Online supplement for

Individual Cardiac Mitochondria undergo rare Transient Permeability Transition Pore openings

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Supplementary Methods

Cardiac Myocyte isolation, dye loading and permeabilization

Cardiac ventricular myocytes were isolated from adult male C57b/6J mice and those lacking MCU [1] or CypD [2] using previously described routine methods [3] and were approved by the UC Davis Institutional Animal Care and Use Committee (IACUC). Freshly isolated myocytes were plated on laminin-coated glass cover slip for >45 min before dye loading. All experiments were performed at room temperature (RT) (22-23°C). Cells were loaded with 10μM Fluo-8 AM for 45 min, or Rhod-2 AM or 1nM TMRM for 30 min, both at 23°C in nominally Ca^{2+} free Tyrode's solution (in mmol/L: HEPES 5, NaCl 140, KCl 140, $MgCl_2$ 1, glucose 10; pH adjusted to 7.4 with NaOH). 30 min were allowed for de-esterification. The $\Delta\Psi_m$ in intact myocyte was measured by TMRM under field stimulation at 1Hz with 1.8 mmol/L extracellular [Ca^{2+}]. For plasma membrane permeabilization, cells were exposed to saponin (50 μg/ml; 30 sec) which was then washed off, in standard intracellular relaxing solution containing (in mmol/L) HEPES 10, K-aspartate 135, $MgCl_2$ 0.7, EGTA 2, reduced glutathione 10, MgATP 5, glucose 10, pH 7.2.

Solutions

A Ca^{2+}-buffered, Ca^{2+}-free, Na^+-free internal solution contained (in mmol/L): EGTA 5, HEPES 20, K-aspartate 100, KCl 40, $MgCl_2$ 1, maleic acid 2, glutamic acid 2, pyruvic acid 5, KH_2PO_4 0.5, pH 7.2 adjusted with trisma base. To control $[Ca^{2+}]_i$, 0.1 M $CaCl_2$ solution was added as calculated with MaxChelator. Free [Ca^{2+}] was confirmed by Ca-sensitive electrode. Na^+-free internal solution with low Ca^{2+} buffering capacity and 100 nM free [Ca^{2+}] contained (in mmol/L): EGTA 0.05, $CaCl_2$ 0.0234, HEPES 20, K-aspartate 100, KCl 40, $MgCl_2$ 0.551, maleic acid 2, glutamic acid 2, pyruvic acid 5, KH_2PO_4 0.5, MgATP 5, pH 7.2 adjusted with trisma base. When NaCl was added to these solutions, equal amounts of KCl were omitted. 80 μM cytochalasin D was included to inhibit myocyte contraction. Ca^{2+} calibration solutions contained 2 μM ionomycin, 10 μM FCCP and 20 μg/ml oligomycin.

Confocal imaging scanning

Mitochondrial Rhod-2 and TMRM fluorescence were measured (Zeiss LSM 5 live confocal microscope, 60X water-immersive objective) in 2-dimensional imaging mode with excitation at 532 nm, with emission at > 560 nm. Fluo-4, Fluo-8 and FAD autofluorescent signals were measured with same confocal (488 nm excitation, emission at 530 ± 15 nm). Calcien signals were collected using excitation at 488 nm and emission at 530 ± 15 nm. Time-lapse x,y images were acquired at 512 bit resolution and at the sampling rate of 507 ms per frame. Region of interest size for analysis of single (or two) mitochondria was ~1×2 μm, centered on an identifiable mitochondrion.

Chemicals and statistics

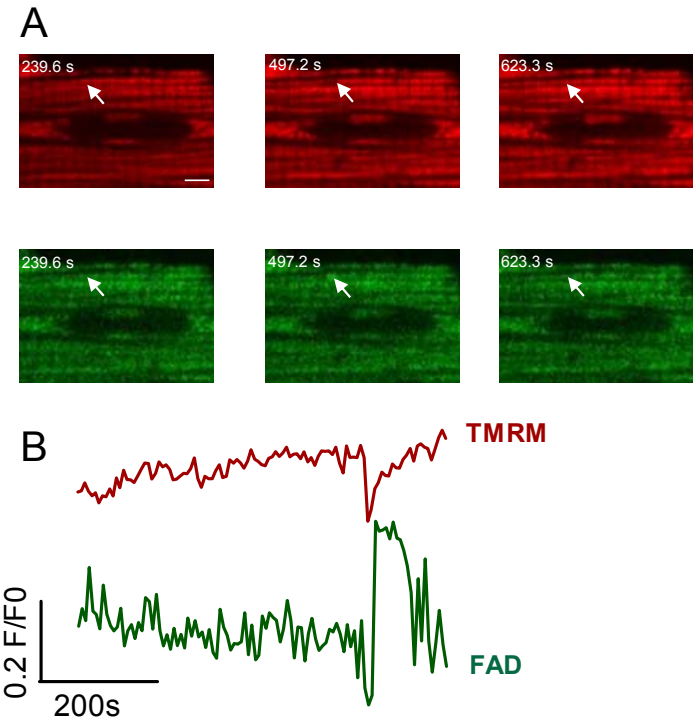
Indicators were obtained from Invitrogen (Eugene, OR), Ru360 from Calbiochem (La Jolla, CA). Data are presented as mean ± SE of n measurements. Comparison between groups used Student's t-test, One-Way ANOVO and Two-Way ANOVO (significant at $p<0.05$)

Heart failure model

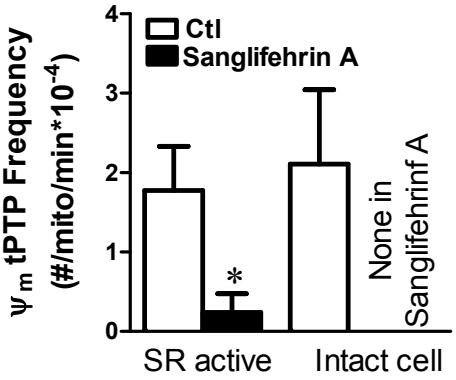
We used transverse aortic constriction in mice to induce HF. Briefly, after a 5-nmol/L thoracotomy is made lateral to the left sternal border, two loose knots were tied around the transverse aorta, and a blunt 27-gauge needle was then placed parallel to the transverse aorta and inside the first loose knot. The first knot was then tied tightly, followed by the second. Immediately after, the needle was taken out, yielding a constriction of 0.4mm in diameter. With this amount of ligation, HF developed in 6-8 weeks after surgery. Experiments with cardiac cells were performed 8 weeks post-surgery.

Echocardiography

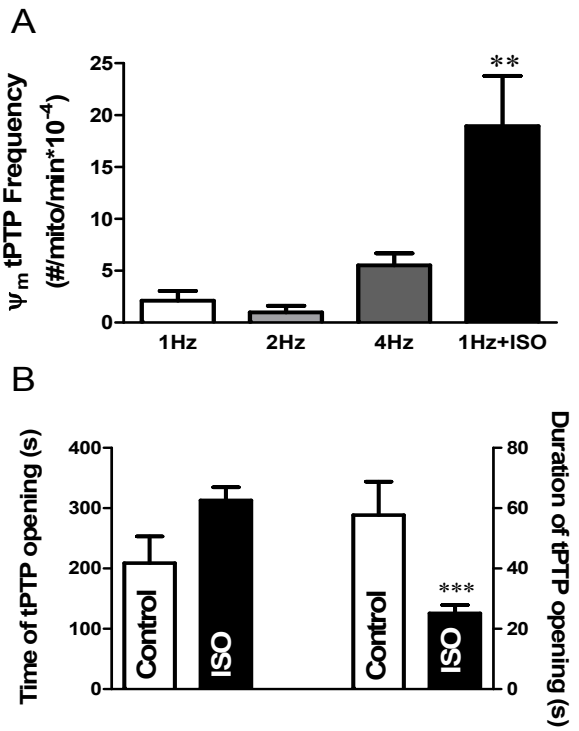
Mice were anesthetized in an isoflurane chamber prior to placing them in the supine position on an ECG platform. The paws of the mouse were taped down with electrode cream onto the ECG sensors, while the nose was connected to a nozzle that delivers 95% O_2 / 5% CO_2 with a concentration of isoflurane that maintains the mouse's heart rate at 550 ± 50 bpm. M-mode echocardiography was performed a day before surgery and eight weeks after surgery using the Visualsonics Vevo 2100 system.



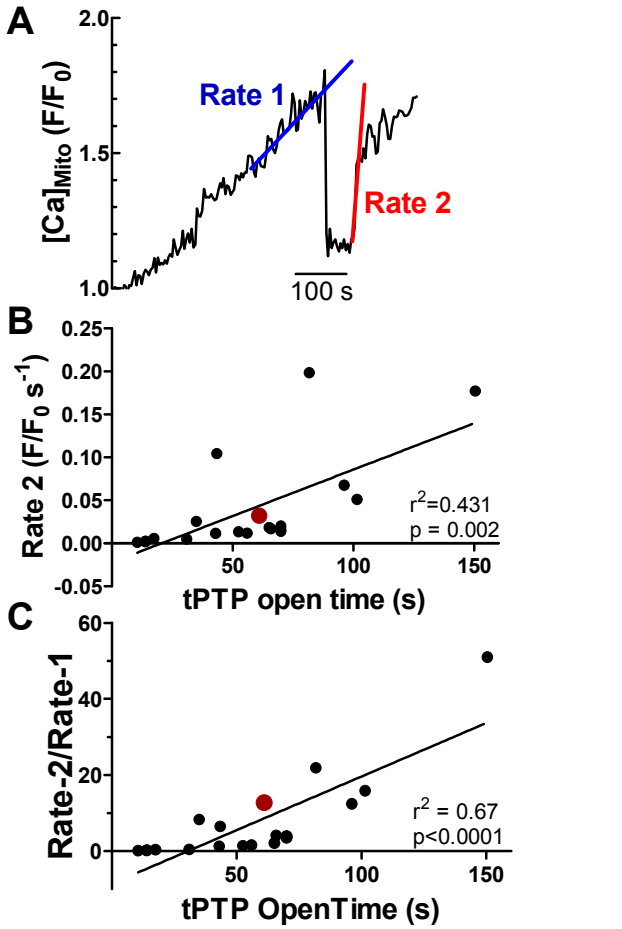
Online Figure I. Transient PTP openings cause temporary mitochondrial depolarization ($\Delta\Psi_m$) and increase oxidation of FAD. (A) Colocalization of $\Delta\Psi_m$ indicator TMRM (red) and auto- fluorescence of FAD (green) during 1Hz pacing in intact cardiac myocytes. (B) Traces were obtained from an individual mitochondrion (or pair), as indicated by white arrows. Depolarization preceded oxidation of FAD and then a gradual increase of reduction of FAD. (Scale bar =4 μm)



Online Figure II. Frequency of tPTP events in the absence and presence of Sangllifehrin A in intact (at 1Hz pacing) and permeabilized cardiac myocytes (*P<0.05, vs Ctl, n=5-7).



Online Figure III. Frequency of transient PTP opening during pacing and β-adrenergic stimulation. (A) Frequency of tPTP openings (using TMRM to assess $\Delta\Psi_m$) in intact cardiac myocytes at different pacing frequency. And the influence of 50 nmol/L ISO on tPTP frequency at 1Hz pacing. (B) The time point at which tPTP opening occurred and its duration of opening (**P<0.01, vs 1Hz, n=6).



Online Figure IV. Correlation of Ca reuptake rate and duration time of the pore opening. (A) Rate of $[Ca^{2+}]_{mito}$ rise before (Rate 1) and after tPTP opening (Rate 2). (B) Rate of $[Ca^{2+}]_{mito}$ rise (Rate 2) as a function of duration of tPTP opening. (C) Rate of $[Ca^{2+}]_{mito}$ rise after tPTP normalized to that before (Rate 2/Rate 1) vs. tPTP open time. Trace in A is the individual mitochondrion indicated by the red dot.

References :

1. Kwong JQ, Lu X, Correll RN, Schwanekamp JA, Vagnozzi RJ, Sargent MA, York AJ, Zhang J, Bers DM, Molkentin JD. The Mitochondrial Calcium Uniporter Selectively Matches Metabolic Output to Acute Contractile Stress in the Heart. *Cell Rep*. 2015;12(1):15-22.

2. Baines CP, Kaiser RA, Purcell NH, Blair NS, Osinska H, Hambleton MA, Brunskill EW, Sayen MR, Gottlieb RA, Dorn GW, Robbins J and Molkentin JD. Loss of cyclophilin D reveals a critical role for mitochondrial permeability transition in cell death. *Nature.* 2005;434:658-62.

3. Pereira L, Cheng H, Lao DH, Na L, van Oort RJ, Brown JH, Wehrens XH, Chen J, Bers DM. Epac2 mediates cardiac β1-adrenergic-dependent sarcoplasmic reticulum Ca^{2+} leak and arrhythmia. *Circulation.* 2013;127(8):913-22.