Supplemental Material

Materials and Methods

Real-Time Polymerase Chain Reaction (RT-PCR)

RNA was isolated from hearts using QIAshredder homogenization and an RNeasy Plus kit (Qiagen). Reverse transcription (RT) reaction was performed using the SuperScript III first-strand synthesis system for RT-PCR (Invitrogen) and oligo-dt primers according to the manufacturer’s instructions. Real-time PCR was performed using the Quantifast Sybrgreen PCR kit (Qiagen). Data were normalized to 18S RNA expression. The following primer sets were used (forward, reverse): 18s 5’- GTAACCCGTTGAACCCATT, 5’-CCATCCAATCGGTAGTACCG; atrial natriuretic factor (ANF) 5’-GCCCTGAGTGACGACTG, 5’-CGGAAGCTGTTGCAGCCTA; brain natriuretic factor (BNP) 5’-CTGCTTGGAGCCTGATAAGAGA, 5’-AGTCAGAACTGGAGTCTC; skeletal muscle actin (SMA) 5’-GCCCTGAGTGAGCAGACTG, 5’-CCATCCAATCGGTAGTACCG; myosin heavy chain alpha (MHCα) 5’-GCCCTGAGTGAGCAGACTG, 5’-CCATCCAATCGGTAGTACCG; myosin heavy chain beta (MHCβ) 5’-GCCCTGAGTGAGCAGACTG, 5’-CCATCCAATCGGTAGTACCG; TRPC1 5’-GCCCTGAGTGAGCAGACTG, 5’-CCATCCAATCGGTAGTACCG; TRPC2 5’-GCCCTGAGTGAGCAGACTG, 5’-CCATCCAATCGGTAGTACCG; TRPC3 5’-GCCCTGAGTGAGCAGACTG, 5’-CCATCCAATCGGTAGTACCG; TRPC4 5’-GCCCTGAGTGAGCAGACTG, 5’-CCATCCAATCGGTAGTACCG; TRPC5 5’-GCCCTGAGTGAGCAGACTG, 5’-CCATCCAATCGGTAGTACCG; TRPC6 5’-GCCCTGAGTGAGCAGACTG, 5’-CCATCCAATCGGTAGTACCG.

Mouse Myocyte Isolation

All animal procedures were approved by the Temple University School of Medicine Institutional Animal Care and Use Committee. Adult mouse myocytes were isolated as previously described. Anesthesia was induced using 3% isoflurane and maintained using 1% isoflurane delivered by nose cone. Adequate induction of anesthesia was confirmed by observation of a negative paw- or tail-pinch reflex. When an unconscious state was induced, mouse hearts were rapidly excised from the thorax, weighed and the aorta was cannulated on a constant-flow Langendorff apparatus. The heart was digested by retrograde perfusion of Tyrode’s solution containing 180 U/mL collagenase (Type II, Worthington Biomedical) and (mmol/L): CaCl$_2$ 0.02, glucose 10, HEPES 5, KCl 5.4, MgCl$_2$ 1.2, NaCl 150, sodium pyruvate 2, pH 7.4. When the tissue softened, the left ventricle was isolated and gently minced, filtered, and equilibrated in Tyrode’s solution with 200umol/L CaCl$_2$, and 1% bovine serum albumin (BSA) at room temperature. Routinely, our initial yield is >90% rod-shaped myocytes, and >80% calcium-tolerant, rod-shaped myocytes survive by the end of the isolation.

Feline Myocyte Isolation, Culture and Infection with Adenovirus

Adult feline left ventricular myocytes (AFMs) were isolated as extensively described. Briefly, felines were anesthetized with sodium pentobarbital and hearts were rapidly excised, cannulated, and mounted on a constant-flow Langendorff apparatus. Hearts were rinsed with a physiological Krebs-Henseleit buffer (KHB) containing (mmol/L): glucose 12.5, KCl 5.4, lactic acid 1, MgSO$_4$ 1.2, NaCl 130, NaH$_2$PO$_4$ 1.2, NaHCO$_3$ 25, Na-pyruvate 2. Solutions were aerated with 95% oxygen and 5% carbon dioxide, pH 7.35 to 7.4, and warmed to 37°C. Following the rinse step,
hearts were retrograde perfused with collagenase-containing KHB (180U/mL) supplemented with 50umol/L CaCl₂. When the tissue softened, the left ventricle was isolated and gently minced, filtered, and equilibrated in KHB with 200umol/M CaCl₂, and 1% bovine serum albumin (BSA) at room temperature. Isolated myocytes were washed with serum-free culture medium (Medium 199, Sigma) supplemented with penicillin-streptomycin-glutamine (PSG, Gibco) and cultured on plates or glass cover-slips coated with laminin (BD Bioscience). For long-term culture (>12hrs), cells were switched to Medium199/PSG with the addition of taurine (5mmol/L), creatine (5mmol/L), and carnitine (2mmmol/L)(Sigma).

Myocytes were infected with adenovirus (Ad) expressing RFP, GFP, TRPC3, dnTRPC6, TRPC6-FLAG, Rem1-Cav (Rem-Cav)⁸, and/or NFATc3-GFP for 12-24 hours at a multiplicity of infection of 100 and then changed to long-term culture media. During the experimental period, culture media was changed once per day. Infection efficiency was determined 36-48 hours after infection by RFP or GFP fluorescence intensity and was typically assessed to be 90-95%.

TRPC Adenovirus Cloning

The TRPC3, TRPC4, dnTRPC4, and dnTRPC6 adenoviruses were all previously described⁹. For generation of TRPC6-Flag adenoviral construct, the pShuttle vector (AdEasy adenoviral system, Agilient Technology, Santa Clara, CA) was modified to contain a C-terminal 3XFLAG peptide sequence downstream of the multiple cloning site. Human TRPC6 cDNA (Open Biosystems, Pittsburgh, PA) was PCR amplified to contain a 5' NotI sequence and to alter the stop codon to a BamHI site. This TRPC6 fragment was subcloned in frame with the FLAG peptide sequence. Adenovirus was made as previously described⁹,¹⁰.

TRPC-Mediated Ca²⁺ Entry

Myocytes were loaded with 5-10 umol/L Fluo-4,AM (Molecular Probes) and placed in a heated chamber (37°C) on the stage of an inverted microscope. Cells were perfused with a Ca²⁺-free normal Tyrode’s solution containing the reversible sarcoplasmic reticulum Ca²⁺ ATPase (SERCA) inhibitor cyclopiazonic acid (CPA, 5umol/L, Sigma) and the TRPC agonist oleoyl-2-acetyl-sn-glycerol (OAG, 10 umol/L, Sigma) for 10 minutes to achieve SR store depletion. Myocytes were then rapidly switched to the same buffer solution supplemented with 1.8mmol/L CaCl₂ to assay for store-operated Ca²⁺ entry measured as a sustained increase in Fluo-4 fluorescence for 5-10 minutes following the addition of Ca²⁺. In some experiments, the LTCC antagonist nifedipine (10umol/L, Sigma) or the TRPC channel inhibitors SKF-96365 (5umol/L, Sigma), GSK503A (GSK833583A, 10umol/L, GlaxoSmithKline), Pyr3 (3umol/L, Sigma), or Pyr10 (3umol/L, Calbiochem) were also included in the solutions throughout the duration of the study. Similar studies were performed using the G-protein coupled receptor agonist angiotensin II (Ang II, 1umol/L, Sigma) rather than OAG (Online Figure I A).

Rest to Pacing Protocol

Myocytes were loaded with 5 umol/L Fluo-4 AM (Molecular Probes) and placed in a heated chamber on the stage of an inverted microscope and perfused with a normal physiological Tyrode’s solution containing (in mmol/L): 150 NaCl, 5.4 KCl, 1.2 MgCl₂, 10 glucose, 2 Na-pyruvate, 1 CaCl₂ and 5 HEPES, pH 7.4 for 5 minutes. Myocytes were then paced at 0.5Hz and fractional shortening data was collected using edge detection. For intracellular Ca²⁺ fluorescence measurements, the F₀ (or F unstimulated) was measured as the average fluorescence of the cell 50 msec prior to stimulation. The maximal Fluo-4 fluorescence (F) was measured at peak amplitude. Background fluorescence was subtracted from each parameter before representing the
peak Ca\(^{2+}\) transient as F/F_0\(^{2,3,11}\). Tau was measured as the decay rate of the average Ca\(^{2+}\) transient trace. The first three fractional shortening and peak Ca\(^{2+}\) transient traces following the rest period were averaged and represent the “initial” magnitude of each parameter following rest. Steady state contractility was defined as the period when the fractional shortening and Ca\(^{2+}\) transients plateaued and was reached within 10-20 stimulations of the myocytes. Five consecutive traces within this plateau period where there was no variation seen between traces were averaged and termed the “steady state” parameter of each individual cell analyzed. Steady-state data points are represented as the steady-state value divided by the initial value to highlight the differences seen.

**Calcium Sparks**

Isolated feline myocytes were plated on laminin coated glass coverslips and infected with adenovirus constructs as described above (Ad-RFP, TRPC3, TRPC6, and/or dnTRPC6 or dnTRPC4). 36-hours after infection, expression was verified by visualization of RFP fluorescence. Myocytes were loaded with 5 umol/L Fluo-4/AM at 37°C for 30 min in Medium 199 (culture medium described above), then prepared for immediate imaging\(^{12,13}\). Images were acquired using a 510 Meta Carl Zeiss confocal microscope through a Plan-Neofluar 40X Oil objective with excitation at 488 nm and emission from 505-550 nm. Whole-frame images at 12.2X digital magnification were acquired every 196.61 msec for 200 cycles using bi-directional raster scanning. Where applicable, myocytes were pre-incubated with the CaMKII inhibitor KN-93 (10umol/L) for 1hr. Images were analyzed and quantified using ZEN 2010 and ImageJ 1.45s software. 100 frames per cell and 20 cells per condition were analyzed.

**Sucrose Density Gradients**

Membrane rafts were fractionated from cultured AFMs or isolated mouse ventricular myocytes as previously described\(^8,14,15\). Briefly, 1x10^6 freshly isolated mouse myocytes or 1-2 x 10^6 cultured AFMs were resuspended in ice-cold, detergent-free Tricine buffer (in mmol/L: 250 sucrose, 1 EDTA, 20 Tricine, pH 7.4) and centrifuged. Cell pellets were resuspended in Tricine buffer and homogenized with a dounce homogenizer. Homogenates were then centrifuged and the supernatant was collected, mixed with 30% Percoll (Sigma) in Tricine buffer, and subjected to ultracentrifugation for 25 minutes (Beckman MLS50 rotor, 77,000 g, 4C). The separated plasma membranes were collected, sonicated and mixed with 60% sucrose to a final concentration of 40% sucrose. This mixture was overlaid with a 30-5% step sucrose gradient and subjected to overnight ultracentrifugation (Beckman MLS50 rotor, 87,000 g, 4C). Fractions were collected every 0.4 mL from the top sucrose layer, and proteins were precipitated using a solution of 0.1% wt/vol deoxycholic acid in 100% wt/vol trichloroacetic acid. Samples were then subjected to SDS-PAGE and immunoblotted using indicated antibodies. Where indicated, methyl-β-cyclodextrin (MβCD) was used at 10mmol/L.

**Caveolae Immunoaffinity Isolations**

Isolation of caveolae organelles was performed according to published methods\(^8,16\). Briefly, sheep anti-mouse IgG-coated magnetic beads (Dynal Biotech) were pre-incubated with a specific monoclonal antibody for caveolin-3 (BD Transduction Laboratories) for 2-4 hours at room temperature. Sonicated plasma membranes prepared as described above were added to coated beads and incubated for 1 hr at 4C. Bound material, representative of caveolae vesicles, was separated magnetically from unbound, non-caveolae membranes, subjected to SDS-PAGE and immunoblotted using the indicated antibodies.
**Immunoprecipitations (IPs)**

Sheep anti-rabbit IgG-coated magnetic beads (Dynal Biotech) were pre-incubated with an antibody for TRPC4 (Alomone Labs), TRPC6 (Alomone Labs) or Cav1.2 (LTCC α1C, Millipore) for 2-4 hours at room temperature. Sonicated plasma membranes prepared from either isolated dnTRPC4 mouse myocytes (Figure 5A), or AFMs infected with Ad-GFP or Ad-TRPC3 and Ad-TRPC6 (Online Figure IIA) for 36hrs (as described above) were added to prepared beads and incubated for 1 hr at 4C. Bound material, representative of TRPC4, TRPC6 or LTCC containing membranes, was separated magnetically from unbound (non-TRPC4, TRPC6 or LTCC containing membranes), subjected to SDS-PAGE and immunoblotted using the indicated antibodies.

For pull-down experiments in AFMs infected with Ad-TRPC6-FLAG (Supplemental Figure 5A), AFMs were infected with Ad-TRPC6-FLAG and plasma membranes were isolated as described above 36hrs after infection. IPs were performed using anti-FLAG M2 Affinity Gel (Sigma) as per the manufacturer’s protocol and bound and unbound samples were subjected to SDS-PAGE and immunoblotted using the indicated antibodies.

For IP experiments looking at interactions of TRPC channel isoforms with dnTRPC4 (Online Figure II B-D), we co-expressed Ad-dnTRPC4 with either RFP, TRPC3, TRPC4 or TRPC6 and pulled down with beads coated with an antibody to TRPC4 (Alomone Labs). We performed westerns with antibodies to TRPC3 (Abcam), TRPC4 or TRPC6 (Alomone Labs). dnTRPC4 was differentiated from full-length TRPC4 by Western based on size.

Immunoprecipitations with non-coated beads were performed side-by-side in each experiment to test for non-specific binding to the solid support used for immunoisolation.

**NFAT Translocation Assay and Confocal Imaging**

AFMs were isolated as described above and plated on laminin coated glass coverslips. Cells were co-infected with Ad-NFATc3-GFP and Ad-RFP, -TRPC3, -TRPC4 and -Rem-Cav, -TRPC4, -TRPC6, and/or Ad-dnTRPC4 or dnTRPC6 for 36hrs. Upon confirmation of adenovirus expression (RFP and/or GFP fluorescence), myocytes were switched to cell culture Medium199 (Sigma) containing 2mmol/L CaCl2 and, where applicable, 10umol/L OAG, 4mmol/L Ca2+, and/or cariporide (10umol/L) for 1hr. For disruption of caveolae by cholesterol sequestration, myocytes were pre-treated with 10mmol/L MβCD for 1hr before the addition of OAG for an additional 1hr. Following 1hr incubation +/- OAG, myocytes were fixed in 4% paraformaldehyde at room temperature for 10 minutes and permeabilized with 1% Triton X-100 for nuclear staining with 4’, 6-diamidino-2-phenylindole (DAPI, Millipore). Fixed cells on coverslips were mounted onto slides and confocal micrographs of cells were acquired using a Nikon Eclipse T1 confocal microscope. NFAT localization was quantified as the normalized nuclear/cytoplasmic ratio of GFP fluorescence intensity using ImageJ 1.45s software.

**Mice and Experimental Protocol**

The methods used to generate the dnTRPC4 mouse have been described previously. Inducible heart-specific expression was achieved with a binary α–myosin heavy chain (α-MHC) promoter–based transgene strategy. The responder transgene permitted expression of dnTRPC4 in the heart only in the presence of the driver transgene encoding the tetracycline transactivator (tTA) protein in the absence of tetracycline/doxycycline (tetracycline/Dox). Experiments were performed based on the National Institutes of Health Guide for the Care and Use of Laboratory Animals,
and all procedures were approved by the Temple University School of Medicine Institutional Animal Care and Use Committee. Myocardial infarction (MI) was induced in mice at the age of 4 months as extensively described\textsuperscript{18, 19}. Mice were anesthetized with 2\% isoflurane inhalation. A skin incision was made over the left thorax, and the pectoral muscles were retracted to expose the ribs. At the level of the fifth intercostal space, the heart was exposed and pumped out through an expanded space between ribs. A permanent knot or slipknot (for reperfusion purpose) was made around the left main descending coronary artery (LCA) 2–3 mm from its origin with a 6-0 silk suture. The heart was immediately placed back into the intrathoracic space after the knot was tied, followed by manual evacuation of pneumothoraces and closure of muscle and the skin suture by means of the previously placed purse-string suture. Sham-operated animals were subjected to the same surgical procedures except that the suture was passed under the LCA but was not tied.

\textit{In-vivo Functional Analysis (Echocardiography, ECHO)}

ECHO was performed with VisualSonics Velvo 770 machine which is specifically designed for mice and rats. Mice were anesthetized with 2\% isoflurane initially and then 1\% during the ECHO procedure. Hearts were viewed in the short-axis between the two papillary muscles and analyzed in M-mode. Parameters were to be measured offline (Velvo software) including end-diastolic diameter (EDD), end-systolic diameter (ESD), posterior wall thickness (PWT), and septal wall thickness (SWT) to determine cardiac morphological changes and ejection fraction (EF), heart rate and fractional shortening (FS).

\textit{Determination of Left Ventricle Area-At-Risk and Infarct Size After MI}

\textit{Area at risk (AAR)} was measured by injecting 0.2 ml of 2\% Evans blue dye into the right ventricle before the heart was excised. Areas of the heart with normal blood flow stained blue. The stained heart was quickly removed and frozen and placed on dry ice. Then the heart was cut into eight 1.0-mm-thick sections perpendicular to the long axis of the heart. The sections were incubated in PBS containing 2\% triphenyltetrazolium chloride (TTC; Sigma) at room temperature for 15 min. The blue areas (area not at risk, ANAR), and non blue areas (AAR) were measured with the NIH ImageJ 1.45s software in at least 7 hearts of each group and the percentage of AAR was calculated (AAR/(AAR+ANAR)). For infarct size measurement, histology tissue slides were prepared. Animals were anesthetized and heparinized intravenously. Hearts were excised, trimmed off excess tissue, weighed, and then perfused with 10\% buffered formalin. The fixed heart tissues were dehydrated embedded in paraffin, longitudinal-sectioned at 5-\textmu m thickness, and then H-E stained. Whole section images were taken by anatomy scope and Image J software were used to do morphology analysis. Myocardial infarction size (expressed as percentage) was calculated using infarct length divided by total circumference of longitudinal tissue sections from post-MI hearts.

\textit{Cellular Functional Analysis}

Myocytes were isolated from sham and post-MI animal hearts to measure cellular fractional shortenting (FS), calcium transients ([Ca\textsuperscript{2+}]) and L-type calcium current (I\textsubscript{CaL}). All experiments were done a 35-37\degree C, in superfused myocyte chambers mounted on fluorescence equipped microscopes. All myocytes were characterized within the same series of experiments.

\textit{I\textsubscript{CaL} Measurement}

\textit{I\textsubscript{CaL}} was measured in a sodium-free and potassium-free solution. Isolated myocytes were placed in a chamber mounted on an inverted microscope (Nikon Diaphot) and perfused with 1mmol/L
calcium-containing Tyrode solution. Both the inflow solution and the chamber were water-heated to maintain the temperature at 36±1°C. A 4-5 MΩ pipette filled with a Cs⁺-containing solution composed of (in mmol/L): Cs-aspartate 130, N-methyl-D-glucamine (NMDG) 10, tetraethylammonium chloride 20, HEPES 10, Tris-ATP 2.5, MgCl₂ 1, and EGTA 10, pH 7.2, was used to obtain gigaseals. Once a gigaseal was formed, the patch was ruptured and the cell was dialyzed for 10 min. The extracellular bath was then changed to a 2mmol/L calcium-containing Cs⁺ substitution bath solution (composition in mmol/L: 4-aminopyridine 2, CaCl₂ 2, CsCl 5.4, glucose 10, HEPES 5, MgCl₂ 1.2, and NMDG 150, pH 7.4 with CsOH). Membrane voltage was controlled by an Axopatch 2A voltage-clamp amplifier and digitized by Digidata 1322 using pClamp8 software (Molecular Devices). Once the signal was converted to digital format, it was stored on a personal computer for off-line analysis with Clampfit 10 (Axon Instruments). The flow of the bathing solution was 2–3 ml/min.1,3,8,20.

**Fractional Shortening and Intracellular Calcium Measurements:**

Myocytes were loaded with 5-10 umol/L Fluo-4 AM (Molecular Probes) and placed in a heated chamber on the stage of an inverted microscope and perfused with a normal physiological Tyrode’s solution containing (in mmol/L): 150 NaCl, 5.4 KCl, 1.2 MgCl₂, 10 glucose, 2 Na-pyruvate, 1 CaCl₂ and 5 HEPES, pH 7.4. Myocytes were paced at 0.5Hz and fractional shortening data was collected using edge detection. For intracellular Ca²⁺ fluorescence measurements, the F0 (or F unstimulated) was measured as the average fluorescence of the cell 50 msec prior to stimulation. The maximal Fluo-4 fluorescence (F) was measured at peak amplitude. Background fluorescence was subtracted from each parameter before representing the peak Ca²⁺ transient as F/F0.2,3,11. Tau was measured as the decay rate of the average Ca²⁺ transient trace. Isoproterenol (Iso, Sigma) was used at 1umol/L.

**Cell Length and Width Measurements**

Myocytes were isolated from mice as described above and imaged with bright field microscopy. Cell length and width were measured using ImageJ 1.45s software for a total of 100 myocytes per animal by identifying the maximal length and width for each individual cell (n=5 mice/genotype).

**Protein Isolation and Western Analysis**

Whole cell lysates, tissue homogenates, or plasma membrane preparations were prepared from isolated feline or mouse myocytes and analyzed by Western as previously described.3,8,21,22 The following primary antibodies were used for detection: TRPC3 (Abcam), TRPC4 (Alamone Labs), TRPC6 (Alamone Labs), Caveolin-3 (BD Transduction Labs), Calcinuerin (protein phosphatase 2B, PP2B, Sigma), total Ryanodine Receptor (RyR₂, Sigma), phosph-RyR₂ (S2808 and S2814, Badrilla Ltd.), SERCA (Sigma), total phospholamban (Millipore), phospho-phospholamban (PS16 and PT17, Badrilla Ltd.), GFP (Sigma), FLAG-M2 (Sigma), NCX (Swant), NHE-1 (Millipore), Glyceraldehyde 3-phosphate dehydrogenase (GAPDH, AbD Serotec). Mouse-HRP and rabbit-HRP secondary antibodies were purchased from GE Healthcare. Where applicable, Western blot band intensities were quantified using ImageJ 1.45s computer software.

**Statistics**

Data are presented as mean±SEM. Unpaired t-test, paired t-test, and one-way ANOVA were performed to detect significance using GraphPad Prism6 software. p<0.05 was considered significant (ns p>0.05; * p≤0.05; ** p≤0.01; *** p≤0.001; **** p≤0.0001). Survival analysis is
presented using a Kaplan-Meier regression and statistical significance was determined using the log-rank test.

References

1. Jaleel N, Nakayama H, Chen X, Kubo H, MacDonnell S, Zhang H, Berretta R, Robbins J, Cribbs L, Molkentin JD, Houser SR. Ca2+ influx through t- and l-type ca2+ channels have different effects on myocyte contractility and induce unique cardiac phenotypes. Circ Res. 2008;103:1109-1119


Online Figure I. TRPC-mediated Ca\textsuperscript{2+} entry. A, TRPC-mediated Ca\textsuperscript{2+} entry in isolated myocytes from sham mice (left) or 1 week post MI (right) in the presence of the G-protein coupled receptor agonist angiotensin II (AngII, 1umol/L) and the SERCA inhibitor CPA (5umol/L). Where indicated, the TRPC antagonist SKF-96365 (5umol/L) or the LTCC inhibitor nifedipine (10umol/L) were used. B-E, Ca\textsuperscript{2+} entry in isolated myocytes from 6-week sham or 6-week MI mice (B, C) or in AFMs infected with the indicated adenoviruses (D, E) in the presence of CPA alone (B, D) or OAG alone (C, E).
Online Figure II. TRPC channel hetero-oligomerization. A, Plasma membranes (PM) were purified from total cell homogenates (H) of isolated myocytes from AFMs infected with Ad-GFP or Ad-TRPC3 and Ad-dnTRPC6. Immunoprecipitations were performed with an antibody for TRPC6 and Westerns were performed with the indicated antibodies (U, unbound fraction; B, bound fraction). Bead controls test for non-specific binding to the solid support used for IP. B-D, Immunoprecipitations were performed with an antibody for TRPC4 on lysates from AFMs infected with dnTRPC4 and RFP or TRPC3 (B), dnTRPC4 and RFP or TRPC4 (C), or dnTRPC4 and RFP or TRPC6 (D). Westerns were performed with the indicated antibodies and show that dnTRPC4 can physically associate with TRPC3, TRPC4 and TRPC6. dnTRPC4 was differentiated from full length TRPC4 by size (C).
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Online Figure III. Fractional shortening and Ca\(^{2+}\) transients from AFMs paced after a period of rest. Representative fractional shortening and Ca\(^{2+}\) transient traces from AFMs infected with the indicated adenoviruses and stimulated to pace after a period of rest.
Online Figure IV. TRPC6 channels induce CaMKII mediated RyR$_2$ and PLN phosphorylation. Whole cell lysates from AFMs infected with Ad-RFP, -TRPC6, -dnTRPC4, or –TRPC6 and –dnTRPC4 at baseline or treated with OAG (10umol/L) were analyzed by Western with the indicated antibodies. KN93 (10umol/L) was used in addition to OAG where noted. Westerns were performed with the antibodies indicated on the left.
Online Figure V. TRPC channels co-localize with LTCCs in caveolae membrane microdomains and their organization can be disrupted by treatment with MβCD. A, Plasma membranes (PM) were purified from total cell homogenates (H) of isolated feline myocytes infected with Ad-TRPC6-FLAG and immunoprecipitations and Westerns were performed with the indicated antibodies (U, unbound fraction; B, bound fraction). B, Sucrose density gradient fractionation on purified PMs from isolated AFMs infected with Ad-TRPC3 and treated with MβCD (10mmol/L). Of importance is the displacement of Cav3 and TRPC3 from more buoyant caveolae enriched fractions (compare to Figure 5B). F1-F11 represent Fractions 1-11.
Online Figure VI. TRPC channel overexpression induces NFAT nuclear translocation. A-D, AFMs were infected with Ad-NFAT-GFP and the indicated adenoviruses and NFAT translocation was monitored in response to the TRPC agonist OAG (10umol/L) or 4mmol/L Ca$^{2+}$ (C) in the presence or absence of MβCD (10mmol/L)(B) or the NHE inhibitor cariporide (10umol/L, D). Scale bar is 10µm.
Online Figure VII. A subpopulation of NHE-1 and NCX localize to caveolae membrane microdomains. A, Plasma membranes (PM) were purified from total cell homogenates (H) of isolated myocytes from AFMs. Caveolae immunoisolutions were performed with an antibody for caveolin-3 (Cav3) and Westerns were performed with the indicated antibodies (U, unbound fraction; B, bound fraction). B, Sucrose density gradient fractionation on purified PMs from isolated AFMs confirm the presence of NHE-1 and NCX in Cav3 enriched lipid raft membrane fractions (Fraction 1-Fraction 11, F1-F11).
Online Figure VIII. TRPC channel expression in WT and dnTRPC4 TG mice. RT-PCR shows an up-regulation of TRPC1/3/4/6 channel isoforms 2-weeks post-MI along with the activation of the fetal gene program compared to sham animals. dnTRPC4 TG show similar levels of TRPC expression levels in sham and 2-week post-MI mice with the exception of TRPC4 which is markedly increased due to transgene overexpression. p<0.05 was considered significant (ns, p>0.05; *p≤0.05, **p≤0.01, ***p≤0.001 vs. WT sham; #p≤0.05, ##p≤0.01, ###p≤0.001 vs. dnTRPC4 sham).
Online Figure IX. A, Area at risk (AAR) measured in WT and dnTRPC4 mice. LV infarct length (B), RV free wall thickness (C), and LV posterior wall thickness (D) were quantified histologically and average values of WT and dnTRPC4 mice 3-weeks post-MI are shown. E, Liver weight (LiverW) normalized to body weight (BW) measured in sham mice and mice 6 weeks post-MI.
Online Figure X. dnTPC4 TG post-MI mice have increased phosphorylated NFAT and reduced cell size.  

**A**, Western blot analysis of lysates from isolated myocytes from WT or dnTRPC4 mice after 6-weeks of sham or MI treatment show elevated levels of phospho-NFAT (inactive form) in dnTRPC4 mice post-MI.  

**B-C**, Average quantified values expressed relative to WT sham myocytes for n=3 experiments.  

**D-E**, Average maximal length (**D**) or width (**E**) of isolated myocytes from WT or dnTRPC4 mice at 2- and 6-week post-MI. p<0.05 was considered significant (ns, p>0.05, *p≤0.05, **p≤0.01, ***p≤0.001).
Online Figure XI. Fractional shortening and Ca$^{2+}$ transients measured in isolated cardiac myocytes from sham and post-MI hearts. Cellular fractional shortening and Ca$^{2+}$ transients were measured in isolated myocytes from sham and 3 week post-MI WT and dnTRPC4 mice. Examples of average traces of fractional shortening (A) and Ca$^{2+}$ transients (B) are shown at baseline and with the addition of isoproterenol (Iso).