Supplemental Material

Detailed Methods

Model Description

Prior to the exercise plus ischemia test, left ventricular (LV) contractile function was evaluated using echocardiography. In agreement with previous studies,[1,2] myocardial infarction (MI) did not significantly alter LV contractile function, and no animal developed heart failure. No significant differences in LV ejection fraction (MI vs. no MI), LV diastolic internal diameter (MI vs. no MI) and LV systolic internal diameter (MI vs. no MI were noted between the dogs that were susceptible to ventricular fibrillation (VF) following infarction compared to sham control (i.e., no infarction) dogs.

Sarcoplasmic reticulum (SR) Ca^{2+} imaging.

Fluo-5N fluorescence was converted to $[Ca^{2+}]$ according to the following equation: $[Ca^{2+}]= K_d*(F - F_{MIN})/(F_{MAX}-F)$, where K_d was 400 μmol/L.[3] F_{MIN} was determined by application of 10 mmol/L caffeine. F_{MAX} was determined by application of 10 mmol/L of $[Ca^{2+}]$ and 0.1% saponin in the presence of 20 mmol/L of BDM. Cytochalasin D (15-40 μmol/L) was used in experiments presented in Figure 2 and 3 (main manuscript) to minimize movement artifacts. It should be noted that since the SR-entrapped Fluo-5N tracks free $[Ca^{2+}]_{SR}$, the Fluo-5 fluorescence signal is not expected to exactly mirror the simultaneously measured fluorescent signal from the cytoslolic Ca^{2+} indicator, Rhod-2, which is proportional to the sum of the total Ca^{2+} released from the SR and Ca^{2+} influx through the Ca^{2+} channels. Thus the differences in the restitution of the luminal Fluo-5N and cytosolic Rhod-2 signals (Figure 3A, main manuscript) can be ascribed to: a) effective buffering of free SR Ca^{2+} by calsequestrin; and b) contribution of the L-type Ca^{2+} current to the cytosolic signal, since initial recovery of the Ca^{2+} current from inactivation occurs faster than restitution of Ca^{2+} release (Online Figure IV).

Myocyte treatment with antioxidant mercaptopropionylglycine

To assess the role of redox modifications in mediating VF-associated alterations in Ca^{2+} signaling myocytes were incubated for 30 min in external solution containing 0.75 mmol/L 2-mercaptopropionylglycine (MPG), a cell-permeable reactive-oxygen species (ROS) scavenger. Our choice to use MPG as a reducing agent in the present study was based on the following reasons: 1) we previously showed that MPG was effective in alleviating ROS-mediated changes in SR Ca^{2+} leak in VF and heart failure myocytes;[4,5] 2) MPG did not have apparent non-specific effects on intracellular Ca^{2+} handling.[4] In the present study we confirmed the apparent lack of non-specific effects of MPG on Ca^{2+} cycling: MPG did not affect amplitude of Ca^{2+} transients recorded in control myocytes in the presence of isoproterenol (Iso), a β-adrenergic agonist (Online Figure VI). MPG treatment also did not affect the frequency of diastolic Ca^{2+} waves (DCWs) recorded in control myocytes field stimulated at 0.3 Hz in a presence of 100 nmol/L Iso [0.12±0.03 waves per cycle in the absence (n=29) *vs.* 0.12±0.02 waves per cycle in the presence of MPG (n=37)]. Consistent with the lack of antioxidant effect on frequency of DCWs in control, we did not observe alterations in the rate of ROS production in control myocytes in the presence of 100 nmol/L Iso (Online Figure VII).

Using caffeine as a sensitizer of ryanodine receptors (RyR2s) to cytosolic Ca^{2+}.

Caffeine is considered to be a classical RyR2 agonist that acts by increasing the sensitivity of RyR2 to cytosolic Ca^{2+}.[6] Recently Kong et al.,[7] reported that the effects of high concentrations of caffeine are mediated by changing the sensitivity of recombinant RyR2 to luminal Ca^{2+}. However a subsequent study of the effects of caffeine on rabbit cardiac RyR2 indicated that the effects of caffeine at <2.5 mmol/L are mediated by sensitization of the RyR2 to cytosolic Ca^{2+},[8]. Therefore we used concentrations of caffeine below 1 mmol/L (0.4-1 mmol/L) to study the effects of sensitizing RyR2s to cytosolic Ca^{2+} on arrythmogenesis.

Sulfhydryl labeling assay

The content of free thiols in RyR2s was determined using the monobromobimane (mBB) fluorescence method.[4] Briefly, LV tissue samples were obtained from VF hearts perfused for 30 min with control buffer or with buffer containing 0.75 mmol/L MPG. Control perfusion buffer contained (mmol/L): 130 NaCl, 5.4 KCl, 3.6 $MgCl_2$, 0.05 $CaCl_2$ 0.5 NaH_2PO_4, 10 Glucose, 5 HEPES, 20 Taurine (pH 7.4). Samples were incubated with 20 mmol/L mBB for 1 hour in the dark at room temperature. To determine the maximal range of mBB signal some samples were treated for 30 min with either 10 mmol/L dithiothreitol, a reducing agent, or 0.2 mmol/L 2,2'-dithiodipyridine, an oxidizing agent. Subsequently proteins were subjected to SDS-PAGE. mBB fluorescence was normalized to the RyR2 levels quantified using Coomassie Blue staining of gels run in parallel. mBB fluorescence was acquired using Epichemi II Darkroom system (UVP Bioimaging Sys., CA). Images were analyzed using ImageJ software (U.S. National Institutes of Health).

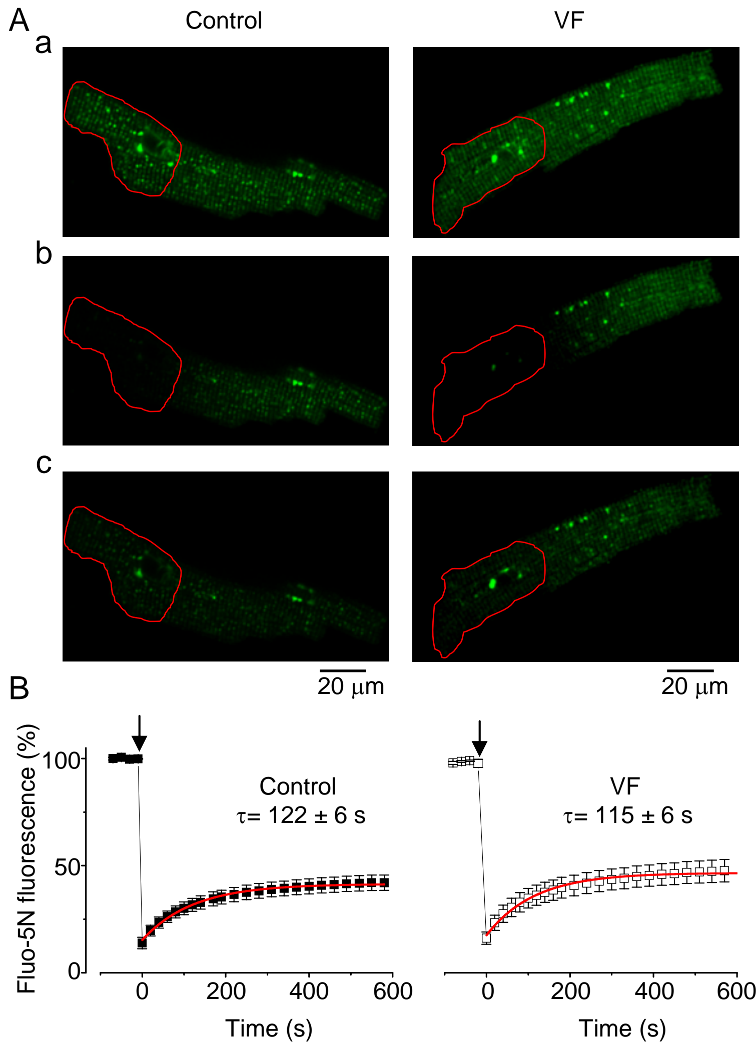
Single RyR2 Channel Measurements

Single RyR2s were reconstituted by fusing canine LV heavy SR microsomes into planar lipid bilayers and single channel currents were recorded with an Axopatch 200A (Molecular Devices, CA) patch clamp amplifier currents at room temperature (21–23°C), as described previously.[9] Data were digitized at 5–10 kHz and filtered at 2 kHz. Acquisition and analysis of data were performed by using PCLAMP 10 software (Molecular Devices). Experimental solutions contained (in mmol/L): 350 $CsCH_3SO_3$, 0.02 $CaCl_2$, 3 MgATP, and 20 HEPES (pH 7.4) on the cytosolic (cis) side of the bilayer, and 20 $CsCH_3SO_3$, 0.02 $CaCl_2$, and 20 HEPES (pH 7.4) on the luminal (trans) side of the bilayer.

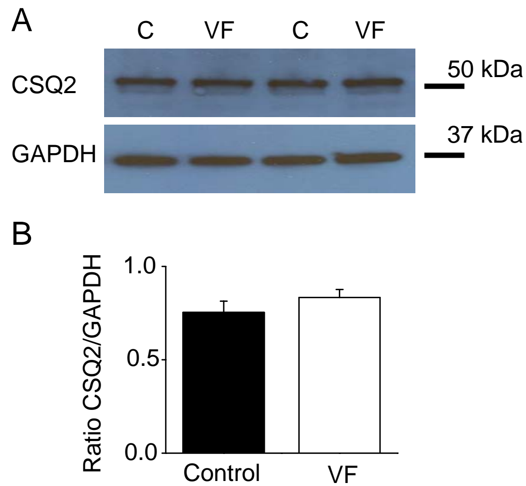
Western Blotting

For Western blot analyses, myocytes were field stimulated at 0.5 Hz for 1 min, then RIPA buffer supplemented with phosphatase, protease and calpain inhibitors was added directly to the cells and then, the samples were instantly frozen with liquid nitrogen. Cell lysate proteins (40 μg) were subjected to 4 to 20% gradient SDS-PAGE and blotted onto nitrocellulose membrane. Phosphorylation of RyR2s was assessed using standard procedures.[10] Anti-phospho-RyR2-Ser-2808 and Ser-2814 antibodies were kindly provided by Dr. X. Wehrens (Baylor College of Medicine, TX). Anti-phospho-RyR2-S2030 antibody was raised against (CG) TIRGRLLS(PO4)LVEKVTYLKKCONH2 (Phosphosolutions, CO). Total RyR2 protein content was assessed in the same samples with anti-RyR2 antibodies (ThermoFisherScientific, MA) on a different blot and used as a control for loading. Protein bands were visualized using the Super Signal Pico Kit. Scans were quantified with Image J software (NIH).

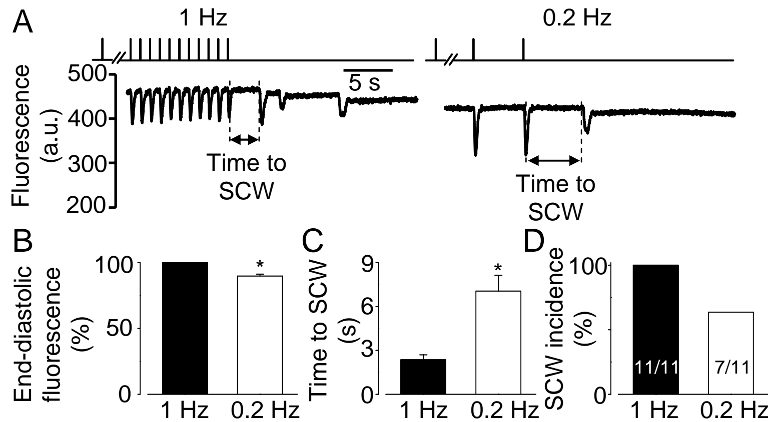
Supplemental Figures and Figure Legends



Online Figure I. Recovery of SR-entrapped fluo-5N fluorescence from photo- bleaching is not different between control and VF myocytes.
Control and VF myocytes were loaded with fluo-5N. After permeabilization with saponine, 30 µmol/L ruthenium red was added to block ryanodine receptors activity. About 35% of myocyte area was bleached using 86 % of laser power for 3.8 s. Fluo-5N fluorescence was acquired using laser power at 0.5%. Kinetics of fluo-5N fluorescence recovery after photo-bleachong (FRAP) were analyzed and compared in control and VF myocytes. A, Representative images of control and VF myocytes are shown before bleaching (a), just after bleaching (b), and at time point when recovery reached steady-state level (c). Bleaching areas are shown in red. B, Average time-course of fluo-5N fluorescence recorded during FRAP experiments in nine control and eleven VF myocytes. Arrows indicate beginning of bleaching period. FRAP data were fitted by monoexponential functions (shown in red).

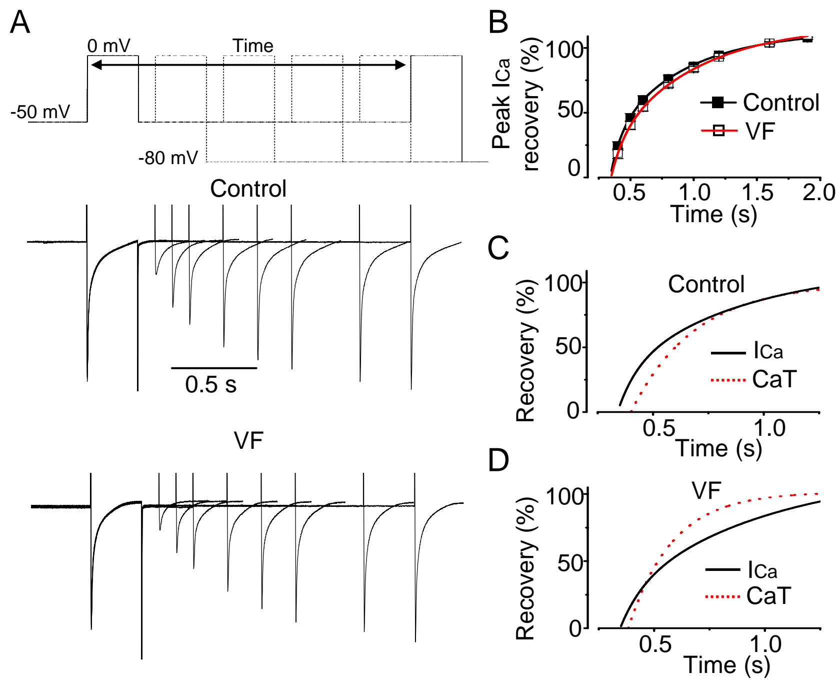


Online Figure II. Protein level of calsequestrin (CSQ2) is not altered in VF. A, Representative Western blots showing levels of CSQ2 and GAPDH in control and in VF hearts. CSQ2 antibody was from ABR Affinity BioReagents, CO and GAPDH antibody was from Abcam, MA. **B,** Graph demonstrates that average levels of CSQ2 normalized to GAPDH recorded in four control and four VF samples are not significantly different ($P>0.3$).

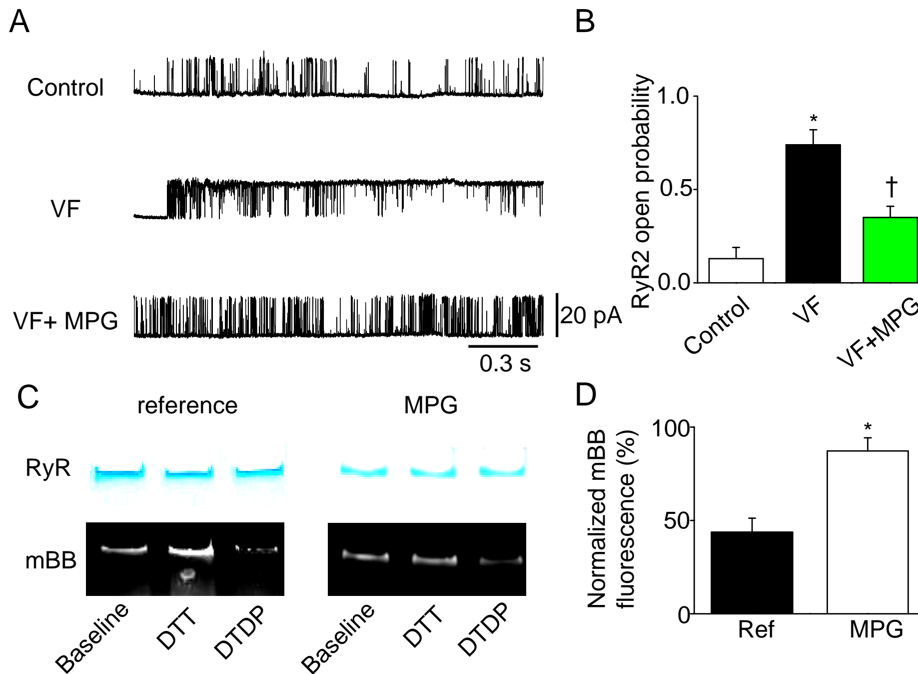


Online Figure III. Reduced [Ca2+]SR is associated with increase in time delay between SR Ca2+ depletion and SCW initiation in control cells.

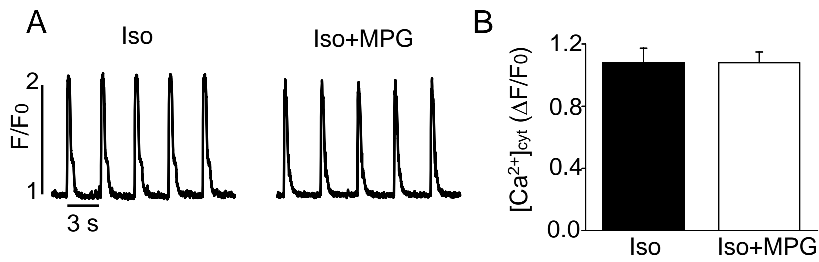
Control myocytes were loaded with fluo-5N. Different levels of SR Ca2+ loading were achieved by stimulating cells at 1 and 02 hz. A, Stimulation protocol and corresponding time-courses of fluo-5N fluorescence. Average end-diastolic levels of fluo-5N fluorescence (B), average time to SCW (C), and SCW incidence during 20s time period after the last stimulus (D) were obtained for control myocytes (n=11) field-stimulated at 1 and 0.2Hz.



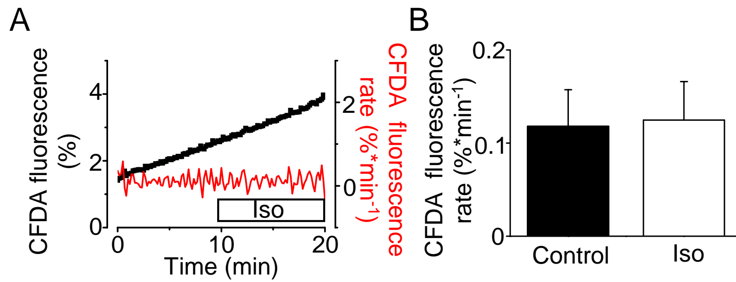
Online Figure IV. L-type Ca current (ICa) recovery from inactivation is not different in control and VF myocytes.
A, Schematic voltage protocol and corresponding traces of ICa in control and VF myocytes. B, Average data showing kinetics of recovery of the amplitude of ICa were obtained from eight control and seven VF myocytes. Data were fit to two exponentials with constants of 85±37 and 576±44 ms in control, 98±21 and 674±34 ms in VF. C and D, Kinetics of recovery from inactivation of the peak ICa and amplitude of Ca transients (CaT) are presented for control and VF myocytes.



Online Figure V. MPG, a reducing agent, normallizes RyR2 activity and increases free thiols content of RyR2s in VF hearts.
Representative recordings (A) and summary data (B) of single RyR2 activity obtained from control (n=8), VF (n=7), and VF plus MPG (n=8) samples C, Representative Coomassie blue-stained gels (upper panels) and corresponding monobrombimane (mBB) fluorescence intensity (lower panels) of RyRs from VF hearts perfused for 30 min with solution contained no MPG (reference) or 0.75 mmol/L MPG. Samples were treated with 0.2 mmol/L 2,2'-dithiodipyridine (DTDP) or 5 mmol/L dithiothreitol (DTT) to determine maximal changes produced by oxidation. D, Relative free thiol content of RyRs from reference vs. MPG samples obtained by normalizing mBB fluorescence to RyR amount determined using Coomassie Blue staining of the gels run in parallel. *P<0.05. Data were obtained from 3 samples for each condition.. *P<0.05 vs control, † P<0.05 vs VF.



Online Figure VI. Mercapto-propionyl-glycine (MPG) does not affect intracellular Ca^{2+} transient in control myocytes. Control myocytes were loaded with Rhod-2 Ca^{2+}-sensitive dye. A, Time-course of Rhod-2 fluorescence recorded during 0.3 hz field stimulation in the presence 100 nmol/L isoproterenol (Iso) in untreated and MPG-treated (0.75 mmol/L, 30 min) control myocytes. B, Average data obtained for the amplitude of Ca transients from twenty eight untreated and twenty four MPG-treated control myocytes.



Online Figure VII. Reactive-oxygen species (ROS) production rate is not affected by beta-adrenergic stimulation in control myocytes.
Control myocytes were loaded with CFDA,a ROS-sensitive dye. A, Time-course of CFDA fluorescence and CFDA fluorescence rate recorded in control myocyte in the absence and in the presence of 100 nmol/L isoproterenol (Iso). Cell was electrically stimulated at 0.3 Hz. Data were acquired every 16 s. At the end of experiment 10 mmol/L H_2O_2 was applied. CFDA fluorescence was normalized to the maximal value recorded in the presence of H_2O_2. B, Average data obtained from five control myocytes. CFDA fluorescence recorded just before and at 10th min of Iso application was used in analyses.

References

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