Supplemental Material

Supplemental Methods

The research protocol (ARC protocol #2003-063-31) was approved by the UCLA Institutional Animal Care and Use Committee following the guidelines of American Heart Association.

Intact heart optical mapping experiments

New Zealand White rabbits (4-6 month old) and male Fisher344 rat (3-4 month old) were used in this study. The hearts of the anesthetized rabbits or rats were removed and the ascending aorta was cannulated for retrograde perfusion through the coronary ostia in the Langendorff fashion as we previously described. [1, 2] The hearts (N=5) were then stained with RH237 for voltage fluorescent optical imaging as described previously.[3] Cytochalasin D (5 μmol/l) was added to the perfusate to eliminate motion artifact during optical recordings. [1, 2] The stained heart was excited with green light (LED) at 532 nm and the emitted fluorescence was collected using CMOS camera (MiCAM Ultima, BrainVision, Tokyo, Japan) at 1 ms/frame and 100x100 pixels with spatial resolution of 0.35x0.35 mm^2 per pixel covering the entire anterior LV epicardial surface.[3] Extracellular electrograms were recorded continuously using unipolar Ag-AgCl electrodes. Intracellular membrane potential was recorded from epicardial cells using glass microelectrodes.

Hypokalemia was induced by lowering [K] of the Tyrode's solution from 5.4 (mild hyperkalemia) to 4.0 (normal), 3.5 (borderline), 2.7 (moderate), 2.0 (severe) or 1.0 mmol/l (extreme). The following interventions were tested: the Ca channel blocker nifedipine (10

μmol/l); reduced extracellular Ca concentration ([Ca_o]) from 1.8 mmol/l to 0.9 mmol/l; the CaMKII inhibitor KN-93 (1 μmol/l) and its inactive form KN-92 (1 μmol/l); and the selective late I_{Na} blocker GS-967 (1 μmol/l) generously provided by Gilead Sciences.

Isolated ventricular myocyte patch clamp studies

Young adult (3- to 4-month-old) New Zealand white male rabbits (1.7-2.0 kg) were injected intravenously once with heparin sulfate (1,000 U) and sodium pentobarbital (100 mg/kg). Following confirmation of adequate anesthesia (absence of pedal withdrawal reflex, corneal reflex, and motor response to pain stimuli by scalpel tip), hearts were rapidly excised and perfused for myocyte isolation.

Freshly isolated single ventricular myocytes were used within 8 hours for whole-cell patch clamp studies as described previously [4]. The pipette solution contained (in mmol/l) K-aspartate 110, KCl 30, NaCl 5, HEPES 10, EGTA 0.0-0.1, MgATP 5, creatine phosphate 5, and cAMP 0.1 (pH 7.2 adjusted with KOH). Cells were superfused at 37°C with standard Tyrode's solution containing (in mmol/l) NaCl 136, KCl 5.4 (reduced in hypokalemia experiments), NaH_2PO_4 0.33, $CaCl_2$ 1.8, $MgCl_2$ 1, HEPES 10, and glucose 10 (pH 7.4 adjusted with NaOH) unless otherwise indicated. Corrections were made for liquid junction potentials. Action potentials were elicited in the current clamp mode at a pacing cycle length (PCL) of 1 or 6 s by 2-ms current pulses of at least twice threshold. Data were acquired and filtered at 2 kHz (Axopatch 200B patch-clamp amplifier; Digidata 1200 acquisition board; and Clampex 8.0, Axon Instruments, Inc.) then analyzed using Clampfit 9.2 (Axon instruments, Inc.) and Origin 7.5 (Microcal Software, Inc.). To induce EADs, $[K]_o$ was reduced from 5.4 to <3.5 mmol/l. To inhibit CaMKII, KN-93 (or KN-92 as control) was added to the superfusate, or CaMKII inhibitor

peptide AIP (2 μM) was added to the patch electrode solution and allowed to equilibrate for 20-30 mins after rupturing the patch before experimental intervention. To selectively block late I_{Na}, GS-967 (l μmol/l) was added to the superperfusate.

CaMKII assay

After an equilibration period of 10-15 min with standard Tyrode's solution containing 5.4 mmol/l [K], isolated rabbit hearts were perfused for an additional 30 min with either 5.4 or 2.7 mmol/l [K]. Left ventricular tissue was then fast frozen in liquid nitrogen and stored at -80^{o}C. Protein lysates were prepared in RIPA cell lysis buffer (Thermo Scientific) supplemented with protease inhibitor (Cell Signaling). Supernatants were collected after centrifugation (14,000g) for 20 minutes at 4^{o}C and used for CaMKII activity measurement. CycLex CaMKII assay kit (MBL International, Woburn, MA, USA) was used to determine CaMKII activity by loading 1.0 μg protein in each well with a reaction mixture without (to measure basal autophosphorylated activity) or with saturating Ca/CaM (to measure maximal activity) and incubated for 7 mins at 30^{o}C. After washing, 100 μl of HRP-conjugated detection antibody was incubated for 60 min at room temperature. After additional washes, 100 μl of substrate reagent was added for a 5 min incubation at room temperature following by 100 μl of stop solution. Serial dilutions of a purified CaMKII positive control (CycLex Co Ltd) was used for standard curve preparation. Results are reported as the ratio of basal (autophosphorylated) CaMKII activity (in the absence of added Ca/CaM) to maximal CaMKII activity (in the presence of Ca/CaM).

Computer modeling

Simulations were carried out using a rabbit ventricular myocyte AP model [5], with the following governing equation for voltage (V):

$$C_m \frac{dV}{dt} = -I_{ion} + I_{sti} ,$$

where C_m=1 μF/cm^2, $I_{ion} = I_{Na} + I_{to,f} + I_{to,s} + I_{Ca,L} + I_{Ks} + I_{Kr} + I_{K1} + I_{NCX} + I_{NaK}$, where I_{ion} is the total ionic current density, and I_{sti} is the stimulation current density. This model has been previously used to study Ca waves, alternans and afterdepolarizations [6-9]. In order to simulate the downstream effects of CaMKII activation on $I_{Ca,L}$ and I_{Na}, we allowed the L-type Ca current conductance, $g_{Ca,L,}$ and late I_{Na} [10] to increase as the mean cytoplasmic free Ca (c_i) increased, according to the following relationships:

$$g_{Ca,L} \rightarrow g_{Ca,L}\,(1 + 0.5f(c_i))$$

$$h, j \rightarrow (0.02 + 0.1f(c_i)) + (1 - (0.02 + 0.1f(c_i)))h, j$$

Here $f(c_i) = \frac{1}{1+\left(\frac{0.1}{CaMK_{trap}(c_i) + CaMK_{bound}(c_i)}\right)^{0.5}}$, and h and j are the linear gating variables for I_{Na}.

CaMKII activation process is controlled by the following ODEs:

$$CaMK_{bound} = CaMK_0\left(1 - CaMK_{trap}\right)\frac{1}{1 + \left(\frac{K_{m,CaM}}{c_i}\right)^5}$$

$$\frac{dCaMK_{trap}}{dt} = \alpha_{CaMK} \cdot CaMK_{bound} \cdot \left(CaMK_{bound} + CaMK_{trap}\right) - \beta \cdot CaMK_{trap},$$

$$\alpha_{CaMK} = 0.05\ ms^{-1};\ \beta_{CaMK} = 0.00068\ ms^{-1}; CaMK_0 = 0.05; K_{m,CaM} = 3\ \mu M.$$

Under these conditions, the CaMKII-induced increases in $I_{Ca,L}$ saturated at 50% [11], and late I_{Na} saturated at 0.8% of the peak I_{Na} value [12, 13], with a half-maximal activation at c_i = 0.35 μM.

Statistical Analyses

Data is summarized as the median with 95% confidence intervals (CI). The conventional percentile bootstrap-resampling approach with 10,000 replications was used for estimating 95% CI as well as examining the significant difference between groups (effect size statistics) [3], with $P \leq 0.05$ considered significant. Differences in the incidence of VT/VF (dichotomous comparisons) were determined using Fisher's exact test, and differences in tissue CaMKII activity using Wilcoxon rank-sum test.

Supplemental Results

Hypokalemia-induced VT/VF in Langendorff rat hearts is suppressed by nifedipine.

Consistent with the key role the L-type Ca current ($I_{Ca,L}$) reactivation in EAD formation, blocking $I_{Ca,L}$ with 10 μmol/l nifedipine eliminated hypokalemia-induced arrhythmias, as illustrated in Fig. S1. In this heart, no episodes of VT/VF occurred during a 90 min exposure to 2.7 mmol/l $[K]_o$ in the presence of nifedipine (panel A), but triggered activity developed within 10 min of washing out nifedipine (panels B-D), culminating in VT/VF after 82 min (panel E), which was suppressed by readmitting nifedipine (panel E). Nifedipine had similar effects in three additional hearts.

Hypokalemia-induced VT/VF in Langendorff rat hearts is suppressed by lowering extracellular [Ca].

If CaMKII activation plays a role in EAD-mediated arrhythmias induced by hypokalemia, then lowering extracellular Ca concentration ($[Ca]_o$) might be expected to minimize CaMKII activation by reducing intracellular Ca loading during hypokalemia. To test this possibility, we lowered $[Ca]_o$ from 1.8 to 0.9 mmol/l during hypokalemia (2.7 mmol/l). In the presence of 0.9 mmol/l $[Ca]_o$, hypokalemia failed to induce VF for up to 80 min (Fig. S2). Upon return to 1.8 mmol/l $[Ca]_o$, however, VF occurred within 45 min, and was suppressed by lowering $[Ca]_o$ to 0.9 mmol/l. Similar effects were observed in four hearts.

Dofetilide sensitization of a rabbit heart to hypokalemia-induced VT/VF.

A rabbit heart which failed to develop VT/VF after 90 mins of moderate hypokalemia (2.7 mmol/l) subsequently developed VT/VF after dofetilide was added (Fig. S3).

Role of CaMKII activation in hypokalemia-induced VF in isolated rat hearts

In 3 out of 4 hearts, treatment with the CaMKII inhibitor KN-93 (1 μmol/l) suppressed hypokalemia-induced VF as shown in Fig. S4A. In the fourth heart, short runs of VT and VF persisted even after 90 min of KN-93 perfusion. In two hearts in which hypokalemia-induced VF was suppressed by KN-93, VF occurred within 10 min when KN-93 was replaced with its inactive congener KN-92 (1 μmol/l), as shown in Fig. S4B.

Supplemental Figures S1-S4

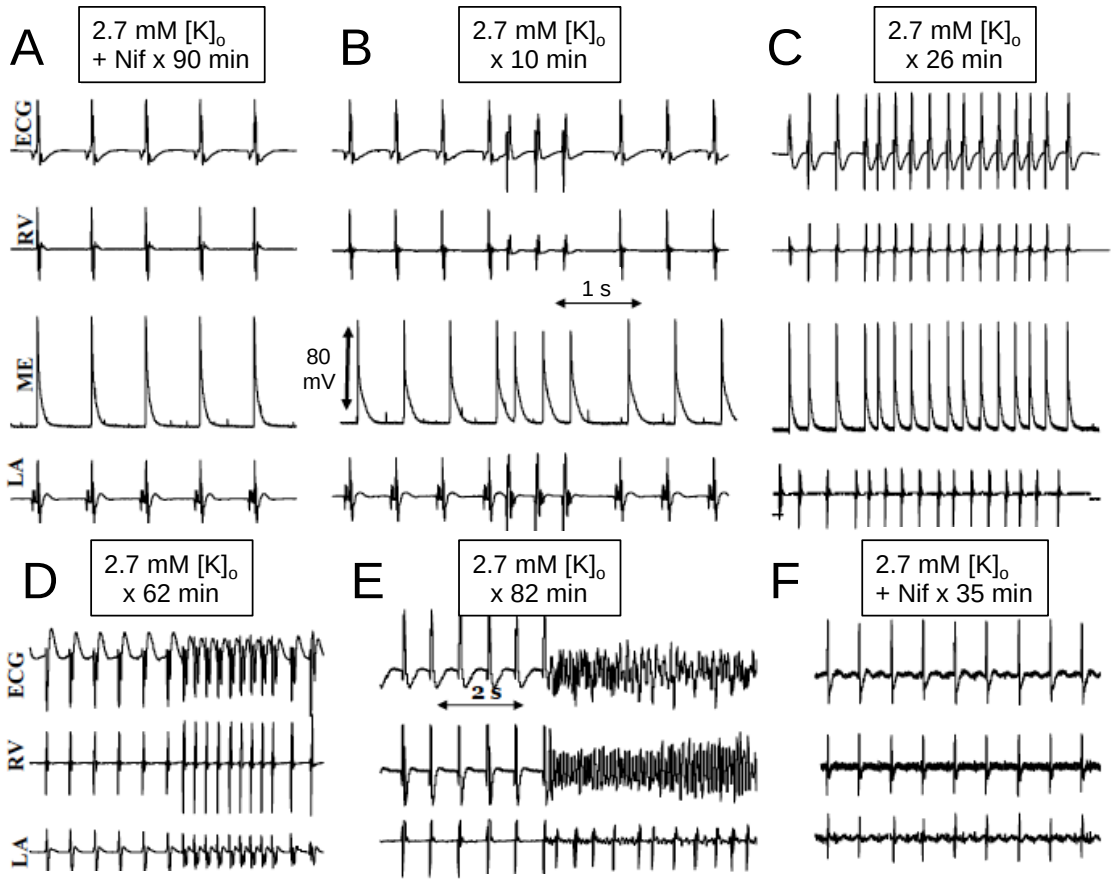

Figure S1. *Protective and suppressive effect of nifedipine (10 μmol/l) against hypokalemia-induced VF is an isolated adult rat heart*. **A.** After 90 min of hypokalemia (2.7 mmol/l $[K]_o$) in the presence of nifedipine, the heart remains in sinus rhythm as indicated by the pseudo-ECG and bipolar electrograms from the RV and LA. **B-E.** After washout of nifedipine, triggered activity (B, C, D) which culminates in VF (E) after 82 mins. **F.** Reapplication of nifedipine restores sinus rhythm.

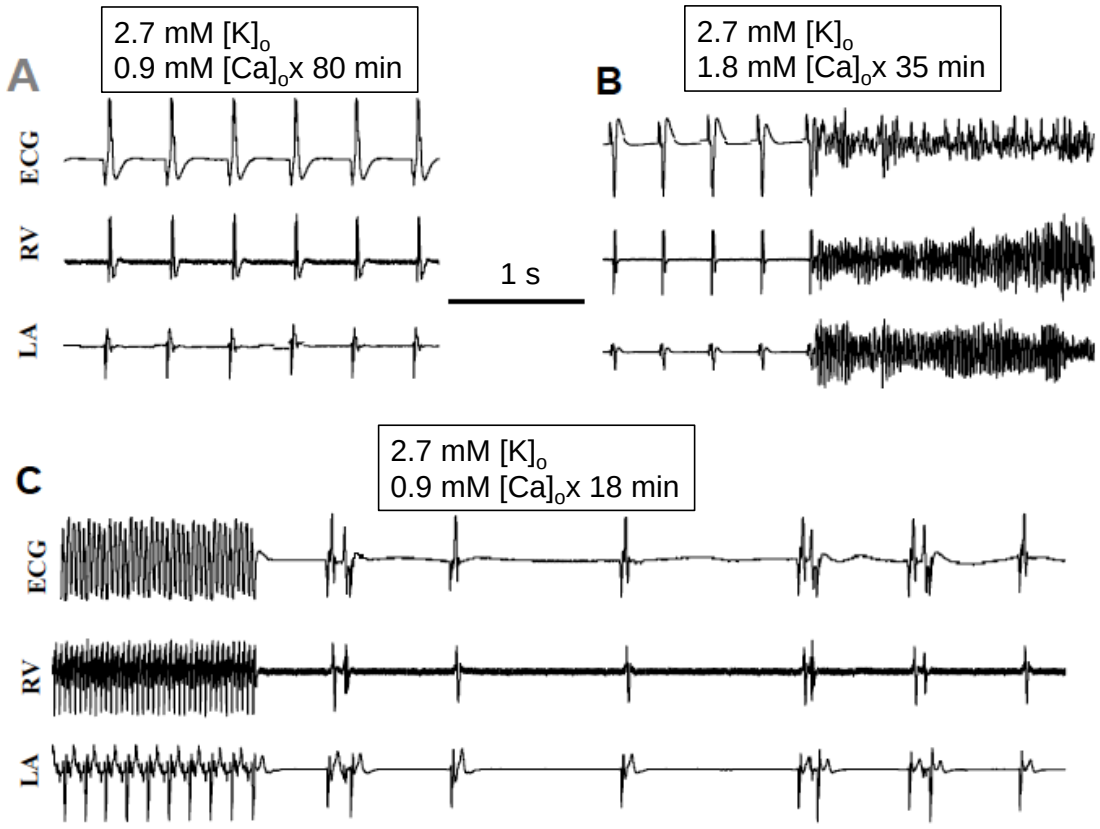

Figure S2. *Protective and suppressive effect of hypocalcemia against hypoalemia-induced VF in an isolated-perfused rat heart.* **A.** After 80 min of hypokalemia (2.7 mmol/l $[K]_o$) and hypocalcemia (0.9 mmol/l $[Ca]_o$), the heart remains in sinus rhythm as indicated by the pseudo-ECG and bipolar electrograms from the RV and LA. **B & C.** Restoring $[Ca]_o$ to 1.8 mmol/l induced VF within 35 minutes (B), which was prevented 18 min after reducing $[Ca]_o$ to 0.9 mmol/l (C).

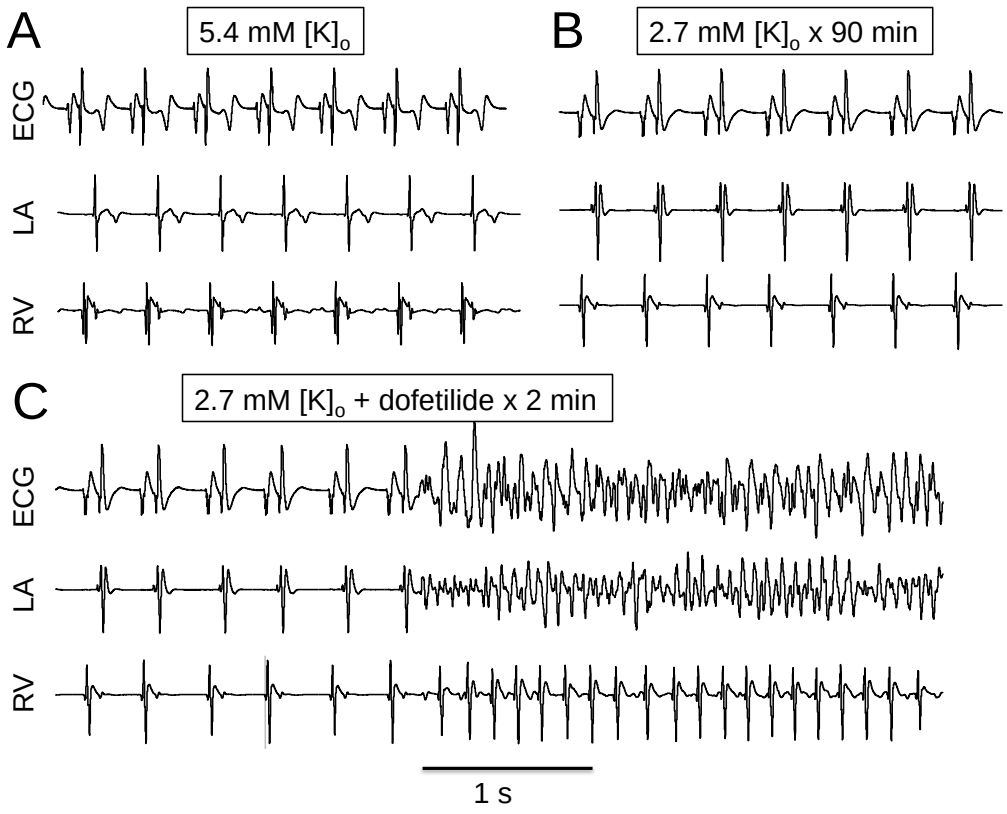

Figure S3. *Dofetilide sensitization of a rabbit heart to hypokalemia-induced VT/VF.* A heart in sinus rhythm (**A**) which failed to develop VT/VF after 90 mins of exposure to 2.7 mmol/l (**B**), which subsequently developed VT/VF within 2 min after dofetilide was added (**C**).

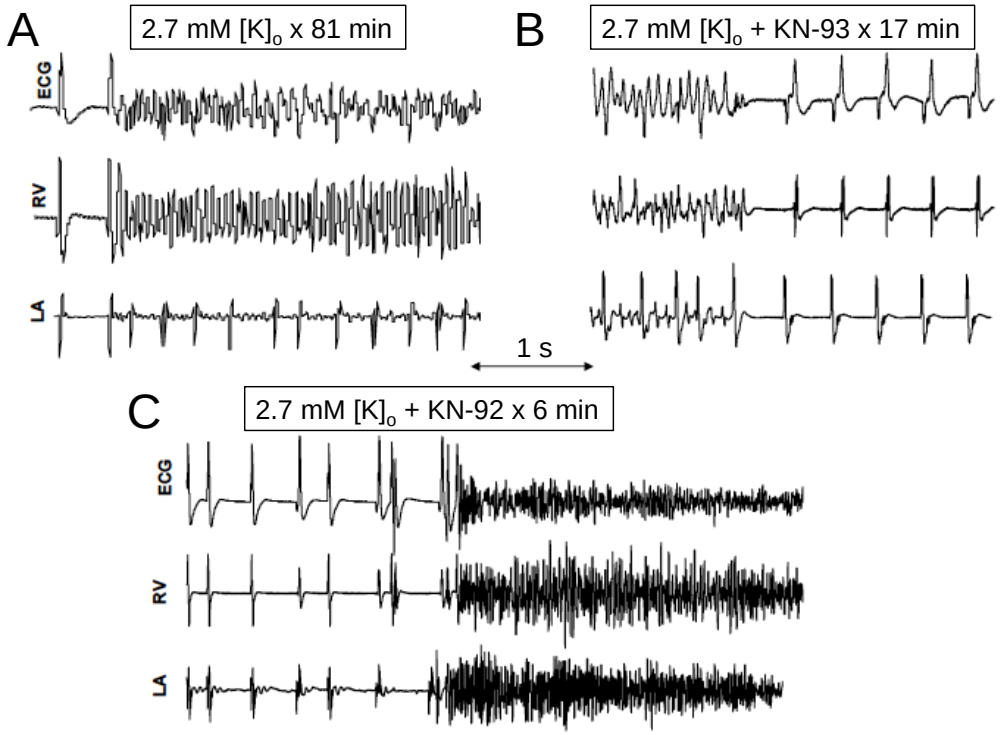

Figure S4. *Suppression of hypokalemia-induced VF by the CaMKII blocker KN-93 (1 μmol/l) but not by its inactive form KN-92 (1 μmol/l).* **A.** VT/VF after 81 minutes of hypokalemia (2.7 mmol/l) **B.** KN-93 suppressed VT/VF after 17 min. **C.** Replacing KN-93 with KN-92 led to recurrence of VT/VF after 6 min.

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