Role of STIM1 (Stromal Interaction Molecule 1) in Hypertrophy-Related Contractile Dysfunction

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Rationale: Pathological increases in cardiac afterload result in myocyte hypertrophy with changes in myocyte electrical and mechanical phenotype. Remodeling of contractile and signaling Ca2+ occurs in pathological hypertrophy and is central to myocyte remodeling. STIM1 (stromal interaction molecule 1) regulates Ca2+ signaling in many cell types by sensing low endoplasmic reticular Ca2+ levels and then coupling to plasma membrane Orai channels to induce a Ca2+ influx pathway. Previous reports suggest that STIM1 may play a role in cardiac hypertrophy, but its role in electrical and mechanical phenotypic alterations is not well understood.

Objective: To define the contributions of STIM1-mediated Ca2+ influx on electrical and mechanical properties of normal and diseased myocytes, and to determine whether Orai channels are obligatory partners for STIM1 in these processes using a clinically relevant large animal model of hypertrophy.

Methods and Results: Cardiac hypertrophy was induced by slow progressive pressure overload in adult cats. Hypertrophied myocytes had increased STIM1 expression and activity, which correlated with altered Ca2+-handling and action potential (AP) prolongation. Exposure of hypertrophied myocytes to the Orai channel blocker BTP2 caused a reduction of AP duration and reduced diastolic Ca2+ spark rate. BTP2 had no effect on normal myocytes. Forced expression of STIM1 in cultured adult feline ventricular myocytes increased diastolic spark rate and prolonged AP duration. STIM1 expression produced an increase in the amount of Ca2+ stored within the sarcoplasmic reticulum and activated Ca2+/calmodulin-dependent protein kinase II. STIM1 expression also increased spark rates and induced spontaneous APs. STIM1 effects were eliminated by either BTP2 or by coexpression of a dominant negative Orai construct.

Conclusions: STIM1 can associate with Orai in cardiac myocytes to produce a Ca2+ influx pathway that can prolong the AP duration and load the sarcoplasmic reticulum and likely contributes to the altered electromechanical properties of the hypertrophied heart. (Circ Res. 2017;121:125-136. DOI: 10.1161/CIRCRESAHA.117.311094.)

Key Words: calcium • myocytes, cardiac • stromal interaction molecule 1

Cardiovascular diseases such as hypertension, valvular disease, and myocardial infarction–induced ventricular dilation all impose a persistent, pathological increase in the work demands (afterload) of the heart.1–3 Neurohumoral systems are activated to increase the contractile demands of the diseased heart.4 Over time, the persistently overworked left ventricle (LV) remodels, increasing wall thickness to at least partially compensate for increased wall stress.5 This hypertrophy occurs at both the organ and cellular levels, with an increase in LV mass and an increase in cardiac myocyte size.5 The signaling processes that produce pathological myocyte hypertrophy have essential Ca2+-dependent signaling steps, but there is some evidence that the sources of Ca2+ for enhanced contractile function and for hypertrophic signaling are distinct.7,9

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Hypertrophied myocytes adopt a modified electromechanical phenotype. The action potential (AP) is prolonged, Ca2+ transients are disrupted with slower kinetics, sarcoplasmic reticulum (SR) Ca2+ reuptake is slowed, Ca2+ leak from the SR is increased to reduce SR Ca2+ stores, and these systems become unresponsive to sympathetic regulation, reducing contractility reserve.10,11 Alterations in Ca2+ regulation in hypertrophy are also linked to increased risk of arrhythmias and HF.12,13 Indeed, LV hypertrophy

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However, the consequence of STIM-mediated Ca\textsuperscript{2+} influx on alterations in the electrical and mechanical properties of the lar to the human heart. In this study, we explore the idea that after pressure overload. There is some evidence from previous reports that these proteins are present in cardiac myocytes and their abundance increases in disease states. \textsuperscript{16,17} This increase has been linked to maladaptive cardiac hypertrophy. \textsuperscript{18,19}

Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>AFM</td>
<td>adult feline myocytes</td>
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<tr>
<td>AP</td>
<td>action potential</td>
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<td>APD</td>
<td>action potential duration</td>
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<tr>
<td>CaMKII</td>
<td>Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II</td>
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<tr>
<td>dn-Orai</td>
<td>dominant negative Orai</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
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<tr>
<td>LV</td>
<td>left ventricular</td>
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<tr>
<td>LTCC</td>
<td>L-type calcium channel</td>
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<tr>
<td>PLB</td>
<td>phospholamban</td>
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<tr>
<td>RFP</td>
<td>red fluorescent protein</td>
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<tr>
<td>SR</td>
<td>sarcoplasmic reticulum</td>
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<tr>
<td>STIM1</td>
<td>stromal interaction molecule 1</td>
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<tr>
<td>TRPC</td>
<td>transient receptor potential, canonical type</td>
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is an independent predictor of hospitalization and sudden cardiac death.\textsuperscript{14,15}

The molecular basis of altered contractile and hypertrophic signaling [Ca\textsuperscript{2+}] are still not fully understood and are the topic of this study.\textsuperscript{2} Here, we specifically study the role of the SR protein, STIM1 (stromal interaction molecule 1), and its sarcolemmal partner Orai in the development of electrical and contractile remodeling of adult ventricular myocytes after pressure overload. There is some evidence from previous reports that these proteins are present in cardiac myocytes and their abundance increases in disease states.\textsuperscript{16,17} This increase has been linked to maladaptive cardiac hypertrophy.\textsuperscript{18,19}

However, the consequence of STIM-mediated Ca\textsuperscript{2+} influx on the function of hypertrophied cardiac myocytes is not clearly defined, especially in large animals with Ca\textsuperscript{2+} regulation similar to the human heart. In this study, we explore the idea that alterations in the electrical and mechanical properties of the hypertrophied heart involve STIM-dependent Ca\textsuperscript{2+} entry and blocking this influx pathway can improve these disturbances.

**What Is Known?**

- STIM1 (stromal interaction molecule 1) in the endo (sarco)plasmic reticulum partners with surface membrane Orai calcium (Ca\textsuperscript{2+}) channels to regulate sarcoplasmic reticulum Ca\textsuperscript{2+} stores in a variety of cell types.
- Pathological cardiac hypertrophy with mechanical dysfunction is associated with disturbed Ca\textsuperscript{2+} regulation.
- Increased STIM1 expression contributes to cardiac hypertrophy.

**What New Information Does This Article Contribute?**

- STIM1 partners with Orai channels in hypertrophied cardiac myocytes to produce Ca\textsuperscript{2+} influx and increase sarcoplasmic reticulum Ca\textsuperscript{2+} stores.
- STIM1-mediated Ca\textsuperscript{2+} influx contributes to action potential prolongation, dyssynchronous Ca\textsuperscript{2+} release (sparks), and cell death in hypertrophied cardiac myocytes.

Ca\textsuperscript{2+} levels in cardiac myocytes regulate contractile function and influence a host of normal and pathological processes. In cardiac hypertrophy, Ca\textsuperscript{2+} regulation is deranged and produces altered electrical and mechanical function. Increased expression of STIM1 in the heart has been linked to the development of pathological hypertrophy, but the contribution of this putative Ca\textsuperscript{2+} influx pathway to hypertrophic electromechanical disturbances is unknown. Here, we used genetic and pharmacologic approaches to define the role of STIM1 to the electromechanical alterations of hypertrophied myocytes. The results of these experiments showed that STIM1 partners with Orai channels to produce a Ca\textsuperscript{2+} influx pathway. STIM1-mediated Ca\textsuperscript{2+} influx contributed to prolonged action potential duration, caused sarcoplasmic reticulum Ca\textsuperscript{2+} overload and associated Ca\textsuperscript{2+} sparks during diastole to shorten diastolic sarcomere length, and could induce necrotic myocyte death. STIM1–Orai was not shown to make significant contributions to the electromechanical properties of normal myocytes. These findings show that STIM1–Orai–mediated Ca\textsuperscript{2+} influx in hypertrophied cardiac myocytes contributes to the electromechanical derangements that can cause lethal cardiac arrhythmias and depressed cardiac pump function.

STIM and Orai proteins are widely known as the key mediators of store-operated Ca\textsuperscript{2+} entry (SOCE), a nearly ubiquitous process in nonexcitable cells.\textsuperscript{20,21} In mammals, there are 2 isoforms of STIM (STIM1 and STIM2) that are present in the endoplasmic reticulum (ER),\textsuperscript{22} and 3 homologs of Orai (Orai1, Orai2, and Orai3) are located in the plasma membrane. SOCE occurs during periods of ER Ca\textsuperscript{2+} depletion, which can physiologically occur after inositol trisphosphate receptor activation of ER Ca\textsuperscript{2+} release. STIM senses reduced ER [Ca\textsuperscript{2+}] via a luminal ejection fraction-hand motif and subsequently undergoes a conformational change resulting in oligomerization.\textsuperscript{23} Activated STIM proteins are then able to bind and gate Orai channels located in the plasma membrane, which are highly selective, low-conductance Ca\textsuperscript{2+} channels.\textsuperscript{24} The resulting Ca\textsuperscript{2+} influx adds Ca\textsuperscript{2+} to the ER and is also thought to activate Ca\textsuperscript{2+}-dependent signaling processes.\textsuperscript{25}

Cardiac myocytes have robust Ca\textsuperscript{2+} entry through the L-type Ca\textsuperscript{2+} channel with each heartbeat. This Ca\textsuperscript{2+} influx induces Ca\textsuperscript{2+} release from the SR to initiate contraction and also provides Ca\textsuperscript{2+} to load the SR.\textsuperscript{26} The SR releases and takes up Ca\textsuperscript{2+} with each heartbeat and is never depleted during normal physiological function. It is, therefore, not surprising that a physiological role for STIM and Orai in cardiac myocytes has not been defined. Yet, many independent groups have found STIM1 to be present in cardiac myocytes\textsuperscript{19} although there are conflicting reports on the function of STIM1 and SOCE in these cells.\textsuperscript{16,27,28} In the diseased heart, SR Ca\textsuperscript{2+} regulation is altered\textsuperscript{18} and increased SOCE activity has been observed in rodent cardiac disease models. Some groups have linked this increased activity with pathological hypertrophy. A recent study\textsuperscript{16} suggested that activated STIM1 can increase SR Ca\textsuperscript{2+} loading by binding to PLB (phospholamban) and thereby regulating SERCA (sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase) activity. This function of STIM1 in normal myocytes was found to be independent of Orai and SOCE. Collectively, these reports suggest that Ca\textsuperscript{2+} influx through STIM and Orai proteins might be involved in the abnormal Ca\textsuperscript{2+} regulation found
in diseased ventricular myocytes. However, this hypothesis has not been tested in a clinically relevant large animal model.

The objective of our study was to explore the if/how STIM1 contributes to the changes in electrical and contractile phenotype of hypertrophied ventricular myocytes from the adult feline heart. We used the feline model because, unlike rodent models, it has electrical and Ca\(^2+\) regulatory properties that are similar to those of human.\(^{20,30}\) Our experiments tested the hypothesis that in myocytes with hypertrophy from persistent pressure overload, SR-based STIM1 is activated and partners with surface membrane Orai to produce Ca\(^2+\) entry that contributes to their altered phenotype. Our results support the idea that STIM1 and Orai are expressed, but do not contribute to the electromechanical function of the healthy heart. In hypertrophied myocytes, STIM1 becomes activated and induces Ca\(^2+\) entry after associating with Orai, resulting in enhanced SR loading and AP prolongation. Excessive STIM1-mediated Ca\(^2+\) entry caused Ca\(^2+\) sparks, spontaneous APs, and cell death. These results suggest that STIM1 activation in disease might help preserve SR Ca\(^2+\) load but predisposes myocytes to arrhythmias and death.

**Methods**

Methods are described in detail in the Online Data Supplement. Briefly, LV hypertrophy was induced in young cats by aortic banding and myocytes were isolated using collagenase as described in detail previously. After isolation, Ca\(^2+\) transients, Ca\(^2+\) currents, Ca\(^2+\) sparks, and cell shortening were measured using standard techniques described in detail in previous reports from our group.\(^{27,31}\) Isolated myocytes from control and banded animals were exposed to 1 µmol/L BTP2 (N-(4-[3,5-bis(trifluoromethyl)-1H-pyrazol-1-yl]phenyl)-4-methyl-1,2,3-thiadiazole-5-carboxamide), in vitro, in cell physiology experiments. Myocytes were also studied in the primary culture with surface membrane Orai to produce Ca\(^2+\) entry that contributed distinct intracellular contents in hypertrophied myocytes.\(^{35,36}\) This procedure caused significant increases of young animals, to induce LV hypertrophy, similar to our previous reports.\(^{35,36}\) This procedure caused significant increases in systolic proximal aortic pressure after 4 months (proximal versus distal pressure: 149.8±15.3 versus 87±9.6 mm Hg; Figure 1A) and a systolic pressure gradient of 63 mm Hg across the aortic constriction. There are no significant decreases in echocardiogram-derived systolic function in this model (Figure 1B). End-diastolic wall thickness was significantly increased by banding as measured by echocardiography (banded versus sham: 7.5±0.5 versus 4.6±0.3 mm; \(P<0.05\); Figure 1C). The heart weight/body weight ratio was significantly increased in banded versus sham animals (9.2±1.13 versus 4.6±0.4; \(P<0.01\); Figure 1D).

Pressure overload was associated with increases in the expression of SOCE-related mRNA. Real-time polymerase chain reaction was used to measure mRNA levels of SOCE proteins in mRNA from isolated myocytes. Myocytes from banded animals had significantly greater amounts of STIM1 and Orai1 mRNA and nonsignificant increases in STIM2 and Orai3 mRNA (Figure 1E). Orai2 was not detected in normal or hypertrophied feline myocytes.

Immunofluorescence staining of isolated myocytes showed that STIM1 exhibited a striated fashion in control myocytes (Figure 1F, top), with what appeared to be uniform intensity along the striations. Co-staining with actinin suggests that STIM1 is localized near the Z-line, where the sarcolemma/SR dyad resides (Online Figure IA). This staining pattern is similar to that seen by others.\(^{38}\) Hypertrophied myocytes from banded animals exhibited a more uneven STIM1 intensity, with a punctate pattern (Figure 1F, bottom). The mean fluorescence intensity of STIM1 staining was increased in banded animals (Figure 1G). STIM1 protein, measured by Western analysis, was also increased in hypertrophied versus normal hearts (Online Figure IB). Collectively, these studies show that STIM1 expression increases in feline ventricular myocytes with pressure overload–induced hypertrophy and suggest that STIM1 may oligomerize in these myocytes.

**Results**

**Pressure Overload Hypertrophy Is Associated With Increased STIM1 Expression and Activity**

Slow progressive pressure overload was induced by aortic banding of young animals, to induce LV hypertrophy, similar to our previous reports.\(^{25,36}\) This procedure caused significant increases in systolic proximal aortic pressure after 4 months (proximal versus distal pressure: 149.8±15.3 versus 87±9.6 mm Hg; Figure 1A) and a systolic pressure gradient of 63 mm Hg across the aortic constriction. There are no significant decreases in echocardiogram-derived systolic function in this model (Figure 1B). End-diastolic wall thickness was significantly increased by banding as measured by echocardiography (banded versus sham: 7.5±0.5 versus 4.6±0.3 mm; \(P<0.05\); Figure 1C). The heart weight/body weight ratio was significantly increased in banded versus sham animals (9.2±1.13 versus 4.6±0.4; \(P<0.01\); Figure 1D).

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The contribution of STIM1–Orai–dependent Ca\(^2+\) influx to steady-state myocyte contractions and Ca\(^2+\) transients was first explored by exposing isolated myocytes to the Orai blocker BTP2. This antagonist was chosen for its relative specificity for Orai over other ion channels.\(^{31}\) Myocytes were incubated with either vehicle or BTP2 and then paced at 1 Hz to measure steady-state fractional shortening and Ca\(^2+\) transients. BTP2 did not significantly affect systolic Ca\(^2+\) transients or contractions in either normal (Online Figure II) or hypertrophied myocytes (Figure 2A through 2I). However, BTP2 did cause an increase in diastolic sarcomere length in hypertrophied myocytes (Figure 2J).

The effects of BTP2 on AP duration (APD) in sham and hypertrophied myocytes were determined using the voltage-sensitive dye di-8-anneps. This method is technically less demanding than patch clamp approaches and allows for study of an increased number of myocytes. In addition, this approach allowed for evaluation of AP waveform, without dialysis that could eliminate distinct intracellular contents in hypertrophied myocytes. Preliminary studies showed that APs had similar waveforms and durations when recorded using either di-8-anneps or patch clamp in myocytes (not shown). BTP2 had no effect on APD in sham myocytes, with a time to 90% repolarization of 285±9.5
ms (Figure 3). In contrast, myocytes from banded animals had longer baseline APDs and had significant APD shortening when incubated with BTP2, as compared with vehicle controls (time to 90% APD: 434±11 versus 390±11.5 ms for vehicle and BTP2, respectively; P≤0.01; Figure 3). In separate experiments, we found that BTP2 did not reduce Ca2+ current at the concentrations used in these experiments (Online Figure IIK).

Ca2+ sparks were quantified during the diastolic interval (1 Hz) in sham and hypertrophied myocytes. Sparks were infrequently observed in sham myocytes but were routinely found in hypertrophied myocytes. BTP2 significantly reduced the number of sparks in hypertrophied myocytes as compared with vehicle control (Figure 4).

Increasing STIM1 Induces Ca2+ Influx That Loads the SR
To further explore the contribution of increased STIM1 expression to altered myocyte Ca2+ regulation in disease, adenoviral gene transfer was used to express STIM1 (human cDNA clone) or RFP (red fluorescent protein) in freshly isolated adult feline myocytes (AFMs). Western blot and immunofluorescence confirmed increased expression of STIM1 within 12 to 18 hours of infection (Online Figure III). In these studies, STIM1 was found to be organized into puncta, similar to the staining pattern seen in hypertrophied myocytes.

AFMs and other myocytes from large mammals are electrically and mechanically quiescent in primary culture, and they maintain low cytoplasmic and SR Ca2+. These properties result from the low cytosolic [Na+] in large mammalian ventricular myocytes, which produces forward mode Na+/Ca2+ exchanger activity and low diastolic and SR [Ca2+] when not paced (Online Figure IVA and IVB).30 These features are distinct from the conditions in rodent myocytes where high cytosolic [Na+] leads to persistent cytosolic and SR Ca2+ overload.30 Unpaced AFMs with STIM1 expression exhibited higher SR loads than RFP myocytes (Online Figure IVA and IVB). When paced, STIM1 myocytes had significantly increased Ca2+ transient amplitudes and decreased diastolic sarcomere lengths but increases in fractional myocyte failed to achieve statistical significance (Figure 5A through 5E).
STIM1 could interact with a variety of sarcomemal proteins to induce increased Ca\textsuperscript{2+} entry.\textsuperscript{23} Our results suggest that STIM1 interaction with Orai is the pathway for Ca\textsuperscript{2+} entry in unpaced myocytes that enhances diastolic SR Ca\textsuperscript{2+} loading. However, previous studies suggest that STIM1 can also directly interact with the L-type calcium channel (LTCC) and modify its behavior,\textsuperscript{42,43} and this could alter myocyte Ca\textsuperscript{2+}. To test whether STIM1 interacted with LTCCs to alter SR loading, we exposed unpaced AFMs±STIM1 to verapamil, a potent LTCC blocker, and then induced SR Ca\textsuperscript{2+} release. Verapamil did not affect SR load in unpaced STIM1-expressing myocytes (Online Figure IV A and IVB). Furthermore, we found that STIM1 expression did not alter LTCC current density, either at basal levels or after BAYK8644 treatment (Online Figure IVC). However, STIM1-infected cells with increased Ca\textsuperscript{2+} transients had greater Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange activity (Online Figure IVD).

It has been recently suggested that 1 function of STIM1 in cardiac myocytes is to interact with PLB to enhance the activity of SERCA. We attempted to replicate this interaction by coimmunoprecipitating STIM1 with PLB. No interaction between STIM1 and PLB was found. We did observe SERCA2a immunoprecipitate with PLB, as expected\textsuperscript{44} (Online Figure V). These results indicate that STIM1 does not interact with PLB in feline myocytes and suggest that effects of STIM1 on Ca\textsuperscript{2+} influx are due to its interaction with sarcomemal Orai channels.
Increased STIM1 Expression Is Sufficient to Induce Diastolic Sparks

STIM1- and RFP-expressing myocytes were paced, and diastolic Ca\(^{2+}\) sparks and sarcomere length were measured. A low rate of spark activity was observed in RFP control myocytes (Figure 5F and 5G). STIM1 myocytes exhibited increased spark rates and a greater reduction in diastolic sarcomere length (Figure 5E). These changes mirrored the increased spark activity and reduced sarcomere length seen during diastole in myocytes from banded animals (Figure 4). BTP2...
eliminated diastolic sparks in STIM1 myocytes (Figure 5F) but had no effects on RFP-infected myocytes (not shown), suggesting a role for STIM1–Orai dependent Ca\(^{2+}\) influx.

To independently test a role for Orai in diastolic Ca\(^{2+}\) sparks, an Orai construct with a mutation in its pore-forming region (E106Q) that blocks Ca\(^{2+}\) permeation was used.\(^{45}\) This mutant will oligomerize with endogenous Orai channels (all isoforms), causing a dominant negative (dn-Orai) effect, blocking all Orai channel activity.\(^{45}\) AFMs were infected with dn-Orai that was tagged with RFP, along with STIM1. These experiments showed that STIM1 was colocalized with dn-Orai in a punctated pattern along the sarcolemma (Figure 5H). Coexpression of dn-Orai with STIM1 prevented the increased diastolic spark rate caused by STIM1 alone (Figure 5F and 5G). Collectively, these results suggest that Orai is a necessary partner for STIM1 to cause Ca\(^{2+}\) influx in AFMs.

**STIM1–Orai–Mediated Ca\(^{2+}\) Influx Causes Ca\(^{2+}\) Sparks and APs.**

AFMs expressing STIM1 exhibit spontaneous Ca\(^{2+}\) sparks and spontaneous contractions (Online Movie I), whereas control myocytes were quiescent (Online Movie II). To explore the bases of these spontaneous contractions, we monitored membrane voltage using di-8-anneps. These experiments showed that STIM1-expressing myocytes demonstrate diastolic depolarization preceding spontaneous APs and contractions (Figure 6A), and these depolarizations are associated with spontaneous SR Ca\(^{2+}\) release. BTP2 treatment of STIM1-expressing myocytes blocked spontaneous APs and shortened the APD of paced myocytes (Figure 6B and 6C). These results are consistent with the idea that increased STIM1 expression produces Ca\(^{2+}\) influx through Orai, increasing SR Ca\(^{2+}\) loading that eventually results in spontaneous SR Ca\(^{2+}\) release (sparks). Spontaneous SR Ca\(^{2+}\) release is known to induce Na\(^{+}\)/Ca\(^{2+}\) exchanger–mediated inward current that can be sufficient to depolarize the membrane potential and trigger an AP\(^{40}\). These experiments also show that Ad-STIM1–infected AFMs could have sufficient numbers of Ca\(^{2+}\) sparks to elicit spontaneous APs (Figure 6A). Spontaneous Ca\(^{2+}\) sparks were not blocked by verapamil, confirming that STIM1 does not raise myocyte Ca\(^{2+}\) levels by altering the activity of LTCCs (Figure 6D and 6E). BTP2 and dn-Orai blocked resting sparks and spontaneous AP generation (Figure 6D and 6E).

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**Figure 6.** STIM1 (stromal interaction molecule 1) causes spontaneous action potentials (APs) by increasing Ca\(^{2+}\) spark rate. **A**, Top, di-8-anneps fluorescence recording of a spontaneously contracting, STIM1-expressing myocyte. Middle, Contraction traces from a spontaneously contracting, STIM1-expressing myocyte. Bottom, Line scan of Ca\(^{2+}\) sparks and transients with corresponding fluorescence intensity trace from a spontaneously contracting, STIM1-expressing myocyte. **B**, Time from stimulus to 90% repolarization from peak (APD\(_{90}\)) of STIM1-expressing cells, with (n=15) and without BTP2 (n=14). **C**, Example trace of APs recorded with di-8-anneps. **D**, Line scans of unpaced myocytes with Ad-RFP or Ad-STIM1, with verapamil, BTP2, or Ad-dn-Orai coexpression. **E**, Quantitated spark rates of unpaced myocytes from **D** (n=15–20 myocytes). **P**≤0.01, ***P**≤0.001, #P≤0.01 vs Ad-STIM1, @P≤0.01 vs Ad-STIM1+verapamil.
STIM1 Activation of Orai Channels Leads to Ca\(^{2+}\)/Calmodulin-Dependent Protein Kinase II Activation and Cell Death

Our experiments showed that STIM1 expression increased the rate of AFM death after 72 hours (20%–40% viability) versus RFP-infected controls (80%–90% viability; Figure 7A and 7B). We next explored the STIM1 membrane signaling partner that is responsible for Ca\(^{2+}\) influx and cell death signaling in STIM1-infected AFMs. Our group and others have documented previously the importance of canonical transient receptor potential channels (TRPC)–mediated Ca\(^{2+}\) influx in cell death signaling myocytes.27,46 Some reports suggest that STIM1 can bind to and activate TRPC channels and induce Ca\(^{2+}\) influx.47

Previous study in our laboratory demonstrated that expression of a dominant negative TRPC4 construct (dn-TRPC4) results in total inhibition of all TRPC activity, through its oligomerization with each of the endogenous TRPC isoforms.27 Using this same construct, we compared the effects of coinfection of STIM1-infected AFMs with either dn-TRPC427 or dn-Orai. STIM1-induced cell death was not altered by dn-TRPC4 but was eliminated by dn-Orai (Figure 7B). BTP2 also rescued the cell death phenotype of STIM1 myocytes (Figure 7B).

The link between increased Ca\(^{2+}\) influx and cell death signaling has also been shown to involve the activation of Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII).48 To test this idea, STIM1 or RFP-infected myocytes were treated with KN-93, a CaMKII inhibitor. KN-93 rescued myocytes from cell death although not as completely as with BTP2 (Figure 7C).

Collectively, these results indicate that increased STIM1-mediated Ca\(^{2+}\) entry can activate CaMKII, which likely contributes to both cell death and changes in myocyte Ca\(^{2+}\) handling. Additional evidence for this idea was that PLB phosphorylation at threonine 17 (a CaMKII-specific site) was found to be increased in STIM1 myocytes (Figure 7D). PLB-threonine 17 phosphorylation also can explain the accelerated tau of Ca\(^{2+}\) transient decay in STIM1-infected myocytes (Online Figure IVE). BTP2 blocked STIM1-mediated PLB-T17 phosphorylation (Figure 7D), consistent with a central role of Orai. No changes in CaMKII autophosphorylation at Thr-286 or Thr-287 were observed by Western blot (data not shown).

CaMKII activity has previously been shown to cause cell death by activating both apoptosis and necrosis in cardiac myocytes.49 To determine whether STIM1 causes apoptosis, caspase 3 cleavage was measured by Western analysis. No differences were found between STIM1-expressing myocytes and RFP controls (Online Figure VI). These results suggest that STIM1 activity can lead to necrotic cell death, similar to what we have seen when excess Ca\(^{2+}\) influx through the LTCC is induced.32

Discussion

SOCE and its effector proteins—STIM and Orai—are present in many tissue types where they regulate many different processes.23 In noncardiac cells, SOCE is activated after ER Ca\(^{2+}\) depletion and the resultant Ca\(^{2+}\) influx refills the ER stores.23 The presence and functional significance of SOCE in
the normal adult heart are not well defined, but STIM expression in cardiac disease has been linked to pathological cardiac hypertrophy. The goal of the present study was to determine whether Ca\textsuperscript{2+} influx through a STIM- and Orai-dependent pathway contributes to the electromechanical phenotype of either normal or hypertrophied ventricular myocytes. A novel aspect of our study was that 2 independent approaches, pharmacological (BTP2) Orai blockade and genetic (dn-Orai) manipulation were used to demonstrate phenotypic features that were dependent on Orai channel function. Our experiments showed that both STIM1/2 and Orai1/3 are expressed in normal feline myocytes but are not major contributors to the phenotypic features we measured in this study (Online Figure II). Therefore, their function in the normal heart is yet to be defined. STIM/Orai contributions to electromechanical remodeling in hypertrophied hearts were studied in ventricular myocytes isolated from feline hearts with slow, progressive pressure overload (aortic banding). Myocytes isolated from hypertrophied hearts exhibited increased STIM1 expression, similar to previous reports. Our novel findings include the observation that STIM1 was organized into puncta, indicating STIM1 activation in hypertrophied myocytes (Figure 1F). Blockade of ion flux through STIM1/Orai with BTP2 in hypertrophied myocytes revealed, for the first time, that STIM1/Orai activity contributes to APD prolongation (Figure 3). In addition, we made the novel observation that STIM1–Orai activity leads to diastolic Ca\textsuperscript{2+} sparks and a shortening of the diastolic sarcomere length (Figures 2 and 4). Additional experiments with culturated AFMs showed that increasing STIM1 expression was sufficient to induce persistent Ca\textsuperscript{2+} influx and recapitulated many of the phenotypic features of hypertrophied myocytes. Specifically, STIM1 expression–induced Ca\textsuperscript{2+} influx caused spontaneous Ca\textsuperscript{2+} sparks, shortening of diastolic sarcomere length, SR Ca\textsuperscript{2+} loading, and spontaneous APs (Figure 5). STIM1 expression was also linked to cell death, in part, by activating CaMKII (Figure 7C). Our studies with BTP2 and dn-Orai, 2 reagents that have not previously been used to investigate Ca\textsuperscript{2+} handling in ventricular cardiac myocytes, demonstrate that Ca\textsuperscript{2+} entry into hypertrophied ventricular myocytes through a STIM1–Orai pathway contribute to those phenotypic features of diseased myocytes that have been linked to lethal arrhythmias, contractile dysfunction, and myocyte death signaling.

Expression and Activity of STIM and Orai in Normal Cardiac Myocytes

The nature of SOCE in normal adult cardiac myocytes from a variety of species has been investigated by many laboratories and found to be small or undetectable. However, myocyte STIM1 knockout leads to aberrant cardiac structure and function. Therefore, STIM1 clearly has roles that are yet to be defined. The experiments performed in the current study support the idea that STIM–Orai activity makes little or no contribution to the electromechanical function of normal ventricular myocytes. BTP2, a potent SOCE (Orai) blocker, did not modify APD, Ca\textsuperscript{2+} transients, or contractions of normal myocytes (Figures 2 and 3; Online Figure II). These data suggest that STIM and Orai proteins, while present in the normal heart, have little or no effects on Ca\textsuperscript{2+} cycling. The reasons for the absence of detectable activation of STIM1–Orai in normal myocytes are unclear and require additional study. For example, there is some evidence that STIM1 can be activated by reactive oxygen species or pH changes, independent of the level of SR [Ca\textsuperscript{2+}] stores and these issues were not examined. It is also possible that STIM and Orai have functional roles in the normal heart beyond the electromechanical features that we primarily examined.

**STIM–Orai Form a Ca\textsuperscript{2+} Influx Pathway That Contributes to Electromechanical Influx Remodeling in Hypertrophy**

STIM1 expression increases after pressure overload and the resultant Ca\textsuperscript{2+} influx is thought to be involved in the induction of pathological hypertrophy. STIM1 may be part of the fetal gene program that becomes reactivated during pathological stress and could contribute a pool of Ca\textsuperscript{2+} that activates pathological hypertrophy signaling. Reduction of STIM1 levels has been shown to reduce hypertrophic signaling in both neonatal and adult rodent myocytes. Increased expression of STIM1 conversely resulted in an exaggerated hypertrophic response to TAC, myocardial infarction, and chronic isoproterenol infusion in rodents. Collectively, these studies support the idea that STIM1 expression and activity increase in myocytes responding to persistent pathological stress and the resultant response includes myocyte hypertrophy.

The direct contribution of STIM1–Orai–dependent Ca\textsuperscript{2+} influx to the distinctive electromechanical phenotype of the hypertrophied ventricular myocytes has not been well studied and was the topic of this study. A feline model of slow progressive pressure overload that has critical features of human diseases, including concentric LV hypertrophy without systolic dysfunction (Figure 1), was used. This model has many features of a human condition called heart failure with preserved ejection fraction, with increases in ventricular wall thickness and heart weight to body weight ratio (Figure 1C and 1D) without cardiac dilation or reductions in systolic function (Figure 1B). In myocytes from banded animals, STIM1 mRNA and protein were increased along with a small but significant increase in Orai3 mRNA (Figure 1E). It is possible that the Orai3 isoform is the major partner for STIM1 in cardiac disease, as found by others. Future studies should focus on how the unique properties of Orai3 contribute to the electromechanical features of hypertrophied myocytes.

In myocytes from normal animals, immunofluorescence staining revealed that STIM1 is localized uniformly in the SR membrane along the Z-line (Online Figure I) but in banded myocytes exhibited a punctated pattern (Figure 1F), indicative of STIM1 oligomerization. These results support the idea that STIM1 is increased in abundance and activated in the hypertrophied heart.

**A Contractile Role for STIM and Orai in Hypertrophy**

Alterations in myocyte Ca\textsuperscript{2+} handling are central to the development of the electromechanical phenotype of the diseased heart. The idea that Ca\textsuperscript{2+} entry through a STIM1–Orai complex contributes to phenotypic adaptation in disease has not, to our knowledge, been studied in detail previously, especially
in animal models with electromechanical properties similar to those in the human heart. To investigate whether increased STIM1–Orai activity contributes to contractile abnormalities in feline hypertrophy, we first used BTP2 to block Orai channels. In comparison to other SOCE antagonists, BTP2 has little affinity for LTCCs (Online Figure IIK) and potassium channels. BTP2 had no effects on APs, Ca$^{2+}$ transients, or contractions in normal myocytes, consistent with little or no STIM1–Orai activity in the normal heart and also documenting the lack of off-target effects under the conditions we used (Online Figure II).

The phenotypic features of hypertrophied AFMs included prolongation of the APD, slowing and prolongation of the Ca$^{2+}$ transient, reductions of diastolic sarcomere length, increases in diastolic spark rate, and prolongation of contraction (Figures 2 through 4). These changes result from modifications in the abundance and behavior of many molecules that participate in contraction. To define a contribution of STIM1–Orai in these complex adaptations, hypertrophied AFMs were treated with BTP2. These studies showed that BTP2 shortened the APD, reduced diastolic spark rates, and produced a lengthening of diastolic sarcomere length, but had no significant effects on steady-state Ca$^{2+}$ transients (Figures 2 through 4). A major advantage of using the feline model to explore STIM–Orai contributions to APD is the low membrane conductance during their human-like AP plateau phase. The high-conductance state during repolarization of rodent APs masks changes in small currents that could make important contributions to changes in APD in human disease.

Our results suggest that BTP2 blocks a small amount of Ca$^{2+}$ entry through a STIM–Orai channel complex in hypertrophied AFMs that contributes to APD prolongation in hypertrophied AFMs. Our results also suggest that this Ca$^{2+}$ influx can contribute to diastolic SR Ca$^{2+}$ leak (Ca$^{2+}$ sparks) that can reduce diastolic sarcomere length. Collectively, these data are consistent with the idea that increased STIM1/Orai activity in hypertrophy contributes to the electromechanical phenotype of hypertrophied AFMs.

**STIM1/Orai Regulate SR Ca$^{2+}$**

Studies in genetically modified mice strongly support the idea that STIM1 expression is linked to cardiac hypertrophy, likely through activation of SOCE. However, these studies do not directly link STIM1 to functional changes because these could be secondary to the hypertrophy rather than directly resulting from STIM1 activity. To directly link the contributions of increased STIM1 expression/activity to myocyte electromechanical properties, we acutely expressed STIM1 in cultured AFMs. This model system allowed us to manipulate levels of STIM1 with minimal compensatory changes in global gene expression, which often occurs in genetic mouse models or in any model of induced disease. STIM1 was organized into puncta (Online Figure IIIB) in STIM1-expressing AFMs, comparable to the pattern observed in myocytes from banded animals. Acute STIM1 expression caused several functional alterations that paralleled changes observed in hypertrophied feline myocytes, including Ca$^{2+}$ sparks during the diastolic period in paced myocytes (Figure 5F), prolonged APs (Figure 6B), and increased Na$^{+}$/Ca$^{2+}$ exchange–mediated Ca$^{2+}$ efflux (Online Figure IVD). These alterations were reduced, both in vitro and in freshly isolated hypertrophied myocytes, when cells were treated with BTP2, supporting a direct role of STIM1–Orai.

Cultured AFMs normally maintain low cytosolic and SR Ca$^{2+}$. Expressing STIM1 caused an increase in SR Ca$^{2+}$ stores (Online Figure IV) and this is the likely basis for their increased Ca$^{2+}$ spark rate and spontaneous, SR Ca$^{2+}$ release–dependent spontaneous APs. STIM1 might also induce abnormal SR Ca$^{2+}$ release by influencing the [Ca$^{2+}$] within the diffusion limiting cleft between the T-tubule and SR membranes or by altering the properties of ryanodine receptors, possibly by CaMKII-mediated phosphorylation, which we showed was increased in STIM1 myocytes. A recent study in cultured rodent ventricular myocytes suggests that expressed STIM1 interacts with PLB to alter SR Ca$^{2+}$ uptake and induce SR Ca$^{2+}$ sparks. We explored this possibility by examining the interaction between PLB and STIM1 using commounprecipitation approaches (Online Figure V) but could find no evidence to support this hypothesis.

**STIM1 Expression Can Cause AFM Necrosis**

Our in vitro studies show that STIM1 expression increased the rate of AFM death (Figure 7). STIM1-infected AFMs did not appear to die via apoptosis, suggestive of a necrotic process. We have previously found that persistent increases in Ca$^{2+}$ influx via the LTCC induces a necrotic cell death in genetically modified mice. The cell death effects of STIM1 were abolished by BTP2 and dn-Orai, documenting a role for Ca$^{2+}$ influx via the STIM–Orai complex. Activation of CaMKII was also shown to be involved in this cell death effect (Figure 7D and 7E). These results warrant further exploration, as increased cell death in vivo would contribute to cardiac fibrosis and compensatory hypertrophy by the remaining myocytes, which may be partly responsible for the hypertrophy induced by STIM1 that others have observed.

In summary, the present study shows that pathological hypertrophy is associated with increases in STIM1 expression in AFMs. Our results are consistent with the idea that STIM1 associates with sarcosomal Orai channels to produce Ca$^{2+}$ influx that prolongs the APD, helps maintain SR Ca$^{2+}$ load, but induces SR Ca$^{2+}$ leak (Ca$^{2+}$ sparks) and reduced diastolic sarcomere length. These changes likely contribute to disturbed Ca$^{2+}$ regulation, Ca$^{2+}$–dependent arrhythmias and cell death in the diseased heart. Our findings suggest that STIM1–Orai activity increases in myocytes from hearts with disease-related increases in the contractile demands of the heart. Collectively, our findings suggest that STIM1–Orai–mediated Ca$^{2+}$ influx directly contributes to both the induction of pathological hypertrophy and the aberrant electromechanical phenotype of the hypertrophied heart, making this complex an attractive therapeutic target.

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**Disclosures**

None.
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Role of STIM1 (Stromal Interaction Molecule 1) in Hypertrophy-Related Contractile Dysfunction

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SUPPLEMENTAL MATERIAL

Detailed Methods

Animal Model
Left ventricular hypertrophy was induced by methods described previously in detail\textsuperscript{1,2}. Briefly, a fixed size (2.8-3.0 mm in diameter) constricting band was placed around the ascending aorta of young male cats (~1.0 kg). Previously, we have shown that as animals grow they develop a concentric, compensated hypertrophy after 4 months. Non-invasive transthoracic echocardiography was performed at baseline and at 4 months after aortic constriction, using a ZONARE z.one ultra sp Ultrasound System. B- and m-mode loops were recorded from a right parasternal short axis view. Chamber geometry and wall thickness were measured. Fractional shortening was derived from m-mode images and expressed as [(diastolic chamber diameter – systolic chamber diameter) / diastolic chamber diameter X 100%]. Hemodynamic studies were completed after 4 months of banding, using a RadiAnalyzer Xpress system that is used for fractional flow reserve (FFR) measurements in clinical routine to assess coronary physiology. Aortic pressures proximal and distal the band were recorded simultaneously using a pressure wire (Certus guidewire) and pressure gradients were calculated. The heart was then immediately excised, weighed and used for myocyte isolations. In total, four banded animals were used and 12 control animals were used for control experiments and for culture experiments.

Isolation and Culture of Feline Myocytes
Adult feline left ventricular myocytes (AFMs) were isolated as extensively described\textsuperscript{3-6}, with similar protocols used for both sham and banded animals. 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\textsubscript{4} 1.2, NaCl 130, NaH\textsubscript{2}PO\textsubscript{4} 1.2, NaHCO\textsubscript{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 (180 U/mL) supplemented with 50umol/L CaCl\textsubscript{2}. When the tissue softened, the left ventricle was isolated and gently minced, filtered, and equilibrated in KHB with 200 µmol/L CaCl\textsubscript{2}, 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). Myocytes were then used for physiologic measurements or 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). Pacing experiments were done 18 hours after adenoviral infection, using an Ion Optix C-Pace system. Voltage and current was adjusted so >90% of myocytes were reliably paced in each culture dish.

Adenovirus
Ad-STIM1 encoding cDNA from human STIM1, was provided by Dr. Jonathan Soboloff. Ad-dnOrai was purchased from Vector BioLabs. It is a human Orai1 cDNA clone with an E106Q mutation which is located in the pore-forming region of Orai1. Additionally, an RFP tag was placed on the C-terminus. A multiplicity of infection (MOI) of 100 was used for each virus.

Western Blot
Whole cell lysates were prepared from isolated feline myocytes and analyzed by Western as previously described\textsuperscript{7-10}, and imaged with a Licor Odyssey Scanner, then analyzed with Image Studio (Li-Cor Biosciences). The following primary antibodies were used for detection: STIM1 (Cell Signaling), total phospholamban (Millipore), phospho-phospholamban (PLB Thr-17, Badrilla Ltd.), calcium/calmodulin kinase II delta (Badrilla Ltd.), calcium/calmodulin kinase II pThr286/287 (Badrilla Ltd.), Caspase (Cell Signaling), Activated Caspase (Cell Signaling). For a positive control in apoptosis and activated caspase
experiments, lysates treated with cytochrome C were used (Cell Signaling).

**Immunofluorescence Staining**
AFMs were placed on laminin coated glass coverslips then washed 1X with PBS and fixed with cold 4% paraformaldehyde for 20 minutes. Cells were permeabilized with PBS containing 1% Triton X-100 and blocked with 1% bovine serum albumin in PBS for 30 minutes. Cells were then incubated overnight at 4°C with primary antibody (1:100), followed by the secondary antibody for 1 hour at 37°C. FITC donkey anti-rabbit IgG, rhodamine red-X donkey anti-mouse IgG, Alexa-647 donkey anti-mouse IgG secondaries were used (Jackson ImmunoResearch Laboratories). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, Millipore). Coverslips were mounted on glass slides with VECTASHIELD HardSet Mounting Medium (Vector Labs). Confocal micrographs were acquired using a Nikon Eclipse T2 confocal microscope (Nikon Inc). Image brightness and contrast were adjusted for visibility in figures using ImageJ (NIH), but identical adjustments were made between groups. ImageJ (NIH) was used to calculate average myocyte staining intensity with 45 myocytes from three banded and three sham animals. Primary antibodies were wheat germ agglutinin-FITC, Rabbit STIM1 (Protein Tech), and mouse actinin (Sigma).

**RT-PCR**
Total RNA was extracted from snap-frozen isolated left ventricular myocytes utilizing TRIzol Reagent (Life Technologies) and cDNA was obtained from 500ng total RNA using SuperScript® III First strand kit (Invitrogen). Real-time RT-PCR was performed using primers listed below. To avoid the influence of genomic contamination, forward and reverse primers for each gene were located in different exons. The StepOnePlus Real-Time PCR system (Applied Biosystems) was employed for quantitative RT-PCR. In each case, cDNA and primers were combined with Quantifast SYBR Green (Qiagen). Cycling conditions were as follow: 95°C for 10 min followed by 40 cycles of amplification (95°C denaturation for 15 sec, 60°C annealing and extension for 1 min). Melting curves were then obtained. Ct values were normalized with respect to GAPDH. Data are expressed as fold change (2^-ΔΔCT method). Primers used are listed below:

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<th>Direction</th>
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<tr>
<td>GAPDH</td>
<td>Reverse</td>
<td>TACTTCTCGTGTTTACGCC</td>
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**Calcium Transients, Contractions, and Action Potentials**
For Ca^{2+} transients, myocytes were loaded with 5-10 μmol/L Fluo-4 AM (Molecular Probes) for 20 minutes. For AP recordings, myocytes were incubated with 1 μmol/L di-8-annepes (Molecular Probes), then washed twice. For recordings, myocytes 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 1 Hz and fractional shortening data was collected by measuring sarcomere length (Ion Optix). For intracellular Ca^{2+} 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/F0\(^{10-12}\). Tau was measured as the decay rate of the average Ca\(^{2+}\) transient trace. For AP recordings, 10-15 paced beats were averaged to get a robust signal, then normalized using Clampfit 10 software (Molecular Devices). APD90 was measured beginning at the initial upstroke to 90% repolarization from the peak of the upstroke (phase 0). Each experiment contained 48-67 myocytes in each group from at least three different animals.

**SR Load**

Myocytes were loaded with 5-10 μmol/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. Fluorescence was monitored and for 1 minute to exclude cells that exhibited spontaneous contractions. Next, rapid application of 10 mM Caffeine in Tyrode’s was applied using a Picospritzer II (Parker Instrumentation). Background fluorescence was subtracted before presenting the data as F/F0. Each experiment contained 11 myocytes in each group from three different culture experiments.

**L-type Channel Calcium Current and Sodium-Calcium Exchange Current**

LTCC current was measured in a sodium-free and potassium-free solution as previously described. Isolated myocytes were placed in a chamber mounted on an inverted microscope (Nikon) and perfused with 1mmol/L calcium-containing Tyrode solution. A 2-4 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, MgCl\(_2\) 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, CaCl\(_2\) 2, CsCl 5.4, glucose 10, HEPES 5, MgCl\(_2\) 1.2, and NMDG 150, pH 7.4 with CsOH). Membrane voltage was controlled by an Axoclamp 900A voltage-clamp amplifier and digitized by Digidata 1440 using pClamp10 software (Molecular Devices). Voltage was held at -50 mV and depolarized in +10 mV step increments for 500 msec, every 20 seconds, while recording current. Currents were normalized to total cell capacitance. Once the signal was converted to digital format, it was stored on a personal computer for off-line analysis. Sodium-calcium exchange current (INCX), myocytes were bathed in a K\(^+\)-free solution containing (in mmol/L): NaCl 145, MgCl\(_2\) 1, HEPES 5, CaCl\(_2\) 2, CsCl 5, glucose 10, ouabain 0.02, nifedipine, 0.01, pH 7.4 adjusted with NaOH. The internal solution contained (in mmol/L): CsCl 65, NaCl 20, Na\(_2\)ATP 5, CaCl\(_2\) 6, MgCl\(_2\) 4, HEPES 10, TEA-Cl 20, EGTA 21, ryanodine 0.0005, pH 7.2 with CsOH. The cell was dialyzed for 10 minutes after rupturing the patch and the membrane current was recorded with a ramp test (+80mV to -80mV at 100mV/s) following a 100-ms depolarization to +80 from the holding potential of -40mV. During the recording, the bath solution then was changed to the K\(^+\)-free solution with 5mmol/L Ni\(^{2+}\). Recording was stopped once a stable effect of Ni\(^{2+}\) was seen and the Ni\(^{2+}\)-sensitive current was INCX.

**Calcium Sparks**

Myocytes were loaded with 5-10 μmol/L Fluo-4 AM (Molecular Probes) and then placed in a heated chamber with Normal Tyrode’s solution on the stage of a confocal microscope (Nikon C2+). Myocytes were paced at 1 Hz using field stimulation. Only myocytes with uniform shortening and clear striations were used to record sparks. Line-scan images were obtained to measure intracellular Ca\(^{2+}\) signals with 488nm excitation and emission from 500 – 580 nm. Data analysis was performed during the last 1/3 of the stimulation period (333 msec) using SparkMaster to determine the spark rate during the diastolic period. Each experiment contained 46-63 myocytes in each group from at least three different animals.

**Reagents**

Verapamil as used at 10 μmol/L (Sigma) diluted in DMSO. Kn-93 (Sigma) in DMSO at 1 μmol/L. BTP2 (Tocris) at 1 μmol/L in ethanol. BayK8644 (sigma) at 1 μmol/L in DMSO. Caffeine (Sigma) at 10 mmol/L diluted in Tyrode’s solution.
**Cell Death**

Cells were cultured in 24 well dishes coated with laminin, and infected with adenovirus as described above. After 24 hours the media was replaced and reagents were added (BTP2, KN-93). After 72 hours total culture, Trypan blue was added to the media and cells that took up the dye were counted as dead. Data is displayed as (Total cells – Dead cells)/Total cells, averaged from three separate culture experiments.

**Statistics**

Data are presented as mean±SEM. Unpaired t-test, one-way ANOVA, and two-way ANOVA were performed to detect significance using GraphPad Prism6 software. p<0.05 was considered significant.

**Supplemental References**

Online Video Legends

Online Video I: Spontaneously contracting AFMs with Ad-STIM1 expression.

Online Video II: Quiescent AFMs with control Ad-RFP expression.
Online Figure I: Localization and expression of STIM1 in AFMs. A, Myocyte immunofluorescence when stained with STIM1, actinin, wheat germ agglutinin (membrane) and DAPI (nucleus), as indicated. B, Expression of STIM1 measured by western blot in sham vs banded heats with GAPDH loading control. (p=0.0456)
Online Figure II: BTP2 Does Not Affect Ca\(^{2+}\) Cycling in Control Myocytes. Effects of BTP2 on freshly isolated myocytes from sham operated animals. A-K, Characteristics of Ca\(^{2+}\) transients and fractional shortening (%FS) in myocytes isolated from sham animals with vehicle or BTP2 exposure. Paced at 1Hz. J, Time to 90% AP repolarization (APD90) of sham myocytes with vehicle or BTP2, paced at 1Hz. Recorded with di-8-anneps fluorescence. (n=20–30 myocytes each group from 3 separate animals.) K, L-type channel Ca\(^{2+}\) currents in sham myocytes with vehicle or BTP2 (n=8 myocytes each group).
Online Figure III: Adenoviral Expression of STIM1 in AVFs: A, STIM1 western blot of Ad-RFP and Ad-STIM1 infected myocytes. B, Immunofluorescence staining with anti-STIM1 and Ad-RFP or Ad-RFP infect myocytes. Scale bar = 25 µm.
Online Figure IV: STIM1 Expression Does Not Modify L-type Channels, but alters Ca\(^{2+}\) decay. A, Unpaced SR load in RFP or STIM1 expressing myocytes, assessed with a 10 second, 10 mmol/L caffeine exposure, measured by peak fluo-4 fluorescence amplitude (n=7–15 myocytes). B, Example fluorescence traces during caffeine exposure. C, Current-Voltage relationship of L-type Ca\(^{2+}\) currents with Ad-RFP or Ad-STIM1, either at baseline or with BayK8644 (n=8–12 myocytes). D, NCX current in RFP vs STIM1 expressing myocytes (n=16 myocytes). E, Decay rate (tau) of electrically evoked calcium transients in RFP (n=19) vs STIM1 (n=21) expressing myocytes. (*p≤0.05)
Online Figure V: STIM1 Does Not Interact with Phospholamban in AVFMs. In a co-immunoprecipitation assay, Ad-RFP or Ad-STIM1 infected AVFM lysates were exposed to anti-STIM1 or anti-PLB for pull-down. Original lysates, unbound proteins and bound proteins were analyzed using standard western blot with anti-STIM1, anti PLB and anti-SERCA2a antibodies.
**Online Figure VI: STIM1 Does Not Activate Caspase-3.** Western blot of AVFMs infected with ad-RFP or ad-STIM1 do not have cleaved caspase-3. A positive control (activated caspase) and a negative control (cell lysate) are included in the blot.