Transient Receptor Potential Channels Contribute to Pathological Structural and Functional Remodeling After Myocardial Infarction

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Rationale: The cellular and molecular basis for post–myocardial infarction (MI) structural and functional remodeling is not well understood.

Objective: Our aim was to determine if Ca\(^{2+}\) influx through transient receptor potential canonical (TRPC) channels contributes to post-MI structural and functional remodeling.

Methods and Results: TRPC1/3/4/6 channel mRNA increased after MI in mice and was associated with TRPC-mediated Ca\(^{2+}\) entry. Cardiac myocyte–specific expression of a dominant-negative (loss-of-function) TRPC4 channel increased basal myocyte contractility and reduced hypertrophy and cardiac structural and functional remodeling after MI while increasing survival in mice. We used adenovirus-mediated expression of TRPC3/4/6 channels in cultured adult feline myocytes to define mechanistic aspects of these TRPC-related effects. TRPC3/4/6 overexpression in adult feline myocytes induced calcineurin (Cn)–nuclear factor of activated T-cells (NFAT)–mediated hypertrophic signaling, which was reliant on caveolae targeting of TRPCs. TRPC3/4/6 expression in adult feline myocytes increased rested state contractions and increased spontaneous sarcoplasmic reticulum Ca\(^{2+}\) sparks mediated by enhanced phosphorylation of the ryanodine receptor. TRPC3/4/6 expression was associated with reduced contractility and response to catecholamines during steady-state pacing, likely because of enhanced sarcoplasmic reticulum Ca\(^{2+}\) leak.

Conclusions: Ca\(^{2+}\) influx through TRPC channels expressed after MI activates pathological cardiac hypertrophy and reduces contractility reserve. Blocking post-MI TRPC activity improved post-MI cardiac structure and function. (Circ Res. 2014;115:567-580.)

Key Words: calcium ▪ calcium channels ▪ cardiomegaly ▪ myocardial infarction ▪ transient receptor potential channels

Cardiac systolic stress is increased in cardiovascular diseases such as hypertension and myocardial infarction (MI), and this requires an increase in contractile Ca\(^{2+}\). Persistent pathological stress usually results in a Ca\(^{2+}\)-dependent pathological hypertrophy with Ca\(^{2+}\)-related contractility defects. Abnormal contractile Ca\(^{2+}\) with depressed contractility reserve is a hallmark of cardiac hypertrophy and heart failure, but this contractile Ca\(^{2+}\) does not seem to be the source for activation of the signaling pathways that cause pathological hypertrophy. Recent data suggest that separate pools of myocyte signaling and contractile Ca\(^{2+}\) are involved in the induction of hypertrophy. The source and cellular location of the signaling Ca\(^{2+}\) is still not clearly defined. The present study explores the hypothesis that the expression of transient receptor potential canonical (TRPC) channels is induced after MI, and Ca\(^{2+}\) influx through these channels within specific microdomains is necessary for the development of pathological hypertrophy, as well as for affecting contractility reserve that ultimately contributes to impaired pump function of the diseased heart.

The function of the TRP family of channels is not well understood in the heart, but it has been implicated in contributing to the initiation of pathological cardiac remodeling. TRP channels are a class of nonselective cation influx channels that are grouped into 7 families and are present in many...
different cell types. The TRPC family includes 7 isoforms (TRPC1–7) that have been divided into 2 structural subfamilies based on functional similarities: TRPC1/4/5 and TRPC3/6/7. In general, TRPC3/6/7 are activated by diacylglycerol generated by G protein–coupled receptors/Goα/ phospholipase C signaling, whereas TRPC1/4/5 can be activated by stretch or depletion of intracellular Ca2+ stores (store-operated Ca2+ entry; SOCE) but, unlike TRPC6, cannot be activated by Ca2+ spark activity. Functional TRPC channels are formed as tetramers of individual 6-transmembrane spanning subunits. Interestingly, the channels can be homomeric or heteromeric assemblies with oligomerization occurring within and between subunits or isoforms.27–30 Studies involving loss of TRPC function suggest that Ca2+ influx through TRPC channels is sufficient for pathological growth.3 Activation of this signaling cascade is thought to be the primary mechanism through which TRPC channels regulate cardiac hypertrophy. A recent in vitro study from our group suggests that TRPCs and LTCC work in a coordinated fashion to activate Cn-NFAT signaling.22 Additional studies from our group showed that a subpopulation of LTCC localized specifically to caveolae membrane signaling microdomains is involved in pathological hypertrophic signaling.25 The present study explores the hypothesis that TRPCs and LTCC function as essential partners in these caveolae signaling microdomains where their activity initiates hypertrophic Cn-NFAT signaling after MI.

The role of TRPC channels within excitation–contraction coupling microdomains has not been clearly defined. There are data in mice associating increased TRPC activity with reduced contractility and loss of TRPC function with increased contractility, but the mechanisms underlying these effects are not understood. The present study explores the hypothesis that Ca2+ influx through TRPC channels within excitation–contraction coupling microdomains results in reduced contractility reserve after MI.

**Methods**

See the extended Materials and Methods section in the Online Data Supplement. In brief, adult mouse and feline myocytes were isolated. TRPC-mediated Ca2+ entry and Ca2+ spark activity were measured in unpaced myocytes loaded with Fluo-4, whereas pacing protocols were implemented for fractional shortening and Ca2+ transient contractility studies. NFAT translocation studies were performed in adult feline myocytes (AFMs) using adenosinomediated expression of NFAT-GFP and immunoprecipitation, sucrose density gradients, and Western blot analysis. Animal procedures were approved by the Temple University Institutional Animal Care and Use Committee. We induced MI in mice by permanent occlusion of the left main coronary artery as previously described, and animals were monitored during the course of the study using in vivo echocardiography.

**Results**

**TRPC Channel Expression and Activity Is Induced After MI**

Individual TRPC channels are expressed at low levels in normal adult heart, but expression and activity of select isoforms seem to be increased in pathological hypertrophy and heart failure. TRPC channels have been suggested as initiators of Ca2+-dependent signaling that leads to pathological cardiac remodeling, hypertrophy, and failure. Transgenic cardiac-specific overexpression of TRPC3 or TRPC6 channels in mice causes re-expression of fetal genes, myocyte hypertrophy, and activation of apoptotic signaling. The prohypertrophic effects of TRPC channels have also been shown in vitro in cultured cardiomyocytes. Studies involving loss of TRPC function suggest a necessary role for these channels in pathological hypertrophy. TRPC3 inhibition with the inhibitor Pyr3 blocks cardiac hypertrophy in mice subjected to pressure overload, and this finding has been supported by data in gene-deleted mice (TRPC1 and TRPC3/6/7) and in mice expressing dominant-negative (dn) mutants of select channels (dnTRPC3/4,6). Interestingly, mice expressing dnTRPC4 also inhibited the activation of the TRPC3/6/7 subfamily in the heart, which suggests that TRPC1/4/5 and 3/6/7 subfamilies function in coordinated complexes, at least when overexpressed. The present study takes advantage of the dnTRPC strategy to define the role of TRPCs in post-MI structural and functional remodeling.
include STIM (stromal interaction molecule) and Orai and potentially TRPC channels on the plasma membrane. Although the role of TRPC channels in SOCE in cardiac myocytes is not well defined, sarcoplasmic reticulum (SR) Ca\(^{2+}\) depletion of isolated myocytes followed by the reintroduction of Ca\(^{2+}\) in the presence of TRPC channel activator

Figure 1. Myocardial infarction induces transient receptor potential canonical (TRPC) channel expression and activity in mice, and overexpression of TRPC channels in feline myocytes leads to increased membrane Ca\(^{2+}\) influx. A, Reverse transcriptase polymerase chain reaction shows an upregulation of TRPC1/3/4/6 channel isoforms at 1, 2, and 6 wks post-myocardial infarction (MI) along with the activation of the fetal gene program. B to D, TRPC-mediated Ca\(^{2+}\) entry in isolated myocytes from sham mice (left) or 1 wk (B), 2 wk (C), or 6 wk (D) post-MI mice (right) in the presence of the TRPC channel agonist OAG (10 umol/L) and the sarcoplasmic reticulum Ca\(^{2+}\) ATPase inhibitor cyclopiazonic acid (CPA; 5 umol/L). Where indicated, the TRPC antagonists SKF-96365 (5 umol/L), GSK503A (GSK; 10 umol/L), Pyr10 (3 umol/L), or the L-type Ca\(^{2+}\) channel inhibitor nifedipine (Nif; 10 umol/L) were used. E, Adult feline myocytes infected with the indicated adenoviruses and assayed for TRPC-mediated Ca\(^{2+}\) entry. \(P<0.05\) was considered significant (ns, \(P>0.05\), *\(P\leq 0.05\), **\(P\leq 0.01\), ***\(P\leq 0.001\) vs sham). ANF indicates atrial natriuretic factor; BNP, brain natriuretic peptide; MHC, myosin heavy chain; and SMA, skeletal muscle actin.
1-oleyl-2-acetyl-sn-glycerol (OAG), a stable cell-permeable analog to the known TRPC agonist diacylglycerol, is an approach that has been used to assess TRPC-mediated Ca\textsuperscript{2+} entry.\textsuperscript{6,20} Myocytes isolated from mice 1, 2, or 6 weeks post-MI showed substantial TRPC-mediated Ca\textsuperscript{2+} entry, whereas myocytes from sham animals showed no detectable activity (Figure 1B–1D). Similar results were seen in these MI myocytes in response to angiotensin II (Online Figure IA). The specificity of TRPC-mediated Ca\textsuperscript{2+} entry in MI myocytes was validated by inhibition with the pan TRPC antagonist SKF-96365 or the TRPC3/6-specific inhibitor GSK503A.\textsuperscript{24} (Figure 1B–1D). TRPC-mediated Ca\textsuperscript{2+} entry was not inhibited by the LTCC antagonist nifedipine, documenting that this Ca\textsuperscript{2+} entry is independent of LTCC-mediated Ca\textsuperscript{2+} entry. Controls were also performed in cells incubated with cyclopiazonic acid alone, which resulted in a transient Ca\textsuperscript{2+} entry in 6-week MI myocytes but no detectable entry in sham myocytes (Online Figure IB) and also with myocytes treated with OAG alone (Online Figure IC), which resulted in Ca\textsuperscript{2+} entry in MI myocytes only and led to spontaneous contractions.

**AFMs Expressing TRPC Channels Have Increased Membrane Ca\textsuperscript{2+} Influx**

To further characterize the properties of TRPC channels in adult cardiac myocytes, we used cultured isolated AFMs because their electrophysiological and Ca\textsuperscript{2+} regulatory properties more closely resemble those of human myocytes (in comparison to rodent myocytes).\textsuperscript{33} AFMs survive in culture without the use of drugs that reduce Ca\textsuperscript{2+} influence and overload. This allows for manipulation of protein expression using adenoviral vectors in an adult myocyte that maintains stable electric and mechanical properties.\textsuperscript{27,34–36}

We performed TRPC-mediated Ca\textsuperscript{2+} entry measurements in AFMs infected with adenovirus (Ad) for red fluorescent protein (RFP; control), TRPC3, TRPC4, TRPC6, or a dnTRPC4 or dnTRPC6. Because of their ability to hetero-oligomerize, the use of a dnTRPC4/6 effectively inhibits the activity of all TRPC subfamilies of channels\textsuperscript{6,21} (Online Figure II). Ad-RFP–infected myocytes showed little or no TRPC-mediated Ca\textsuperscript{2+} entry, whereas myocytes infected with Ad-TRPC3, TRPC4, or TRPC6 showed significant Ca\textsuperscript{2+} entry, which was inhibited by the expression of Ad-dnTRPC4 or dnTRPC6 but unaffected by nifedipine (Figure 1E). TRPC-mediated Ca\textsuperscript{2+} entry was inhibited by the pan TRPC antagonist SKF-96365 in TRPC3/4/6-infected cells, whereas the more targeted TRPC3/6 inhibitor GSK503A only inhibited TRPC-mediated Ca\textsuperscript{2+} entry in TRPC3/6-infected cells. Similarly, the TRPC3 inhibitor Pyr10\textsuperscript{37} was able to inhibit TRPC-mediated Ca\textsuperscript{2+} entry in TRPC3-infected cells. Controls were also performed in cells incubated with cyclopiazonic acid alone, which resulted in a transient Ca\textsuperscript{2+} entry in TRPC4-infected myocytes only but no detectable entry in control or TRPC3/6 expressing myocytes (Online Figure ID). This is likely because of the ability of TRPC4 to participate in SOCE, whereas TRPC3/6 tends to be more agonist-induced channels. Similar to our findings in mouse myocytes, TRPC-infected AFMs treated with OAG alone (Online Figure IE) resulted in Ca\textsuperscript{2+} entry and led to spontaneous contractions.

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**Figure 2. Transient receptor potential canonical channel overexpression in adult feline myocytes (AFMs) enhances sarcoplasmic reticulum Ca\textsuperscript{2+} during resting conditions.** A, Representative fractional shortening and Ca\textsuperscript{2+} transient traces from AFMs infected with the indicated adenoviruses and stimulated to pace after a period of rest. Fractional shortening (B) and peak Ca\textsuperscript{2+} transients (C) are represented as the average raw values of the initial beat (left) and as the ratio of the steady-state raw values divided by the initial beat raw value (right). *P<0.05 was considered significant (ns, P>0.05; *P≤0.05 vs red fluorescent protein (RFP) control; #P<0.05 vs raw value of first beat of the same experimental group). All statistical analysis was done on raw values.
TRPC Channel Overexpression in AFMs Enhances SR Ca\textsuperscript{2+} in AFMs

TRPC3/6 overexpression in the adult mouse heart is linked to cardiac hypertrophy and depressed cardiac contractility.\textsuperscript{7,20} Myocyte contractions (fractional shortening) and Ca\textsuperscript{2+} transients were measured in AFMs infected with Ad-RFP, -TRPC3, -TRPC4, -TRPC6 or -dnTRPC4 or -dnTRPC6 after periods of rest (Figure 2A–2C). One of the hallmark contractile characteristics of large mammalian myocytes, including AFMs, is a positive contractile staircase when stimulation is reinstated after a period of rest.\textsuperscript{26,38} AFMs, as well as those of other large mammals including humans, have a lower cytoplasmic [Na\textsuperscript{+}] than found in rodents.\textsuperscript{34} This promotes forward-mode Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange (NCX), which in the absence of pacing results in low cytoplasmic [Ca\textsuperscript{2+}] and very small amounts of Ca\textsuperscript{2+} stored in the SR. Therefore, in normal AFMs, the first post-rest contraction and Ca\textsuperscript{2+} transient are small and then increase in subsequent beats as the SR is progressively loaded with Ca\textsuperscript{2+} to a new steady state. After rest periods, control (Ad-RFP) AFMs showed a beat-dependent increase in their contractions and Ca\textsuperscript{2+} transients (Figure 2A). Conversely, the first post-rest beat in Ad-TRPC3, -TRPC4, or -TRPC6–infected cells was larger and similar to the steady-state contraction. Intracellular Ca\textsuperscript{2+} was elevated in Ad-TRPC3, -TRPC4, and -TRPC6–infected cells compared with Ad-RFP cells as evidenced by increased fractional shortening (Figure 2A and 2B) and increased Ca\textsuperscript{2+} transient amplitude (F/F0; Figure 2A and 2C) in the first paced contraction after a nonpaced interval. These TRPC-mediated effects on rested state contractions and Ca\textsuperscript{2+} transients were inhibited by coexpression with dnTRPC4 or dnTRPC6 (Figure 2; Online Figure III). These results suggest that Ca\textsuperscript{2+} influx through TRPC channels maintains SR Ca\textsuperscript{2+} stores in the absence of LTCC-mediated Ca\textsuperscript{2+} entry, supporting a role for TRPC-mediated Ca\textsuperscript{2+} entry in disease when the normal
pathway for Ca\textsuperscript{2+} influx (through LTCC) is reduced and the SR Ca\textsuperscript{2+} load is diminished, although the nondiseased adult heart probably does not use this pathway because TRPC channels are not appreciably expressed.

**Ca\textsuperscript{2+} Influx Through TRPC Channels Induces Ca\textsuperscript{2+} Spark Activity in AFMs**

TRPC3/4/6 expressing AFMs had enhanced Ca\textsuperscript{2+} influx at rest that promotes SR Ca\textsuperscript{2+} loading, but steady-state contractions were not increased. Therefore, we tested the idea that persistent Ca\textsuperscript{2+} influx through TRPC can lead to excess spontaneous SR Ca\textsuperscript{2+} release (SR Ca\textsuperscript{2+} leak). Spontaneous Ca\textsuperscript{2+} sparks were measured to address this idea.\textsuperscript{39} Ca\textsuperscript{2+} sparks are local spontaneous Ca\textsuperscript{2+} release events caused by the opening of a cluster of ryanodine receptor (RyR) channels in the absence of LTCC opening.\textsuperscript{40,41} These events are common in quiescent rodent myocytes because of their high [Na\textsuperscript{+}]\textsubscript{i} that promotes Ca\textsuperscript{2+} entry via reverse-mode NCX activity culminating in SR Ca\textsuperscript{2+} overload.\textsuperscript{34,42} As discussed above, AFMs maintain low [Na\textsuperscript{+}]\textsubscript{i} and they do not exhibit Ca\textsuperscript{2+} accumulation or spontaneous SR Ca\textsuperscript{2+} release in long-term culture.\textsuperscript{34} We infected AFMs with Ad-RFP (control), -TRPC3, -TRPC6, -TRPC3 and -dnTRPC6, or -TRPC6 and dnTRPC4 and measured Ca\textsuperscript{2+} spark activity in the presence and absence of the TRPC channel agonist OAG. Control myocytes rarely exhibited Ca\textsuperscript{2+} sparks but did show a low level of Ca\textsuperscript{2+} spark activity with the addition

![Figure 4](http://circres.ahajournals.org/)

**Figure 4.** Transient receptor potential canonical (TRPC) channels induce Ca\textsuperscript{2+}/calmodulin protein kinase II–mediated ryanodine receptor 2 (RyR2) and phospholamban (PLN) phosphorylation. Whole-cell lysates from adult feline myocytes infected with Ad-RFP, -TRPC3, -dnTRPC6, or -TRPC3 and -dnTRPC6 at baseline or treated with OAG (10 umol/L) were analyzed by Western blot with the indicated antibodies. KN93 (10 umol/L) was used in addition to OAG where noted. A representative Western blot is shown in A. B to G. Average quantified values expressed relative to Ad-RFP control cells at baseline for n=3 experiments. P<0.05 was considered significant (ns, P>0.05, *P≤0.05, **P≤0.01).
of OAG (Figure 3A and 3B). AFMs infected with TRPC3 or TRPC6 showed robust Ca\(^{2+}\) spark activity under baseline conditions, and this was increased further with OAG stimulation. The majority of detectable Ca\(^{2+}\) spark activity was blocked by dnTRPC6, even in the presence of OAG. TRPC3- or TRPC6-mediated Ca\(^{2+}\) spark activity was also significantly inhibited by the Ca\(^{2+}\)/calmodulin protein kinase II (CaMKII) inhibitor KN93, suggesting that this process may in part result from local activation of CaMKII and phosphorylation of RyR\(_{2}\). To address this issue, we measured phosphorylation of RyR at S2814, which is a CaMKII phosphorylation site, in AFMs infected with TRPC3±dnTRPC6 and ±OAG (Online Figure IV). These experiments show that TRPC3 and TRPC6 induce RyR S2814 and phospholamban T17 phosphorylation (Figure 4C and 4F) without modifying RyR S2808 or phospholamban S16 phosphorylation (Figure 4B and 4E) or total RyR or phospholamban expression (Figure 4D and 4G). OAG-mediated increases in RyR S2814 and phospholamban T17 phosphorylation was reduced by CaMKII inhibition (KN93; Figure 4C and 4F; Online Figure IV). We also found that TRPC3 or TRPC6 expression in AFMs was associated with diminished contractile response to catecholamines as evidenced by a reduction in maximal amplitude of fractional shortening and peak Ca\(^{2+}\) transients in the presence of isoproterenol (Figure 3C and 3D).

TRPC Channels Localize to Caveolae Where Their Organization Is Essential for Hypertrophic Signaling

Previous work from our group showed that Ca\(^{2+}\) influx through both TRPCs and LTCC contributes to the activation of Cn-NFAT signaling and indicated that there may be a potential interaction between the channels.\(^{22}\) This interaction might take place within subcellular signaling microdomains such as caveolae,\(^{25}\) and it is known that nearly all TRPC isoforms contain a putative caveolin-binding motif.\(^{43}\) To explore this further, we
characterized the biochemical interactions between TRPCs, LTCC, and caveolin-3, the major structural protein of myocyte caveolae, using immunoprecipitation with purified plasma membranes from isolated ventricular myocytes of dnTRPC4 transgenic mice (Figure 5A) or AFMs infected with a FLAG-tagged version of TRPC6 (Ad-TRPC6-FLAG; Online Figure VA). Our immunoprecipitation data show that caveolin-3, LTCC, and TRPC channels are all complexed together in caveolae. This was further substantiated using sucrose density gradient fractionation of plasma membrane preparations from AFMs infected with Ad-TRPC3 (Figure 5B).

To examine the functional relevance of TRPC channels localized to caveolae, we assessed their role in pathological hypertrophic signaling using an NFAT-GFP reporter assay. AFMs were infected with Ad-NFAT-GFP and either Ad-RFP (control), -TRPC3, -TRPC4, -TRPC6, -TRPC3 and -dnTRPC6, or -TRPC6 and -dnTRPC4. Essentially all of the NFAT-GFP was localized to the cytoplasm in control AFMs (Figure 5C and 5D). Ad-TRPC3, -TRPC4, and -TRPC6–infected cells showed a small but significant increase in baseline nuclear NFAT signal, which was inhibited by coinfection with dnTRPC6 or dnTRPC4 (Figure 5C and 5D; Online Figure VI). Exposing myocytes to OAG caused a very slight increase in NFAT translocation in control cells and a significant increase in nuclear NFAT in TRPC3- or TRPC6–infected cells (Figure 5C and 5D). Incubating TRPC4–infected cells with high Ca²⁺ (4 mmol/L) also led to a significant increase in NFAT translocation (Online Figure VI).
The TRPC3/4/6 effect was eliminated with coinfec-
tion with dnTRPC6 or dnTRPC4 (Figure 5C and 5D). To
further assess whether organizing Ca2+ influx pathways in
caveolae is essential for NFAT regulation, we subjected
myocytes to treatment with methyl-β-cyclodextrin, which
disrupts caveolae by depleting cholesterol and displaces
the macromolecular signaling complexes usually organized
in caveolae microdomains (Online Figure V). Methyl-
β-cyclodextrin inhibited TRPC3/4/6-mediated NFAT nuclear
translocation in the presence of OAG or high Ca2+ (Figure
5C and 5D; Online Figure VI), suggesting that the organization
of LTCC and TRPC channels together in caveolae signaling
microdomains is necessary for them to activate hypertro-
phic signaling.

A previous study from our group characterized a cave-
olae-targeted LTCC inhibitor, Rem1-265-Cav, which could
specifically inhibit LTCC with caveolin-3 microdomains,
to reduce NFAT nuclear translocation without affecting
contractility.25 When we coinfected NFAT-GFP–expressing
AFMs with Ad-TRPC3 and -Rem1-265-Cav and treated
these cells with OAG, we saw a significant inhibition of
NFAT translocation (Figure 5C and 5D). These results
suggest that TRPC channels and LTCC housed together
in caveolae membrane microdomains provide a source of
Ca2+ that induces calcineurin activation and nuclear NFAT
translocation.

TRPCs are relatively nonselective cation channels that
allow both Na+ and Ca2+ entry. Others have shown that Na+
entry through the Na+/H+ exchanger (NHE-1) increases local
Ca2+ via NCX, leading to NFAT activation.45,46 Using both ca-
veolin-3 immunoisolations and sucrose density gradients, we
found the presence of both NHE-1 and NCX in caveolae mem-
brane microdomains (Online Figure VII). To look specifically
at a functional role for NHE-1 in our model, we performed
NFAT-GFP assay in TRPC3/4/6 in the presence of the NHE-1
inhibitor cariporide (Online Figure VID) and found that there
was no effect on NFAT translocation in these cells. These data
suggest that although NHE-1 colocalizes with TRPC, it is not
contributing to TRPC-mediated NFAT activation under our
conditions.

Figure 7. Cardiac function and survival was improved and pathological remodeling attenuated in dominant-negative
transient receptor potential canonical isoform 4 (dnTRPC4) mice vs wild-type (WT) animals post–myocardial infarction (MI).
A, Representative M-mode tracings from sham and MI animals at 6 wk post-MI. Average cardiac ejection fraction (B), posterior wall
thickness (C), and left ventricular (LV) internal diameter (D) were measured by echocardiography in sham and MI mice at baseline and
2 and 6 wk post-MI. E, Six-week survival data analyzed using a Kaplan–Meier regression of WT vs dnTRPC4 mice. Significance was
determined using the log-rank test. Heart weight (HW; F) or lung weight (LungW; G) normalized to body weight (BW) measured in sham
and MI mice after 6 wks. P<0.05 was considered significant (ns, P>0.05, *P≤0.05, **P≤0.01, ***P≤0.001).
Loss of TRPC Function Protects Against Cardiac Dysfunction Progression After MI and Improves Survival

After MI, myocytes develop pathological hypertrophy, myocyte function is altered, and TRPC channel expression increases. Overexpression of TRPC channels in the mouse heart is sufficient to induce hypertrophy and cardiomyopathy, and mice expressing dominant-negative versions of the channel have less hypertrophy in response to pressure overload or neuroendocrine agonist infusion. Taken together with our in vitro findings, these data support the idea that inhibiting TRPC function in the heart could be beneficial after MI. To test this idea, we used a transgenic mouse with cardiac-specific expression of a truncated dnTRPC4 that reduces the activity of both the TRPC1/4/5 and TRPC3/6/7 subfamilies of TRPC channels. TRPC mRNA levels were not significantly different between wild-type (WT) and dnTRPC4 mice both in sham and MI groups (Figure 6; Online Figure VIII) with the exception of TRPC4, which was markedly increased in dnTRPC4 animals because of overexpression of the transgene. TRPC-mediated Ca\(^{2+}\) entry seen in WT 6-week MI myocytes, which could be partially inhibited by the selective TRPC3/6 antagonist GSK503A or the TRPC3 inhibitors Pyr3 or Pyr10 or completely inhibited with SKF-96365, was not present in dnTRPC4 mice (Figure 6). Echocardiography measurements revealed a slightly increased baseline ejection fraction in dnTRPC4 mice compared with WT animals (75.5% versus 68.2%; Figure 7A and 7B), consistent with the inotropic effects of dnTRPC6 observed in AFMs. The area at risk after MI was identical in WT and dnTRPC4 mice (41.3±3.8% versus 43.5±4.9%; Online Figure IIIA). Infarct length measured 3 weeks after MI was not significantly different in dnTRPC4 than in WT mice (dnTRPC4 versus WT: 28.7±1.7% versus 33.2±0.9%; Online Figure IXB). In addition, the dilation seen in WT mice 3 weeks post-MI was attenuated in dnTRPC4 hearts (Online Figure IXC and IXD).

Serial echocardiography was used to measure left ventricular structure and function after MI. WT (ejection fraction: sham versus MI, 68.2% versus 37.2%) and dnTRPC4 (ejection fraction: sham versus MI, 75.5% versus 41.8%) animals had equivalent reductions in cardiac pump function 2 weeks after MI (Figure 7B). Left ventricular function remained depressed at 6 weeks post-MI in WT animals, whereas there was a significant improvement in cardiac pump function in dnTRPC4 hearts 6 weeks after MI (Figure 7A and 7B).

There were significant pathological changes in ventricular geometry and wall thickness in all hearts after MI (Figure 7C and 7D). The magnitude of these changes increased with time in WT animals but was attenuated in dnTRPC4 mice. After MI, posterior wall thickness was decreased in both WT and dnTRPC4 hearts (WT pre-MI versus post-MI: 0.97 versus 0.87 mm; dnTRPC4: 1.06 versus 0.84 mm; Figure 7C). At 6 weeks post-MI, posterior wall thickness returned to values near pre-MI levels (or close to shams) in dnTRPC4 hearts, whereas posterior wall thickness remained thinner in WT hearts. All hearts showed some evidence of dilation after MI;
however, left ventricular internal diameter increased significantly more in WT than in dnTRPC4 hearts increased significantly more in WT than in dnTRPC4 hearts increased significantly more in WT than in dnTRPC4 hearts increased significantly more in WT than in dnTRPC4 hearts increased significantly more in WT than in dnTRPC4 hearts in the first 2 weeks after MI (WT pre-MI versus post-MI: 3.8 versus 4.9 mm; dnTRPC4: 3.7 versus 4.5 mm; Figure 7D). By 6 weeks post-MI, left ventricular internal diameter was significantly more dilated in WT hearts than in dnTRPC4 (WT versus dnTRPC4: 5.3 versus 4.8 mm).

dnTRPC4 mice had significantly greater survival after MI (53.7%) than WT animals (27.5%) during the 6-week post-MI study period (Figure 7E). Heart weight/body weight and lung weight/body weight were significantly increased (Figure 7F and 7G) in WT versus dnTRPC4 mice 6 weeks post-MI. There were minimal changes in liver weight/body weight ratio before and after MI, and there was no significant difference between dnTRPC4 and WT mice (Online Figure IXE).

Dephosphorylation of NFAT induces cytoplasmic to nuclear translocation. Six-week MI dnTRPC4 mice had increased levels of phosphorylated NFAT as compared with WT 6-week MI mice, indicating reduced NFAT dephosphorylation in dnTRPC4 mice (Online Figure XA–XC). In accordance with this, isolated myocytes from 2- and 6-week post-MI WT had significantly greater length and width than in dnTRPC4 mice (Online Figure XD andXE). Collectively, these results show that dnTRPC4 animals have less pathological remodeling after MI, improved cardiac pump function, and enhanced survival.

Myocytes From dnTRPC4 Mice Retain Their Hypercontractile Phenotype After MI

One potential mechanism for improved cardiac function after MI in dnTRPC4 versus WT hearts is that myocyte function and adrenergic responsiveness are better preserved. To address this idea, we first measured twitch contractions and 

\[ [Ca^{2+}] \]

transients in the absence and presence of isoproterenol in dnTRPC4 and WT myocytes after sham or MI procedures. Representative data are shown in Online Figure XIA and XIB. Fractional shortening of dnTRPC4 was significantly greater than in WT (sham) myocytes (dnTRPC4 versus WT: 11.0±0.6 versus 8.4±0.6%; Figure 8A). After MI, contractions remained significantly greater in dnTRPC4 than in WT myocytes (dnTRPC4 versus WT: 13.4±1.1% versus 8.9±0.4%).

Myocyte contractions in both WT and dnTRPC4 cells increased with isoproterenol (WT sham:isoproterenol: 8.4±0.6 versus 11.7±0.8%; dnTRPC4 sham:isoproterenol: 11.0±0.6 versus 13.5±0.6%) with baseline contractions in dnTRPC4 being greater than in WT (Figure 8A). Post-MI, dnTRPC4 myocytes again had greater baseline contractions and isoproterenol response than WT myocytes (post-MI WT:isoproterenol: 8.9±0.4 versus 13.0±0.4%; post-MI dnTRPC4:isoproterenol: 13.4±1.1 versus 16.0±0.8%).

Peak systolic 

\[ [Ca^{2+}] \]

in dnTRPC4 (sham) myocytes was significantly greater than in WT myocytes (Figure 8B), explaining their greater twitch contractions. After MI, 

\[ [Ca^{2+}] \]

transients in dnTRPC4 myocytes remained significantly greater than in WT myocytes (peak 

\[ F/F_0 \]

in dnTRPC4 sham versus WT sham: 3.0±0.3 versus 2.1±0.3; post-MI dnTRPC4 versus WT: 3.6±0.5 versus 2.5±0.2). Isoproterenol significantly increased 

\[ Ca^{2+} \]

transient amplitude in both WT and dnTRPC4 myocytes, with 

\[ Ca^{2+} \]

transient amplitude being significantly greater in dnTRPC4 myocytes (WT [sham]:isoproterenol: 2.1±0.3 versus 3.5±0.4; dnTRPC4 [sham]:isoproterenol: 3.0±0.3 versus 4.4±0.5; post-MI WT:isoproterenol: 2.5±0.2 versus 3.6±0.6; post-MI DNTRPC4:isoproterenol: 3.6±0.5 versus 6.0±0.8; Figure 8B).

Contraction half width and the time constant of decay (Tau) of 

\[ Ca^{2+} \]

transients were also measured. Half width (isoproterenol conditions) was significantly less in dnTRPC4 (sham) versus WT (sham) myocytes (dnTRPC4 [sham]:isoproterenol: 220±11 versus 209±9 ms; WT [sham]:isoproterenol: 280±20 versus 261±17 ms; Figure 8C). MI induced changes (half width increase) in the duration of contractions in both dnTRPC4 and WT myocytes. After MI, half width of contractions (isoproterenol conditions) remained significantly less in dnTRPC4 versus WT myocytes (dnTRPC4 [MI]:isoproterenol: 247±16 versus 230±14 ms; WT [MI]:isoproterenol: 315±15 versus 279±11 ms; Figure 8C). Isoproterenol induced significant decreases in Tau in both WT and dnTRPC4 myocytes after sham or MI (Figure 8D). There were no significant differences in Tau between WT and dnTRPC4 myocytes after sham or MI:isoproterenol conditions (Figure 8D). Collectively, these data show that myocytes from dnTRPC4 MI hearts retain a hypercontractile phenotype after MI.

LTCC Current (\( I_{\text{Ca,L}} \)) Was Not Different Between dnTRPC4 and WT Myocytes After Sham or MI

Altered function of LTCC and loss of adrenergic regulation is a common feature of diseased cardiac myocytes. We next examined if loss of TRPC function influenced the behavior of LTCC either before or after MI. LTCC currents were measured in single isolated myocytes from WT and dnTRPC4 hearts with or without MI. \( I_{\text{Ca,L}} \) density was not significantly different in sham dnTRPC4 versus WT myocytes (peak \( I_{\text{Ca,L}} \) in dnTRPC4 versus WT: −13.0±1.4 versus −12.1±0.85 pA/pF; Figure 8E). Isoproterenol increased \( I_{\text{Ca,L}} \) density in both sham dnTRPC4 myocytes (pre-isoproterenol versus after isoproterenol: −13.0±1.4 versus −22.5±2.8 pA/pF) and sham WT myocytes (−12.1±0.85 to −20.9±1.9 pA/pF). After MI, \( I_{\text{Ca,L}} \) density was decreased to a similar extent in all myocytes (dnTRPC4 versus WT: −11.8±1.7 versus −10.4±1.1 pA/pF; Figure 8E). However, only dnTRPC4 myocytes showed a significant increase in \( I_{\text{Ca,L}} \) with isoproterenol after MI (pre-isoproterenol versus after isoproterenol: −11.8±1.7 versus −18.3±2.5 pA/pF). Cell capacitance (Figure 8F) and cell size (Online Figure XD andXE) were similar in sham WT and dnTRPC4 animals, but a significant increase in cell capacitance was seen in WT cells after MI (Figure 8F) but not in dnTRPC4 myocytes.

Discussion

This study explored the idea that \( Ca^{2+} \) influx through TRPC channels expressed after MI contributes to altered myocyte contractility and hypertrophic signaling. Our studies revealed a low level of TRPC isoform expression in normal adult mouse and AFMs, with a significant increase in select TRPC isoform expression after MI (Figure 1). TRPC was shown to induce SOCE, which was abolished with a dnTRPC6-expressing adenovirus. Using AFMs, we showed that \( Ca^{2+} \) entry through TRPC3 could load the SR when SR \( Ca^{2+} \) stores were naturally depleted but could overload the SR and cause
spontaneous SR Ca\(^{2+}\) release (Ca\(^{2+}\) sparks, leak) if SOCE was persistent or excessive, and these effects seem to be because of CaMKII-mediated RyR S2814 phosphorylation (Figure 4). TRPC expression was associated with reduced contractile effects of catecholamines. Our studies also showed that a fraction of TRPC channels is localized to caveolae, where together with LTCCs they activate pathological hypertrophic signaling. Finally, mice with cardiac myocyte-specific expression of dn-TRPC4 had less cardiac dysfunction and adverse remodeling after MI.

### TRPC Expression in Disease

A clear link between Ca\(^{2+}\) influx and cardiac hypertrophy has been established,\(^1,48\) and activation of Cn-NFAT signaling is known to initiate the coordinated expression of maladaptive hypertrophic genes, and overstimulation of this pathway can lead to heart failure.\(^3,49,50\) Multiple in vitro\(^5,22,24,51,52\) and in vivo\(^2,20,21,51,53\) TRPC expression systems have documented a role for these channels in the induction of Cn-NFAT signaling and subsequent hypertrophic remodeling. TRPC loss-of-function\(^6,21,24,54\) and selective inhibition\(^23,24,53\) animal models are protected against cardiac hypertrophy and indices of heart failure after either pressure overload or neurohormonal stress. In accordance with these studies, we found that cardiac-specific overexpression of dn-TRPC4 resulted in reduced pathological remodeling in an MI model of injury (Figure 7) and a cardioprotective phenotype that increased survival post-MI.

### TRPC, SOCE, and Myocyte Contractility

Progressive deterioration of cardiac contractility is a central feature of heart failure, and alterations of intracellular Ca\(^{2+}\) regulation are primarily responsible for this depression in contractility reserve.\(^1,47\) In this study, we explored the hypothesis that TRPC channels expressed in diseased myocytes contribute to their deteriorating contractility. Others have found that cardiac-specific overexpression of TRPC6 in mice resulted in an exaggerated hypertrophic response to pressure overload with decreased systolic function,\(^7\) and a similar study showed that TRPC3 transgenic mice developed a loss of ventricular contractility reserve after MI.\(^20\) We found enhanced cardiac pump function in cardiac-specific dn-TRPC4 mice (Figure 7A and 7B) and increased myocyte fractional shortening and peak Ca\(^{2+}\) transients (Figure 8A and 8B). These are somewhat curious findings, because TRPC is a pathway for Ca\(^{2+}\) influx, and this would be expected to increase rather than decrease contractile Ca\(^{2+}\). To examine if/how TRPC channels cause alterations in myocyte contractility, TRPC3, TRPC4, TRPC6 or dnTRPC6 and dnTRPC4 were expressed in AFMs. We found that the changes in AFM contractile function were dependent on the experimental conditions. When normal or RFP-infected AFMs were unpaced for a period of time, their SR Ca\(^{2+}\) stores became depleted (Figure 2A–2C). TRPC3/4/6 expression resulted in enhanced rested-state contractions (Figure 2A–2C), suggesting that when SR Ca\(^{2+}\) stores are depleted, TRPC channels can supply Ca\(^{2+}\) for refilling. However, in paced AFMs, TRPC3/6 resulted in reduced steady-state contractile function and reduced responsiveness to catecholamines (Figure 3C). TRPC3/6 overexpression induced Ca\(^{2+}\) sparks (Figure 3A and 3B), suggesting that altered contractility was because of enhanced SR Ca\(^{2+}\) leak. Finally, we showed that increased TRPC3/6 activity was associated with RyR S2814 phosphorylation that was reduced by CaMKII inhibition (Figure 4A and 4C). These results suggest that although Ca\(^{2+}\) influx through TRPC channels can replenish depleted SR Ca\(^{2+}\) stores, excess TRPC channel activity causes local activation of CaMKII and phosphorylation of RyR at S2814, resulting in abnormal RyR function, producing spontaneous diastolic SR Ca\(^{2+}\) release leading to depressed contractility reserve.

### TRPC and Hypertrophy Signaling

Many groups have shown that TRPC channels contribute to the activation of Cn-NFAT signaling and hypertrophic response.\(^6,21,22,55\) Data from our study are in accordance with this and expand on what is known to show that the organization of TRPC channels along with LTCC in caveolae membrane microdomains influences their ability to orchestrate Cn-NFAT signaling. TRPC channels are known to allow influx of both Na\(^{+}\) and Ca\(^{2+}\), and it has been shown by others that Na\(^{+}\) entry via NHE-1 and induce Ca\(^{2+}\) entry via the NCX to activate NFAT activation.\(^45,46,56\) We found both NHE-1 and NCX in caveolae membrane microdomains (Online Figure VII), suggesting that they could contribute to TRPC-mediated NFAT activation in these signaling microdomains. We addressed this by inhibiting NHE-1 with cariporide (Online Figure VDI) but saw no change in TRPC-mediated NFAT nuclear translocation, indicating that NHE-1 is not playing a central role in this process in our system. However, our experimental design is unable to definitively rule out a role for Na\(^{+}\) in this process, and it is possible that TRPC-mediated Na\(^{+}\) entry is also a contributing factor to Cn-NFAT.

### TRPC Inhibition Post-MI

Finally, an in-depth characterization of an MI model of injury in dnTRPC4 transgenic mice was used to determine if reducing TRPC channel activity after MI reduced structural and functional remodeling and had a beneficial outcome. We found that dnTRPC4 mice did not exhibit TRPC-mediated Ca\(^{2+}\) entry after MI and had less pathological hypertrophy, better cardiac performance, less progression of heart failure, and increased survival after MI compared with WT animals (Figures 6 and 7; Online Figures IX and X).

Collectively, our studies show that TRPC channels are stress-response molecules that are upregulated in chronic cardiac disease states. Mechanistically, our data suggest that TRPC channels disrupt normal SR Ca\(^{2+}\) storage by inducing SR Ca\(^{2+}\) leak to contribute to depressed contractility reserve in disease. These effects are accompanied by coordinated Ca\(^{2+}\)-activated Cn-NFAT signaling through caveolae membrane microdomains. These data suggest that targeted inhibition of cardiac myocyte TRPC channels might be an effective strategy for attenuating pathological structural remodeling and for maintaining contractility reserve after MI.

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Disclosures

None.

References


What Is Known?

- Transient receptor potential canonical (TRPC) channels are present in low abundance in the normal heart, and their expression is increased by pressure overload.
- TRPC channels contribute to cardiac hypertrophy and disease progression in pressure overload.

What New Information Does This Article Contribute?

- TRPC channel expression increases after myocardial infarction (MI) and is associated with pathological remodeling and contractility reserve defects; inhibition of TRPC channels post-MI increased survival, reduced pathological remodeling, and improved cardiac function.
- 
  Ca2+ influx through TRPC channels activates Ca2+/calmodulin protein kinase II resulting in Ca2+ leak from sarcoplasmic reticulum stores and reduces contractility reserve.
- TRPC channels organized in caveolae membrane signaling microdomains provide a local Ca2+ signal that activates calcineurin-nuclear factor of activated T-cells signaling, a well-established upstream mediator of pathological hypertrophy.

Novelty and Significance

TRPC channel expression and activity is increased in models of pathological hypertrophy and heart failure. We asked if and how Ca2+ influx through TRPC channels contributes to the structural remodeling and contractility defects seen after MI. We found that isoforms of the TRPC channel were upregulated after MI. The biological activity of TRPC channels was linked to reduced sarcoplasmic reticulum Ca2+ stores using an in vitro system. Our studies showed that TRPC activity triggered spontaneous sarcoplasmic reticulum Ca2+ release that was linked to Ca2+/calmodulin protein kinase II activation and downstream modification of ryanodine receptors making them more prone to leak. These changes resulted in a reduction of contractility reserve. Our results showed that TRPC channels localized to caveolae membrane domains are involved in stress-mediated activation of calcineurin-nuclear factor of activated T-cells signaling. TRPC channel inhibition with a cardiac-specific dominant-negative TRPC construct reduced pathological structural and functional remodeling after MI and improved survival. These studies suggest that after MI, the biological activity of TRPC channels perpetuates cardiac hypertrophy and contributes to depression of contractility reserve.
Transient Receptor Potential Channels Contribute to Pathological Structural and Functional Remodeling After Myocardial Infarction

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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'-GTAACCCGTTGAACCCCAT, 5'-CCATCCAATCGGTAGTACCG; atrial natriuretic factor (ANF) 5'-GCCCTGAGTGACAGACTG, 5'-CGGAAGCTGTTGAGACAGCCCT; brain natriuretic factor (BNP) 5'-CTGCTGGAGCTGATAAGAGA, 5'-CCATCCAATCGGTAGTACCG; skeletal muscle actin (SMA) 5'-TACACGTCACAGCTCTG, 5'-ATGTATACAACCAGCTCTATCTTG; myosin heavy chain alpha (MHCα) 5'-ACCTACAGACAGAGGAAGAT, 5'-ATTGTGTATTGGCCACAGCG; myosin heavy chain beta (MHCβ) 5'-ACCTACAGACAGAGGAAGAT, 5'-TTGCAAAAGTGCCAGCTTGAG; TRPC1 5'-GGCGGAGGTCACGGAACGGA, 5'-GGGGCCACCATGTTTGGCACAT; TRPC2 5'-GCCACCCCACCTCAATCTTG, 5'-GGCCACCATGTTTGGCACAT; TRPC3 5'-GGGGCCACCATGTTTGGCACAT, 5'-GCCACCCCCAATCTTG; TRPC4 5'-TTCGGGACATGTTGGCGGCGT, 5'-CCACCCAGGCGAGGCCAT; TRPC5 5'-CCGATCCCCCTCAATCTTG, 5'-GGCTGGGAACCTGCTTCTTCCA; TRPC6 5'-GCTACTACCCCCAGCTCTCGGGG, 5'-TGGATGGTTGAGGATTGCTCCACA.

**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-pinching 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): CaCl₂ 0.02, glucose 10, HEPES 5, KCl 5.4, MgCl₂ 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 200μmol/L CaCl₂, 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, MgSO₄ 1.2, NaCl 130, NaH₂PO₄ 1.2, NaHCO₃ 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 (2mmol/L)(Sigma).

Myocytes were infected with adenovirus (Ad) expressing RFP, GFP, TRPC3, dnTRPC6, TRPC6-FLAG, Rem₁²⁶⁵-Cav (Rem-Cav)⁸, and/or NFATc3-GFP for 12-16 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, Agilent 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 (GSK83583A, 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 1A).

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 Tricene buffer (in mmol/L: 250 sucrose, 1 EDTA,20 Tricene, pH 7.4) and centrifuged. Cell pellets were resuspended in Tricene buffer and homogenized with a dounce homogenizer. Homogenates were then centrifuged and the supernatant was collected, mixed with 30% Percoll (Sigma) in Tricene buffer, and subjected to ultracentrifugation for 25 minutes (Beckman ML150 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 ML150 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 IIB-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, -TRPC3 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 CaCl$_2$ and, where applicable, 10umol/L OAG, 4mmol/L Ca$^{2+}$, 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$^9$. 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)$^{17}$. 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.

*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).

*Determination of Left Ventricle Area-At-Risk and Infarct Size After MI*

*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, washed, and then perfused with 10\% buffered formalin. The fixed heart tissues were dehydrated embedded in paraffin, longitudinal-sectioned at 5-\(\mu\)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.

*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_{CaL}). All experiments were done a 35-37\(^\circ\)C, in superfused myocyte chambers mounted on fluorescence equipped microscopes. All myocytes were characterized within the same series of experiments.

*I_{CaL} Measurement*

I_{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-Dglucamine (NMDG) 10, tetaethylammonium 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.

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. 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. 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), phospho-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).
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 RyR2 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 immunoisolations 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).