Published in final edited form as:
Nat Med. 2014 February ; 20(2): 184–192. doi:10.1038/nm.3440.

The Ryanodine Receptor Store Sensing Gate Controls Ca^{2+} Waves and Ca^{2+} Triggered Arrhythmias

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Abstract

Spontaneous Ca^{2+} release from intracellular stores is important for various physiological and pathological processes. In cardiac muscle cells, spontaneous store overload-induced Ca^{2+} release (SOICR) can result in Ca^{2+} waves, a major cause of ventricular tachyarrhythmias (VTs) and sudden death. The molecular mechanism underlying SOICR has been a mystery for decades. Here, we show that a point mutation E4872A in the helix bundle crossing (the proposed gate) of the cardiac ryanodine receptor (RyR2) completely abolishes luminal, but not cytosolic, RyR2 Ca^{2+} activation. Introducing metal-binding histidines at this site converts RyR2 into a luminal Ni^{2+} gated channel. Mouse hearts harboring an RyR2 mutation at this site ($E4872Q^{+/-}$) are resistant to store overload-induced Ca^{2+} waves and completely protected against Ca^{2+}-triggered VTs. These data show that the RyR2 gate directly senses store Ca^{2+}, explaining RyR2 store Ca^{2+} regulation,

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#WC, XT, XZ are recipients of the Alberta Innovates-Health Solutions (AIHS) Studentship Award; PPJ, AIHS and Heart and Stroke Foundation Fellowship Awards; MLO, Canadian Institutes of Health Research (CIHR) Fellowship Award; HJD, DPT, and SRWC, AIHS Scientist Awards.

AUTHOR CONTRIBUTIONS WC, RW, BC, XZ, HK, YB, QZ, AG, XT, PPJ, MLO, JC, DPT, AMG, HJD, MF, LSS, SRWC, designed research; WC, RW, BC, XZ, HK, YB, QZ, CX, JZ, AG, XT, PPJ, MLO, YL, TM, LZ, JB, LS, HC, JZ performed research; WC, RW, BY, XZ, HK, YB, QZ, CX, JZ, AG, XT, PPJ, MLO, YL, TM, LZ, JB, JZ, LSS, SRWC, analyzed data; and WC, XZ, YB, PPJ, MLO, MF, LSS, SRWC, wrote the paper.

The authors declare no competing financial interests.

Ca^{2+} wave initiation, and Ca^{2+}-triggered arrhythmias. This novel store-sensing gate structure is conserved in all RyRs and inositol 1,4,5-trisphosphate receptors.

INTRODUCTION

Ca^{2+} release from intracellular stores drives many cellular processes [1–4]. This release is generally mediated by two homologous Ca^{2+} channels: ryanodine receptors (RyRs) and inositol 1,4,5- trisphosphate receptors (IP3Rs). Cytosolic Ca^{2+} activation of RyRs and IP3Rs is commonly called Ca^{2+}-induced Ca^{2+} release (CICR)[3–6]. The possibility of release regulation by store (luminal) Ca^{2+} was first proposed to explain IP3R function [7–10]. Since then, it has become clear that luminal Ca^{2+} also critically controls the cardiac RyR (RyR2)[11–19]. In cardiac muscle cells, sarcoplasmic reticulum (SR) Ca^{2+} overload triggers spontaneous RyR2-mediated Ca^{2+} release [5, 20–24]. This store-overload-induced Ca^{2+} release (SOICR) can result in Ca^{2+} waves and triggered activities, a major cause of ventricular tachyarrhythmias (VTs) and sudden death [16, 25–29]. Analogous mechanisms appear to operate in many other types of cells where spontaneous Ca^{2+} release play an important role in a variety of cellular processes [1, 2, 4, 8, 9, 30–33]. Despite its physiological and pathological significance, the molecular mechanism underlying spontaneous Ca^{2+} release remains largely unknown.

A key SOICR feature is that it occurs when store Ca^{2+} reaches a critical level where RyR2 channels begin to open [12, 15, 34, 35], but how elevating store/luminal Ca^{2+} activates RyR2 is unclear. One proposed mechanism, the “feed-through” hypothesis, suggests that luminal Ca^{2+} passes through an open RyR2 and acts on its own cytosolic Ca^{2+} activation site [14, 36]. However, there is an accumulating body of evidence indicating that luminal Ca^{2+} activation of single RyR2 is mediated by some luminal Ca^{2+} sensing mechanism(s) that is (or are) structurally distinct from the RyR’s cytosolic Ca^{2+} activation site [13, 19, 37–40]. The molecular nature of the luminal Ca^{2+} sensing mechanism(s) is poorly understood. It is commonly believed that cardiac calsequestrin (CASQ2), a SR luminal Ca^{2+} binding protein, serves as the key SR luminal Ca^{2+} sensor [19, 41]. However, the RyR2s in CASQ2-null cardiomyocytes still sense SR luminal Ca^{2+} changes [42], indicating that other luminal Ca^{2+} sensing mechanisms exist. Indeed, purified native and recombinant RyRs that lack CASQ2 can sense changes in luminal Ca^{2+} [14, 43, 44]. Thus, RyR2 is also regulated by a luminal Ca^{2+} sensing mechanism that does not require CASQ2. In the present study, we identified an essential element of this non-CASQ2 based store/luminal Ca^{2+} sensing mechanism on the RyR2’s helix bundle crossing (its proposed gate). We show that this store Ca^{2+} sensing gate controls RyR2 luminal Ca^{2+} regulation, the initiation of Ca^{2+} waves, and consequently Ca^{2+}-triggered VTs. Interestingly, this store-sensing gate is conserved in all types of RyRs and IP3Rs.

RESULTS

Residue E4872 is an essential element of the RyR2 luminal Ca^{2+} sensing mechanism

A large number of functional and structural studies [45–54] suggest that the COOH-terminal part of the RyR’s predicted inner helix (the helix bundle crossing region) constitutes the ion

gate of the channel (Fig. S1), based on analogy to the intracellular gates of potassium and sodium channels [55–57]. Interestingly, there are a number of negatively charged residues that are clustered in or near the RyR's proposed ion gate (Fig. S1). The functional significance of these negatively charged residues in RyR2 Ca^{2+} regulation was assessed using site-directed mutagenesis and single channel recordings in planar lipid bilayers with K^+ as the charge carrier at −20 mV (cytosolic). Only mutation E4872A (not D4875A, E4878A, or E4882A, see Fig. S2) completely abolished luminal Ca^{2+} activation of single RyR2 channels. As shown in Fig. 1, single RyR2 (wt) channels are substantially activated by luminal Ca^{2+} in the presence of ATP and caffeine (Fig. 1A, E). Note that the RyR2 (wt) channel can also be activated by luminal Ca^{2+} with [18, 44] or without [18, 38, 44, 58] caffeine present. Since both ATP and caffeine individually enhance RyR2 luminal Ca^{2+} activation [38, 44], we used ATP and caffeine together to maximize RyR2 Ca^{2+} sensitivity so that even very small (or residual) luminal Ca^{2+} response of the mutant channels could be better detected. In sharp contrast to single RyR2 (wt) channels, luminal Ca^{2+} (up to 40 mM) did not activate single E4872A mutant channels at all with (red triangles) or without (blue diamonds) caffeine present (Fig. 1B, E). The E4872A mutant was completely unresponsive to luminal Ca^{2+} even with 10-fold higher cytosolic Ca^{2+} (491 nM) present (Fig. 1C, E). Incredibly, introducing a negative charge (G4871E) next to E4872A (double mutant G4871E/E4872A) largely restored the missing luminal Ca^{2+} activation of E4872A mutant channels (Fig. 1D, E). Like the E4872A mutation, the isosteric mutation E4872Q also abolished or dramatically reduced RyR2's response to luminal Ca^{2+} activation (Fig. S3). Removing fixed negative charges in the internal pore might be expected to alter RyR2 permeation properties. However, the E4872A mutation did not affect the single channel conductance in the presence (2.5 mM) or near absence (45 nM) of luminal Ca^{2+} (Fig. 1F). These results show that the negative charge at (or near) residue E4872 is essential for RyR2 luminal Ca^{2+} activation.

The effect of the E4872A and E4872Q mutations on cytosolic Ca^{2+} activation of RyR2 in lipid bilayers is shown in Fig. 2. Although totally unresponsive to luminal Ca^{2+}, single E4872A/Q mutant channels remained sensitive to cytosolic Ca^{2+} (Fig. 2A–C). However, the E4872A/Q mutants were less sensitive to activation by cytosolic Ca^{2+} (Fig. 2D), and had reduced mean open times (To) (Fig. 2E) and increased EC_{50} (~3-fold) of Ca^{2+} activation of [^{3}H]ryanodine binding (from 0.22 μM to 0.72μM and 0.58μM, wt to mutant, respectively) (Fig. 2F). This indicates that the E4872A/Q mutations also affect the cytosolic Ca^{2+} activation process. We have previously shown that the E3987 residue is critically involved in RyR2 cytosolic Ca^{2+} activation [59]. Mutating this residue (E3987A) dramatically diminished cytosolic Ca^{2+} sensitivity and increased the EC_{50} of Ca^{2+} activation of [^{3}H]ryanodine binding by ~270-fold [59]. However, unlike the E4872A/Q mutant, single E3987A mutant channels remained sensitive to luminal Ca^{2+} activation albeit at a reduced sensitivity (Fig. S4). Therefore, the complete lack of luminal Ca^{2+} response of the E4872A/Q mutant is unlikely to be attributable to its slightly reduced cytosolic Ca^{2+} sensitivity, because luminal Ca^{2+} activation persists even there is little cytosolic Ca^{2+} sensitivity (as seen in E3987A). Collectively, our results demonstrate that the E4872A/Q mutation uniquely and selectively abolishes luminal Ca^{2+} sensing, and that luminal and cytosolic Ca^{2+} activation processes are different but interactive.

Molecular mechanism of luminal Ca^{2+} sensing

To gain insight into how residue E4872 might be involved in luminal Ca^{2+} sensing, we constructed a RyR2 pore model (Fig. S5) based on the crystal structure of the K^+ channel KcsA, as done by others previously [48]. This model is very similar to the crystal structure of the NaK channel pore (Fig. S6) [60]. Our model indicates that the E4872 residue lies inside the internal pore, suggesting that luminal Ca^{2+} sensing may take place within the internal pore. If so, then luminal Ca^{2+} would need to enter the internal pore when the RyR2 is closed (Fig. S5B). Electrostatic calculations (Fig. 3) predict that the strong negative potential inside the internal pore, when the gate is closed, would be more than sufficient to draw luminal Ca^{2+} through the selectivity filter and into the internal pore. To begin to experimentally explore this possibility, we closed single RyR2 (wt) channels by reducing cytosolic Ca^{2+} (to 45 nM) with cytosolic Mg^{2+} present. Closed WT RyR2s were activated by elevating luminal Ca^{2+} to 2.5 mM (Fig. 3A), but closed E4872A RyR2s were not (Fig. 3B). This indicates that luminal Ca^{2+} can indeed access to the E4872-dependent activation mechanism when the RyR2 is closed. Activation of a closed WT RyR2 by luminal Ca^{2+} persisted even when cytosolic Ca^{2+} was reduced to extremely low levels (0.33 nM or 0.046 nM) (Fig. S7A). This is consistent with the notion that luminal Ca^{2+} can gate the RyR2 channel in the absence of cytosolic Ca^{2+} activation.

To determine whether E4872 and/or nearby residues in the helix bundle crossing directly interact with luminal cations, we next used site-directed mutagenesis to manipulate the local cation binding properties of this region. To this end, we generated double histidine RyR2 mutants to create local Ni^{2+} binding sites in this region. One of the mutants, G4871H/E4872H, was found to be sensitive to luminal Ni^{2+}. Unlike WT RyR2s that are insensitive to luminal Ni^{2+} (Fig. 3C), closed G4871H/E4872H channels were activated by luminal Ni^{2+} (14 nM) (Fig. 3D). Thus, luminal Ni^{2+} was able to access and interact with the inserted Ni^{2+} binding site, and activate the closed mutant channel. Further, luminal Ni^{2+} activated closed G4871H/E4872H channels even when there was extremely little cytosolic Ca^{2+} present (0.32 nM or 0.046 nM; Fig. S7B). These results are consistent with the view that cation binding at or near E4872 in the helix bundle crossing can control the RyR2 gate in the absence of cytosolic Ca^{2+} activation. If the Ni^{2+} can access and bind to this site (when the RyR2 is closed), then the smaller mass Ca^{2+} could very well do the same. In other words, the E4872-dependent luminal Ca^{2+} sensing mechanism may involve a direct interaction of the cation with negative charges in the helix bundle crossing.

Our RyR2 pore model also suggests that the positively charged residue R4874 on one subunit of the RyR2 tetramer will likely interact with the negatively charged residues D4868 and/or E4872 on the neighboring subunit (Fig. S5A). To experimentally test this possibility, we reversed the charge polarity of these individual residues by generating single mutations D4868R, E4872R or R4874D/E. These single mutations dramatically reduced or abolished RyR2 function (Figs.3E, S8). We also generated double mutants where the positive charged residue was made negative and potentially interacting negative charge partner made positive (D4868R/R4874D and E4872R/R4874E). Remarkably, the D4868R/R4874D double mutation restored caffeine responsiveness and the E4872R/R4874E mutation partially restored it (Figs.3E, S8). The E4872R/R4874E partial restoration may be related to the

E4872 residue that is involved in both electrostatic interactions and luminal Ca^{2+} sensing. To determine whether the electrostatic interaction is intra- or inter-subunit, we co-expressed two single mutations (D4868R and R4874D or E4872R and R4874E) to produce heterotetrameric RyR2s comprised of both mutants. Co-expression of the listed pairs also restored RyR2's caffeine response (Fig. S8). Thus, these electrostatic interactions are between residues on neighboring subunits (i.e. inter-subunit interactions) as predicted by our modeling (Fig. S5A). These data demonstrate that there are local electrostatic interactions within the helix bundle crossing (the putative RyR2 gate). By virtue of their electrostatic interactions, residues D4868 and R4874 may play a vital role in stabilizing the helix bundle crossing region and thus the operation of the RyR2 gate. Residue E4872, which is essential for luminal Ca^{2+} sensing, is also part of these electrostatic interactions. Therefore, we propose that the RyR2 helix bundle crossing encompasses a "store Ca^{2+} sensing gate", because it contains structural elements critical for both luminal Ca^{2+} sensing and channel gating. Interestingly, residues D4868, E4872, and R4874 are conserved in all types of RyRs and IP3Rs (Fig. S9), implying that this store sensing gate structure may be a common feature of Ca^{2+} release channels.

Residue E4872 is a critical determinant of store-overload triggered Ca^{2+} waves

In the next series of experiments, we assessed the role of this RyR2 store-sensing gate in store overload induced Ca^{2+} oscillations or Ca^{2+} waves (i.e. SOICR). As shown in Fig. 4, the E4872A and E4872Q mutations abolished SOICR in HEK293 cells (Figs.4A–C). In the HL-1 mouse cardiac cells, expression of E4872A/Q (but not WT) essentially abolished SOICR (Figs.4D, S10). Thus, the E4872 residue essential for RyR2 luminal Ca^{2+} activation is also critical for SOICR.

We next explored the significance of the RyR2 store-sensing gate in the context of adult cardiomyocytes and intact hearts by generating a knock-in mouse model that harbors the RyR2 E4872Q mutation (Fig. S11). We chose the isosteric E4872Q mutation because it is slightly more conservative than E4872A and thus would likely produce a less severe phenotype as suggested by our single channel studies (Figs.2D, S3). Heterozygous E4872Q ($E4872Q^{+/-}$) embryos survived and $E4872Q^{+/-}$ adult mice had no gross structural and functional abnormalities detectable by echocardiography (Table S1). However, the homozygous $E4872Q^{+/+}$ mutation was embryonically lethal with most embryos dying between embryonic day 10.5–11.5 (Fig. S12), indicating that the E4872-based RyR2 luminal Ca^{2+} regulation is important for normal embryonic development.

Cardiomyocytes isolated from $E4872Q^{+/-}$ mice had a reduced propensity for SOICR compared to WT cells (Figs.4E, S13). We also determined the SOICR propensity in the ventricles of intact WT and mutant hearts (*ex vivo*) using line-scan confocal imaging. Elevating extracellular Ca^{2+} (2–10mM) or application of isoproterenol (Iso) increased the frequency of spontaneous Ca^{2+} waves in intact WT hearts (Fig. 4F, H). However, the same experimental maneuvers resulted in very few or no Ca^{2+} waves in intact $E4872Q^{+/-}$ hearts (Fig. 4G, H). Thus, the E4872-dependent luminal Ca^{2+} sensing mechanism determines the likelihood of SOICR in HEK293 cells, HL-1 cardiac cells, freshly isolated cardiomyocytes, and intact hearts.

Impact of the E4872Q$^{+/-}$ mutation on excitation-contraction (EC) coupling

To determine whether the E4872Q mutation affects normal cardiac EC coupling, we examined depolarization- and caffeine-induced intracellular Ca^{2+} transients. We found that there were no significant differences in the amplitude of depolarization-induced Ca^{2+} transients (Fig. 5A–F), the resting cytosolic Ca^{2+} level or the SR Ca^{2+} content (Fig. 5C) between WT and E4872Q$^{+/-}$ cardiomyocytes. However, the L-type Ca^{2+} channel current ($I_{Ca,L}$; Fig. 5G) in E4872Q$^{+/-}$ cardiomyocytes was significantly increased compared to that in WT cells. This resulted in a reduced EC coupling gain (Fig. 5H). Further, the time-to-peak (T_{peak}) of the Ca^{2+} transient was significantly increased, while the late decay times (at T_{75} and T_{90}, but not at T_{50}) were significantly shorter in E4872Q$^{+/-}$ cardiomyocytes (Figs. 5I, S14). The exact reasons for the faster late decay are unclear. It may be, in part, due to enhanced Na^+/Ca^{2+} exchanger activity in the E4872Q$^{+/-}$ cardiomyocytes (Fig. S15). Overall, our results indicate that the E4872Q mutation desensitizes RyR2 to normal CICR. This desensitization may be related to the shorter open times and decreased cytosolic Ca^{2+} sensitivity of the E4872Q mutant (Figs.2D–F). In turn, this may promote a compensatory increase in the systolic Ca^{2+} influx (Fig. 5G) and diastolic Ca^{2+} extrusion (Fig. S15) that helps normalize the amplitude of Ca^{2+} transients in the E4872Q$^{+/-}$ cardiomyocytes.

The E4872Q$^{+/-}$ mutation completely protects against stress-induced VTs

We previously showed that the naturally occurring RyR2-R4496C mutation that is associated with catecholaminergic polymorphic ventricular tachycardia (CPVT) in humans enhances SOICR [16]. In the RyR2-R4496C knock-in mice, CPVT can be readily induced by the administration of caffeine and epinephrine (Fig. 6A) [61]. To determine whether the E4872Q$^{+/-}$ mutation is able to prevent CPVT, we generated a compound mutant mouse model (R4496C$^{+/-}$/E4872Q$^{+/-}$) in which one RyR2 allele has the R4496C mutation and the other the E4872Q mutation. We found that the E4872Q$^{+/-}$ mutation completely eliminated the CPVT phenotype of the R4496C$^{+/-}$ mice (Fig. 6B–D).

Since VTs in the RyR2-R4496C mutant mice are caused by triggered activities induced by spontaneous Ca^{2+} waves [29, 61–63], it seems reasonable to propose that the E4872Q mutation prevents VTs in the R4496C mutant mice by suppressing the occurrence of spontaneous Ca^{2+} waves.

DISCUSSION

Spontaneous Ca^{2+} release during store Ca^{2+} overload (SOICR) has long been observed in cardiac cells [5, 20–24]. This SOICR occurs as a result of RyR2 opening when the store Ca^{2+} content exceeds a threshold level [12, 15, 34, 35], but the molecular mechanism responsible has been a longstanding mystery. Here, we demonstrate for the first time that the RyR2's helix bundle crossing (its proposed gate) encompasses an essential component of the store/luminal Ca^{2+} sensing mechanism that controls SOICR and thus Ca^{2+} triggered VTs. This store Ca^{2+} sensing gate also governs normal luminal Ca^{2+} regulation of RyR2 and EC coupling gain. Thus, this uniquely positioned store Ca^{2+} sensing mechanism plays an important role in both health and disease.

Our results demonstrate that the E4872 residue located in the helix bundle crossing is essential for luminal Ca^{2+} activation of RyR2. However, it is unlikely that E4872 is the only residue involved in the RyR2 luminal Ca^{2+} sensing mechanism, as Ca^{2+} binding often involves ion coordination with multiple oxygen donors that form a Ca^{2+} binding pocket. Identification and characterization of all the residues involved in the formation of the luminal Ca^{2+} binding pocket will be a daunting task, but our discovery that E4872 is an essential element represents an important first step toward understanding the underlying mechanism.

Besides its essential role in luminal Ca^{2+} sensing, the E4872 residue is also part of a network of inter-subunit salt bridges within the RyR2 helix bundle crossing. This is interesting because the helix bundle crossing is thought to be the RyR2's gate. We propose that when luminal Ca^{2+} associates with E4872, the inter-subunit electrostatic interactions between residues D4868/E4872/R4874 are disrupted, and this increases the likelihood that the channel transitions from the closed to open state. Additional detailed and comprehensive studies of course will be required to verify this working model of luminal Ca^{2+} control of RyR2 gating.

The E4872Q mutation completely abolishes luminal Ca^{2+} activation of RyR2, despite the presence of luminal-to-cytosolic Ca^{2+} flux and cytosolic Ca^{2+} activation albeit with a reduced sensitivity. On the other hand, the E3987A mutant channel that exhibits dramatically diminished cytosolic Ca^{2+} response [59] remains responsive to luminal Ca^{2+} activation. Further, luminal Ca^{2+} (or luminal Ni^{2+}) is able to open a closed single RyR2 WT (or G4871H/E4872H mutant) channel in the absence of cytosolic Ca^{2+} activation. Taken together, these observations indicate that luminal and cytosolic Ca^{2+} activation of RyR2 are mediated by different but interacting mechanisms, consistent with early observations [64, 65]. Cytosolic RyR2 Ca^{2+} activation involves cytosolic Ca^{2+} sensor [59]. Luminal RyR2 Ca^{2+} activation clearly involves a luminal Ca^{2+} sensor. However, luminal and cytosolic Ca^{2+} activation of RyR2 are inherently interactive, because both of these Ca^{2+} sensors govern the same gate.

Pharmacological agents that reduce RyR2 open time suppress Ca^{2+} waves and Ca^{2+} triggered VTs [61, 66, 67]. Interestingly, the E4872Q mutation also reduced the duration of RyR2 openings, decreased SOICR likelihood and completely suppressed VTs in CPVT-prone mouse hearts. Thus, limiting RyR2 open duration appears to be a common and effective means to suppress Ca^{2+}-mediated arrhythmias. This makes the RyR2 store Ca^{2+} sensing gate a potential therapeutic target for anti-arrhythmic therapies.

The novel E4872-based luminal Ca^{2+} sensing mechanism defined here does not require the CASQ2 protein. CASQ2-based RyR2 luminal Ca^{2+} regulation is well established [19, 41]. In our single channel and cell-line studies, the E4872A/Q mutations altered RyR2 luminal Ca^{2+} regulation in the absence of CASQ2. When CASQ2 was present, as in our cardiomyocyte and intact heart studies, E4872A/Q mutations significantly altered RyR2 luminal Ca^{2+} sensing. Although the relationship between the E4872- and CASQ2-based luminal Ca^{2+} sensing mechanisms is not entirely clear yet, it is clear that the E4872-based mechanism can operate in the absence of CASQ2. Thus, the existence of the E4872-based mechanism may

explain why SR Ca^{2+} release is still governed by luminal Ca^{2+} in CASQ2-null mouse cardiomyocytes [42].

Lastly, the amino acid sequence surrounding the RyR2-E4872 residue is completely conserved in all three mammalian RyR isoforms across different species. This implies that all RyRs have the store Ca^{2+} sensing gate structure that we identified here. This is consistent with the reported luminal Ca^{2+} sensitivity of single skeletal muscle RyRs (RyR1) [36, 68] as well as the SR Ca^{2+} load-dependent spontaneous Ca^{2+} release in isolated skeletal muscle SR vesicles [64] and skeletal muscle fibers [69, 70]. Spontaneous Ca^{2+} release during SR Ca^{2+} overload has also been observed in RyR3-containing smooth muscle cells [71]. The key RyR2 residues (D4868, E4872, and R4874) are also conserved in all types of IP3Rs (Fig. S3), raising the intriguing possibility that IP3Rs luminal Ca^{2+} sensitivity [7–10] may also be governed by a store-sensing gate.

METHODS

Please see the Supplementary Information for extended, detailed experimental procedures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by research grants from the Canadian Institutes of Health Research to HJD, DPT and SRWC, the National Institutes of Health to JC, LSS, MF, and SRWC, the Heart and Stroke Foundation of Alberta to AMG, HJD, and SRWC. The authors would also like to thank the generous donations from the King family, the Howarth family, and the Libin Cardiovascular Institute of Alberta.

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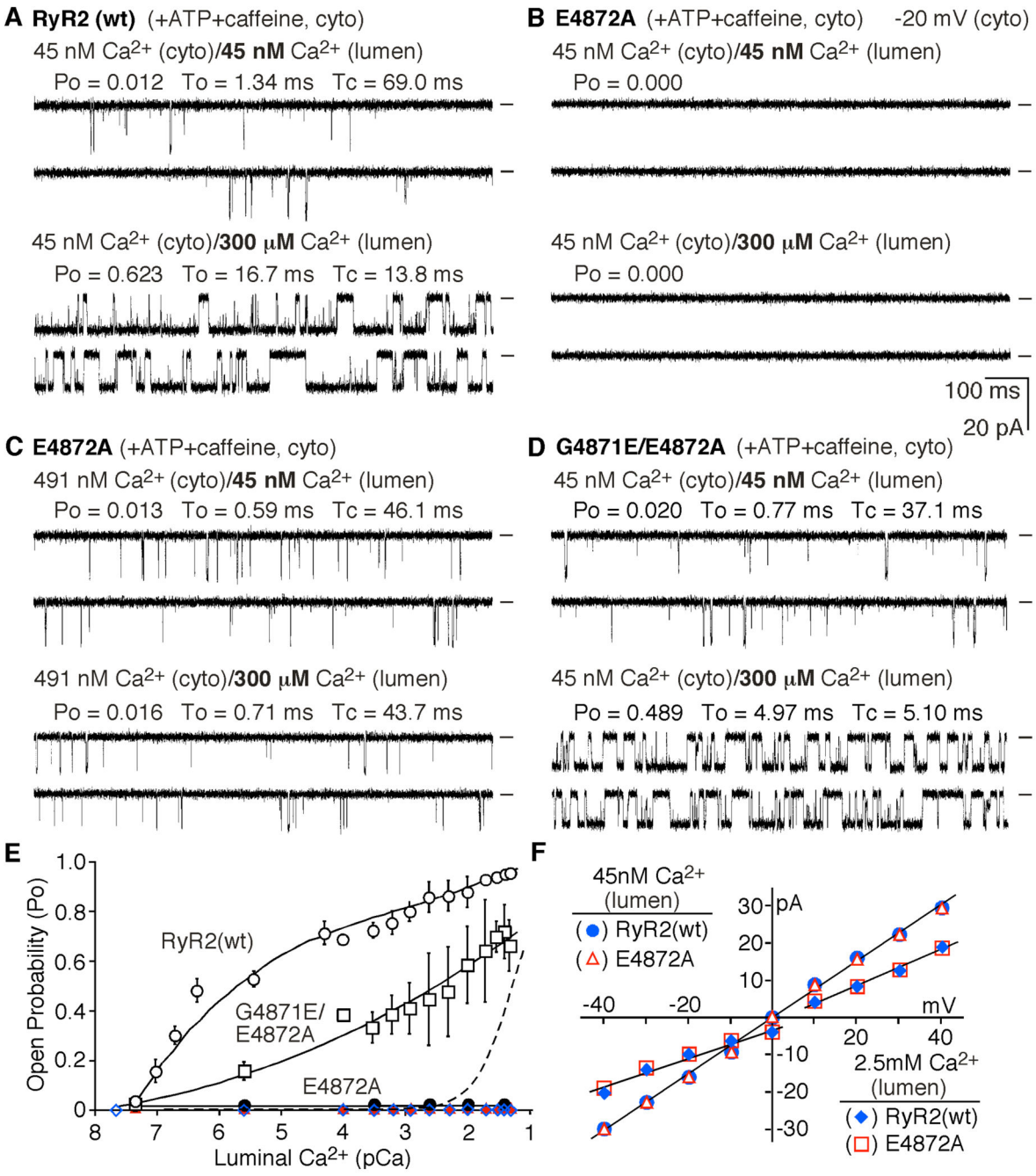

Fig. 1. The E4872A mutation abolishes luminal, but not cytosolic, Ca^{2+} activation of RyR2

Single channel activities of RyR2 (wt) (**A**), the E4872A mutant (**B**, **C**), and the double mutant G4871E/E4872A (**D**) in the presence of cytosolic Ca^{2+} (45 or 491 nM), ATP (2.5 mM) and caffeine (2.5 mM) as well as luminal Ca^{2+} (45 or 300 μM). Recording potentials, −20mV. Openings downward and zero current baselines are indicated (short bars). Open probability (Po), mean open time (To), and mean closed time (Tc) are shown. (**E**) Po-luminal Ca^{2+} relationships for single RyR2 (wt) (white circles) (n=11), G4871E/E4872A (white squares) (n=8), and E4872A (red triangles) (n=10) channels with cytosolic Ca^{2+} (45

nM), ATP and caffeine; single E4872A channels with cytosolic Ca^{2+} (491 nM), ATP and caffeine (black circles) (n=6); and single E4872A channels with cytosolic Ca^{2+} (45 nM) and ATP but no caffeine (blue diamonds) (n=6). Dash-line indicates the response of single RyR2 (wt) channels to luminal Ca^{2+} in the absence of ATP and caffeine [44]. (**F**) Current-voltage relationships for single RyR2 (wt) (n=5) and E4872A mutant (n=7) channels at 45nM or 2.5 mM luminal Ca^{2+}. Data shown are mean ± SEM.

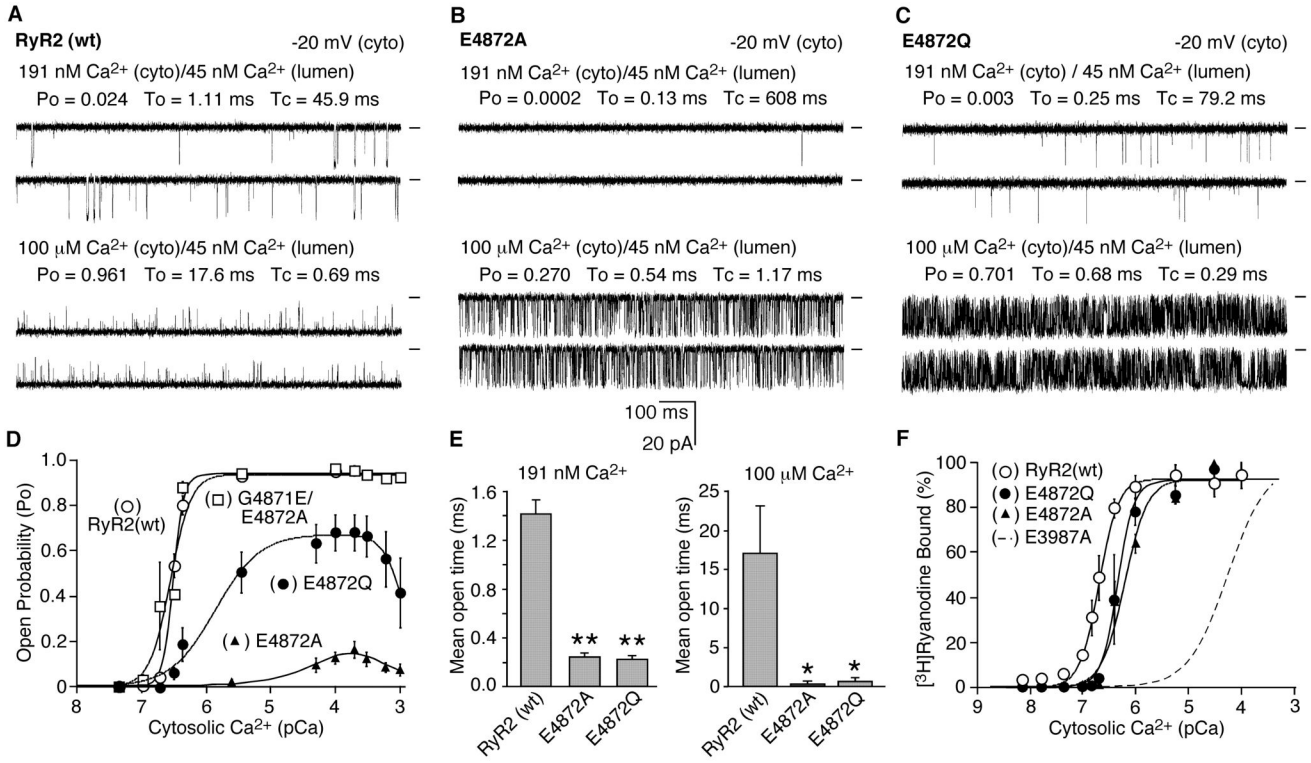

Fig. 2. Effect of E4872A and E4872Q on cytosolic Ca^{2+} activation of single RyR2 channels

Single channel activities of RyR2 (wt) (**A**), the E4872A mutant (**B**), and the E4872Q mutant (**C**) were recorded in a symmetrical recording solutions containing 250 mM KCl and 25 mM HEPES (pH 7.4) in the presence of 191nM or 100 μM cytosolic Ca^{2+} and 45 nM luminal Ca^{2+}. Recording potentials were −20mV. Openings downward and zero current baselines are indicated (short bars). Open probability (Po), mean open time (To), and mean closed time (Tc) are shown. (**D**) Po-cytosolic Ca^{2+} relationships for single WT (n=5), G4871E/ E4872A (n=4), E4872Q (n=6), and E4872A (n=8) channels with 45 nM luminal Ca^{2+}. (**E**) Comparison of the mean open time of single wt, E4872A, and E4872Q channels at 191 nM (n=5–11) and 100 μM (n=4–7) cytosolic Ca^{2+} (*$P < 0.05$, ** $P < 0.01$, vs WT). (**F**) Ca^{2+} dependent [^{3}H]ryanodine binding to RyR2 (wt) (n=3), E4872A (n=7), and E4872Q (n=4). Dash-line represents the binding curve for the cytosolic Ca^{2+} sensor mutant E3987A (EC_{50} = 59μM). The EC_{50} values are 0.22 ± 0.02 μM for WT, 0.72 ± 0.10 μM for E4872A, and 0.58 ± 0.12 for E4872Q. Data shown are mean ± SEM.

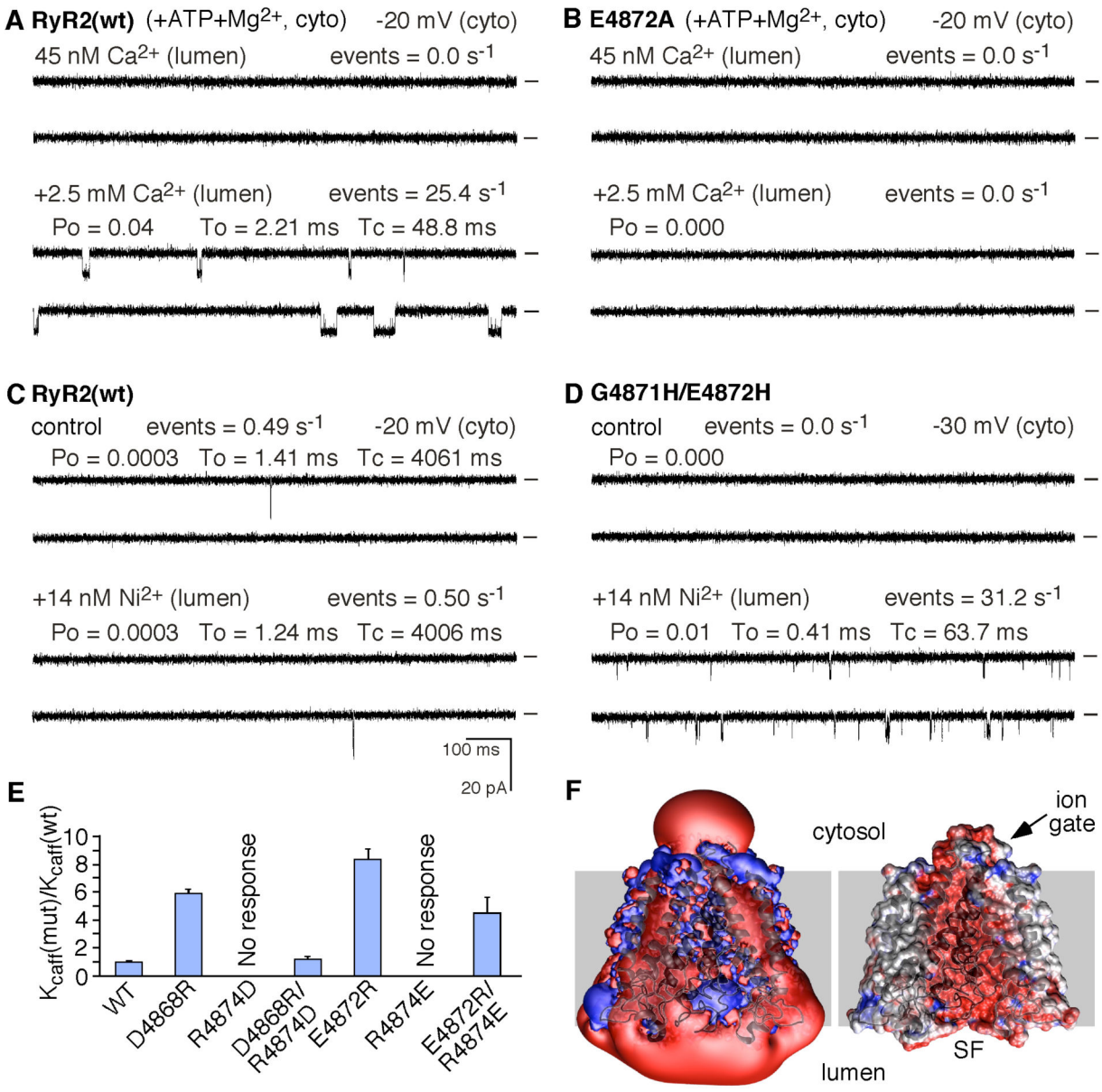

Fig. 3. Mechanism of activation of RyR2 by luminal Ca^{2+}

(**A**, **B**) Response of single RyR2 (wt) (**A**) and E4872A mutant (**B**) channels to luminal Ca^{2+} (2.5 mM) in the presence of 45 nM cytosolic Ca^{2+}, 3.0 mM ATP, and 1.5 mM Mg^{2+} (1.6mM free ATP and 0.11mM free Mg^{2+}). Open event frequency of WT channels increased from 0.02±0.01 to 22±9.0 events/s (n=5), while open frequency (0.00 event/s) of E4872A channels did not change (n=6). (**C**, **D**) Response of single RyR2 (wt) (**C**) and G4871H/ E4872H (**D**) channels to luminal Ni^{2+} (14 nM) in the presence of 45 nM cytosolic Ca^{2+} and 600μM luminal Ca^{2+}. Open event frequency of G4871H/E4872H channels increased from 0.03±0.01 to 36±20 events/s (n=7), while open frequency of WT did not increase (from 0.40±0.07 to 0.34±0.06 events/s) (n=5). (**E**) Relative caffeine sensitivity of single or double mutants. The relative caffeine sensitivity for WT and each mutant was defined by

determining the cumulative caffeine concentration (K_{caff}) that produces 50% of the maximal caffeine-induced Ca^{2+} release and normalizing it to that of the WT to yield a ratio of $K_{caff}(mut)/K_{caff}(wt)$. Note that the R4874A mutation completely abolished the caffeine response. Data shown are mean ± SEM (n=3–4). (**F**) Electrostatic potential isosurface (positive in blue, negative in red) of the RyR2 channel pore model (left) and electrostatic potential mapped onto the cross-section of the pore (right).

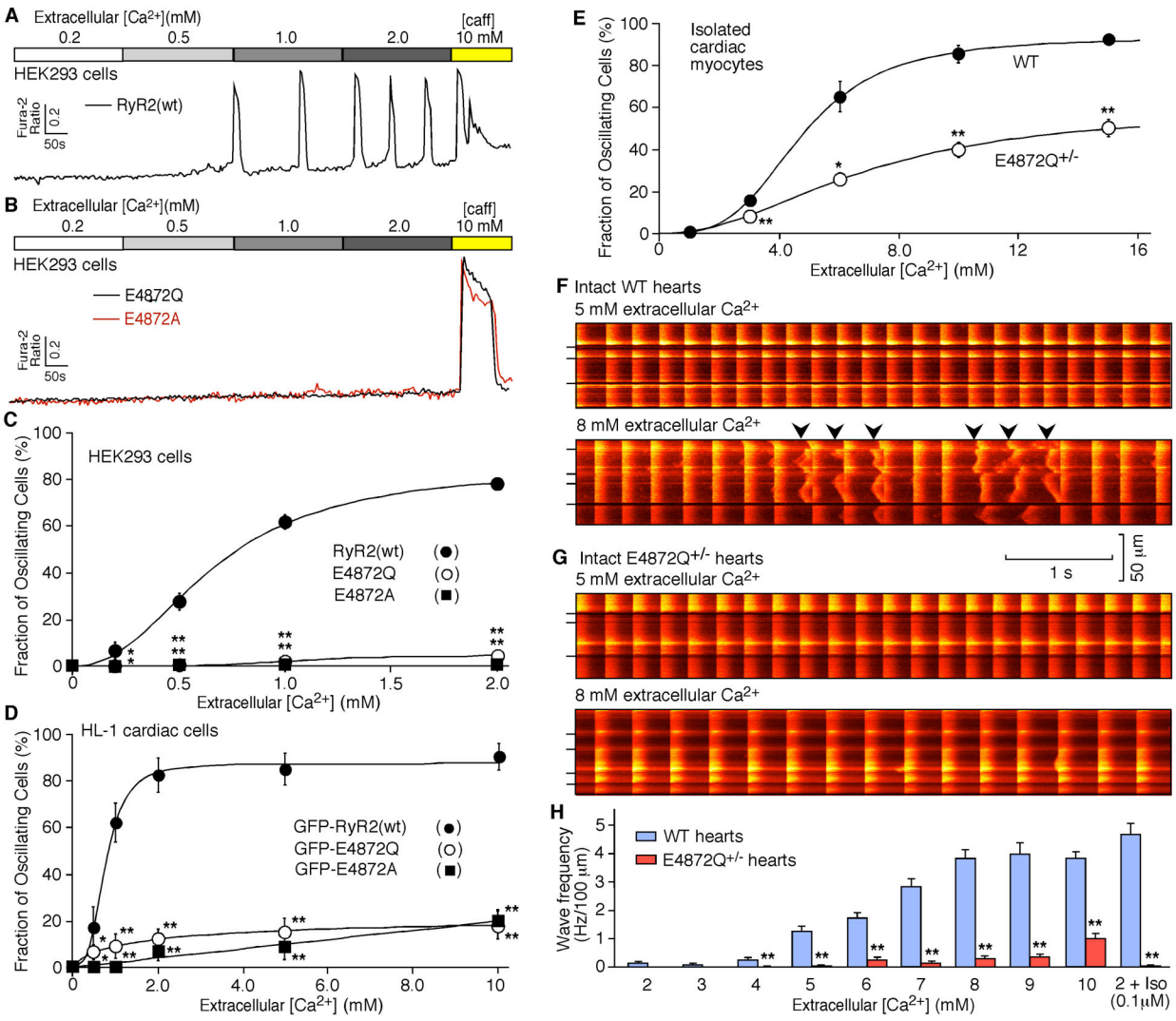

Fig. 4. Effect of E4872 mutations on SOICR

HEK293 cells expressing RyR2 (wt) (**A**) as well as the E4872Q and E4872A mutants (**B**) were perfused with increasing levels of extracellular Ca^{2+} (this overloads the store and triggers SOICR). (**C**) The percentage of RyR2 (wt), E4872Q, or E4872A cells (337–674) that display Ca^{2+} oscillations (n=4). (**D**) The percentage of mouse HL-1 cardiac cells (25–65) transfected with GFP-tagged RyR2 (wt), GFP-tagged E4872Q, or GFP-tagged E4872A that display Ca^{2+} oscillations (n=5–6). (**E**) The percentage of isolated WT (445) or E4872Q$^{+/-}$ (506) ventricular myocytes displaying SOICR (n=14–15). In situ line-scan confocal imaging of Ca^{2+} transients in intact WT (**F**) and E4872Q$^{+/-}$ mutant (**G**) hearts perfused with 5 or 8 mM extracellular Ca^{2+}. Arrow heads show the occurrence of spontaneous Ca^{2+} waves. Short bars on the left indicate cell boundaries. (**H**) Ca^{2+} wave frequency in ventricular myocytes in intact WT (n=4) or E4872Q$^{+/-}$ mutant (n=3) hearts at various extracellular Ca^{2+} concentrations (2–10 mM) or plus 0.1 µM isoproterenol (Iso). Data shown are mean ± SEM from 40–100 images (*$P < 0.05$, ** $P < 0.01$, vs WT).

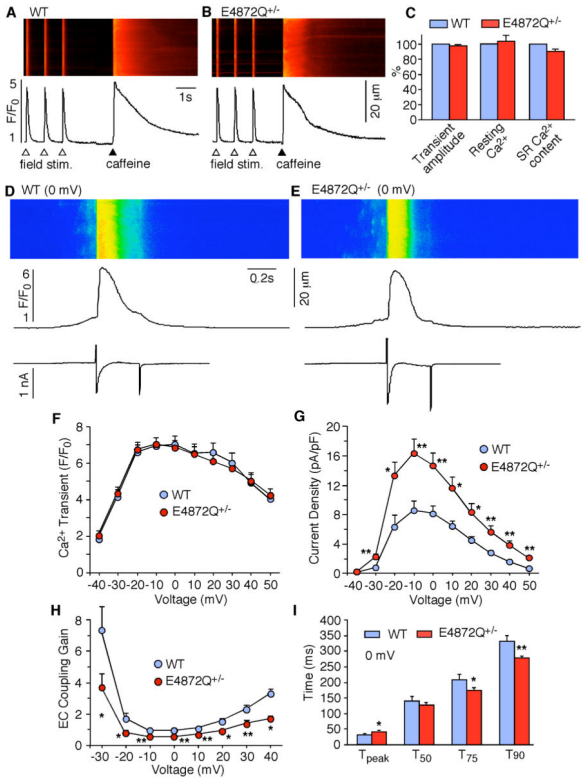

Fig. 5. Impact of the E4872Q mutation on EC coupling

Line-scan confocal imaging of field-stimulated and caffeine-induced Ca^{2+} transients in isolated WT (**A**) and E4872Q$^{+/-}$ (**B**) ventricular myocytes loaded with Rhod-2 AM. (**C**) Field-stimulated Ca^{2+} transient amplitudes, resting Ca^{2+} levels, and caffeine-induced Ca^{2+} release (SR Ca^{2+} contents) in WT and E4872Q$^{+/-}$ myocytes. Data shown are mean ± SEM (n=37–47). Simultaneous recordings of depolarization (0 mV)-induced Ca^{2+} transients and L-type Ca^{2+} current ($I_{Ca,L}$) in isolated WT (**D**) and E4872Q$^{+/-}$ (**E**) myocytes (holding potential −80 mV). (**F**–**I**) Depolarization-induced Ca^{2+} transient amplitudes (**F**), $I_{Ca,L}$ (**G**), EC coupling gains (**H**), and the kinetics of Ca^{2+} transients (at 0 mV) (**I**) in WT and E4872Q$^{+/-}$ ventricular myocytes (T_{peak}, time to peak; T_{50}, T_{75} and T_{90}, time from peak to 50%, 75%, and 90% decay in Ca^{2+} transients, respectively). Depolarization (above) was from −40 to 50 mV. Data shown are mean ± SEM from 14 WT and 17 E4872Q$^{+/-}$ cells (*P < 0.05, ** P < 0.01, vs WT).

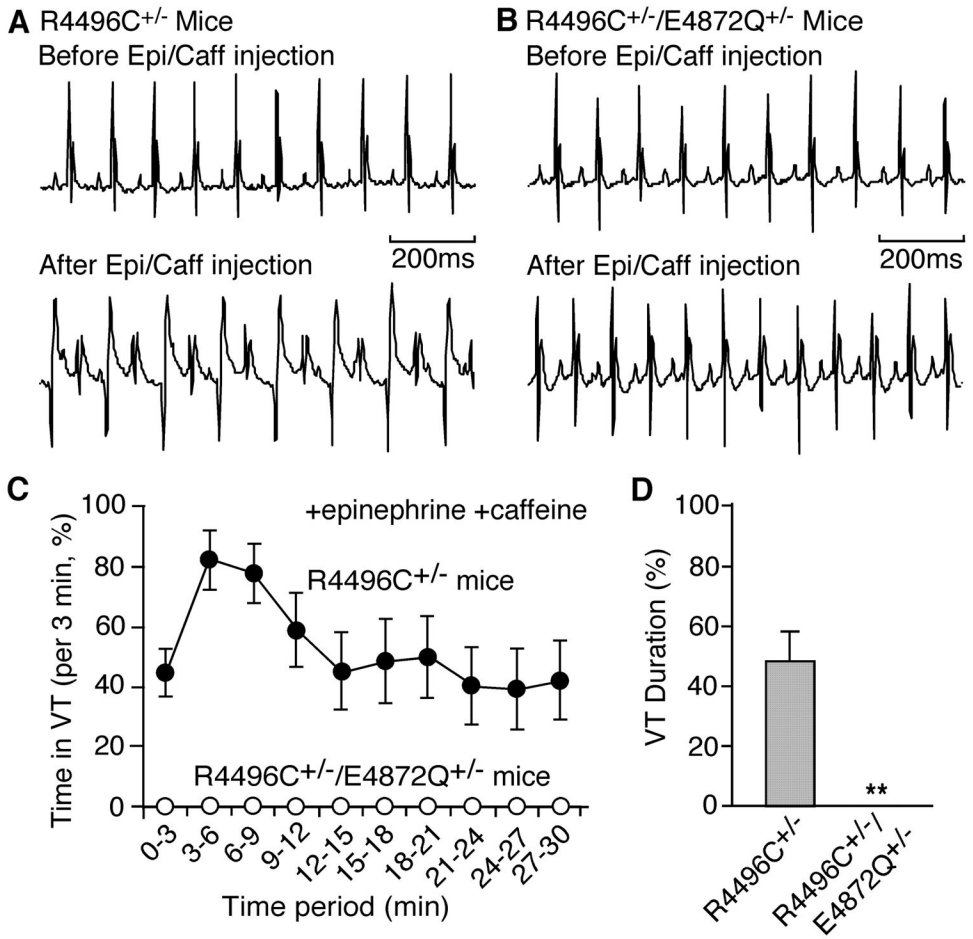

Fig. 6. E4872Q$^{+/-}$ mouse hearts are resistant to SOICR-induced VTs

Representative ECG recordings of R4496C$^{+/-}$ (**A**) and R4496C$^{+/-}$/E4872Q$^{+/-}$ (**B**) mice before and after injection of epinephrine (1.6 mg/kg) and caffeine (120 mg/kg). VT duration (%) in R4496C$^{+/-}$ or R4496C$^{+/-}$/E4872Q$^{+/-}$ mice within each 3-min (**C**) or 30-min (**D**) period of ECG recordings ($P < 0.05$ for all points, vs R4496C$^{+/-}$).