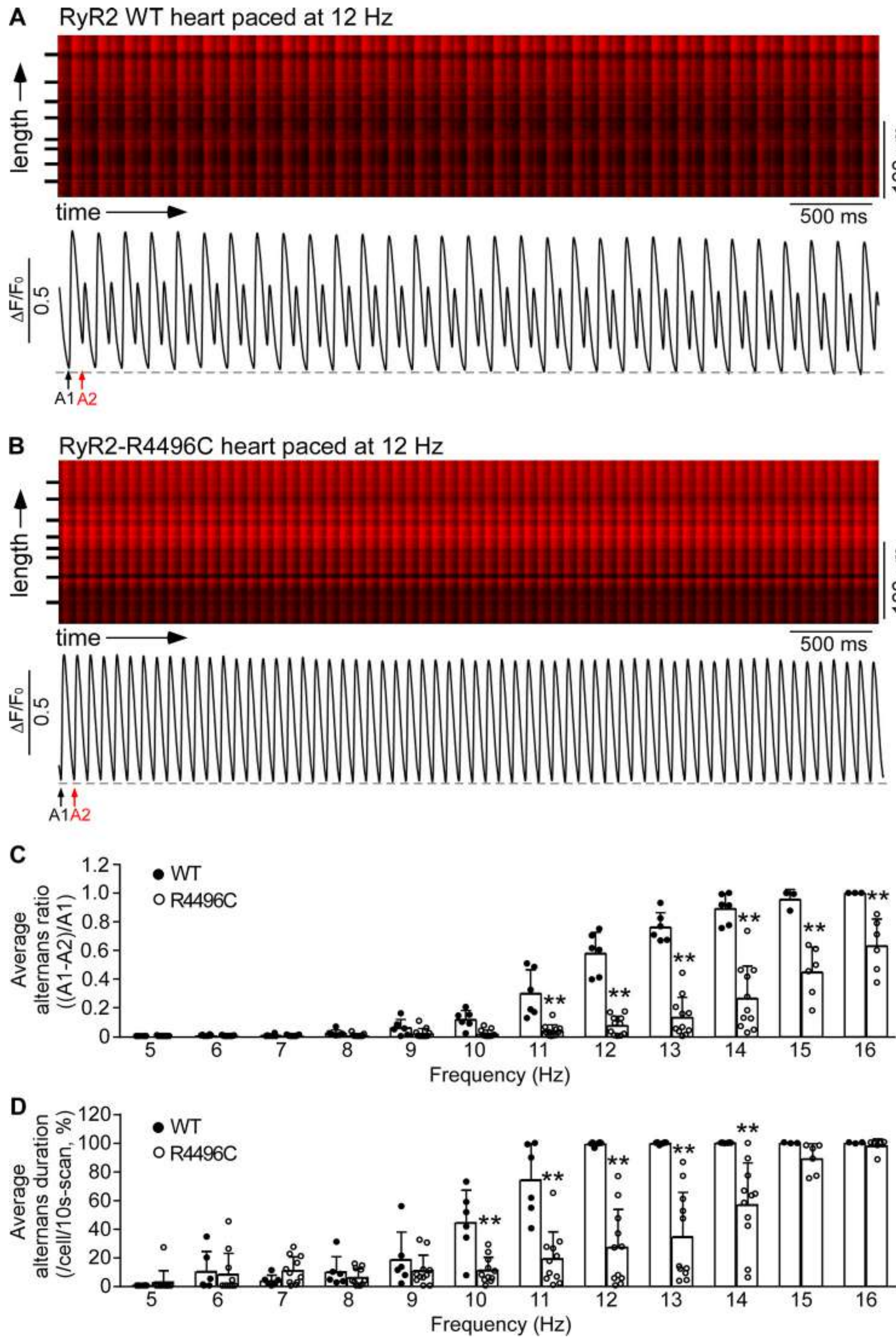

Figure 2. Ca^{2+} transient alternans in intact RyR2 WT and RyR2-R4496C hearts. Langendorff-perfused intact RyR2 WT (*A*) and R4496C mutant (*B*) hearts were loaded with Rhod-2 AM. Ca^{2+} transients in intact Rhod-2 AM loaded RyR2 WT and RyR2-R4496C mutant hearts were elicited by pacing at different frequencies (5–16 Hz) and recorded using line-scanning confocal imaging. Cell boundaries are indicated by *short bars* to the *left*. The $\Delta F/F_o$ traces depict the average fluorescence signal of the scan area. Alternans ratio for each cell that displayed alternans in the scan area and alternans duration for each cell in the same scan area were determined and averaged per cell to yield the average alternans ratio (*C*) and average alternans duration (*D*). Alternans ratio is defined as the ratio of the difference in amplitude between the large and small Ca^{2+} transients over the amplitude of the large Ca^{2+} transient. Alternans duration is defined as the percentage of time in alternans over the 10-s scanning period. Data shown are mean ± S.D. (*error bars*) ($n = 6$ hearts for WT at stimulation frequencies 5–14 Hz, and $n = 3$ hearts for WT at 15–16 Hz; $n = 11$ hearts for R4496C at stimulation frequencies 5–14 Hz, and $n = 6$ hearts for RC for 15–16 Hz); two-way ANOVA with a Bonferroni's post hoc test (**, $p < 0.01$). For the analysis of alternans ratios, the F statistics are as follows: for the row factor (pacing frequency), $F = 112.763$, $p < 0.01$; for the column factor (genotypes), $F = 282.836$, $p < 0.01$; interaction between the column and row factors, $F = 26.7164$; $p < 0.01$. For the analysis of alternans durations, the F statistics are as follows: for the row factor (pacing frequency), $F = 59.165$, $p < 0.01$; for the column factor (genotypes), $F = 83.86$, $p < 0.01$; interaction between the column and row factors, $F = 11.127$; $p < 0.01$.

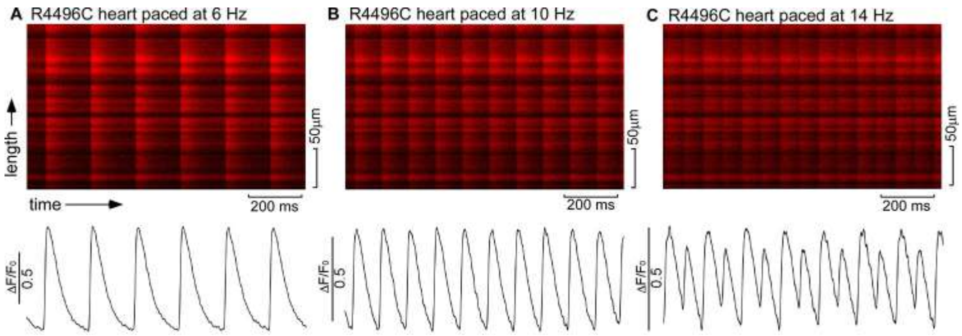

Figure 3. Intact working RyR2-R4496C hearts exhibit no detectable spontaneous Ca^{2+} release events during pacing. Intact Rhod-2 AM–loaded R4496C mutant hearts were stimulated at increasing frequencies (6–14 Hz) and recorded using line-scanning confocal imaging. *A*, Ca^{2+} transients at 6 Hz. *B*, Ca^{2+} transients at 10 Hz. *C*, Ca^{2+} transients at 14 Hz. There were no spontaneous Ca^{2+} sparks or Ca^{2+} waves detected during pacing.

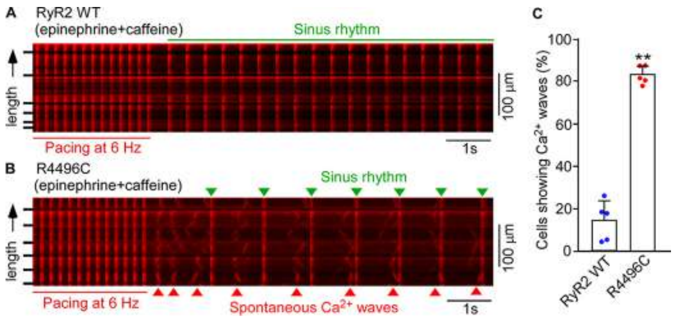

Figure 4. Intact working RyR2-R4496C hearts display spontaneous Ca^{2+} waves after cessation of stimulation. Intact Rhod-2 AM–loaded RyR2 WT (*A*) and R4496C mutant (*B*) hearts were stimulated at 6 Hz (indicated by a *red line*) and recorded using line-scanning confocal imaging during stimulation and after the cessation of stimulation. *C*, percentage of cells in intact WT and R4496C hearts that displayed spontaneous Ca^{2+} waves (indicated by *red triangles*). Spontaneous sinus rhythm is indicated by a *green line* or *green triangles*. Data shown are mean ± S.D. (*error bars*) ($n = 5$ hearts for RyR2 WT; $n = 5$ hearts for R4496C; **, $p < 0.01$).

SERCA2a function either has a relatively minor impact on Ca^{2+} alternans or can lead to marked suppression of Ca^{2+} alternans.

We also assessed the impact of tBHQ on Ca^{2+} alternans in intact RyR2 E4872Q mutant hearts (32, 58). Similar to those observed in RyR2 WT hearts, tBHQ at 3 and 10 μM significantly reduced the amplitude of Ca^{2+} transients. It also prolonged the T_{50} of Ca^{2+} transients by 8% (at 3 μM) and 16% (at 10 μM) (Fig. S6). As with intact WT hearts, tBHQ at 10 μM also significantly reduced the average alternans ratio and duration in intact RyR2 E4872Q mutant hearts (Fig. 8). On the other hand, tBHQ at 3 μM had a minor effect on the average alternans ratio or duration in intact E4872Q hearts (Fig. 8). Collectively, these data indicate that compared with RyR2, SERCA2a plays a relatively minor role in Ca^{2+} alternans in intact hearts.

Discussion

Beat-to-beat alternations in the amplitude of the cytosolic Ca^{2+} transient (Ca^{2+} alternans) are thought to be the primary cause of cardiac alternans (1–11), which is a major risk factor for ventricular arrhythmias and sudden cardiac arrest (12–19). Despite its important role in arrhythmogenesis, the molecular mechanism underlying Ca^{2+} alternans remains undefined. An increased body of evidence suggests that Ca^{2+} alternans results from abnormal SR Ca^{2+} cycling (9, 11, 21–25). Because SR Ca^{2+} cycling is governed by SR Ca^{2+} release via RyR2 and SR Ca^{2+} reuptake by SERCA2a (20), altered RyR2 or SERCA2a function would be expected to affect SR Ca^{2+} cycling, thus leading to Ca^{2+} alternans. However, how changes in the activity of RyR2 or SERCA2a affect Ca^{2+} alternans is unclear. To address this question, here we determined the impact of genetically or pharmacologically enhancing or suppressing RyR2 or SERCA2a function on Ca^{2+} alternans in intact working hearts. We found that altering RyR2 function, but not SERCA2a function, has a major impact on Ca^{2+} alternans. These findings shed new insights into the molecular mechanism of Ca^{2+} alternans and have important therapeutic implications for cardiac alternans.

Recent studies have consistently shown that suppressing the function of RyR2 prolongs the refractoriness of SR Ca^{2+} release and promotes Ca^{2+} alternans (26–30, 32). However, the impact of enhanced RyR2 function on Ca^{2+} alternans is unclear. On the one hand, enhancing RyR2 function would increase spontaneous Ca^{2+} release (Ca^{2+} sparks/Ca^{2+} waves), which would promote Ca^{2+} alternans (25, 28, 38, 41–46). On the other hand, enhancing RyR2 function would shorten SR Ca^{2+} release refractoriness, which would suppress Ca^{2+} alternans (10, 31, 32, 34, 35, 39). To ascertain these seemingly paradoxical effects of enhanced RyR2 function on Ca^{2+} alternans, we determined the impact of a disease-causing RyR2 mutation (R4496C) with enhanced channel activity on Ca^{2+} alternans in the setting of intact working hearts. We found that, despite the enhanced RyR2 activity, intact working RyR2 R4496C mutant hearts displayed little or no spontaneous Ca^{2+} sparks or waves during electrical stimulation, similar to what was reported previously (47). This is also consistent with the observation that increased heart rate alone (as in programmed electrical stimulation) can rarely trigger VTs in patients with CPVT (61). In contrast, accelerating heart rate (in the absence of excessive adrenergic stress) suppresses spontaneous Ca^{2+} release and prevents VTs in both CPVT animal models and patients (62). Furthermore, we found that, in the absence of Ca^{2+} sparks/waves, enhancing RyR2 function shortens SR Ca^{2+} release refractoriness and suppresses Ca^{2+} alternans. Hence, contrary to depressed RyR2 function, which promotes Ca^{2+} release refractoriness and Ca^{2+} alternans and suppresses stress-provoked CPVT, enhanced RyR2 function protects against Ca^{2+} alternans but promotes CPVT. These observations suggest that the mechanisms underlying stress-provoked CPVT and Ca^{2+} alternans are different. It is of interest to note that, contrary to our findings, enhanced

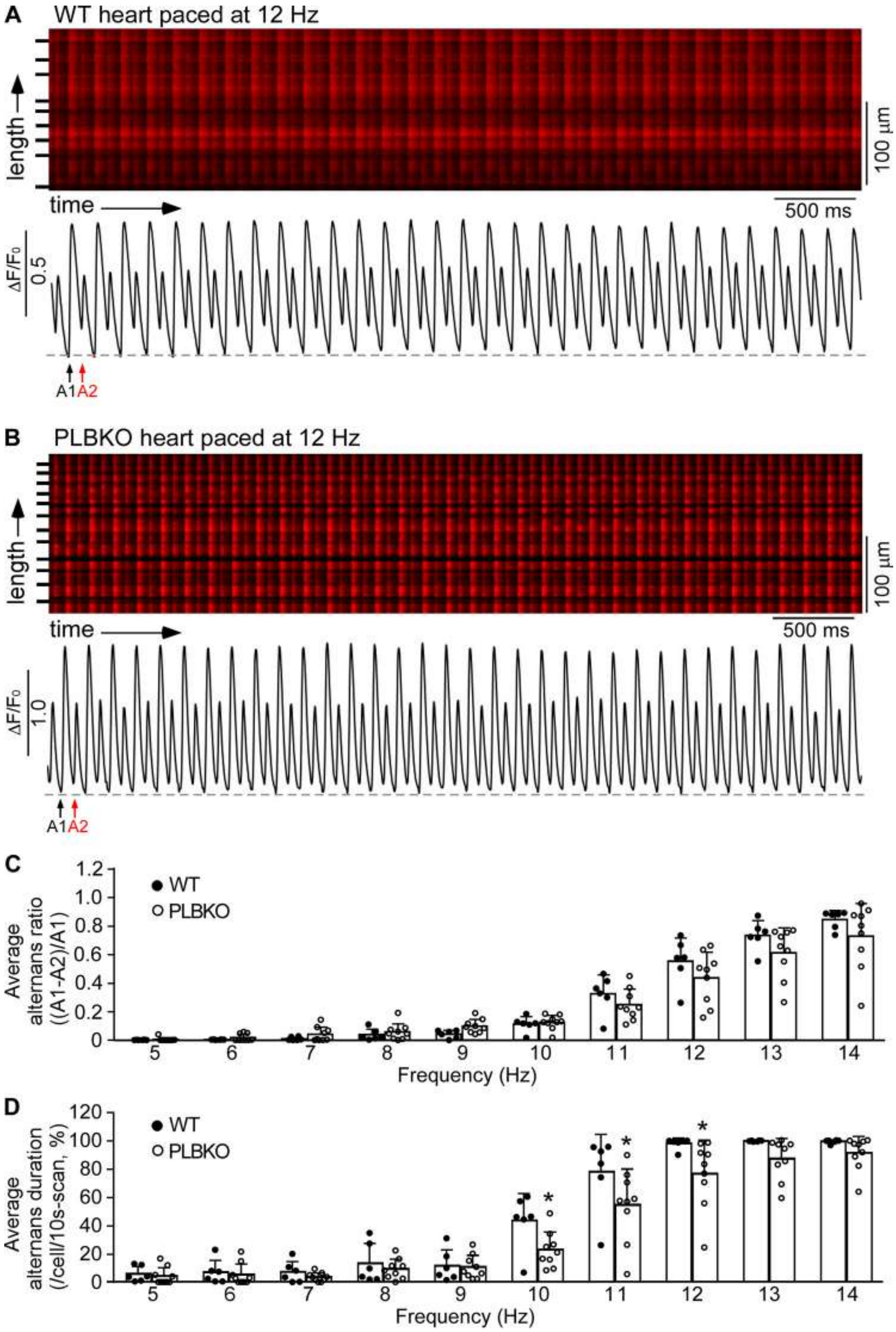

Figure 5. Ca^{2+} transient alternans in intact WT and PLB-KO hearts. Langendorff-perfused intact RyR2 WT (*A*) and PLB-KO (*B*) hearts were loaded with Rhod-2 AM. Ca^{2+} transients were elicited by pacing at different frequencies (5–14 Hz) and recorded using line-scanning confocal imaging. Cell boundaries are indicated by *short bars* to the *left*. The $\Delta F/F_o$ *traces* depict the average fluorescence signal of the scan area. The average alternans ratio (*C*) and average alternans duration (*D*) in intact RyR2 WT and PLB-KO hearts at different stimulation frequencies are shown. Data shown are mean ± S.D. (*error bars*) ($n = 6$ hearts for WT, $n = 9$ hearts for PLB-KO); two-way ANOVA with Bonferroni's post hoc test (*, $p < 0.05$). For the analysis of alternans ratios, the F statistics are as follows: for the row factor (pacing frequency), $F = 113.179$, $p < 0.01$; for the column factor (genotypes), $F = 2.859$, $p = 0.0932$; interaction between the column and row factors, $F = 1.589$; $p = 0.1249$. For the analysis of alternans durations, the F statistics are as follows: for the row factor (pacing frequency), $F = 124.8$, $p < 0.01$; for the column factor (genotypes), $F = 18.94$, $p < 0.01$; interaction between the column and row factors, $F = 1.65$; $p = 0.1215$.

RyR2 function has been shown to promote Ca^{2+} alternans in isolated cardiomyocytes where spontaneous Ca^{2+} release is present (36, 38, 40). These observations suggest that the presence or absence of spontaneous SR Ca^{2+} release may influence whether enhanced RyR2 function will promote or suppress Ca^{2+} alternans and that the nature and mechanisms of Ca^{2+} alternans with or without spontaneous SR Ca^{2+} release may be different. Further studies are needed to fully understand the role of spontaneous Ca^{2+} release in the genesis of Ca^{2+} alternans.

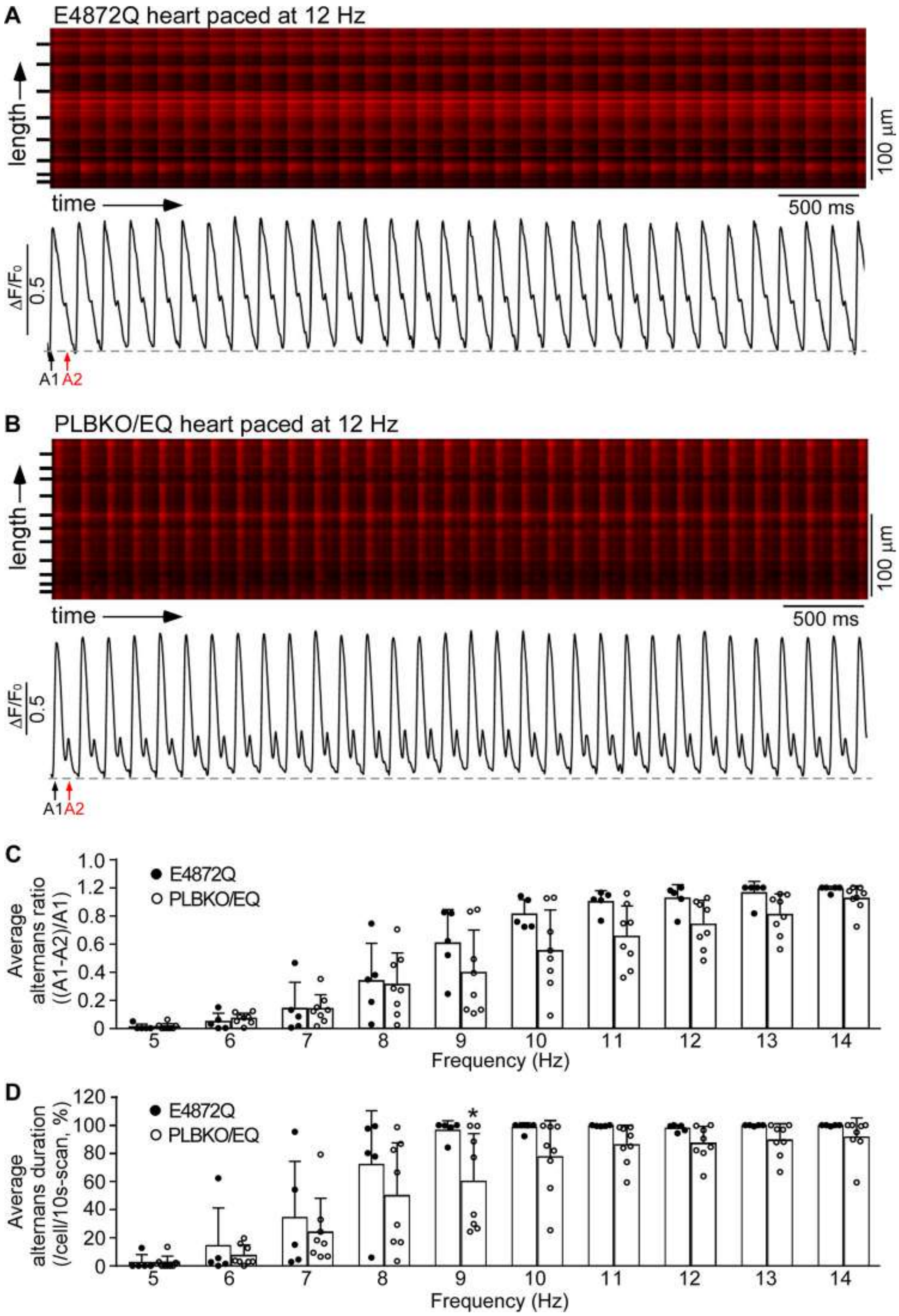

Figure 6. Ca^{2+} alternans in intact RyR2-E4872Q hearts with or without PLB-KO. Langendorff-perfused intact RyR2-E4872Q (*A*) and PLB-KO/RyR2-E4872Q$^{+/-}$ (PLB-KO/EQ) (*B*) hearts were loaded with Rhod-2 AM. Ca^{2+} transients were elicited by pacing at different frequencies (5–14 Hz) and recorded using line-scanning confocal imaging. Cell boundaries are indicated by *short bars* to the *left*. The $\Delta F/F_o$ traces depict the average fluorescence signal of the scan area. The average alternans ratio (*C*) and average alternans duration (*D*) intact RyR2-E4872Q and PLB-KO/EQ at different stimulation frequencies are shown. Data shown are mean ± S.D. (*error bars*) (n = 5 hearts for E4872Q, n = 8 hearts for PLB-KO/EQ); two-way ANOVA with a Bonferroni's post hoc test (*, $p < 0.05$). For the analysis of alternans ratios, the F statistics are as follows: for the row factor (pacing frequency), $F = 54.76$, $p < 0.01$; for the column factor (genotypes), $F = 13.26$, $p < 0.01$; interaction between the column and row factors, $F = 1.276$; $p = 0.257$. For the analysis of alternans durations, the F statistics are as follows: for the Row factor (pacing frequency), $F = 38.66$, $p < 0.01$; for the column factor (genotypes), $F = 13.874$, $p < 0.01$; interaction between the column and row factors, $F = 0.726$; $p = 0.6834$.

The role of SERCA2a in Ca^{2+} alternans is complex. On the one hand, reduced SERCA2a function would decrease SR Ca^{2+} content and thus SR Ca^{2+} release, which would suppress Ca^{2+} alternans. On the other hand, reduced SERCA2a function would prolong Ca^{2+} transient decay and elevate cytosolic Ca^{2+} concentration, which would promote Ca^{2+} alternans (25, 31, 46, 52). Similarly, enhanced SERCA2a function would increase SR Ca^{2+} content and thus SR Ca^{2+} release, which would promote Ca^{2+} alternans. In contrast, enhanced SERCA2a function would hasten Ca^{2+} transient decay and reduce cytosolic Ca^{2+}

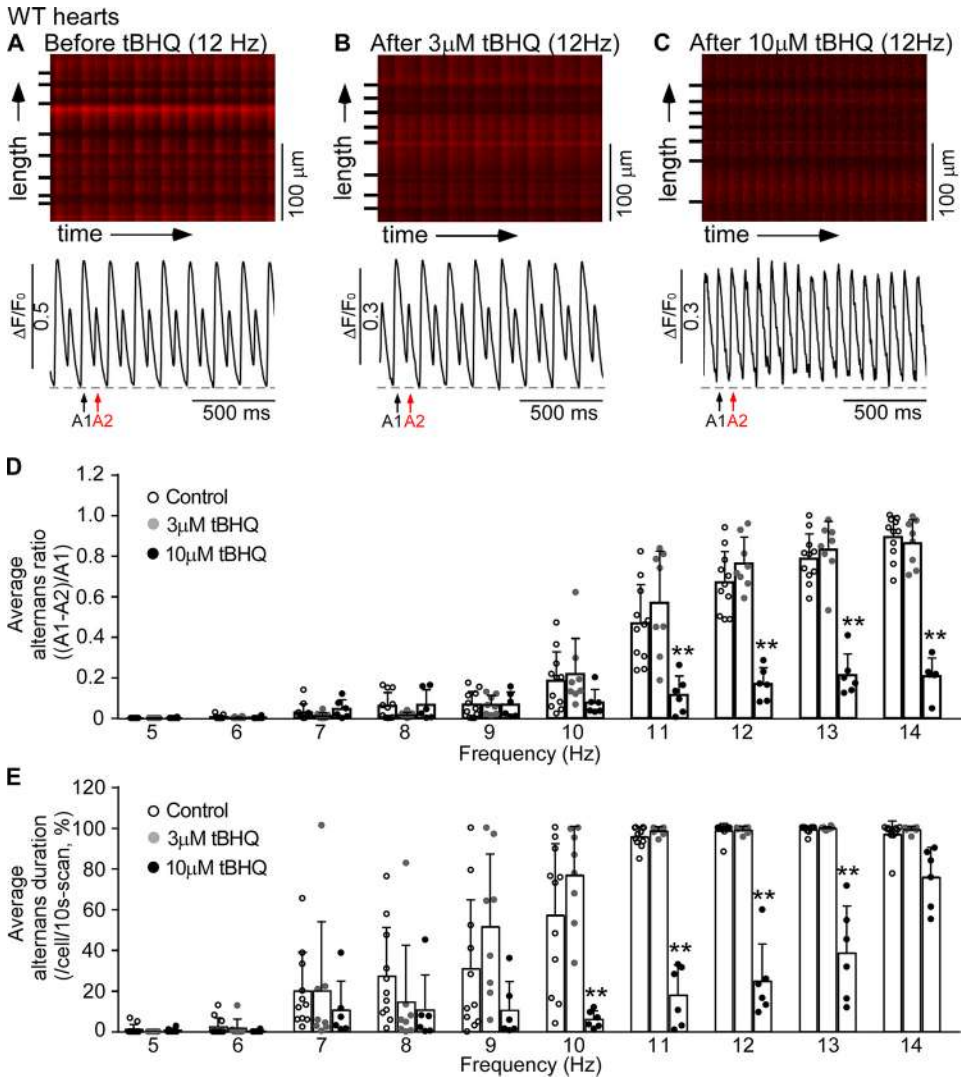

Figure 7. Effect of tBHQ on Ca^{2+} alternans ratio and duration in intact RyR2 WT hearts. Langendorff-perfused intact RyR2 WT hearts were loaded with Rhod-2 AM. Ca^{2+} transients were elicited by pacing at different frequencies (5–14 Hz), and recorded using line-scanning confocal imaging before (*A*) and after the treatment of 3 μM (*B*) or 10 μM (*C*) tBHQ. Cell boundaries are indicated by *short bars* to the *left*. The $\Delta F/F_o$ traces depict the average fluorescence signal of the scan area. The average alternans ratio (*D*) and average alternans duration (*E*) at different stimulation frequencies are shown. Data shown are mean ± S.D. (*error bars*) ($n = 11$ hearts before tBHQ treatment, $n = 8$ hearts after 3 μM tBHQ, $n = 6$ hearts after 10 μM tBHQ); two-way ANOVA with a Dunnett's post hoc test (**, $p < 0.01$). For the analysis of alternans ratios, the *F* statistics are as follows: for the row factor (pacing frequency), $F = 153.906$, $p < 0.01$; for the column factor (tBHQ treatment), $F = 108.024$, $p < 0.01$; interaction between the column and row factors, $F = 17.8275$; $p < 0.01$. For the analysis of alternans durations, the *F* statistics are as follows: for the row factor (pacing frequency), $F = 84.08$, $p < 0.01$; for the column factor (tBHQ treatment), $F = 86.24$, $p < 0.01$; interaction between the column and row factors, $F = 7.386$; $p < 0.01$.

concentration, which would suppress Ca^{2+} alternans (25, 31, 46, 52). Thus, changes in the activity of SERCA2a would promote or suppress Ca^{2+} alternans, depending on the relative changes in the cytosolic Ca^{2+} concentration and the SR Ca^{2+} content. Furthermore, because the activity of SERCA2a oppositely affects the cytosolic Ca^{2+} concentration and SR Ca^{2+} content, changes in the SERCA2a activity would be expected to have only a minor impact on Ca^{2+} alternans due to the resultant opposite changes in the cytosolic Ca^{2+} concentration and SR Ca^{2+} content. Indeed, consistent with this view, we found that genetically enhancing the SERCA2a function by PLB-KO significantly increased the amplitude of SR Ca^{2+} release, which would promote Ca^{2+} alternans, but decreased the decay time of Ca^{2+} transients, which would suppress Ca^{2+} alternans. As a result of these opposing effects, PLB-KO did not markedly alter Ca^{2+} alternans in intact WT hearts. We also found that PLB-KO did not rescue the enhanced Ca^{2+} alternans in intact RyR2 E4872Q mutant hearts.

We also investigated the impact of reduced SERCA2a function on Ca^{2+} alternans. Partially reducing SERCA2a activity using a low concentration of tBHQ (3 μM) reduced the amplitude of SR Ca^{2+} release, which would suppress Ca^{2+} alternans, but increased the decay time of Ca^{2+} transients, which would promote Ca^{2+} alternans. As a result, these opposing actions of tBHQ in the amplitude and decay time of Ca^{2+} transients led to no marked alteration in Ca^{2+} alternans in intact WT hearts. We also found that tBHQ (3 μM) had no major impact on the enhanced Ca^{2+} alternans in intact RyR2 E4872Q mutant hearts. Interestingly, further inhibition of SERCA2a activity using a higher concentration of tBHQ (10 μM) significantly suppressed Ca^{2+} alternans in intact WT or E4872Q mutant hearts. This suppression on Ca^{2+} alternans probably resulted from the

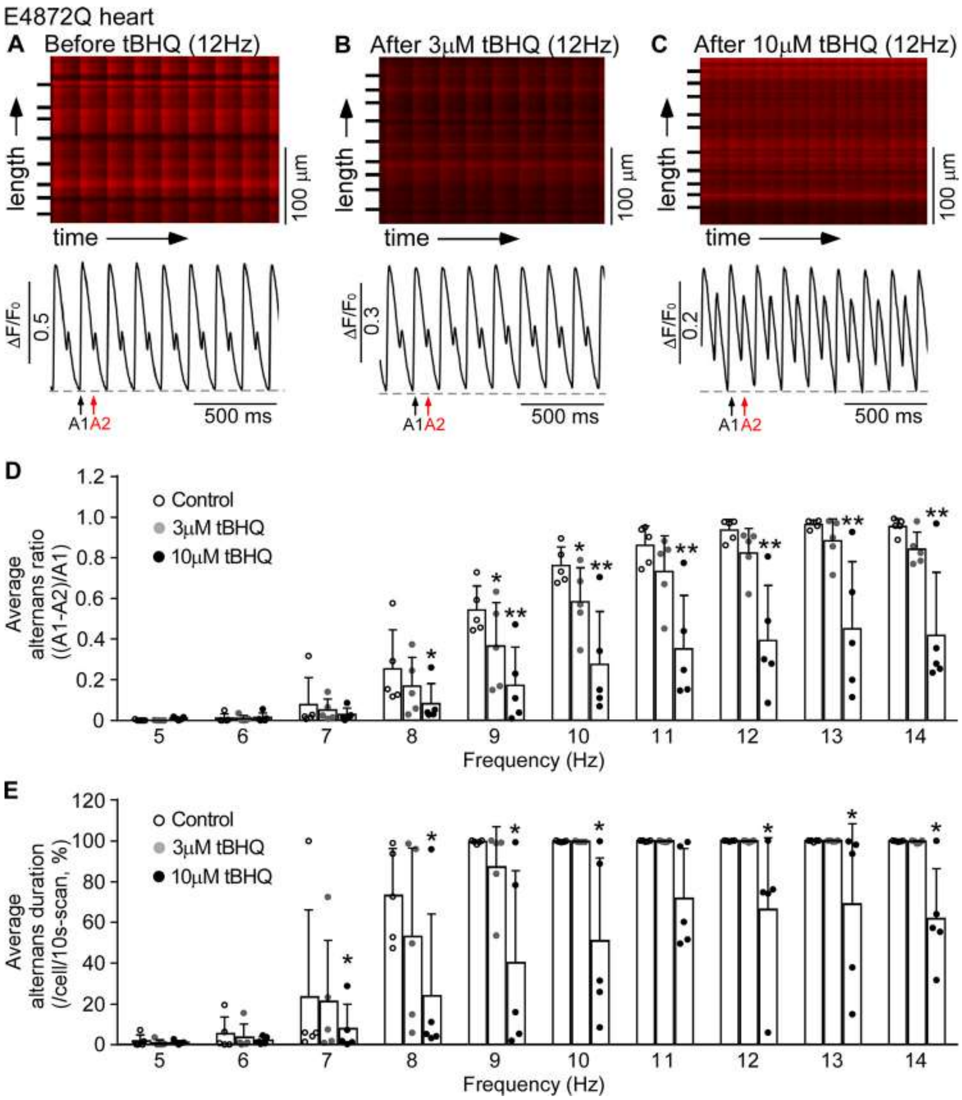

Figure 8. Effect of tBHQ on Ca^{2+} alternans ratio and duration in intact RyR2-E4872Q hearts. Langendorff-perfused intact RyR2-E4872Q hearts were loaded with Rhod-2 AM. Ca^{2+} transients were elicited by pacing at different frequencies (5–14 Hz) and recorded using line-scanning confocal imaging before (*A*) and after the treatment of 3 μM (*B*) or 10 μM (*C*) tBHQ. Cell boundaries are indicated by *short bars* to the *left*. The $\Delta F/F_o$ *traces* depict the average fluorescence signal of the scan area. The average alternans ratio (*D*) and average alternans duration (*E*) at different stimulation frequencies are shown. Data shown are mean ± S.D. (*error bars*) ($n = 5$ hearts each for control, 3 μM, and 10 μM tBHQ groups); two-way ANOVA with Dunnett's post hoc test (*, $p < 0.05$; **, $p < 0.01$). For the analysis of alternans ratios, the F statistics are as follows: for the row factor (pacing frequency), $F = 32.41$, $p < 0.01$; for the column factor (tBHQ treatment), $F = 107.4$, $p < 0.01$; interaction between the column and row factors, $F = 6.527$; $p < 0.01$. For the analysis of alternans durations, the F statistics are as follows: for the row factor (pacing frequency), $F = 28.754$, $p < 0.01$; for the column factor (tBHQ treatment), $F = 35.61$, $p < 0.01$; interaction between the column and row factors, $F = 1.538$; $p = 0.1035$.

stronger effect of 10 μM tBHQ on the reduction in SR Ca^{2+} content, as a result of stronger inhibition of SERCA2a activity, than on the increase in Ca^{2+} transient decay. Taken together, our findings indicate that moderate changes in the SERCA2a function have a relatively minor impact on Ca^{2+} alternans in intact working hearts. However, it is important to note that altered SERCA2a function also affects the propensity for spontaneous Ca^{2+} release. Thus, changes in SERCA2a function may play an important role in Ca^{2+} alternans in the setting of disease hearts where spontaneous Ca^{2+} release is enhanced.

Although genetically engineered mouse models harboring a RyR2 GOF or loss-of-function mutation or a PLB deletion allow us to determine the effect of specifically reducing or enhancing RyR2 or SERCA2a function on Ca^{2+} alternans, whether our findings from these mouse hearts could be translated into the human hearts is unclear. It is known that intracellular Ca^{2+} handling and electrophysiological properties of the mouse hearts are substantially different from those of the human hearts. Hence, the significance and relative contribution of RyR2 and SERCA2a function to Ca^{2+} alternans in human hearts has yet to be determined.

In summary, the present study demonstrates for the first time that genetically enhancing RyR2 function shortens SR Ca^{2+} release refractoriness and protects against Ca^{2+} alternans in intact working hearts. On the other hand, enhancing or suppressing SERCA2a function has a relatively minor impact on Ca^{2+} alternans in intact working hearts. These findings indicate that the activity of RyR2, but not SERCA2a, is a major determinant of Ca^{2+} alternans. Thus, RyR2 represents a promising therapeutic target for cardiac alternans.

Experimental procedures

Animal studies

All animal studies were approved by the institutional animal care and use committee at the University of Calgary and performed in accordance with National Institutes of Health guidelines. The phospholamban (PLB) knockout, RyR2-R4496C, and RyR2-E4872Q knock-in mutant mice were generated as described previously (56, 58, 60). The RyR2-E4872Q mutant mice were cross-bred with the PLB-KO mice to produce a PLB-deficient mouse line expressing the heterozygous RyR2-E4872Q$^{+/-}$ mutation (PLB-KO/EQ$^{+/-}$). Adult RyR2-R4496C$^{+/-}$, RyR2-E4872Q$^{+/-}$, PLB-KO, and PLB-KO/EQ$^{+/-}$ mutant and WT control mice (8–12 weeks) were used for all experiments.

Determination of refractoriness of SR Ca^{2+} release

The refractoriness of voltage-induced release of Ca^{2+} from the SR was determined by using the S1S2 stimulation protocol as described previously with some modifications (10, 32). Briefly, Ca^{2+} transients in Rhod-2 AM–loaded hearts were first induced at 5 Hz for 5 s (S1), followed by a single S2 stimulation at a specific interval. The hearts were repeatedly stimulated by a series of S1S2 protocols with progressively decreased S1S2 intervals (from 200 to 40 ms). Ca^{2+} transients before and after S2 stimulation were continuously recorded by using the Nikon-A1R confocal microscope in the line-scan mode.

Laser-scanning confocal Ca^{2+} imaging of intact hearts

WT and mutant mice were sacrificed by cervical dislocation. Their hearts were quickly removed and loaded with 4.4 μM Rhod-2 AM (Biotium, Inc., Hayward, CA) in oxygenated Tyrode's buffer (118 mM NaCl, 5.4 mM KCl, 25 mM $NaHCO_3$, 1 mM $MgCl_2$, 0.42 mM NaH_2PO_4, 11.1 mM glucose, 10 mM taurine, 5 mM creatine, and 1.8 mM $CaCl_2$, pH 7.4) via the retrograde Langendorff perfusion system at 25 °C for 45 min (47, 63). The Langendorff-perfused hearts were placed in a recording chamber mounted onto the Nikon A1R microscope for *in situ* confocal imaging (line-scan) of Ca^{2+} signals from epicardial ventricular myocytes. The temperature of the heart was kept at 35 °C throughout the experiment with 5 μM blebbistatin (Toronto Research Chemicals, Toronto, Canada) to prevent motion artifact. The pixel size of the resulting line-scan images ranged between 1.8 and 2 ms in the temporal dimension and between 0.1 and 0.4 μm in the spatial dimension. Ca^{2+} alternans in the WT and mutant hearts in the absence or presence of tBHQ (3 or 10 μM) was induced by rapid electrical stimulation at increasing frequencies (5–14 Hz, 6 V).

Image and signal processing

The signal and image processing methods were implemented using MATLAB (The Mathworks Inc., Boston, MA) as described previously (32). Briefly, line-scan fluorescence images were filtered according to the noise level estimated by the median absolute deviation of the pixel intensities. Individual cells in the images were manually marked, and the average fluorescence in each cell was obtained for further analysis. A wavelet peak detection algorithm was used to detect individual calcium release events in the average fluorescence signals. For each event detected in each cell, we determined the peak amplitude (local minimum-maximum difference) and the alternans ratio (relative amplitude difference between consecutive peaks). The presence of alternans periods was established when six consecutive peaks presented an alternans ratio above 0.05. Alternans duration was defined as the percentage of alternans periods over the total line-scan duration. Average magnitudes were obtained by taking the mean over each line scan.

Statistical analysis

GraphPad Prism version 6.0 was used for statistical analyses. All values shown are mean ± S.D. unless indicated otherwise. To test for differences between groups, we used Student's *t* test (two-tailed) or one- or two-way ANOVA with Dunnett's or Bonferroni's post hoc test when appropriate. A p value <0.05 was considered to be statistically significant.

Author contributions—B. S., J. W., X. Z., W. G., J. Y., R. W., R. B., L. H.-M., S. R. W. C. designed the research; B. S., J. W., X. Z., W. G., J. Y., and R. W. performed the research; B. S., J. W., X. Z., W. G., A. V., R. B., L. H.-M., and S. R. W. C. analyzed data; and B. S., X. Z., A. V., R. B., L. H.-M., and S. R. W. C. wrote the paper.

Acknowledgments—We thank Dr. Evangelia G. Kranias (University of Cincinnati) for kindly providing the phospholamban knockout mice and Dr. Long-Sheng Song (University of Iowa) for continued support and helpful discussion on intact heart Ca^{2+} imaging.

References

1. Kihara, Y., and Morgan, J. P. (1991) Abnormal Cai^{2+} handling is the primary cause of mechanical alternans: study in ferret ventricular muscles. *Am. J. Physiol.* **261,** H1746–H1755 Medline
2. Chudin, E., Goldhaber, J., Garfinkel, A., Weiss, J., and Kogan, B. (1999) Intracellular Ca^{2+} dynamics and the stability of ventricular tachycardia. *Biophys. J.* **77,** 2930–2941 CrossRef Medline
3. Díaz, M. E., O'Neill, S. C., and Eisner, D. A. (2004) Sarcoplasmic reticulum calcium content fluctuation is the key to cardiac alternans. *Circ. Res.* **94,** 650–656 CrossRef Medline
4. Goldhaber, J. I., Xie, L. H., Duong, T., Motter, C., Khuu, K., and Weiss, J. N. (2005) Action potential duration restitution and alternans in rabbit ventricular myocytes: the key role of intracellular calcium cycling. *Circ. Res.* **96,** 459–466 CrossRef Medline
5. Wan, X., Laurita, K. R., Pruvot, E. J., and Rosenbaum, D. S. (2005) Molecular correlates of repolarization alternans in cardiac myocytes. *J. Mol. Cell. Cardiol.* **39,** 419–428 CrossRef Medline
6. Eisner, D. A., Li, Y., and O'Neill, S. C. (2006) Alternans of intracellular calcium: mechanism and significance. *Heart Rhythm* **3,** 743–745 CrossRef Medline
7. Laurita, K. R., and Rosenbaum, D. S. (2008) Cellular mechanisms of arrhythmogenic cardiac alternans. *Prog. Biophys. Mol. Biol.* **97,** 332–347 CrossRef Medline
8. Laurita, K. R., and Rosenbaum, D. S. (2008) Mechanisms and potential therapeutic targets for ventricular arrhythmias associated with impaired cardiac calcium cycling. *J. Mol. Cell. Cardiol.* **44,** 31–43 CrossRef Medline
9. Xie, L. H., Sato, D., Garfinkel, A., Qu, Z., and Weiss, J. N. (2008) Intracellular Ca alternans: coordinated regulation by sarcoplasmic reticulum release, uptake, and leak. *Biophys. J.* **95,** 3100–3110 CrossRef Medline
10. Wang, L., Myles, R. C., De Jesus, N. M., Ohlendorf, A. K., Bers, D. M., and Ripplinger, C. M. (2014) Optical mapping of sarcoplasmic reticulum Ca^{2+} in the intact heart: ryanodine receptor refractoriness during alternans and fibrillation. *Circ. Res.* **114,** 1410–1421 CrossRef Medline

11. Kanaporis, G., and Blatter, L. A. (2015) The mechanisms of calcium cycling and action potential dynamics in cardiac alternans. *Circ. Res.* **116,** 846–856 CrossRef Medline
12. Rosenbaum, D. S., Jackson, L. E., Smith, J. M., Garan, H., Ruskin, J. N., and Cohen, R. J. (1994) Electrical alternans and vulnerability to ventricular arrhythmias. *N. Engl. J. Med.* **330,** 235–241 CrossRef Medline
13. Armoundas, A. A., Tomaselli, G. F., and Esperer, H. D. (2002) Pathophysiological basis and clinical application of T-wave alternans. *J. Am. Coll. Cardiol.* **40,** 207–217 CrossRef Medline
14. Narayan, S. M. (2006) T-wave alternans and the susceptibility to ventricular arrhythmias. *J. Am. Coll. Cardiol.* **47,** 269–281 CrossRef Medline
15. Wilson, L. D., Jeyaraj, D., Wan, X., Hoeker, G. S., Said, T. H., Gittinger, M., Laurita, K. R., and Rosenbaum, D. S. (2009) Heart failure enhances susceptibility to arrhythmogenic cardiac alternans. *Heart Rhythm* **6,** 251–259 CrossRef Medline
16. Qu, Z., Xie, Y., Garfinkel, A., and Weiss, J. N. (2010) T-wave alternans and arrhythmogenesis in cardiac diseases. *Front. Physiol.* **1,** 154 Medline
17. Verrier, R. L., and Nieminen, T. (2010) T-wave alternans as a therapeutic marker for antiarrhythmic agents. *J. Cardiovasc. Pharmacol.* **55,** 544–554 CrossRef Medline
18. Verrier, R. L., and Malik, M. (2013) Electrophysiology of T-wave alternans: mechanisms and pharmacologic influences. *J. Electrocardiol.* **46,** 580–584 CrossRef Medline
19. Escobar, A. L., and Valdivia, H. H. (2014) Cardiac alternans and ventricular fibrillation: a bad case of ryanodine receptors reneging on their duty. *Circ. Res.* **114,** 1369–1371 CrossRef Medline
20. Bers, D. M. (2002) Cardiac excitation-contraction coupling. *Nature* **415,** 198–205 CrossRef Medline
21. Cordeiro, J. M., Malone, J. E., Di Diego, J. M., Scornik, F. S., Aistrup, G. L., Antzelevitch, C., and Wasserstrom, J. A. (2007) Cellular and subcellular alternans in the canine left ventricle. *Am. J. Physiol. Heart Circ. Physiol.* **293,** H3506–H3516 CrossRef Medline
22. Aistrup, G. L., Shiferaw, Y., Kapur, S., Kadish, A. H., and Wasserstrom, J. A. (2009) Mechanisms underlying the formation and dynamics of subcellular calcium alternans in the intact rat heart. *Circ. Res.* **104,** 639–649 CrossRef Medline
23. Alvarez-Lacalle, E., Cantalapiedra, I. R., Peñaranda, A., Cinca, J., Hove-Madsen, L., and Echebarria, B. (2013) Dependency of calcium alternans on ryanodine receptor refractoriness. *PLoS One* **8,** e55042 CrossRef Medline
24. Edwards, J. N., and Blatter, L. A. (2014) Cardiac alternans and intracellular calcium cycling. *Clin. Exp. Pharmacol. Physiol.* **41,** 524–532 CrossRef Medline
25. Qu, Z., Liu, M. B., and Nivala, M. (2016) A unified theory of calcium alternans in ventricular myocytes. *Sci. Rep.* **6,** 35625 CrossRef Medline
26. Orchard, C. H., McCall, E., Kirby, M. S., and Boyett, M. R. (1991) Mechanical alternans during acidosis in ferret heart muscle. *Circ. Res.* **68,** 69–76 CrossRef Medline
27. Hüser, J., Wang, Y. G., Sheehan, K. A., Cifuentes, F., Lipsius, S. L., and Blatter, L. A. (2000) Functional coupling between glycolysis and excitation-contraction coupling underlies alternans in cat heart cells. *J. Physiol.* **524,** 795–806 CrossRef Medline
28. Díaz, M. E., Eisner, D. A., and O'Neill, S. C. (2002) Depressed ryanodine receptor activity increases variability and duration of the systolic Ca^{2+} transient in rat ventricular myocytes. *Circ. Res.* **91,** 585–593 CrossRef Medline
29. Kockskämper, J., Zima, A. V., and Blatter, L. A. (2005) Modulation of sarcoplasmic reticulum Ca^{2+} release by glycolysis in cat atrial myocytes. *J. Physiol.* **564,** 697–714 CrossRef Medline
30. Kapur, S., Wasserstrom, J. A., Kelly, J. E., Kadish, A. H., and Aistrup, G. L. (2009) Acidosis and ischemia increase cellular Ca^{2+} transient alternans and repolarization alternans susceptibility in the intact rat heart. *Am. J. Physiol. Heart Circ. Physiol.* **296,** H1491–H1512 CrossRef Medline
31. Shkryl, V. M., Maxwell, J. T., Domeier, T. L., and Blatter, L. A. (2012) Refractoriness of sarcoplasmic reticulum Ca^{2+} release determines Ca^{2+} alternans in atrial myocytes. *Am. J. Physiol. Heart Circ. Physiol.* **302,** H2310–H2320 CrossRef Medline
32. Zhong, X., Sun, B., Vallmitjana, A., Mi, T., Guo, W., Ni, M., Wang, R., Guo, A., Duff, H. J., Gillis, A. M., Song, L. S., Hove-Madsen, L., Benitez, R., and Chen, S. R. (2016) Suppression of ryanodine receptor function prolongs Ca^{2+} release refractoriness and promotes cardiac alternans in intact hearts. *Biochem. J.* **473,** 3951–3964 CrossRef Medline
33. Kornyeyev, D., Petrosky, A. D., Zepeda, B., Ferreiro, M., Knollmann, B., and Escobar, A. L. (2012) Calsequestrin 2 deletion shortens the refractoriness of Ca^{2+} release and reduces rate-dependent Ca^{2+}-alternans in intact mouse hearts. *J. Mol. Cell Cardiol.* **52,** 21–31 CrossRef Medline
34. Picht, E., DeSantiago, J., Blatter, L. A., and Bers, D. M. (2006) Cardiac alternans do not rely on diastolic sarcoplasmic reticulum calcium content fluctuations. *Circ. Res.* **99,** 740–748 CrossRef Medline
35. Lugo, C. A., Cantalapiedra, I. R., Peñaranda, A., Hove-Madsen, L., and Echebarria, B. (2014) Are SR Ca content fluctuations or SR refractoriness the key to atrial cardiac alternans?: insights from a human atrial model. *Am. J. Physiol. Heart Circ. Physiol.* **306,** H1540–H1552 CrossRef Medline
36. Belevych, A. E., Terentyev, D., Viatchenko-Karpinski, S., Terentyeva, R., Sridhar, A., Nishijima, Y., Wilson, L. D., Cardounel, A. J., Laurita, K. R., Carnes, C. A., Billman, G. E., and Gyorke, S. (2009) Redox modification of ryanodine receptors underlies calcium alternans in a canine model of sudden cardiac death. *Cardiovasc. Res.* **84,** 387–395 CrossRef Medline
37. Sabir, I. N., Ma, N., Jones, V. J., Goddard, C. A., Zhang, Y., Kalin, A., Grace, A. A., and Huang, C. L. (2010) Alternans in genetically modified Langendorff-perfused murine hearts modeling catecholaminergic polymorphic ventricular tachycardia. *Front. Physiol.* **1,** 126 Medline
38. Xie, W., Santulli, G., Guo, X., Gao, M., Chen, B. X., and Marks, A. R. (2013) Imaging atrial arrhythmic intracellular calcium in intact heart. *J. Mol. Cell Cardiol.* **64,** 120–123 CrossRef Medline
39. Ferrantini, C., Coppini, R., Scellini, B., Ferrara, C., Pioner, J. M., Mazzoni, L., Priori, S., Cerbai, E., Tesi, C., and Poggesi, C. (2016) R4496C RyR2 mutation impairs atrial and ventricular contractility. *J. Gen. Physiol.* **147,** 39–52 CrossRef Medline
40. Kang, G., Giovannone, S. F., Liu, N., Liu, F. Y., Zhang, J., Priori, S. G., and Fishman, G. I. (2010) Purkinje cells from RyR2 mutant mice are highly arrhythmogenic but responsive to targeted therapy. *Circ. Res.* **107,** 512–519 CrossRef Medline
41. Wasserstrom, J. A., Sharma, R., Kapur, S., Kelly, J. E., Kadish, A. H., Balke, C. W., and Aistrup, G. L. (2009) Multiple defects in intracellular calcium cycling in whole failing rat heart. *Circ. Heart Fail.* **2,** 223–232 CrossRef Medline
42. Xie, L. H., and Weiss, J. N. (2009) Arrhythmogenic consequences of intracellular calcium waves. *Am. J. Physiol. Heart Circ. Physiol.* **297,** H997–H1002 CrossRef Medline
43. Rovetti, R., Cui, X., Garfinkel, A., Weiss, J. N., and Qu, Z. (2010) Spark-induced sparks as a mechanism of intracellular calcium alternans in cardiac myocytes. *Circ. Res.* **106,** 1582–1591 CrossRef Medline
44. Kapur, S., Aistrup, G. L., Sharma, R., Kelly, J. E., Arora, R., Zheng, J., Veramasuneni, M., Kadish, A. H., Balke, C. W., and Wasserstrom, J. A. (2010) Early development of intracellular calcium cycling defects in intact hearts of spontaneously hypertensive rats. *Am. J. Physiol. Heart Circ. Physiol.* **299,** H1843–H1853 CrossRef Medline
45. Llach, A., Molina, C. E., Fernandes, J., Padró, J., Cinca, J., and Hove-Madsen, L. (2011) Sarcoplasmic reticulum and L-type Ca^{2+} channel activity regulate the beat-to-beat stability of calcium handling in human atrial myocytes. *J. Physiol.* **589,** 3247–3262 CrossRef Medline
46. Qu, Z., Nivala, M., and Weiss, J. N. (2013) Calcium alternans in cardiac myocytes: order from disorder. *J. Mol. Cell Cardiol.* **58,** 100–109 CrossRef Medline
47. Chen, B., Guo, A., Gao, Z., Wei, S., Xie, Y. P., Chen, S. R., Anderson, M. E., and Song, L. S. (2012) *In situ* confocal imaging in intact heart reveals stress-induced Ca^{2+} release variability in a murine catecholaminergic polymorphic ventricular tachycardia model of type 2 ryanodine receptor$^{R4496C+/-}$ mutation. *Circ. Arrhythm. Electrophysiol.* **5,** 841–849 CrossRef Medline
48. Kameyama, M., Hirayama, Y., Saitoh, H., Maruyama, M., Atarashi, H., and Takano, T. (2003) Possible contribution of the sarcoplasmic reticulum Ca^{2+} pump function to electrical and mechanical alternans. *J. Electrocardiol.* **36,** 125–135 CrossRef Medline
49. Cutler, M. J., Wan, X., Laurita, K. R., Hajjar, R. J., and Rosenbaum, D. S. (2009) Targeted SERCA2a gene expression identifies molecular mecha-

nism and therapeutic target for arrhythmogenic cardiac alternans. *Circ. Arrhythm. Electrophysiol.* **2,** 686–694 CrossRef Medline
50. Cutler, M. J., Wan, X., Plummer, B. N., Liu, H., Deschenes, I., Laurita, K. R., Hajjar, R. J., and Rosenbaum, D. S. (2012) Targeted sarcoplasmic reticulum Ca^{2+} ATPase 2a gene delivery to restore electrical stability in the failing heart. *Circulation* **126,** 2095–2104 CrossRef Medline
51. Stary, V., Puppala, D., Scherrer-Crosbie, M., Dillmann, W. H., and Armoundas, A. A. (2016) SERCA2a upregulation ameliorates cellular alternans induced by metabolic inhibition. *J. Appl. Physiol.* **120,** 865–875 CrossRef Medline
52. Weiss, J. N., Karma, A., Shiferaw, Y., Chen, P. S., Garfinkel, A., and Qu, Z. (2006) From pulsus to pulseless: the saga of cardiac alternans. *Circ. Res.* **98,** 1244–1253 CrossRef Medline
53. Nassal, M. M., Wan, X., Laurita, K. R., and Cutler, M. J. (2015) Atrial SERCA2a overexpression has no affect on cardiac alternans but promotes arrhythmogenic SR Ca^{2+} triggers. *PLoS One* **10,** e0137359 CrossRef Medline
54. Jiang, D., Xiao, B., Zhang, L., and Chen, S. R. (2002) Enhanced basal activity of a cardiac Ca^{2+} release channel (ryanodine receptor) mutant associated with ventricular tachycardia and sudden death. *Circ. Res.* **91,** 218–225 CrossRef Medline
55. Jiang, D., Xiao, B., Yang, D., Wang, R., Choi, P., Zhang, L., Cheng, H., and Chen, S. R. W. (2004) RyR2 mutations linked to ventricular tachycardia and sudden death reduce the threshold for store-overload-induced Ca^{2+} release (SOICR). *Proc. Natl. Acad. Sci. U.S.A.* **101,** 13062–13067 CrossRef Medline
56. Zhou, Q., Xiao, J., Jiang, D., Wang, R., Vembaiyan, K., Wang, A., Smith, C. D., Xie, C., Chen, W., Zhang, J., Tian, X., Jones, P. P., Zhong, X., Guo, A., Chen, H., *et al.* (2011) Carvedilol and its new analogs suppress arrhythmogenic store overload-induced Ca^{2+} release. *Nat. Med.* **17,** 1003–1009 CrossRef Medline
57. Fernández-Velasco, M., Rueda, A., Rizzi, N., Benitah, J. P., Colombi, B., Napolitano, C., Priori, S. G., Richard, S., and Gómez, A. M. (2009) Increased Ca^{2+} sensitivity of the ryanodine receptor mutant RyR2R4496C underlies catecholaminergic polymorphic ventricular tachycardia. *Circ. Res.* **104,** 201–209, 12p following 209 CrossRef Medline
58. Chen, W., Wang, R., Chen, B., Zhong, X., Kong, H., Bai, Y., Zhou, Q., Xie, C., Zhang, J., Guo, A., Tian, X., Jones, P. P., O'Mara, M. L., Liu, Y., Mi, T., *et al.* (2014) The ryanodine receptor store-sensing gate controls Ca^{2+} waves and Ca^{2+}-triggered arrhythmias. *Nat. Med.* **20,** 184–192 CrossRef Medline
59. Zhang, J., Chen, B., Zhong, X., Mi, T., Guo, A., Zhou, Q., Tan, Z., Wu, G., Chen, A. W., Fill, M., Song, L. S., and Chen, S. R. (2014) The cardiac ryanodine receptor luminal Ca^{2+} sensor governs Ca^{2+} waves, ventricular tachyarrhythmias and cardiac hypertrophy in calsequestrin-null mice. *Biochem. J.* **461,** 99–106 CrossRef Medline
60. Luo, W., Grupp, I. L., Harrer, J., Ponniah, S., Grupp, G., Duffy, J. J., Doetschman, T., and Kranias, E. G. (1994) Targeted ablation of the phospholamban gene is associated with markedly enhanced myocardial contractility and loss of beta-agonist stimulation. *Circ. Res.* **75,** 401–409 CrossRef Medline
61. Priori, S. G., Napolitano, C., Memmi, M., Colombi, B., Drago, F., Gasparini, M., DeSimone, L., Coltorti, F., Bloise, R., Keegan, R., Cruz Filho, F. E., Vignati, G., Benatar, A., and DeLogu, A. (2002) Clinical and molecular characterization of patients with catecholaminergic polymorphic ventricular tachycardia. *Circulation* **106,** 69–74 CrossRef Medline
62. Faggioni, M., Hwang, H. S., van der Werf, C., Nederend, I., Kannankeril, P. J., Wilde, A. A., and Knollmann, B. C. (2013) Accelerated sinus rhythm prevents catecholaminergic polymorphic ventricular tachycardia in mice and in patients. *Circ. Res.* **112,** 689–697 CrossRef Medline
63. Bai, Y., Jones, P. P., Guo, J., Zhong, X., Clark, R. B., Zhou, Q., Wang, R., Vallmitjana, A., Benitez, R., Hove-Madsen, L., Semeniuk, L., Guo, A., Song, L. S., Duff, H. J., and Chen, S. R. (2013) Phospholamban knockout breaks arrhythmogenic Ca^{2+} waves and suppresses catecholaminergic polymorphic ventricular tachycardia in mice. *Circ. Res.* **113,** 517–526 CrossRef Medline