Expanded Materials & Methods

Embryonic ventricular myocyte (EVM) tissue harvest or cell culture.

All animals & animal procedures used in this study were approved by the University of Kentucky Institutional Animal Care and Use Committee. E10 or E16 mouse (ICR outbred strain, Harlan) hearts were dissected free of connective tissues, and ventricles were separated from conotruncus and sinus venosus, or atria. Cells were enzymatically dispersed and cultured as previously described[1]. Briefly, 10-40 embryos were minced and quickly transferred to nominally Ca^{2+}-free digestion buffer containing 0.5mg/mL collagenase (type II, Worthington) and 1 mg/mL pancreatin for two 15-minute cycles. Digested tissue yielded a large fraction of single spontaneously beating cells in culture media consisting of DMEM+10% FBS. Cells were transfected in 24 well plates using Lipofectamine transfection reagent (Invitrogen). Cells were transfected with either a GFP-Rem fusion construct, or GFP (control).

Cytosolic Calcium Imaging

Cardiac myocytes were loaded with 2 μM fura-2-AM for 10 minutes in a 5% CO_2 incubator and then de-esterified in Tyrodes (140 NaCl2, 1.8 CaCl2, 1 MgCl2, 10 Hepes (free acid), 5.4 KCl, 10 Glucose, pH = 7.4, NaOH) solution for ~20 minutes. Spontaneous Ca^{2+} transients were recorded from the annulus of the photometry tube focused on a single cell or a cell cluster. All recordings were performed at 37^{o}C. The cells were excited with light of 340 nm and 380 nm wavelengths. The images obtained at 340 nm and 380 nm were divided pixel by pixel and the ratio data was reported. Data was collected and analyzed with IonOptix (Milton, MA) hardware and software. Additional off-line transient analysis was performed with custom routines written in MatLab (Northampton, MA). Exponential fitting was performed using PClamp 9.2 (Axon Instruments, Union City, CA). The function used for exponential fitting of caffeine-induced Ca^{2+} transient (CaffTr) relaxations was $f(t) = (n, i = 1) \Sigma A_i * \exp(-t/\tau_i) + C$. Background fluorescence was subtracted. Then, the measurement of diastolic calcium was made

subtracting the baseline of recording in 1.8 mM bath Ca^{2+} solution (diastolic) from lowest point between 20 and 25 seconds after 0 bath Ca^{2+} (140 NaCl2, 0 CaCl2, 1 MgCl2, 10 Hepes (free acid), 5.4 KCl, 10 Glucose, 5 EGTA, pH = 7.4, NaOH was introduced (basal calcium level). Calibrating the system to give exact concentrations of cytosolic calcium concentrations were attempted by using various methods such as ionophores and permeablizing the cells with detergents. We determined that these measurements were unreliable and not reproducible. Therefore we chose the subtraction of the diastolic from the basal level to give the most accurate measure of diastolic calcium levels. GFP-transfected or unstransfected cells had indistinguishable Ca transients, and responses to bath manipulations.

Real-time RT-PCR

Total RNA was isolated using the RNAqueous -4PCR kit (Ambion) and quantitated spectrophotometrically at 260nm. Contaminating genomic DNA was eliminated by DNase treatment (Ambion). A portion of the resulting RNA (1 μg) was immediately used as a template for cDNA synthesis. Reverse transcription was performed using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). Removal of genomic DNA was confirmed by preparing a no reverse transcription control for each sample. cDNA was then stored at -20^{o}C.

Real-time PCR was performed in 96-well optical plates in triplicate using an ABI 7700 Sequence Detector. Samples (0.2 ng cDNA) were prepared using the TaqMan PCR core Reagent Kit (Applied Biosystems) using the following primer and probe sets: Rem: Forward 5'-GCGCCTACGTCATCGTGTACT-3', Reverse 5'-TCCGAGGCGCTCTCAAAG-3', Probe 5'-FAM-CATAGCGGATCGCAGCA-TAMRA-3'; NCX1: Forward 5'GACGGTGAGAATCTGGAACGA-3', Reverse 5'-AAGATCCCAGGGCCATCAA-3', Probe 5'-FAM-ACTGTGTCGAACCTGAC-

TAMRA-3'; Cyclophilin A: Forward 5'-GGATGAGAACTTCATCCTAAAGCA-3', Reverse 5'-CCACAGTCGGAAATGGTGA-3', Probe 5'-FAM-ACTGAATGGCTGGATGGCAAGCATGT-TAMRA-3'. Samples were cycled for 50 cycles using an ABI 7700 Sequence Detector (Applied Biosystems). Cycle conditions were as follows: 2 minutes at 50°C, 10 minutes at 95°C followed by 50 cycles of 15 seconds at 95°C and 1 minute at 60°C. Primers were chosen which provided an efficiency of 90 to 100%. In all cases a single amplicon of the appropriate size was detected using gel electrophoresis. Results are expressed normalized to Cyclophilin A according to the $2^{-\Delta Ct}$ method[2]. No RT controls were prepared for each sample and showed no amplification.

SDS-PAGE and Western Blot Analysis of NCX1.1

Whole cell lysates were prepared from transfected (RFP Rem and RFP) E10 cells 3 days post transfection. SDS-PAGE (7.5% separating gel, Biorad) and immunoblotting were carried out following routine protocols. Mouse monoclonal NCX1 antibody (Novus Biologicals) was visualized with a horseradish peroxidase-conjugated secondary antibody (Molecular Probes) and Super Signal West Pico Chemiluminescence (Pierce). Each lane contained protein 10 µg total protein. All Western blot experiments were repeated at least 3 times to ensure that experimental observations were reproducible. Loading was confirmed by stripping (Restore Western Blot Stripping Buffer, Pierce) and reprobing blots with GAPDH monoclonal antibody (Ambion). Immunoblots were scanned on an Epson Perfection 1650 and quantified using densitometry (Scion Image, Scion Corporation).

Computer simulations

To simulate calcium transients in a myocyte, we used the Shannon-Bers Model[3] which can be downloaded from: (http://www.luhs.org/depts/physio/personal_pages/bers_d/index.html). The Shannon-Bers model is a set of ordinary differential equations which simulate excitation-contraction coupling in a mature rabbit ventricular myocyte. The downloaded file contains the CVODE package (http://www.netlib.org/ode/index.html) which solves the system of ordinary differential equations. This entire package was compiled on a linux (Mandrake 9.2) workstation.

To reproduce some features of the experimental results, we removed the reverse mode of the SERCA pump, and we adjusted the following parameters: GCaB (0.00025 -> 0.0015 mS/μF); INaCaX_Vmf(9.0 -> 18.0 A/F); and ISRCaP_Vmf(286 -> 200 μmol/l cytosol/s). To model LCC block, we decreased the permeability of ions through LCC by a factor of 0.01.

References cited in supplemental Materials & Methods

1. Cribbs LL, Martin BL, Schroder EA, Keller BB, Delisle BP, Satin J. Identification of the T-Type Calcium Channel $Ca_V3.1d$ in Developing Mouse Heart. *Circ Res*. 2001;88:403-407.

2. Gaborit N, Steenman M, Lamirault G, Le Meur N, Le Bouter S, Lande G, Leger J, Charpentier F, Christ T, Dobrev D, Escande D, Nattel S and Demolombe S. Human atrial ion channel and transporter subunit gene-expression remodeling

associated with valvular heart disease and atrial fibrillation. *Circulation* 112: 471-481, 2005.

3. Shannon TR, Wang F, Puglisi J, Weber C, Bers DM. A mathematical treatment of integrated Ca dynamics within the ventricular myocyte. *Biophys J*. 2004;87:3351-71.

Figure Legend for Supplemental Figure

Scattergram of time constants of CaffTr relaxation. Note that fast component is eliminated by 10 mM Ni^{2+} .