Mitochondrial Ca^{2+} flux modulates spontaneous electrical activity in ventricular cardiomyocytes

An Xie[1], Anyu Zhou[1], Hong Liu[1], Guangbin Shi[1], Man Liu[1], Kenneth R. Boheler[2], Samuel C. Dudley, Jr[1] *

1 Department of Medicine, Lillehei Heart Institute, University of Minnesota, Minneapolis, MN, United States of America, **2** Stem Cell and Regenerative Medicine Consortium, LKS Faculty of Medicine, Hong Kong University, Hong Kong, P.R. China

* sdudley@umn.edu

Abstract

Introduction

Ca^{2+} release from sarcoplasmic reticulum (SR) is known to contribute to automaticity via the cytoplasmic Na$^+$-Ca^{2+} exchanger (NCX). Mitochondria participate in Ca^{2+} cycling. We studied the role of mitochondrial Ca^{2+} flux in ventricular spontaneous electrical activity.

Methods

Spontaneously contracting mouse embryonic stem cells (ESC)-derived ventricular cardiomyocytes (CMs) were differentiated from wild type and ryanodine receptor type 2 (RYR2) knockout mouse ESCs and differentiated for 19–21 days. Automaticity was also observed in human induced pluripotent stem cell (hiPSC)-derived ventricular CMs differentiated for 30 days, and acute isolated adult mouse ventricular cells in ischemic simulated buffer. Action potentials (APs) were recorded by perforated whole cell current-clamp. Cytoplasmic and mitochondrial Ca^{2+} transients were determined by fluorescent imaging.

Results

In mouse ESC-derived ventricular CMs, spontaneous beating was dependent on the L-type Ca^{2+} channel, cytoplasmic NCX and mitochondrial NCX. Spontaneous beating was modulated by SR Ca^{2+} release from RYR2 or inositol trisphosphate receptors (IP$_3$R), the pacemaker current (I$_f$) and mitochondrial Ca^{2+} uptake by the mitochondrial Ca^{2+} uniporter (MCU). In RYR2 knockout mouse ESC-derived ventricular CMs, mitochondrial Ca^{2+} flux influenced spontaneous beating independently of the SR Ca^{2+} release from RYR2, and the mitochondrial effect was dependent on IP$_3$R SR Ca^{2+} release. Depolarization of mitochondria and preservation of ATP could terminate spontaneous beating. A contribution of mitochondrial Ca^{2+} flux to automaticity was confirmed in hiPSC-derived ventricular CMs and ischemic adult mouse ventricular CMs, confirming the findings across species and cell maturity levels.

Conclusions

Mitochondrial and sarcolemma NCX fluxes are required for ventricular automaticity. Mitochondrial Ca^{2+} uptake plays a modulatory role. Mitochondrial Ca^{2+} uptake through MCU is influenced by IP_3R-dependent SR Ca^{2+} release.

Introduction

Adult ventricular cardiomyocytes (CMs) show spontaneous electrical activity known as abnormal automaticity when injured. This spontaneous activity is thought to contribute to arrhythmic risk. Mouse embryonic stem cell (ESC)-derived and human induced pluripotent stem cell (hiPSC)-derived ventricular-like CMs show similar spontaneous electrical activity that allows for more detailed the study of the ion currents underlying this ventricular automaticity. In these cells, spontaneous electrical activity is modulated strongly by sarcoplasmic reticulum (SR) Ca^{2+} release through ryanodine receptor type 2 (RYR2). This Ca^{2+} release activates the Na^+/Ca^{2+} exchanger (NCX) to produce an inward current that can accelerate the rate of spontaneous membrane depolarization[1–4]. Targeted inactivation of the NCX type 1 results in the lack of a spontaneous heart beat in NCX type 1-null embryos[5], making Ca^{2+} cycling a critical requirement of spontaneous electrical activity in whole hearts. Other ion channels and receptors that are involved in spontaneous electrical activity include the inward rectifier potassium current (I_{K1})[6], the I_f pacemaker current[7], and the inositol trisphosphate receptor (IP_3R)-mediated SR Ca^{2+} release[8]. These mechanisms of automaticity in ESC-derived working CMs are similar to those in the cardiac pacemaker cells in the terms of the "M-clock (voltage clock)" and the "Ca^{2+}-clock"[9, 10].

Mitochondria are involved in the regulation of cellular Ca^{2+}. It has been reported that mitochondria Ca^{2+} uptake contributes to buffering cytoplasmic Ca^{2+} peaks in cardiomyocytes[11]. Mitochondrial Ca^{2+} cycling modulates cardiac pacemaker and atrial cell automaticity[12, 13]. Nevertheless, the role of mitochondrial Ca^{2+} cycling in automaticity of cardiac ventricular cells is unclear. Mitochondrial Ca^{2+} homeostasis involves Ca^{2+} influx mainly through the mitochondrial Ca^{2+} uniporter (MCU) driven by the mitochondrial membrane potential (Ψm)[14]. Other mitochondrial Ca^{2+} uptake pathways include the ryanodine receptor type 1[15]. Mitochondrial Ca^{2+} efflux occurs mainly through the mitochondrial NCX[16].

Because of the role of spontaneous electrical activity in initiating ventricular arrhythmias, we tested whether mitochondrial Ca^{2+} flux influenced ventricular CM spontaneous electrical activity in the presence and absence of the SR Ca^{2+} release channel, using ESC-derived mouse ventricular CMs. Results were confirmed in adult mouse ventricular and hiPSC-derived CMs to attempt to ensure applicability of the results to human pathophysiology.

Materials and methods

All relevant data are available from the Figshare repository at the following DOI: 10.6084/m9.figshare.6260555.

Culture and differentiation of mouse ESCs and hiPSC

The mouse R1 ESC cell line transfected with a neomycin resistance gene (ATCC, Manassas, VA, USA) was used throughout the present study. To knock out RYR2, gene targeting of the R1 cells was performed by sequential homologous recombination of the two chromosomal

alleles of RYR2 in R1 ESCs. PCR analysis and Western blotting of ESCs, embryonic bodies, and CM-derived RNA or protein confirmed no evidence of RYR2 transcript or protein, respectively[3]. Undifferentiated ESCs were maintained and expanded as described previously [17–19]. A suspension containing about 500 ESCs in 30 μL media enriched in FBS (20%) was placed as hanging drops on the lids of petri dishes filled with 10 mL PBS. After two days in hanging drops, embryoid bodies were removed and plated onto 0.1% gelatin-coated petri dishes. The 7 day old embryoid bodies were used for the final stages of CM differentiation. Usually after 9–12 days of plating, embryoid bodies showed areas of spontaneous beating. Embryoid bodies differentiated after 18 days were used to isolate single CMs. Eight to ten embryoid bodies containing spontaneously beating areas were used for CM isolation using a procedure described previous. The isolated CMs were plated on 35 mm dishes coated with 0.1% gelatin and 20 μg/mL laminin in cultivation medium and incubated in 37˚C. After over-night incubation, spontaneously beating CMs were used for electrophysiological studies within 1–3 days after the cell isolation.

The hiPSC-derived CMs (iCell® Cardiomyocytes²) differentiated for 30 days were pur-chased from Cellular Dynamics International, Inc. (Madison, WI, USA). Cells were plated and maintained according to the manufacturer's instructions. Ventricular CMs were selected according to the action potential (AP) morphology[20].

Isolation and induction of abnormal automaticity in adult mouse ventricular CMs

All animal protocols were in accordance with the guidelines of the Animal Care and Use Com-mittee of the University of Minnesota and conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. Adult mouse ventricular CMs were isolated from 11–12 weeks old C57BL/6 mice (22–25 g; Charles River, Wilmington, MA) as described. Randomly selected mice of either sex were anaesthetized using inhaled iso-flurane (3% for induction and 1.5–2% for maintenance). After the anesthetized mouse was non-responsive to toe pinch, a thoracotomy was performed. The rib cage was cut bilaterally and the heart exposed. The heart was then excised and placed immediately in a petri dish filled with ice cold Tyrode's solution. The heart was then perfused with buffer (in mmol/L: NaCl/113, KCl 4.7, Na_2HPO_4 0.6, KH_2PO_4 0.6, $MgSO_4$ 1.2, Phenol Red 0.032, NaHCO3 12, $KHCO_3$ 10, HEPES 10, Taurine 30, and 2-3-butanedione monoxime 10) and digested with collagenase II (Worthington Biochemical Co. Lakewood, NJ). CMs were washed with control buffers (in mmol/L: NaCl 133.5, KCl 4, Na_2HPO_4 1.2, HEPES 10, and $MgSO_4$ 1.2) with serially increasing Ca^{2+} concentrations (0.2, 0.5, and 1 mmol/L). Then, myocytes were incubated in MEM medium (modified Eagle's medium with 1% insulin–transferrin–selenium, 0.1% bovine serum albumin, 1% l-glutamine, and 1% penicillin/streptomycin) in a 95% O_2/5% CO_2 incubator at 37˚C for 1 h prior to being used for Ca^{2+} transient measurements. During experiments, the acute isolated mouse ventricular CMs were exposed to a simulated ischemic buffer containing (in mM: 110 NaCl, 10 KCl, 1 $MgSO_4$, 10 Na^+-HEPES, 2.5 $CaCl_2$, 20 2′-deoxyglucose, 20 Na^+-lactate and 1 NaCN, at pH 6.6) at 37˚C for 10 min to induce spontaneous beating[21].

AP recordings from the ESC-derived CMs

APs were recorded at using perforated whole-cell current-clamp as described before[1]. To measure spontaneous APs, the culture medium was replaced with Tyrode solution contained (in mmol/L) 140 NaCl, 5.4 KCl, 1 $MgCl_2$, 10 HEPES, 1.8 $CaCl_2$, 0.33 NaH_2PO_4 and 5.5 glucose (pH 7.4). Glass patch pipettes (World Precision Instrument, Sarasota, FL, USA) were pulled to a resistance of 2–5 MΩ. For AP measurements, the intracellular solution consisted of (in

mmol/L) 120 potassium gluconate, 20 KCl, 5 NaCl, 5 HEPES, and 5 MgATP (pH 7.2). APs were recorded using perforated whole-cell current-clamp and an Axopatch-200B amplifier (Molecular Devices, Sunnyvale, CA, USA). Membrane potential recordings performed in the current-clamp configuration were low pass filtered at 10 kHz and digitized at 20 kHz using a gap-free acquisition mode. For perforated-patch experiments, β-escin (50 μmol/L) was added to pipette solution. Only CMs with a ventricular-like AP were further studied. Criteria for selection of Wt ventricular CMs were a resting membrane potential ($<$-70 mV) and AP duration at 90% repolarization ($>$ 180 ms)[18].

Intracellular Ca²⁺ measurements

To visualize changes in the intracellular Ca²⁺ transients, mouse ESC-derived CMs and acutely isolated mouse ventricular CMs were loaded with Indo-1 AM (ThermoFisher Scientific, Minneapolis, MN, USA 2 μmol/L) for 40 min followed by 20 min of de-esterification at room temperature[22]. The CMs were transferred onto the stage of a real-time fluorescence microscope (IonOptix LLC, Milton, MA, USA). Ca²⁺ transients were acquired at a rate of 1 kHz. Analysis of the signals was performed using IonWizard. Indo-1was excited at 405 nm and emitted fluorescence was collected at wavelengths of 470–490 nm. Selective experiments using Fluo-4 as the fluorescent reporter with mESC-derived cardiomyocytes showed comparable results to Indo-1 (data not shown). Fluo-4 AM (ThermoFisher Scientific), 3 μmol/L, was loaded in hiPSC-derived CMs for 20 min followed with 20 min de-esterification. The excitation wavelength was 488 nm and the absorption wavelength was 500 nm. Cells were transferred onto the stage of a real-time florescence microscope (Olympus IX81, Japan). Analysis of the signals was performed with the software MetaMorph (version 7.8.11.0, Nashville, TN). Cytoplasmic Ca²⁺ transients were presented as the ratio of intensities normalized by background fluorescence (F/F_0).

Mitochondrial Ca²⁺ recordings

Mitochondrial Ca²⁺ transients were monitored by loading cells with Rhod-2 AM (Thermo-Fisher Scientific, 1 μM, load for 1 h at 37˚C), washing the cells twice, and allowing for de-esterification for 1 h in Rhod-2-free DMEM. In these measurements, the ruptured current-clamp technique was used with 20 μM EGTA added in the pipette solution. Minor cytosolic traces of Rhod-2 were eliminated by whole-cell dialysis by the pipette solution[23, 24]. We used very big glass pipette to do this experiment with a resistance ~2.5 MΩ. This helps a lot to reduce the cytoplasmic Ca²⁺ contaminate. Rhod-2 intensity was sampled at a rate of 1 kHz. Mitochondrial Ca²⁺ transients were presented as background-subtracted normalized fluorescence (F/F_0).

Silencing MCU

To silence MCU, specific MCU siRNAs were synthesized by Life Technologies[25]. siRNA-MCU1 targets nucleotides 899–917 of the corresponding mRNA (5'-GCCAGAGACAGACAA UACUtt-3' and 3'-ttCGGUCUCUGUCUGUUAUGA-5'. siRNA-MCU2 targets nucleotides 360–378 of the corresponding mRNA (5'-GGGAAUUGACAGAGUUGCUtt-3' and 3'-ttC CCUUAACUGUCUCAACGA-5'). The non-targeting siRNA (scrambled) was used as a control (5'-GCCUAAGAACGACAAAUCAtt-3' and 3'-ttCGGAUUCUUGCUGUUUAGU-5'). Cells were transfected by Lipofectamine RNAiMAX reagent (ThermoFisher Scientific) for 72 h with a siRNA concentration of 30 pmol/L. MCU mRNA was measured after 72 h by qPCR. Single Cell-to-CT™ Kit (PN 4458237) and primers (MCU primers: Mm01168773_m1, Cat.# 4351372; β-actin primers: Mm00607939_s1, Cat.# 4331182) were from ThermoFisher Scientific.

All data are presented as mean ± SE. The t-tests and Chi-tests were employed for statistical analysis. All experiments were carried out at 37˚C.

Results and discussion

Ventricular spontaneous electrical activity or abnormal automaticity is thought to be a cause of arrhythmias, especially after ischemic insult[26]. Automaticity is thought to be dependent on sarcolemmal and SR Ca^{2+} oscillations. For example, RYR2 loss contributes to bradycardia, arrhythmia, and sudden death in adult mice[27]. Major ion channels contributors to automaticity include the cardiac Na$^+$ channel, L-type Ca^{2+} channel, T-type Ca^{2+} channel, HCN channel, K$^+$ channel and NCX1[28].

Mitochondria are known to participate in Ca^{2+} oscillations[12, 29]. Mitochondrial Ca^{2+} cycling modulates cardiac pacemaker and atrial cell automaticity. In pacemaker cells, inhibition of mitochondrial Ca^{2+} influx into (Ru360) or Ca^{2+} efflux from (CGP) mitochondria modulated the spontaneous AP frequency in opposing directions (111 ± 1% control or 89 ± 2% control), respectively. In atrial cells, NCLX knockdown using siRNA results in slowing of spontaneous electrical activity, an effect similar to that seen in pacemaker cells[12, 13]. Therefore, it stands to reason that mitochondria may be able to influence automaticity of ventricular CMs. Nevertheless, the mechanisms of how sarcolemmal, sarcoplasmic reticular, and mitochondrial Ca^{2+} affect spontaneous activity and whether these mechanisms are similar in pacemaker, atrial, and ventricular cardiomyocytes are unknown.

Spontaneous electrical activity was modulated by mitochondrial Ca^{2+} cycling

Wild type (Wt) ESC-derived ventricular-like CMs showed spontaneous beating (Fig 1A). Spontaneous electrical activity was absolutely dependent on sarcolemmal Ca^{2+} flux since blocking the L-type Ca^{2+} channel with 10 μmol/L nifedipine for 2 min or blocking the sarcolemmal NCX with the NCX1 specific blocker[30], KB-R7943 (10 μmol/L, for 2 min), arrested CM electrical activity (Fig 1B). While spontaneous electrical activity was dependent on L-type Ca^{2+} channel activity, it is unclear whether the L-type Ca^{2+} channel provides directly Ca^{2+} to the NCX or indirectly through the SR. Spontaneous beating was modulated by the pacemaker current through hyperpolarization-activated cyclic nucleotide (HCN)-gated channels, designated as I$_f$. The specific blocker, 10 μmol/L ivabradine[31], had a modest inhibitory effect on the beating rate (from 1.7 ± 0.1 to 1.2 ± 0.2 Hz, $p<0.05$, Fig 1B and 1E) at 2 min after application. Consistent with a role for SR Ca^{2+} cycling in the modulation of spontaneous electrical activity[4, 8], the beating rate could be substantially reduced by treating with 10 μmol/L ryanodine more than 30 min, which inhibited the main SR Ca^{2+} release pathway through the RYR2, and by treating with 2 μmol/L 2-aminoethyl diphenylborinate (2-APB) for more than 15 min, which inhibited SR Ca^{2+} release pathway through IP$_3$Rs. The spontaneous beating rates were greatly reduced to 54% and 71% by ryanodine and 2-APB, respectively (Fig 1C and 1E).

Mitochondrial Ca^{2+} flux also influenced spontaneous beating in Wt CMs. Inhibiting mitochondrial Ca^{2+} uptake by intracellular application of a MCU-specific blocker, 1 μmol/L Ru360, had a similar effect on beating rate as inhibiting SR Ca^{2+} release from IP$_3$Rs (Fig 1D and 1E). The mitochondrial NCX blocker, 3 μmol/L CGP-37157 (CGP)[32], terminated action potential (AP) firings (Fig 1D) after 2 min of application. In order to avoid nonspecific effects of the antagonists, the concentrations used of these compounds were less than or equal to the reported concentrations that were specific for the intended targets[22, 30–34].

The experimental results of cytoplasmic Ca^{2+} transients confirmed the effects of above antagonists on the spontaneous rhythm in ESC-derived ventricular-like cells (Fig 2).
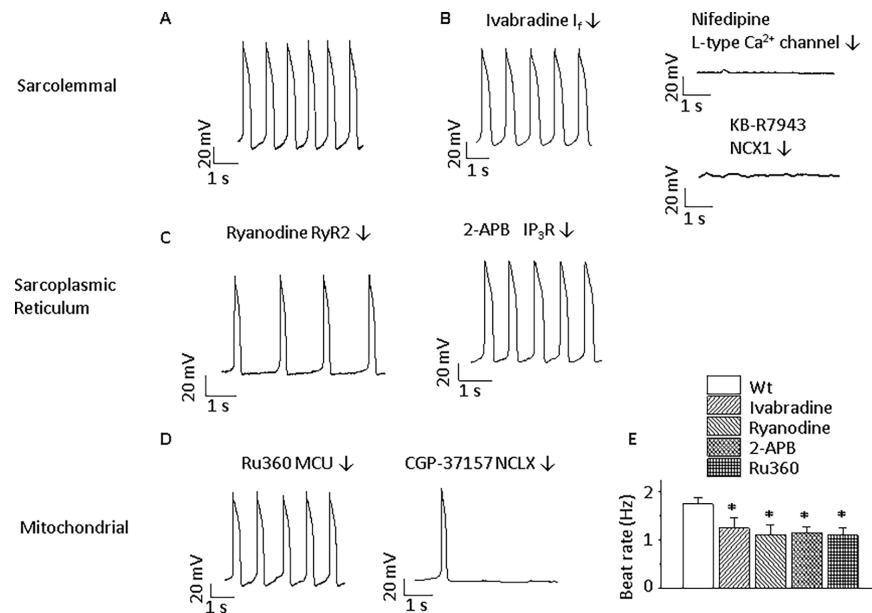
Fig 1. Modulation of spontaneous beating by blocking ion channels and receptors in Wt ventricular-like ESC-derived CMs. A) Typical APs of Wt ventricular-like ESC-derived CM recorded by perforated whole-cell current-clamp technique (n = 15). **B**) Effects of sarcolemmal ion channel or transporter blockers. Beating rate is reduced by the I$_f$ specific blocker, 10 µmol/L ivabradine (n = 6). Automaticity is eliminated by the L-type Ca^{2+} channel blocker, 10 µmol/L nifedipine (n = 6), and the NCX blocker, 10 µmol/L KB-R7943 (n = 5). **C**) Effects of sarcoplasmic reticulum ion channel blockers. Spontaneous beating is modulated by the SR Ca^{2+} release blocker, 10 µmol/L Ryanodine (n = 5), and the IP$_3$R blocker, 2 µmol/L 2-APB (n = 4). **D**) Effects of altering mitochondrial Ca^{2+} handling. Spontaneous beating is modulated by mitochondrial Ca^{2+} influx blocker, 1 µmol/L Ru360 (in pipette solution, n = 5), and mitochondrial NCX (NCLX) blocker, 3 µmol/L CGP-37157 (n = 6). **E**) Summary of effects on spontaneous beating. *p<0.05, compared with control group.

Mitochondrial Ca^{2+} cycling affected spontaneous electrical activity in the absence of RYR2 Ca^{2+} release

In order to isolate the effect of mitochondrial Ca^{2+} cycling, we utilized cells lacking RYR2 receptor. ESC-derived ventricular-like RYR2 knockout (RYR2$^{-/-}$) CMs showed the expected reduction in spontaneous beating similar to the effect of ryanodine on Wt CMs (Figs 1A, 1C, 3A and 3E). To demonstrate the lack of SR Ca^{2+} release, 10 mmol/L caffeine was unable to induce Ca^{2+} release from SR in RYR2$^{-/-}$ CMs (Fig 4). TTX at a concentration of 30 µM could totally block the spontaneous beating of ESC-derived ventricular-like CMs (Fig 5B). It is known that phase 0 of the AP in ventricular cells is mediated by inward Na$^+$ current. Consistent with this idea, TTX totally blocked the spontaneous beating of ESC-derived ventricular-like CMs, an effect differing from that of TTX on pacemaker cells[28].

In ESC-derived ventricular-like RYR2$^{-/-}$ CMs, 1 µmol/L Ru360 significantly inhibited pacemaker activity by 34.8% (Fig 3D and 3E, p<0.05), and Ru360 showed a similar effect to that in Wt CMs, reducing the spontaneous beating rate by 45.0% (Fig 1D and 1E). MCU silencing by MCU-1 or MCU-2 siRNA for 72 h relative to scrambled siRNA controls resulted in reduced MCU mRNA abundance by 96%, decreased mitochondrial Ca^{2+} transients, and a substantial slowing of the spontaneous beating rate (Fig 6). After MCU silencing by MCU-1 or MCU-2 siRNA for 72 h, the peak mitochondrial Ca^{2+} transient (F/F$_0$) was significantly reduced from 2.7 ± 0.6 to 1.3 ± 0.4 while the Ca^{2+} transient rise time constant substantially increased from 78.8 ± 19.5 to 155.0 ± 24.4 ms and the decay time constant increased from 129.8 ± 25.4 to
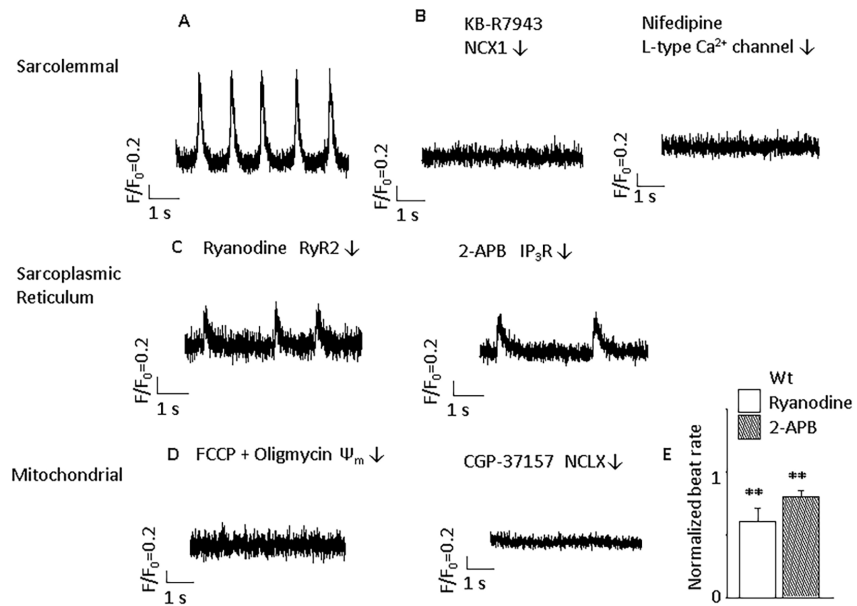
Fig 2. Cytosolic Ca²⁺ traces in Wt ventricular-like ESC-derived CMs. A) Typical cytoplasmic Ca²⁺ transients in a Wt ventricular-like CM. **B**) Cytoplasmic Ca²⁺ transients are abolished by the NCX blocker, 5 μmol/L KB-R7943 (n = 4) and the L-type Ca²⁺ channel blocker, 10 μmol/L nifedipine (n = 4). **C**) Blocking RyR and IP₃Rs with 10 μmol/L ryanodine (n = 5) and 2 μmol/L 2-APB (n = 4), respectively, reduces but does not eliminate the cytoplasmic Ca²⁺ transients. **D**) Cytoplasmic Ca²⁺ transients are abolished by mitochondrial depolarization with 300 nmol/L FCCP and 1 μmol/L oligomycin (n = 5). Inhibiting mitochondrial Ca²⁺ release with 3 μmol/L CGP-37157 also terminates cytoplasmic Ca²⁺ transients (n = 6). **E**) The summary of ryanodine and 2-APB's effects on spontaneous beating. Data were normalized with those before blockers were used. **p<0.01, compared with those before compounds applied.

224.3 ± 31.7 ms. Basal mitochondrial Ca²⁺ (F/F₀) was slightly decreased from 2.3 ± 0.3 to 1.7 ± 0.9 (Fig 6B).

To confirm the role of mitochondrial Ca²⁺ influx in the spontaneous electrical activity, mitochondria were depolarized and ATP preserved with the combination of carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), a protonophoric uncoupler, and oligomycin, an inhibitor of F1F0-ATP synthase[35]. FCCP causes a rapid depletion of the cellular bulk ATP pool, but this process is not observed if oligomycin (1 μmol/L) is applied[36]. FCCP (300 nmol/L)/oligomycin (1 μmol/L) terminated cytoplasmic Ca²⁺ transients and spontaneous activity (Fig 3D) after 2 min. This effect was reversible by washout (data not shown). FCCP/oligomycin inhibited intracellular Ca²⁺ transients and spontaneous beating (Figs 2D, 3D and 5D). The lack of ATP depletion during this timeframe was confirmed as shown in the Supplementary Material.

Reduced mitochondrial Ca²⁺ sequestration could decrease mitochondrial Ca²⁺ release, reduce the activity of sarcolemmal NCX, and lead to reduced automaticity. The mitochondrial NCX blocker, 3 μmol/L CGP, terminated AP firings in RYR2⁻/⁻ CMs (Fig 3D) within 2 min, which is consistent with the previous report in HL-1 CMs[12]. This result suggested that mitochondrial Ca²⁺ efflux was an indispensable step for automaticity when SR Ca²⁺ handling is depressed. At the same time, cytoplasmic Ca²⁺ handling was totally inhibited by CGP (Fig 5D). Confirming the requirement for sarcolemmal NCX in spontaneous activity, the sarcolemmal NCX1 specific blocker, KB-R7943 (5 μmol/L), could terminate CM electrical firing and cytosolic Ca²⁺ transients in RYR2⁻/⁻ CMs (Fig 3B and Fig 5B).
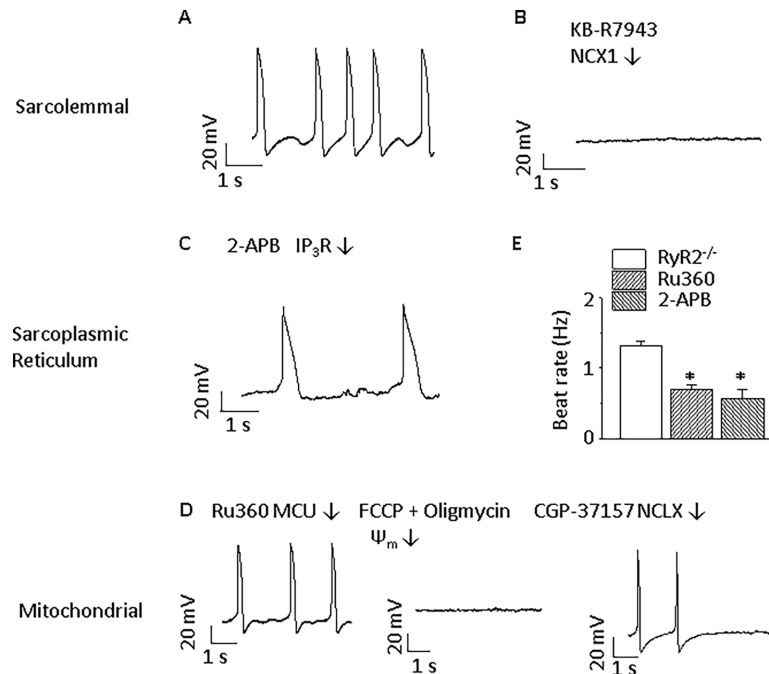
Fig 3. Regulation of automaticity by blocking ion channels and transporters in RYR2$^{-/-}$ ventricular-like ESC-derived CMs. A) In the absence of ryanodine receptors, there is still spontaneous beating (n = 14). **B**) Effects of sarcolemmal alterations. Spontaneous beating is eliminated by the NCX blocker, 5 μmol/L KB-R7943 (n = 5). **C**) Effect of sarcoplasmic reticulum ion channel blocker. Blocking IP$_3$Rs with 2 μmol/L 2-APB could inhibit spontaneous beating (n = 7). **D**) Effects of mitochondrial alterations. Spontaneous beating is modulated by mitochondrial Ca^{2+} influx blocker, 1 μmol/L Ru360 (in pipette solution, n = 7). Mitochondria depolarization with 300 nmol/L Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) and 1 μmol/L oligomycin arrests spontaneous activity (n = 5). Inhibiting mitochondrial Ca^{2+} release with 3 μmol/L CGP-37157 also terminates spontaneous beating (n = 10). **E**) Summary of effects on spontaneous beating. $^{*}p < 0.05$ compared with control group. Ψ_m: mitochondrial membrane potential.

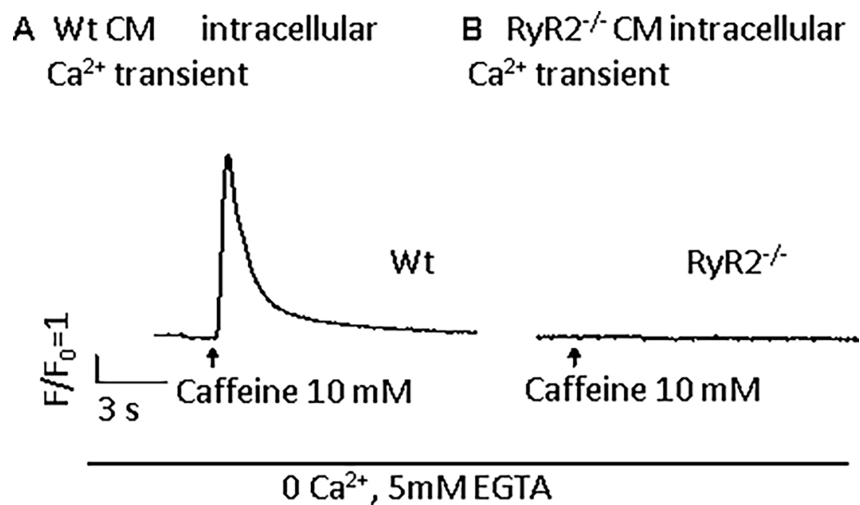https://doi.org/10.1371/journal.pone.0200448.g003

Fig 4. RYR2$^{-/-}$ ventricular-like ESC-derived CMs have no Ca^{2+} release with caffeine exposure. Left panel: A representative Wt ventricular-like CM with Ca^{2+} release induced by 10 mol/L caffeine. **Right panel**: Caffeine (10 mmol/L) does not induce SR Ca^{2+} release from RYR2$^{-/-}$ CMs. n = 5 for each group.
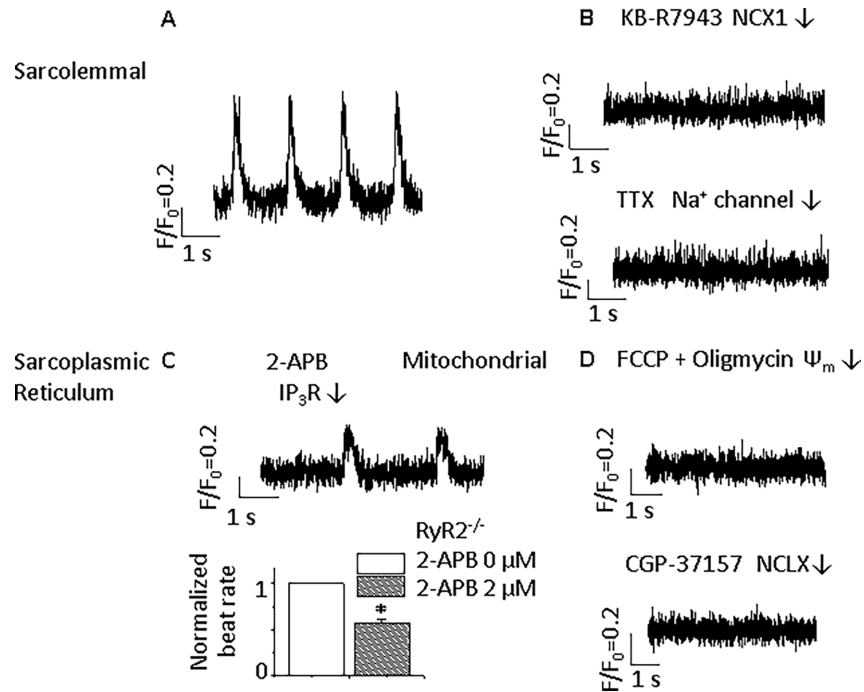
https://doi.org/10.1371/journal.pone.0200448.g004

Fig 5. Cytoplasmic Ca^{2+} transients in RYR2$^{-/-}$ ventricular-like ESC-derived CMs. A) Typical cytoplasmic Ca^{2+} transients in a RYR2$^{-/-}$ CM. **B)** Cytoplasmic Ca^{2+} transients are eliminated by the NCX blocker, 5 μmol/L KB-R7943 and the Na$^+$ channel blocker, 30 μmol/L TTX (n = 4 for each group). **C)** Blocking IP$_3$Rs with 2 μmol/L 2-APB reduces but does not eliminate the cytoplasmic Ca^{2+} transients (n = 8). Data were normalized with those before blocker was used. *$p<0.05$, compared with those before antagonist applied. **D)** Cytoplasmic Ca^{2+} transients are abolished by mitochondrial depolarization with 300 nmol/L FCCP and 1 μmol/L oligomycin (n = 5). Inhibiting mitochondrial Ca^{2+} release with 3 μmol/L CGP-37157 also terminates cytoplasmic Ca^{2+} transients (n = 6).

The features of APs and cytoplasmic Ca^{2+} transients recorded in Wt and RYR2$^{-/-}$ mouse ESC-derived ventricular-like CM are listed in Table 1 and Table 2. Compared to Wt CMs, RYR2 knockout (RYR2$^{-/-}$) CMs showed a smaller AP amplitude, a shorter AP duration, a more depolarized minimum diastolic membrane potential, a smaller cytoplasmic Ca^{2+} transient amplitude, a slower rate of rise of the Ca^{2+} transient, and a slower Ca^{2+} decay rate. The basal Ca^{2+} concentrations were similar, however.

Beating frequency was significantly slowed down by 2 μmol/L 2-APB from 1.3 ± 0.1 to 0.6 ± 0.1 Hz ($p<0.05$, Fig 3C and 3E). In RyR$^{-/-}$ ESC-derived CMs, the average beating rate was 1.39 ± 0.15 Hz and decreased to 0.52 ± 0.20 Hz after 2 μmol/L 2-APB was applied, suggesting that both SR Ca^{2+} release pathways are involved in generating spontaneous electrical activity. Cytoplasmic Ca^{2+} transients were similarly affected (Fig 5C). Inhibition of IP$_3$Rs by 2-APB did not decrease the beating rate further when MCU was silenced in ESC-derived ventricular-like RYR2$^{-/-}$ CMs (Fig 6D, 6E and 6F).

Our experiments show that mitochondrial Ca^{2+} cycling can influence spontaneous electrical activity of ventricular-like CMs, especially in states of reduced SR Ca^{2+} release. Blocking mitochondrial Ca^{2+} uptake pharmacologically or by knockdown resulted in reduced spontaneous beating. The effect was more pronounced in RYR2$^{-/-}$ CMs. Eliminating all mitochondrial Ca^{2+} flux in the absence of RYR2 prevented automaticity entirely, suggesting mitochondrial Ca^{2+} flux helps sustain spontaneous beating when SR Ca^{2+} release is inhibited. Reduced SR Ca^{2+} release and increased mitochondrial Ca^{2+} efflux are hallmarks of heart failure[29], implying that mitochondria may play a more prominent role in automatic rhythms occurring in heart failure.
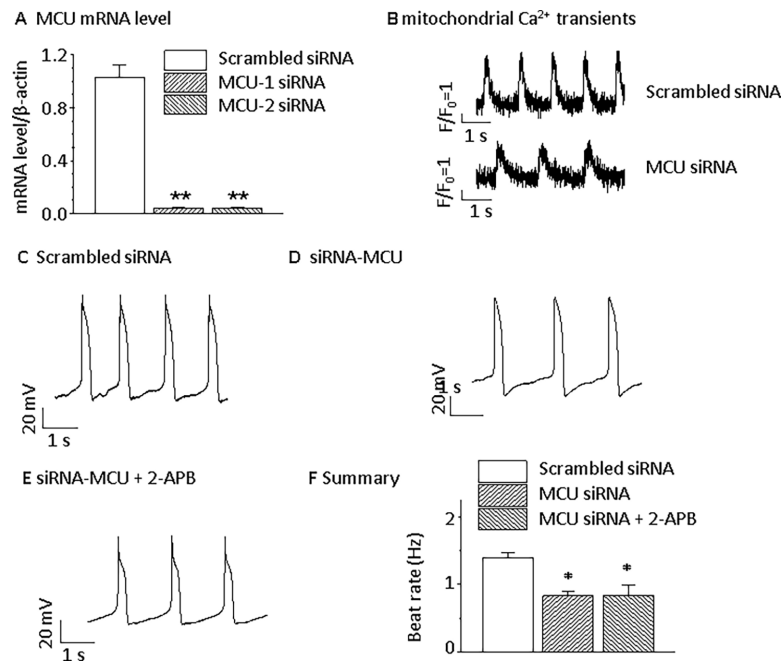
Fig 6. MCU knockdown alters spontaneous beating in RYR2$^{-/-}$ ventricular-like ESC-derived CMs. A) MCU mRNA was decreased by 96% after either of two MCU siRNAs for 72 h. $^{**}p<0.01$, compared with the scrambled siRNA group. **B**) MCU silencing by MCU-1 or MCU-2 siRNA for 72 h relative to scrambled siRNA controls results in reduced mitochondrial Ca^{2+} transients (n = 5 for each group). **C**) Representative APs recorded from a RYR2$^{-/-}$ ventricular-like CM after scrambled siRNA transfection and incubation for 72 h (n = 14). **D**) Spontaneous beating is depressed after MCU-1 or MCU-2 siRNA silence for 72 h (n = 16). **E**) Inhibition of IP$_3$R has no effect on spontaneous beating after MCU knockdown (n = 7). **F**) The summary of MCU siRNA effects on spontaneous beating. $^{*}p<0.05$, compared with scrambled siRNA groups.

Under normal conditions, SR Ca^{2+} release through RYR2 appears to play a modulatory role in spontaneous beating. Despite electrical remodeling with RYR2 knockout, eliminating SR Ca^{2+} release through RYR2 either pharmacologically or by knockout, resulted in slowing but not elimination of automaticity. The L-type Ca^{2+} channel and RYR2 form a tightly coupled pair when influencing spontaneous beating. This is consistent with other reports[12].

SR IP$_3$Rs also appeared to have a significant role in modulating spontaneous beating. Blocking IP$_3$R in the presence or absence of RYR2 slowed spontaneous beating. Knockdown of the mitochondrial Ca^{2+} uptake pathway, eliminated the effect of IP$_3$R inhibition on the spontaneous beating rate. These observations are most easily explained if SR Ca^{2+} release from IP$_3$R is tightly linked to mitochondrial Ca^{2+} handling. This is consistent with previous observations that Ca^{2+} is transferred directly between endoplasmic reticulum and mitochondria[37, 38]. It is well known that Ca^{2+} influx from the L-type Ca^{2+} channel can induce a larger Ca^{2+} release

Table 1. The characteristics of APs recorded in Wt and RYR2$^{-/-}$ ventricular-like CM.

	Wt	n	RYR2$^{-/-}$	n
APD90 (ms)	279 ± 25	9	196 ± 11*	6
APA (mV)	115 ± 4	9	92 ± 3*	6
RMP (mV)	-80.4 ± 2.6	9	-66.3 ± 2.0*	6

AP: action potential. APD90: AP duration at 90% repolarization. APA: AP amplitudes. RMP: resting membrane potentials. n: number of cells in each group. Data were presented as mean ± SE.

$^{*}p<0.05$ compared with that in WT group.

Table 2. The parameters of cytoplasmic Ca²⁺ transients recorded in Wt and RYR2⁻/⁻ ventricular-like CM.

	Amplitude (F/F₀)	Baseline (F/F₀)	time to 90% peak (ms)	TD50 (ms)
Wt Control	0.74 ± 0.09	1.23 ± 0.03	52 ± 1	211 ± 19
Wt Ryanodine	$0.40 \pm 0.05^{\dagger}$	1.14 ± 0.02	$189 \pm 3^{\dagger\dagger}$	$741 \pm 73^{\dagger}$
Wt 2-APB	$0.19 \pm 0.01^{\dagger\dagger}$	1.09 ± 0.01	$92 \pm 5^{\dagger}$	$326 \pm 13^{\dagger}$
RYR2⁻/⁻ Control	$0.38 \pm 0.02^{*}$	1.25 ± 0.03	$192 \pm 3^{**}$	$613 \pm 43^{*}$
RYR2⁻/⁻ Ryanodine	0.33 ± 0.02	1.17 ± 0.03	196 ± 4	601 ± 19
RYR2⁻/⁻ 2-APB	0.29 ± 0.03	1.24 ± 0.02	$254 \pm 6^{*}$	$615 \pm 23^{*}$

SR Ca²⁺ ryanodine receptor blocker, 10 μmol/L Ryanodine, and the IP₃R blocker, 2 μmol/L 2-APB, were used. TD50: transient duration at 50% amplitude. The number of cells in Wt Control, Wt Ryanodine, Wt 2-APB, RYR2⁻/⁻ Control, RYR2⁻/⁻ Ryanodine and RYR2⁻/⁻ 2-APB groups are 28, 21, 26, 21, 15 and 19 respectively. Data are presented as mean ± SE.

$^{*}p<0.05$

$^{**}p<0.01$ compared with that in WT group.

$^{\dagger}p<0.05$

$^{\dagger\dagger}p<0.01$ compared with that in control group.

from the SR. Normal cytoplasmic Ca²⁺ is then restored in part by the sarcolemmal NCX, which generates a depolarizing current. This can explain why blocking the L-type Ca²⁺ channel in ventricular-like ESC-derived Wt CMs can eliminate spontaneous beating.

The effect of mitochondrial Ca²⁺ flux on the automaticity of hiPSC-derived CMs

Inhibiting mitochondrial Ca²⁺ influx by 1 μmol/L Ru360 could decrease mitochondrial Ca²⁺ release, reduce the activity of sarcolemmal NCX, and reduce the rate of spontaneous beating in hiPSC-derived ventricular CMs (Fig 7A and 7B). The spontaneous beating rate was reduced
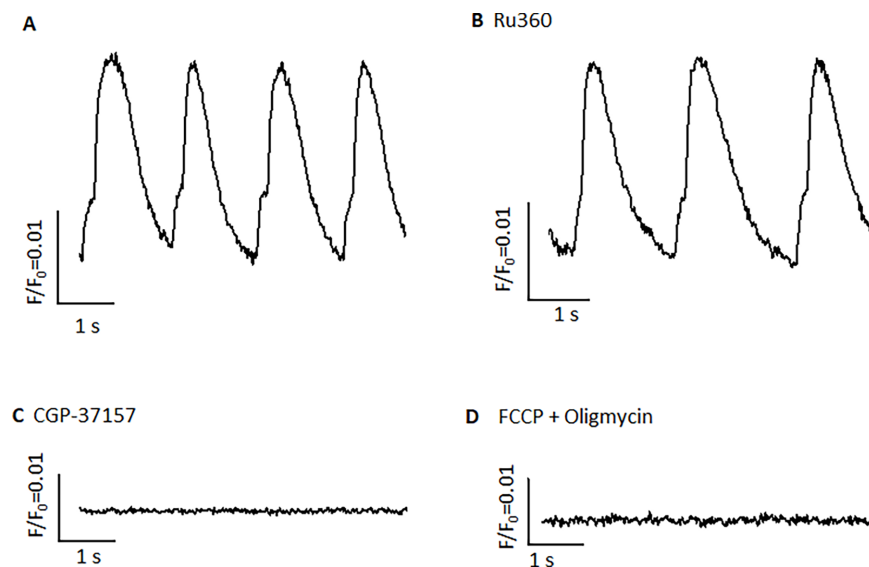

Fig 7. The effect of mitochondrial Ca²⁺ flux on spontaneously beating in hiPSC-derived ventricular-like CMs. A) Typical cytoplasmic Ca²⁺ transients in a hiPSC-derived CM. **B)** Inhibition effects of mitochondrial Ca²⁺ influx blocker, 1 μmol/L Ru360 (in pipette solution), on the beating rate of cytoplasmic Ca²⁺ transients. The spontaneous beating rate is reduced by 21.2 ± 4.3% (n = 10). **C)** The mitochondrial NCX (NCLX) blocker, 3 μmol/L CGP-37157, abolishes the automaticity of CMs (n = 11). **D)** Cytoplasmic Ca²⁺ transients are abolished by mitochondrial depolarization by 300 nmol/L FCCP and 1 μmol/L oligomycin (n = 9).

by 21.2 ± 4.3% (n = 10) after Ru360 within 2–5 min of when the drug was applied. The mitochondrial NCX blocker, 3 µmol/L CGP, eliminated Ca²⁺ transients in these CMs (Fig 7C) within 2–5 min of application. Depolarization of mitochondria and preservation of ATP by the combination of 300 nmol/L FCCP and 1 µmol/L oligomycin abolished the automaticity of hiPSC-derived ventricular CMs (Fig 7D). The [ATP]$_i$ level was not altered significantly during the combination of FCCP and oligomycin treatment (S1 Fig). These results were consistent with those obtained from mouse ESC-derived ventricular CMs.

Mitochondrial Ca²⁺ flux regulated spontaneous beating in acutely isolated, adult CMs

Mouse ES cell-derived ventricular CMs were chosen because of their stable nature in culture, the presence of spontaneous beating, and genetic tractability. Human iPSC-derived ventricular CMs were used to ensure the results applied across species. Now, we have added experiments with acutely isolated, adult mouse ventricular cardiac myocytes, showing that the results apply to mature cardiac cells. Unlike the ESC-derived or hiPSC-derived ventricular CMs, normal adult mouse ventricular CMs do not show spontaneous electrical activity[39].Nevertheless, abnormal automaticity can be elicited in matured ventricular CMs by acute ischemia[40]. In this study, simulated ischemic buffer was used to induce spontaneous beating in isolated adult mouse ventricular cells. Inhibiting mitochondrial Ca²⁺ influx by 1 µmol/L Ru360 could significantly reduce the beating rate from 0.051 ± 0.003 Hz to 0.037 ± 0.003 Hz in mouse ventricular CMs (Fig 8).

The electrophysiology of ventricular-like ESC-derived or hiPSC-derived CM may not recapitulate exactly the electrophysiology of intact ventricular cells. For example, they have reduced I$_{K1}$ and increased I$_f$ when compared to acutely isolated ventricular cells[20]. On the other hand, our findings that MCU inhibition alters spontaneous beating in adult ventricular CMs reinforces our central finding that mitochondrial Ca²⁺ handling plays a role in spontaneous electrical activity. This result is consistent with previous studies showing a role for mitochondrial Ca²⁺ handling in pacemaker/atrial cell automaticity and extends these results to
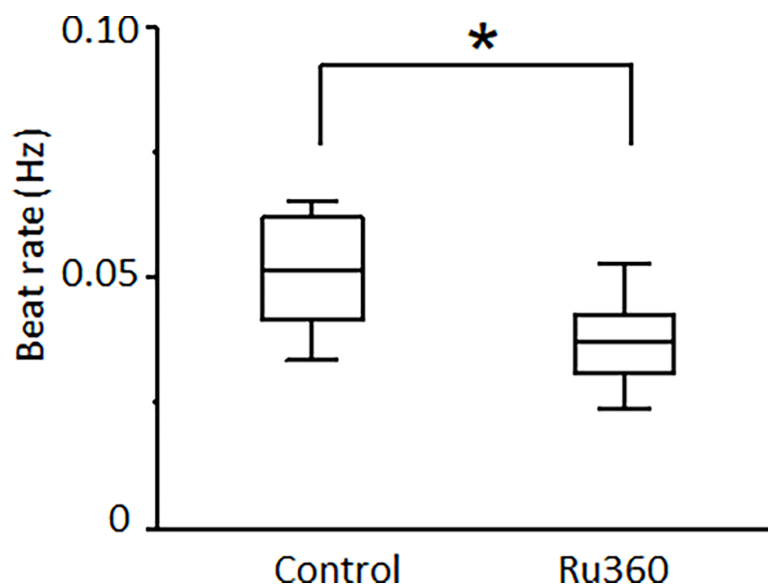

Fig 8. Regulation of abnormal automaticity by mitochondrial Ca²⁺ flux in ischemic mouse ventricular CMs. MCU blocker, 1 µmol/L Ru360 (in the pipette solution), reduces the ischemia-induced spontaneous beating rate of cytoplasmic Ca²⁺ transients in mouse ventricular CMs (*$p < 0.05$, compared with control groups. n = 11).

https://doi.org/10.1371/journal.pone.0200448.g008

ventricular cells. In the case of pacemaker cells, inhibition of MCU or the mitochondrial NCX had opposing effects on beating rate, with MCU inhibition accelerating beating. These effects were ascribed to a mitochondrial Ca^{2+} buffering effect that ultimately led to mitochondrial ATP production changes that balanced the metabolic needs of SR Ca^{2+} flux. In the case of ventricular-like CMs, automaticity was influenced differently from pacemaker cells by changes in mitochondrial Ca^{2+} handling, and inhibition of MCU or mitochondrial NCX had similar effects to reduce beating rate. Based on our data, it is reasonable to speculate that reduced mitochondrial Ca^{2+} handling caused reduced NCX activity as shown in Fig 9, with the effect of mitochondrial Ca^{2+} handling changes on spontaneous beating being independent of SR Ca^{2+} handling. Whether the influence of mitochondrial Ca^{2+} flux was independent of the M-clock or metabolic demand needs further study. Our findings suggest that the role of the mitochondria in spontaneous beating differs between pacemaker cells and ventricular-like CMs. The fact that mouse and human CMs showed similar responses suggests that the role of mitochondria in spontaneous beating is species independent and relevant to the human condition.

The state of mitochondrial maturity in our experiments is unknown. Nevertheless, the experiments show proof of principle that mitochondrial Ca^{2+} flux may contribute to spontaneous beating and possibly arrhythmic risk. In support of this idea, SR Ca^{2+} release in adult mouse ventricular myocytes contributes 90% of the Ca^{2+} transient [41]. The contribution of the SR Ca^{2+} flux to the Ca^{2+} transient was 72% in our ventricular-like ESC-derived CMs [8]. The later contribution is close to that in adult mouse ventricular cells after myocardial infarction[42]. Therefore, the effect of mitochondrial flux on spontaneous beating in adult mouse ventricular cells was studied in simulated ischemia condition.
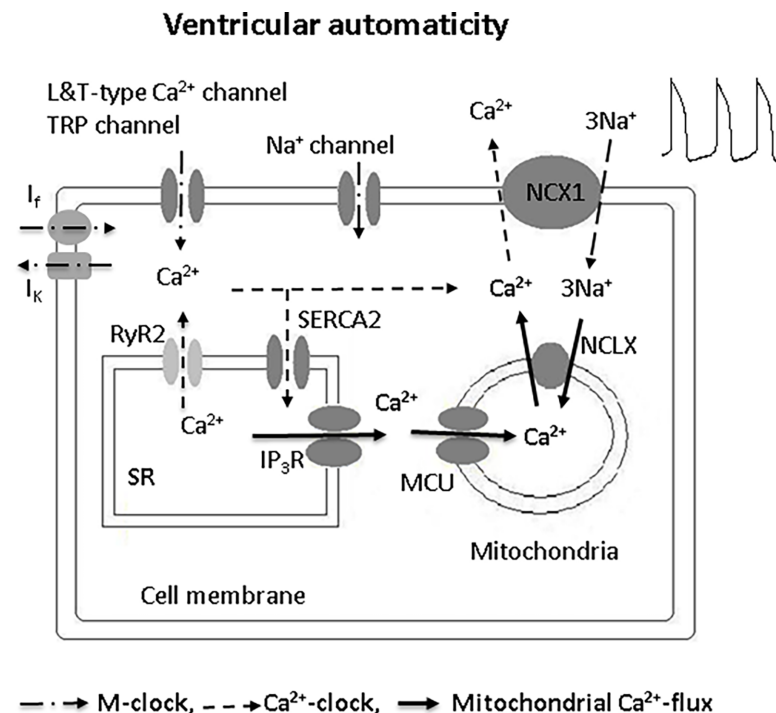

Fig 9. Proposed model of ion channels and transporters modulating spontaneous beating in ventricular-CMs. Sarcolemmal and mitochondrial NCXs are necessary for spontaneous beating. The mitochondrial membrane potential, L-type Ca^{2+} channels, and Na^{+} channels are also required. NCLX: mitochondrial NCX. NCX1: NCX type 1. Solid lines represent mitochondrial Ca^{2+} flux pathways for automaticity. Dashed lines show Ca^{2+} clock and dash dot line is M-clock (Voltage clock).

MCU activity is increased in ischemia and cardiomyopathy[43, 44]. Although none of these studies looked at arrhythmic risk, based on our results, the increased MCU activity in these disease states might be expected to enhance spontaneous beating in ventricular cells. Therefore, inhibiting MCU activity might reduce the risk of arrhythmias based on the expected enhanced or abnormal automaticity.

Conclusions

Spontaneous beating in ventricular cells involves mitochondrial Ca^{2+} flux and sarcolemmal NCX currents. RYR2/L-type Ca^{2+} channel and IP$_3$R/MCU pairs appear to form functional units of Ca^{2+} handling. These results suggest that mitochondria may influence ventricular spontaneous depolarization and abnormal automaticity by altering mitochondrial Ca^{2+} handling (Fig 9).

Supporting information

S1 Fig. Intracellular free ATP level was not altered significantly during the combination of FCCP and oligomycin treatment. The average effect of FCCP (w/wo oligomycin) on [Mg^{2+}]$_i$ in hiPSC-derived ventricular-like CMs (n = 10 for each group).
(PDF)

Acknowledgments

Author Contributions

Conceptualization: Kenneth R. Boheler, Samuel C. Dudley, Jr.

Data curation: An Xie, Anyu Zhou, Man Liu.

Formal analysis: An Xie, Hong Liu, Samuel C. Dudley, Jr.

Funding acquisition: Samuel C. Dudley, Jr.

Investigation: An Xie, Anyu Zhou, Hong Liu, Guangbin Shi, Man Liu.

Methodology: An Xie, Anyu Zhou, Hong Liu, Guangbin Shi, Man Liu.

Project administration: Samuel C. Dudley, Jr.

Resources: Kenneth R. Boheler.

Supervision: Samuel C. Dudley, Jr.

Validation: Samuel C. Dudley, Jr.

Visualization: An Xie.

Writing – original draft: An Xie, Samuel C. Dudley, Jr.

Writing – review & editing: An Xie, Anyu Zhou, Hong Liu, Guangbin Shi, Man Liu, Kenneth R. Boheler.

Writing – review . . . editing: Samuel C. Dudley, Jr.

References

1. Bogdanov KY, Vinogradova TM, Lakatta EG. Sinoatrial nodal cell ryanodine receptor and Na$^+$-Ca^{2+} exchanger: molecular partners in pacemaker regulation. Circ Res. 2001; 88: 1254–8. PMID: 11420301

2. Huser J, Blatter LA, Lipsius SL. Intracellular Ca^{2+} release contributes to automaticity in cat atrial pacemaker cells. J Physiol. 2000; 524 Pt 2: 415–22.

3. Yang HT, Tweedie D, Wang S, Guia A, Vinogradova T, Bogdanov K, et al. The ryanodine receptor modulates the spontaneous beating rate of cardiomyocytes during development. Proc Natl Acad Sci USA. 2002; 99: 9225–30. https://doi.org/10.1073/pnas.142651999 PMID: 12089338

4. Zahanich I, Sirenko SG, Maltseva LA, Tarasova YS, Spurgeon HA, Boheler KR, et al. Rhythmic beating of stem cell-derived cardiac cells requires dynamic coupling of electrophysiology and Ca cycling. J Mol Cell Cardiol. 2011; 50: 66–76. https://doi.org/10.1016/j.yjmcc.2010.09.018 PMID: 20920509

5. Koushik SV, Wang J, Rogers R, Moskophidis D, Lambert NA, Creazzo TL, et al. Targeted inactivation of the sodium-calcium exchanger (Ncx1) results in the lack of a heartbeat and abnormal myofibrillar organization. FASEB J. 2001; 15: 1209–11. PMID: 11344090

6. Lieu DK, Fu JD, Chiamvimonvat N, Tung KC, McNerney GP, Huser T, et al. Mechanism-based facilitated maturation of human pluripotent stem cell-derived cardiomyocytes. Circ.Arrhythm.Electrophysiol. 2013; 6: 191–201. https://doi.org/10.1161/CIRCEP.111.973420 PMID: 23392582

7. Satoh H, Sperelakis N. Hyperpolarization-activated inward current in embryonic chick cardiac myocytes: developmental changes and modulation by isoproterenol and carbachol. Eur.J.Pharmacol. 1993; 240: 283–90. PMID: 8243543

8. Kapur N, Banach K. Inositol-1,4,5-trisphosphate-mediated spontaneous activity in mouse embryonic stem cell-derived cardiomyocytes. J.Physiol. 2007; 581: 1113–27. https://doi.org/10.1113/jphysiol.2006.125955 PMID: 17379641

9. Yaniv Y, Tsutsui K, Lakatta EG. Potential effects of intrinsic heart pacemaker cell mechanisms on dysrhythmic cardiac action potential firing. Front Physiol. 2015; 6: 47. https://doi.org/10.3389/fphys.2015.00047 PMID: 25755643

10. Yaniv Y, Lakatta EG, Maltsev VA. From two competing oscillators to one coupled-clock pacemaker cell system. Front Physiol. 2015; 6: 28. https://doi.org/10.3389/fphys.2015.00028 PMID: 25741284

11. Drago I, De SD, Rizzuto R, Pozzan T. Mitochondrial Ca^{2+} uptake contributes to buffering cytoplasmic Ca^{2+} peaks in cardiomyocytes. Proc.Natl.Acad.Sci.U.S.A. 2012; 109: 12986–91. https://doi.org/10.1073/pnas.1210718109 PMID: 22822213

12. Takeuchi A, Kim B, Matsuoka S. The mitochondrial Na^{+}-Ca^{2+} exchanger, NCLX, regulates automaticity of HL-1 cardiomyocytes. Sci.Rep. 2013; 3: 2766. https://doi.org/10.1038/srep02766 PMID: 24067497

13. Yaniv Y, Spurgeon HA, Lyashkov AE, Yang D, Ziman BD, Maltsev VA, et al. Crosstalk between mitochondrial and sarcoplasmic reticulum Ca^{2+} cycling modulates cardiac pacemaker cell automaticity. PLoS.One. 2012; 7: e37582. https://doi.org/10.1371/journal.pone.0037582 PMID: 22666369

14. Saotome M, Katoh H, Satoh H, Nagasaka S, Yoshihara S, Terada H, et al. Mitochondrial membrane potential modulates regulation of mitochondrial Ca^{2+} in rat ventricular myocytes. Am.J.Physiol Heart Circ.Physiol. 2005; 288: H1820–8. https://doi.org/10.1152/ajpheart.00589.2004 PMID: 15563537

15. Yang KC, Bonini MG, Dudley SC, Jr. Mitochondria and arrhythmias. Free Radic Biol Med. 2014; 71C: 351–61.

16. Campanella M, Pinton P, Rizzuto R. Mitochondrial Ca^{2+} homeostasis in health and disease. Biol.Res. 2004; 37: 653–60. PMID: 15709694

17. Wobus AM, Guan K, Yang HT, Boheler KR. Embryonic stem cells as a model to study cardiac, skeletal muscle, and vascular smooth muscle cell differentiation. Methods Mol.Biol. 2002; 185: 127–56. PMID: 11768985

18. Zhang YM, Hartzell C, Narlow M, Dudley SC Jr. Stem cell-derived cardiomyocytes demonstrate arrhythmic potential. Circulation. 2002; 106: 1294–9. PMID: 12208808

19. Zhang YM, Shang L, Hartzell C, Narlow M, Cribbs L, Dudley SC Jr. Characterization and regulation of T-type Ca^{2+} channels in embryonic stem cell-derived cardiomyocytes. Am.J.Physiol Heart Circ.Physiol. 2003; 285: H2770–9. https://doi.org/10.1152/ajpheart.01114.2002 PMID: 12919937

20. Ma J, Guo L, Fiene SJ, Anson BD, Thomson JA, Kamp TJ, et al. High purity human-induced pluripotent stem cell-derived cardiomyocytes: electrophysiological properties of action potentials and ionic currents. Am J Physiol Heart Circ Physiol. 2011; 301: H2006–17. https://doi.org/10.1152/ajpheart.00694.2011 PMID: 21890694

21. Wang J, Drake L, Sajjadi F, Firestein GS, Mullane KM, Bullough DA. Dual activation of adenosine A1 and A3 receptors mediates preconditioning of isolated cardiac myocytes. Eur J Pharmacol. 1997; 320: 241–8. PMID: 9059860

22. Mackenzie L, Bootman MD, Laine M, Berridge MJ, Thuring J, Holmes A, et al. The role of inositol 1,4,5-trisphosphate receptors in Ca^{2+} signalling and the generation of arrhythmias in rat atrial myocytes. J. Physiol. 2002; 541: 395–409. https://doi.org/10.1113/jphysiol.2001.013411 PMID: 12042347

23. Kohlhaas M, Liu T, Knopp A, Zeller T, Ong MF, Bohm M, et al. Elevated cytosolic Na⁺ increases mitochondrial formation of reactive oxygen species in failing cardiac myocytes. Circulation. 2010; 121: 1606–13. https://doi.org/10.1161/CIRCULATIONAHA.109.914911 PMID: 20351235

24. Maack C, Cortassa S, Aon MA, Ganesan AN, Liu T, O'Rourke B. Elevated cytosolic Na⁺ decreases mitochondrial Ca²⁺ uptake during excitation-contraction coupling and impairs energetic adaptation in cardiac myocytes. Circ.Res. 2006; 99: 172–82. https://doi.org/10.1161/01.RES.0000232546.92777.05 PMID: 16778127

25. De SD, Raffaello A, Teardo E, Szabo I, Rizzuto R. A forty-kilodalton protein of the inner membrane is the mitochondrial calcium uniporter. Nature. 2011; 476: 336–40. https://doi.org/10.1038/nature10230 PMID: 21685888

26. Di Diego JM, Antzelevitch C. Ischemic ventricular arrhythmias: experimental models and their clinical relevance. Heart Rhythm. 2011; 8: 1963–8. https://doi.org/10.1016/j.hrthm.2011.06.036 PMID: 21740880

27. Bround MJ, Asghari P, Wambolt RB, Bohunek L, Smits C, Philit M, et al. Cardiac ryanodine receptors control heart rate and rhythmicity in adult mice. Cardiovasc.Res. 2012; 96: 372–80. https://doi.org/10.1093/cvr/cvs260 PMID: 22869620

28. Dobrzynski H, Boyett MR, Anderson RH. New insights into pacemaker activity: promoting understanding of sick sinus syndrome. Circulation. 2007; 115: 1921–32. https://doi.org/10.1161/CIRCULATIONAHA.106.616011 PMID: 17420362

29. Luo M, Anderson ME. Mechanisms of altered Ca²⁺ handling in heart failure. Circ.Res. 2013; 113: 690–708. https://doi.org/10.1161/CIRCRESAHA.113.301651 PMID: 23989713

30. Shigekawa M, Iwamoto T. Cardiac Na⁺-Ca²⁺ exchange: molecular and pharmacological aspects. Circ. Res. 2001; 88: 864–76. PMID: 11348995

31. Bucchi A, Baruscotti M, DiFrancesco D. Current-dependent block of rabbit sino-atrial node I_f channels by ivabradine. J.Gen.Physiol. 2002; 120: 1–13. https://doi.org/10.1085/jgp.20028593 PMID: 12084770

32. Cox DA, Conforti L, Sperelakis N, Matlib MA. Selectivity of inhibition of Na⁺-Ca²⁺ exchange of heart mitochondria by benzothiazepine CGP-37157. J.Cardiovasc.Pharmacol. 1993; 21: 595–9. PMID: 7681905

33. Brennan JP, Berry RG, Baghai M, Duchen MR, Shattock MJ. FCCP is cardioprotective at concentrations that cause mitochondrial oxidation without detectable depolarisation. Cardiovasc.Res. 2006; 72: 322–30. https://doi.org/10.1016/j.cardiores.2006.08.006 PMID: 16979603

34. Matlib MA, Zhou Z, Knight S, Ahmed S, Choi KM, Krause-Bauer J, et al. Oxygen-bridged dinuclear ruthenium amine complex specifically inhibits Ca²⁺ uptake into mitochondria in vitro and in situ in single cardiac myocytes. J.Biol.Chem. 1998; 273: 10223–31. PMID: 9553073

35. Zhao Z, Gordan R, Wen H, Fefelova N, Zang WJ, Xie LH. Modulation of intracellular calcium waves and triggered activities by mitochondrial ca flux in mouse cardiomyocytes. PLoS.One. 2013; 8: e80574. https://doi.org/10.1371/journal.pone.0080574 PMID: 24348912

36. Zima AV, Pabbidi MR, Lipsius SL, Blatter LA. Effects of mitochondrial uncoupling on Ca²⁺ signaling during excitation-contraction coupling in atrial myocytes. Am J Physiol Heart Circ Physiol. 2013; 304: H983–93. https://doi.org/10.1152/ajpheart.00932.2012 PMID: 23376829

37. Collins S, Meyer T. Cell biology: A sensor for calcium uptake. Nature. 2010; 467: 283. https://doi.org/10.1038/467283a PMID: 20844529

38. Perocchi F, Gohil VM, Girgis HS, Bao XR, McCombs JE, Palmer AE, et al. MICU1 encodes a mitochondrial EF hand protein required for Ca²⁺ uptake. Nature. 2010; 467: 291–6. https://doi.org/10.1038/nature09358 PMID: 20693986

39. Liao SY, Liu Y, Siu CW, Zhang Y, Lai WH, Au KW, et al. Proarrhythmic risk of embryonic stem cell-derived cardiomyocyte transplantation in infarcted myocardium. Heart Rhythm. 2010; 7: 1852–9. https://doi.org/10.1016/j.hrthm.2010.09.006 PMID: 20833268

40. Bouchard S, Jacquemet V, Vinet A. Automaticity in acute ischemia: bifurcation analysis of a human ventricular model. Phys Rev E Stat Nonlin Soft Matter Phys. 2011; 83: 011911. https://doi.org/10.1103/PhysRevE.83.011911 PMID: 21405717

41. Li L, Chu G, Kranias EG, Bers DM. Cardiac myocyte calcium transport in phospholamban knockout mouse: relaxation and endogenous CaMKII effects. Am.J.Physiol. 1998; 274: H1335–H47. PMID: 9575939

42. Louch WE, Hake J, Jolle GF, Mork HK, Sjaastad I, Lines GT, et al. Control of Ca²⁺ release by action potential configuration in normal and failing murine cardiomyocytes. Biophys.J. 2010; 99: 1377–86. https://doi.org/10.1016/j.bpj.2010.06.055 PMID: 20816049

43. Joiner ML, Koval OM, Li J, He BJ, Allamargot C, Gao Z, et al. CaMKII determines mitochondrial stress responses in heart. Nature. 2012; 491: 269–73. https://doi.org/10.1038/nature11444 PMID: 23051746

44. Kwong JQ, Lu X, Correll RN, Schwanekamp JA, Vagnozzi RJ, Sargent MA, et al. The mitochondrial calcium uniporter selectively matches metabolic output to acute contractile stress in the heart. Cell Rep. 2015; 12: 15–22. https://doi.org/10.1016/j.celrep.2015.06.002 PMID: 26119742