Critical Role for Stromal Interaction Molecule 1 in Cardiac Hypertrophy

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Jean-Sébastien Hulot, MD, PhD; Jérémy Fauconnier, PhD; Deepak Ramanujam, MSc; Antoine Chaanine, MD; Fleur Aubart, MD; Yassine Sassi, PhD; Sabine Merkle, PhD; Olivier Cazorla, PhD; Aude Ouillé, PhD; Morgan Dupuis, PhD; Lahouaria Hadri, PhD; Dongtak Jeong, PhD; Silke Mühlstedt, MSc; Joachim Schmitt, MD; Attila Braun, PhD; Ludovic Bénard, PhD; Youakim Saliba, PhD; Bernhard Laggerbauer, PhD; Bernhard Nieswandt, PhD; Alain Lacampagne, PhD; Roger J. Hajjar, MD; Anne-Marie Lompré, PhD; Stefan Engelhardt, MD, PhD

Background—Cardiomyocytes use Ca^{2+} not only in excitation-contraction coupling but also as a signaling molecule promoting, for example, cardiac hypertrophy. It is largely unclear how Ca^{2+} triggers signaling in cardiomyocytes in the presence of the rapid and large Ca^{2+} fluctuations that occur during excitation-contraction coupling. A potential route is store-operated Ca^{2+} entry, a drug-inducible mechanism for Ca^{2+} signaling that requires stromal interaction molecule 1 (STIM1). Store-operated Ca^{2+} entry can also be induced in cardiomyocytes, which prompted us to study STIM1-dependent Ca^{2+} entry with respect to cardiac hypertrophy in vitro and in vivo.

Methods and Results—Consistent with earlier reports, we found drug-inducible store-operated Ca^{2+} entry in neonatal rat cardiomyocytes, which was dependent on STIM1. Although this STIM1-dependent, drug-inducible store-operated Ca^{2+} entry was only marginal in adult cardiomyocytes isolated from control hearts, it increased significantly in cardiomyocytes isolated from adult rats that had developed compensated cardiac hypertrophy after abdominal aortic banding. Moreover, we detected an inwardly rectifying current in hypertrophic cardiomyocytes that occurs under native conditions (ie, in the absence of drug-induced store depletion) and is dependent on STIM1. By manipulating its expression, we found STIM1 to be both sufficient and necessary for cardiomyocyte hypertrophy in vitro and in the adult heart in vivo. *Stim1* silencing by adeno-associated viruses of serotype 9–mediated gene transfer protected rats from pressure overload–induced cardiac hypertrophy.

Conclusion—By controlling a previously unrecognized sarcolemmal current, STIM1 promotes cardiac hypertrophy.

Key Words: calcium ■ myocytes, cardiac ■ hypertrophy ■ gene therapy ■ STIM1 protein, human

Cardiomyocytes exhibit dramatic fluctuations of cytosolic Ca^{2+} in alternating cycles of excitation, contraction, and relaxation. In each cycle, depolarization of the plasma membrane causes an influx of Ca^{2+} into the cytoplasm through the sequential opening of plasma membrane L-type Ca^{2+} channels (LTCC) and sarcoplasmic reticulum (SR) ryanodine receptors.[1,2] The coupling of ryanodine receptor activity to LTCC gating is structurally based on their clustering in diads, ie, small cellular compartments in which these molecules are in close proximity.[1,2] Originating from this compartment, Ca^{2+} then rapidly diffuses to adjacent sarcomeres, where it binds to troponin C, thus facilitating actin-myosin interaction for muscle contraction. In addition to cardiomyocyte contraction, Ca^{2+} is fundamental for the transcriptional activation that occurs during the cardiac growth response (hypertrophy) to various stressors. The 2 major Ca^{2+}-dependent signaling pathways in this respect result in the activation of nuclear factor of activated T cells (NFAT) through calcineurin or in the regulation of histone deacetylase activity through CaM-kinase II and/or protein kinase D.[3–5]

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For the Ca^{2+}-dependent regulation of nuclear histone deacetylase activity, a mechanism involving inositol

From the Université Pierre et Marie Curie-Paris 6, INSERM UMR S 956, Paris, France (J.-S.H., F.A., Y. Sassi, Y. Saliba, A.-M.L.); Pharmacology Department, Pitié-Salpêtrière University Hospital, Paris, France (J.-S.H.); Cardiovascular Research Center, Mount Sinai School of Medicine, New York, NY (J.-S.H., A.C., L.H., D.J., L.B., R.J.H.); Université Montpellier 1 & 2, INSERM U 1046, Montpellier, France (J.F., O.C., A.O., A.L.); Institut für Pharmakologie und Toxikologie, Technische Universität München, Munich, Germany (D.R., Y. Sassi, S. Merkle, B.L., S.E.); Rudolf-Virchow-Zentrum, Universität Würzburg, Würzburg, Germany (S. Merkle, M.D., S. Mühlstedt, A.B., B.N.); Institut für Pharmakologie und Toxikologie, Universität Würzburg, Würzburg, Germany (J.S.); and Munich Heart Alliance, Munich, Germany (S.E.).

Correspondence to Stefan Engelhardt, MD, PhD, or Jean-Sébastien Hulot, MD, PhD, Institute of Pharmacology and Toxicology, Technische Universität München, Biedersteiner Strasse 29, 80802 Munich, Germany. E-mail stefan.engelhardt@tum.de or jean.hulot@mssm.edu

triphosphate-dependent release of Ca^{2+} from the nuclear envelope and/or CaM-kinase II–dependent activation of protein kinase D has been demonstrated.[6,7] In contrast, it is largely unclear how the CaN-NFAT axis is activated in the presence of the Ca^{2+} fluctuations that occur during cardiomyocyte excitation-contraction coupling.[8] The Ca^{2+} involved here seems to be released independently of the myofilament-activating Ca^{2+} transients. Candidate gates for Ca^{2+} that induce downstream signaling are the LTCC, the ryanodine receptor, or dysfunctional SR Ca^{2+} ATPase (SERCA).

A potential mechanism for Ca^{2+}-dependent NFAT activation in cardiomyocytes is store-operated Ca^{2+} entry (SOCE), which has been largely described in nonexcitatory cells but also observed in cardiomyocytes on drug-induced inhibition of the endoplasmic reticulum (ER)/SERCA.[9,10] In nonexcitable cells, 2 groups of proteins, called stromal interaction molecules (STIMs) and Ca^{2+} release–activated channel modulators (ORAIs), have been identified as key mediators of SOCE-mediated Ca^{2+} signaling.[11,12] The characterization of STIM1 has unraveled a complex mechanism for Ca^{2+}-dependent signal transduction. On depletion of the ER from Ca^{2+}, STIM1 forms homo-oligomers and relocalizes within the ER to regions that are in close vicinity to the cytoplasmic membrane. Thus, STIM1 promotes the opening of ORAI1 in the plasma membrane to induce the entry of extracellular Ca^{2+},[13] resulting in transcriptional activation through NFAT.[14,15] In addition, STIM1 has also been reported to interact with the canonical transient receptor protein channel (TRPC) family in various cell types,[16–20] and contacts between STIM1 and TRPC1 have been mapped in both proteins.[16,21] This is one of the arguments why TRPC channels have likewise been regarded as store-operated channels,[20] despite the fact that they are less selective for Ca^{2+} than ORAI proteins.[22,23] It seems currently unresolved whether store-operated currents activated by STIM1 are based on its interaction with 1 or several ORAI proteins, TRPC proteins, or even a combination of both protein families.[19,24]

Even less is known about the potential function of STIM1 in the heart. Two recent studies carried out in neonatal rat cardiomyocytes (NRCMs) suggested that it translates prohypertrophic stimuli into a growth response,[25,26] but the underlying mechanism remained elusive, and it remained unclear whether STIM-dependent Ca^{2+} entry occurs in the adult heart.

In the present study, we have expanded the experimental scope to cardiomyocytes isolated from an in vivo model for cardiac hypertrophy (transaortic constriction in adult rats) and ultimately to the rat heart in vivo. We analyzed transmembrane currents and intracellular Ca^{2+} concentrations in the presence and absence of drugs that induce SR store depletion and investigated their dependence on expression of STIM.

Our data provide evidence for an inwardly rectifying current that occurs in the absence of store-depleting drugs, which increases significantly in cardiomyocyte hypertrophy and requires STIM1. On the basis of this finding and our finding that reduced expression of STIM1 in vivo partially protects from cardiac hypertrophy, we conclude that STIM1 is a key player in cardiac hypertrophy.

Methods

Please refer to the expanded Methods section in the online-only Data Supplement for a detailed description.

Cardiomyocyte Isolation and Cellular Electrophysiology

Whole-cell patch-clamp experiments were performed at room temperature (≈24°C) with an Axopatch 200B (Axon Instruments, Burlingham, CA). Patch pipettes had a resistance of 2 to 3 mol/LΩ. Currents were normalized to the cell membrane capacitance and presented as current density (pA/pF). Patch pipettes were filled with a solution containing 137 mmol/L cesium aspartate, 2 mmol/L CsCl, 8 mmol/L $MgSO_4$, 15 mmol/L HEPES, and 5 mmol/L EGTA adjusted to pH 7.2 (with CsOH) and 310 mOsm (with d-mannitol). The external solution consisted of 150 mmol/L NaCl, 2 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, 10 mmol/L HEPES, 10 mmol/L glucose, and 20 mmol/L sucrose adjusted to pH 7.4 (with NaOH) and 320 mOsm (with d-mannitol); in the *N*-methyl-D-glucamine solution, Na^+ was replaced with an equimolar amount of *N*-methyl-D-glucamine (pH 7.4 adjusted with HCl); in the Ba^{2+} solution, Ba^{2+} replaced Ca^{2+}; the divalent cation–free solution was supplemented with 10 mmol/L EDTA. To block the LTCC, verapamil (10 μmol/L) was added in external solutions, K^+ channels were blocked by Cs^+ in the internal solution, and the voltage-dependent Na^+ channel was inactivated by the stimulation protocol. Currents were recorded with an Axopatch 200A amplifier with a Digidata 1200 interface and analyzed with pCLAMP software. Currents were induced by 1-second voltage ramp protocols every 2 seconds (from 50 to −120 mV) at a holding potential of −80 mV. As quality controls of the patch-clamp configuration, access resistance was required to stay below 6.5 mol/LΩ and to be stable throughout analysis, and leak current should not have exceeded 100 pA at −80 mV in external standard solution (with Ca^{2+} and Na^{2+}) for 5 minutes before switching solution. The fraction of cells which did not meet these criteria and were thus excluded was 17% of cardiomyocytes from sham-operated animals and 23% of cells from AAB animals.

Transgenic Mice

The generation of *Stim1*$^{-/-}$ mice has been described elsewhere.[27] Wild-type or heterozygous *Stim1*$^{+/-}$ mice served as controls.

Statistical Analysis

All quantitative data are reported as mean±SEM. Statistical analysis was performed with the Prism software package (GraphPad version 4). One-way ANOVA was used to compare each parameter. Post hoc *t* test comparisons were performed to identify which group differences accounted for significant overall ANOVA results. Differences were considered significant when $P<0.05$.

Results

Store-Operated Ca^{2+} Entry Requires Stromal Interaction Molecule 1 in Both Neonatal and Adult Cardiomyocytes and Is Strongly Enhanced in Cardiomyocytes From Hypertrophic Hearts

We initially analyzed the effects of manipulated STIM1 expression on intracellular Ca^{2+} concentrations in isolated NRCMs and in cardiomyocytes from *Stim1*$^{-/-}$ mice. In each case, cells were exposed to Ca^{2+}-free buffer and thapsigargin, a SERCA inhibitor that causes depletion of the SR from Ca^{2+}. Then, $CaCl_2$ was added as an extracellular source for SOCE, typically resulting in Ca^{2+} influx, as measured by Fura-2 (Figure 1A and 1B, black tracings). Partial silencing of *Stim1* (by adenoviral infection with a short hairpin RNA matching *Stim1* mRNA) significantly reduced this response, as also observed in *Stim1*$^{-/-}$ cardiomyocytes (Figure 1B, green tracings). A reciprocal effect was obtained on

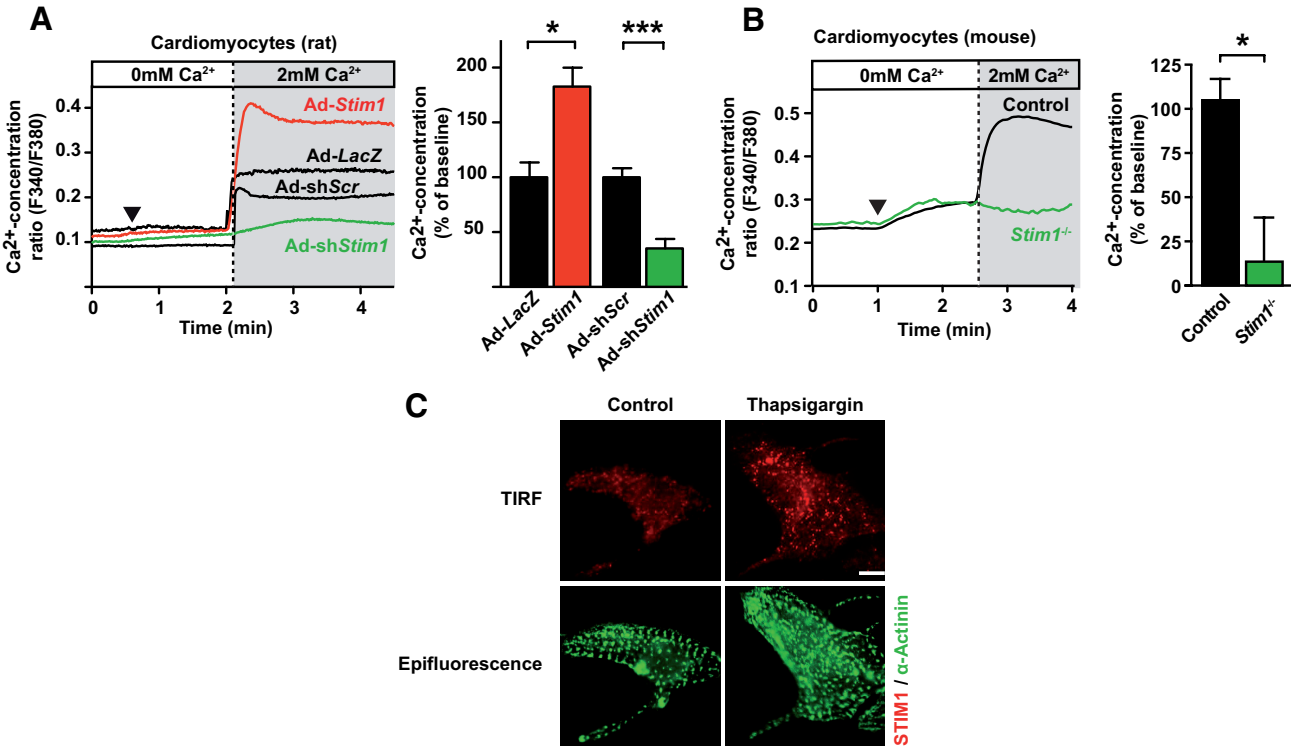

Figure 1. Role of stromal interaction molecule 1 (STIM1) in store depletion–induced Ca^{2+} entry in neonatal cardiomyocytes. **A**, Fluorescence analysis of Ca^{2+} entry in neonatal rat cardiomyocytes (NRCMs). Left, Representative recordings of the Fura-2 emission ratio (F340/380) in cardiomyocytes after sarcoplasmic reticulum (SR) Ca^{2+} depletion (by Ca^{2+} removal and thapsigargin addition) and subsequent switch to Ca^{2+}-containing buffer. Seventy-two hours before measurement, cells were infected with adenovirus encoding STIM1 (Ad-*Stim1*), a short hairpin to reduce STIM1 (Ad-sh*Stim1*), and controls expressing β-galactosidase (Ad-*LacZ*) and scrambled shRNA (Ad-sh*Scr*). **B**, Ca^{2+} entry in cardiomyocytes isolated from *Stim1*$^{-/-}$ mice. On extracellular addition of Ca^{2+} (2 mmol/L), store-operated Ca^{2+} entry occurred in control (n=53) cardiomyocytes but not in *Stim1*$^{-/-}$ cardiomyocytes (n=11). Data are mean±SEM. **C**, Microscopic detection (total internal fluorescence microscopy) of endogenous STIM1 in isolated NRCMs. Immunofluorescent staining for STIM1 (red) and α-actinin (green) before and after Ca^{2+} depletion of the SR. Scale bar=5 μm. *P<0.05, **P<0.01, ***P<0.005 in this and other figures.

adenovirus-driven *Stim1* overexpression in NRCMs (Figure 1A, red tracing). As detected by immunostaining with an anti-STIM1 antibody, thapsigargin treatment of wild-type rat and mouse cardiomyocytes enhanced the clustering of STIM1 into puncta, a typical phenomenon of SOCE (Figure 1C and Figure IA in the online-only Data Supplement). Given the short time until cell fixation (10 minutes), we attributed the increased signal to the clustering and redistribution of STIM1 rather than to upregulated *Stim1* expression. A similar pattern was obtained with an alternative antibody raised against the N terminus of STIM1 (Figure IB in the online-only Data Supplement). We also controlled for a potential contribution of noncardiomyocyte cells to these results. Our isolates typically contain <20% of noncardiomyocyte cells, the majority of which are cardiac fibroblasts (Figure IIA in the online-only Data Supplement). Ca^{2+} fluxes in these cells were smaller and not affected by *Stim1* silencing (Figure IC in the online-only Data Supplement), excluding a significant contribution of nonmyocyte cells to the above findings. Together, these data recapitulate and expand the existing knowledge that STIM1 is required for SOCE in the presence of an SERCA inhibitor.

We then analyzed whether SOCE likewise exists in adult rat cardiomyocyte and whether it is affected by prohypertrophic conditions. Because *Stim1*$^{-/-}$ mice die perinatally,[27] a knockdown strategy to reduce expression of *Stim1* was chosen. We used a rat model of compensated cardiac hypertrophy resulting from pressure overload by abdominal aortic banding. Sham-operated rats served as controls. Twenty-four days after surgery, both groups were infected with an adenovirus that carries a short hairpin RNA (shRNA) directed against *Stim1*, in addition to cDNA encoding the dsRed fluorescent protein (Ad-sh*Stim1*-dsRed), and adult rat cardiomyocytes were isolated 4 days later (at day 28; Figure 2A). This strategy allowed us to distinguish unaltered from reduced *Stim1* expression in cardiomyocytes isolated from the same rat heart. Echocardiography was performed on all animals before adenovirus injection, confirming left ventricular hypertrophy with preserved ejection fraction in the pressure-overloaded group (Table I in the online-only Data Supplement). Suitability of the adenoviral constructs was validated in NRCMs (residual *Stim1* mRNA in dsRed-positive cardiomyocytes was 10% to 15% of the mean control value; see also Reference 14). The sh*Stim1* sequence was selected from several sequences tested, which were all found to efficiently prevent hypertrophy of cardiomyocytes (data not shown). Marked differences between cardiomyocytes from adult healthy or hypertrophic rats were observed in patch-clamp recordings of sarcolemmal store-operated currents (I_{SOC}). As depicted in Figure 2B, thapsigargin induced

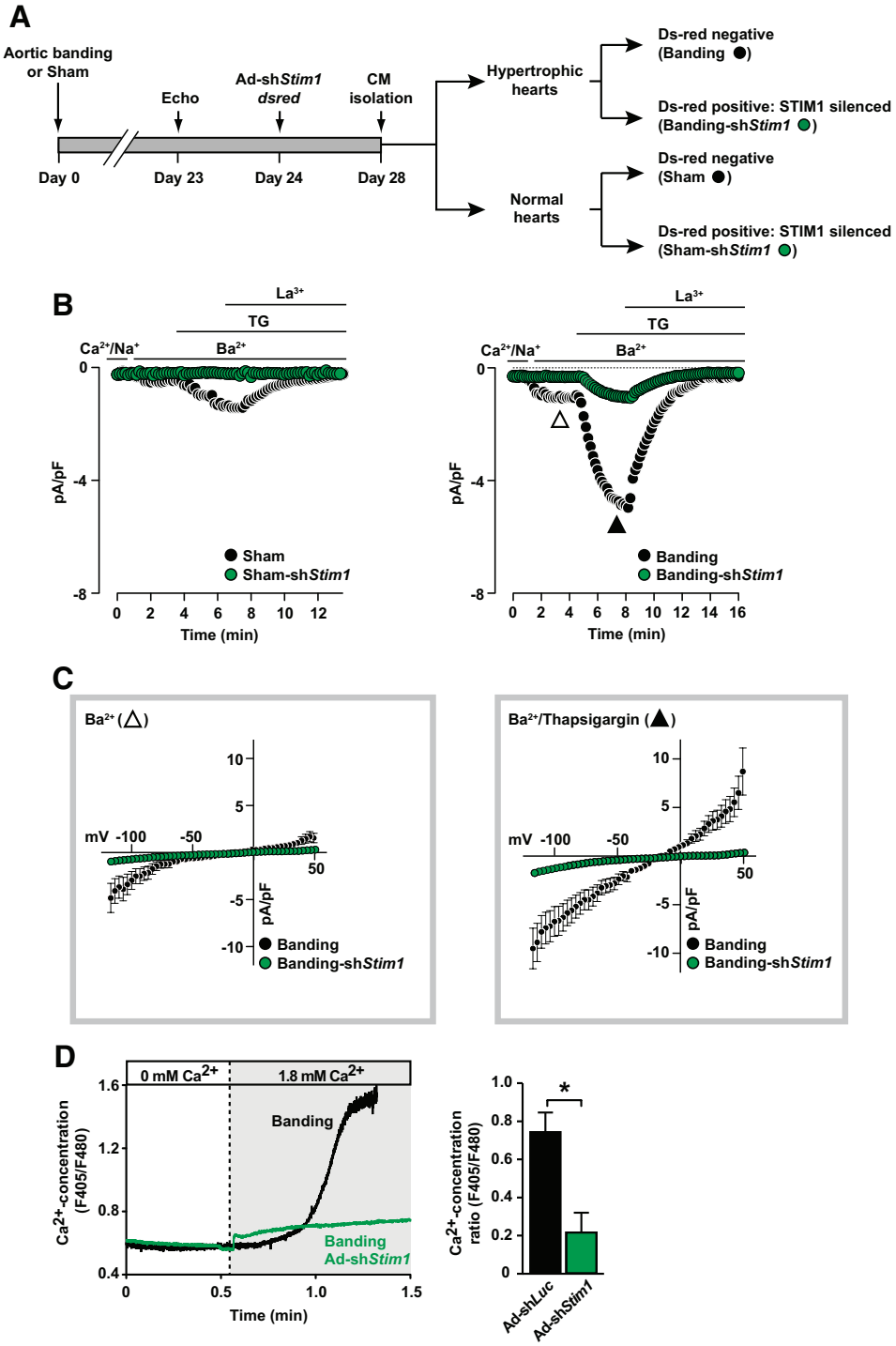

Figure 2. Stromal interaction molecule 1 (STIM1)–dependent cation currents in adult cardiomyocytes (CM). **A**, Schematic timescale and experimental strategy to analyze store-operated Ca^{2+} currents in adult rat cardiomyocytes (ARCMs). Cells were isolated 28 days after abdominal aortic constriction or sham treatment. The Ad-sh*Stim1*-dsRed vector was administered on day 24 after surgery. Isolated ARCMs were assigned to the sh*Stim* or the control (ie, noninfected) group on the basis of dsRed fluorescence, thus allowing us to compare the effects of reduced and normal *Stim1* expression in a collection cells from the same animal. **B**, Whole-cell patch-clamp recordings in ARCMs before and after sarcoplasmic reticulum (SR) Ca^{2+} store depletion by thapsigargin (TG). Left, Recordings of Ba^{2+} current (I_{SOC}) in the presence or absence of thapsigargin-induced SR store depletion in cardiomyocytes from sham-treated rats. Cells in which STIM1 expression had been reduced by Ad-sh*Stim1*-dsRed (green symbols) are shown next to tracings of nonsilenced cardiomyocytes (black symbols). Right, The same recordings conducted on ARCMs isolated after pressure overload. Recordings were performed at −80 mV. Analyses are from 3 animals per group with >10 cells per animal. The open and filled triangles refer to the corresponding current-to-voltage relations depicted in **C**. **C**, I_{SOC} current-to-voltage relation after Ba^{2+} (asterisk) and Ba^{2+}/thapsigargin perfusion as indicated in **A** in noninfected (black) or Ad-sh*Stim1*-dsRed–infected (green) ARCMs from pressure-overloaded hearts. Tracings display the average of 12 cells (banding) and 6 cells (banding sh*Stim1*). **D**, Fluorescence analysis of Ca^{2+} entry in hypertrophic ARCMs. Cardiomyocytes were isolated from hypertrophic hearts as in **A** and loaded with the Ca^{2+} sensor Indo-1. Store-operated entry of Ca^{2+} was induced as described in **A** and was detected as increased Indo-1 emission ratio (F405/480). Left, Representative tracings recorded from hypertrophic cardiomyocytes with or without silencing of *Stim1*. Right, Quantification from 3 independent experiments with 3 animals per group and >10 cells analyzed per animal.

a divalent cation current that was small in cardiomyocytes from sham-treated adult rats but markedly increased in cardiomyocytes isolated from banded adult rats (Figure 2B, black tracings). In both cases, experimentally reduced STIM1 expression dramatically decreased this current (green tracings in Figure 2B). This is further supported by Indo-1 measurement of intracellular Ca^{2+} on store depletion. Whereas adult rat cardiomyocytes from sham-treated animals allowed only a moderate entry of extracellular Ca^{2+} after SR depletion (not shown), a strong increase was observed in the analogous experiment with cardiomyocytes from banding-treated animals (Figure 2D, black tracing). Reduced expression of STIM1 potently diminished this response (Figure 2D, green tracing and column).

Caffeine-induced Ca^{2+} release, which allows the evaluation of SR Ca^{2+} concentrations, suggested that the different currents of the 4 groups of cardiomyocytes did not originate from different SR Ca^{2+} loads (Figure IIIC in the online-only Data Supplement). Together, these data demonstrate that STIM1 is crucially involved in thapsigargin-induced SOCE in adult cardiomyocytes.

Stromal Interaction Molecule 1 Also Promotes a Current in the Absence of Drug-Induced Store Depletion

Intriguingly, we also observed a current in the absence of drug-induced store depletion. Although marginal in cardiomyocytes from healthy adult rats, this current is clearly visible and enhanced in cardiomyocytes from hypertrophic hearts (Figure 2B; compare the black tracings in the section marked by the empty triangle). This current is completely abolished in sh*Stim1*-infected cells from the same cardiomyocyte population (Figure 2B, green tracing in this section) but did not affect excitation contraction coupling because neither cellular Ca^{2+} transients nor cell shortening was altered in Ad-sh*Stim1*-dsRed–infected cells (Figure IIIC and IIID in the online-only Data Supplement).

Currents recorded in the absence or presence of thapsigargin share I_{SOC} features such as the amplification in a divalent cation–free environment or the inhibition by SKF or by La^{3+} (Figure IIIA and IIIB in the online-only Data Supplement). In contrast, differences became evident on plotting the current-voltage relation that underlies these currents. Perfusion of hypertrophic adult rat cardiomyocytes with thapsigargin induced a double rectifying current (Figure 2C, black tracing, right panel). The store-operated Ca^{2+} current was as permeable to barium as to calcium (Figure 2B and Figure IIIB in the online-only Data Supplement).

In contrast to the above, the current-voltage curve recorded in the absence of thapsigargin is predominantly an inward rectifying current (Figure 2C, black tracing, left panel). With Ba^{2+}- (Figure 2B) or a Na^{+}-free Ca^{2+} solution (Figure IIIA in the online-only Data Supplement), it developed slowly and reached steady state after ≈2 minutes. This curve shape displays characteristics of gating by ORAI proteins (see the work by Vig et al[28]), but the current did not show the pronounced selectivity for Ca^{2+} over Ba^{2+} that has been reported for ORAI1 but not for ORAI 2 and ORAI3[29] (Figure IIIA in the online-only Data Supplement).

Effects of Stromal Interaction Molecule 1 Overexpression or Silencing on the Growth of Isolated Cardiomyocytes

Our finding that STIM1 deficiency reduces sarcolemmal Ca^{2+} fluxes prompted us to ask whether altered STIM1 expression affects cardiomyocyte growth and intracellular signaling and whether it would involve store-operated channels. Indeed, NRCMs overexpressing STIM1 were significantly larger than LacZ-overexpressing controls, as detected by α-actinin staining and by automated size detection (Figure 3A). In presence of SKF96365, cell sizes were comparable to LacZ controls, suggesting that STIM1 confers hypertrophy through an interaction with ORAI or TRPC proteins. In line with STIM1 being an activator of the NFAT pathway, a reporter assay indicated that STIM1 overexpression enhanced NFAT activity (Figure 3B).

Additional parameters were assessed to test for the functional impact of STIM1 on NFAT activation and hypertrophy. Reduction of *Stim1* expression in NRCMs (to ≈25% of control; see Figure 3C, left) partially prevented phenylephrine-induced hypertrophy (as determined by size or ^{3}H-leucine integration; Figure 3C). Furthermore, NFAT activity and mRNAs encoding atrial natriuretic factor or modulatory calcineurin interacting protein 1 were reduced (Figure 3D). Finally, cellular capacitance as a means of cardiomyocyte size was higher in cardiomyocytes from pressure-overloaded hearts compared with sham, and *Stim1* silencing (see also Figure 2A) resulted in significantly lower values (Figure 3E). These data indicate that STIM1 is both sufficient and necessary for the cardiomyocyte hypertrophic response.

Adeno-Associated Virus-Mediated Silencing of Stromal Interaction Molecule 1 In Vivo Prevents Cardiac Hypertrophy

We also asked whether endogenous STIM1 levels change under hypertrophic conditions. We found increased amounts of *Stim1* mRNA and protein in NRCMs 48 hours after stimulation by endothelin 1 or phenylephrine (Figure IVA in the online-only Data Supplement). *Stim1* expression was observed to be moderate in the adult heart (Figure IVB in the online-only Data Supplement) but was significantly upregulated in left ventricular myocardium from rats after pressure overload (Figure IVC in the online-only Data Supplement). To determine the effects of silencing *Stim1* on cardiac hypertrophy in vivo, we generated recombinant cardiotropic adeno-associated viruses of serotype 9 (AAV9), allowing for cardiomyocyte-targeted RNAi against *Stim1* (AAV9-sh*Stim1*) under control of the U6 promoter (Figure V in the online-only Data Supplement). An AAV9 encoding an shRNA directed against luciferase served as a negative control (AAV9-sh*Luc*). Vectors were injected into rats (n=9 per group, 5×10^{11} genomes per animal), and 28 days later, abdominal aortic constriction was applied (Figure 4A). Eight of 9 animals survived in both groups.

Consistent with the cardiotropic serotype of AAV9,[30] cardiac expression of an AAV9–green fluorescent protein

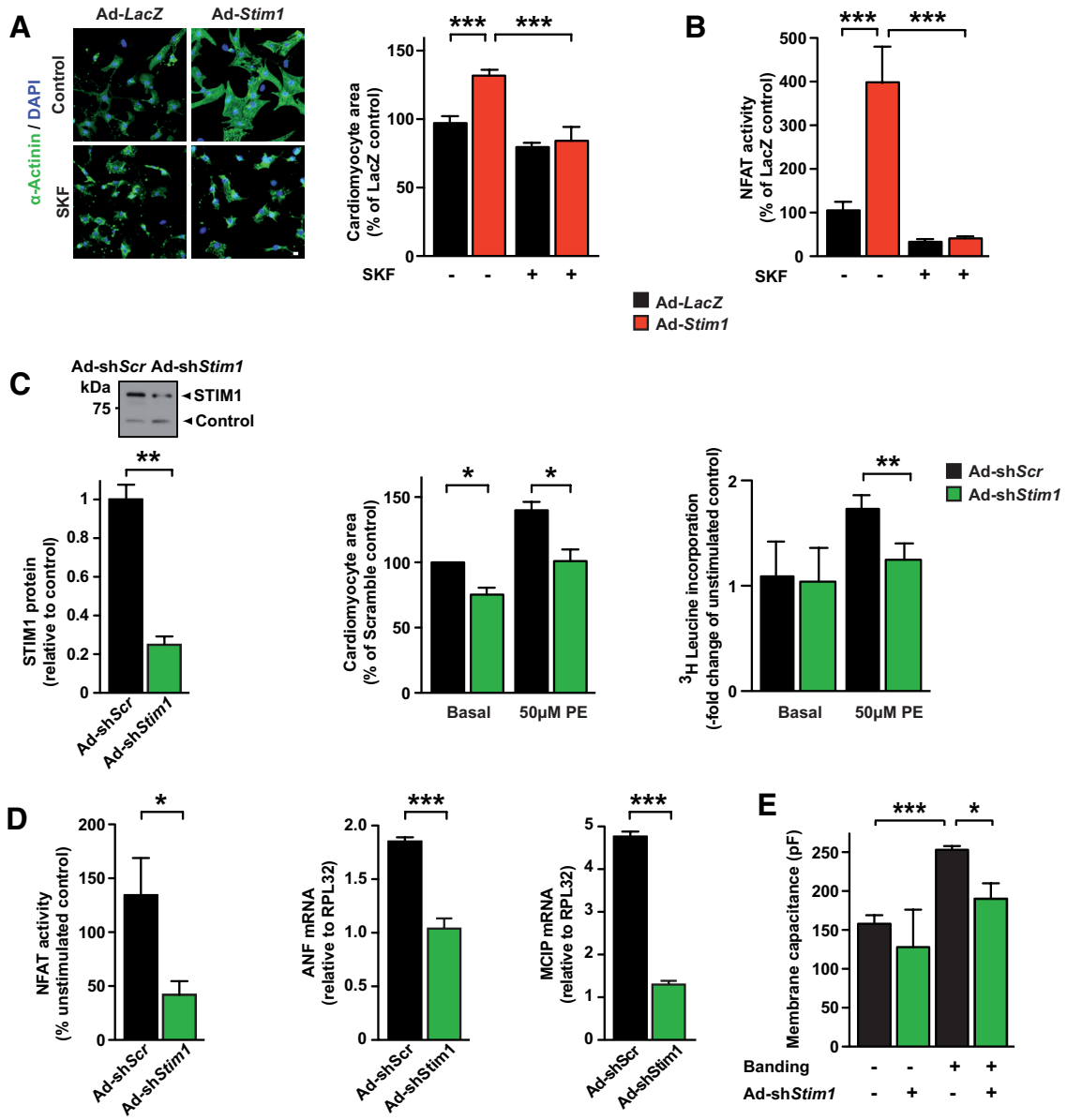

Figure 3. Role of stromal interaction molecule 1 (STIM1) in cardiomyocyte growth and signaling. **A**, Effects of *STIM1* overexpression on the growth of neonatal rat cardiomyocytes. Left, Immunofluorescence analysis of neonatal rat cardiomyocytes (NRCMs) infected with adenoviral vectors (multiplicity of infection, 10) encoding β-galactosidase (Ad-*LacZ*) or STIM1 (Ad-*Stim1*) and treated with the store-operated Ca^{2+} entry inhibitor SKF96365 (SKF) or control (dimethyl sulfoxide). Immunofluorescence analysis was carried out 48 hours after stimulation with an antibody against α-actinin (green). Nuclei were stained with DAPI. Scale bar=5 μm. Right, Quantification of cardiomyocyte area. **B**, Determination of nuclear factor of activated T cells (NFAT) activity by luciferase reporter assay in NRCMs that have been treated as in **A**. **C**, Effects of *Stim1* silencing on cardiomyocyte hypertrophy. Left, Western blot detecting STIM1 in cardiomyocytes 72 hours after infection with an adenoviral silencing vector (Ad-sh*Stim1*) or a control (Ad-sh*Scr*) and quantitative analysis of the results. Center, Surface area determination of the cardiomyocytes. Right, ^{3}H-leucine incorporation during 48 hours of stimulation with phenylephrine (50 μmol/L) 3 days after infection. **D**, Effects of STIM1 silencing on phenylephrine-induced NFAT activation and expression of atrial natriuretic factor (ANF) and modulatory calcineurin-interacting protein 1 (MCIP1). **E**, Membrane capacitance of cardiomyocytes as a measure of cell size. The ARCMs infected with Ad-sh*Stim1* were isolated as depicted in Figure 2 (n≥6 for all groups. Data are from ≥3 independent experiments with ≥3 replicates each, except that 2 independent experiments were performed for ^{3}H-leucine detection in **C** and ANF and MCIP mRNA determination in **D**. In **A** and **C**, >500 cells were analyzed in each independent experiment.

control construct led to exclusive green fluorescent protein detection in cardiomyocytes, as opposed to endothelial cells or interstitial cells, which we presume to be mainly fibroblasts (Figure VC in the online-only Data Supplement). Furthermore, we determined whether our viral constructs would affect lymphocyte infiltration or capillary density (rather than STIM1 activity in cardiomyocytes). For this, we analyzed tissue sections of rat hearts for the presence of $CD8^+$ and $CD45^+$ cells and for vessels stained positive for von Willebrand factor. Moderately enhanced infiltration by $CD45^+$ lymphocytes and a decrease in the capillary density were observed in hypertrophic hearts but were independent of manipulated *Stim1* expression (Figure VI in the online-only Data Supplement).

Reduced *Stim1* expression, occurring after infection with AAV9-sh*Stim1* (Figure 4A), significantly prevented cardiac

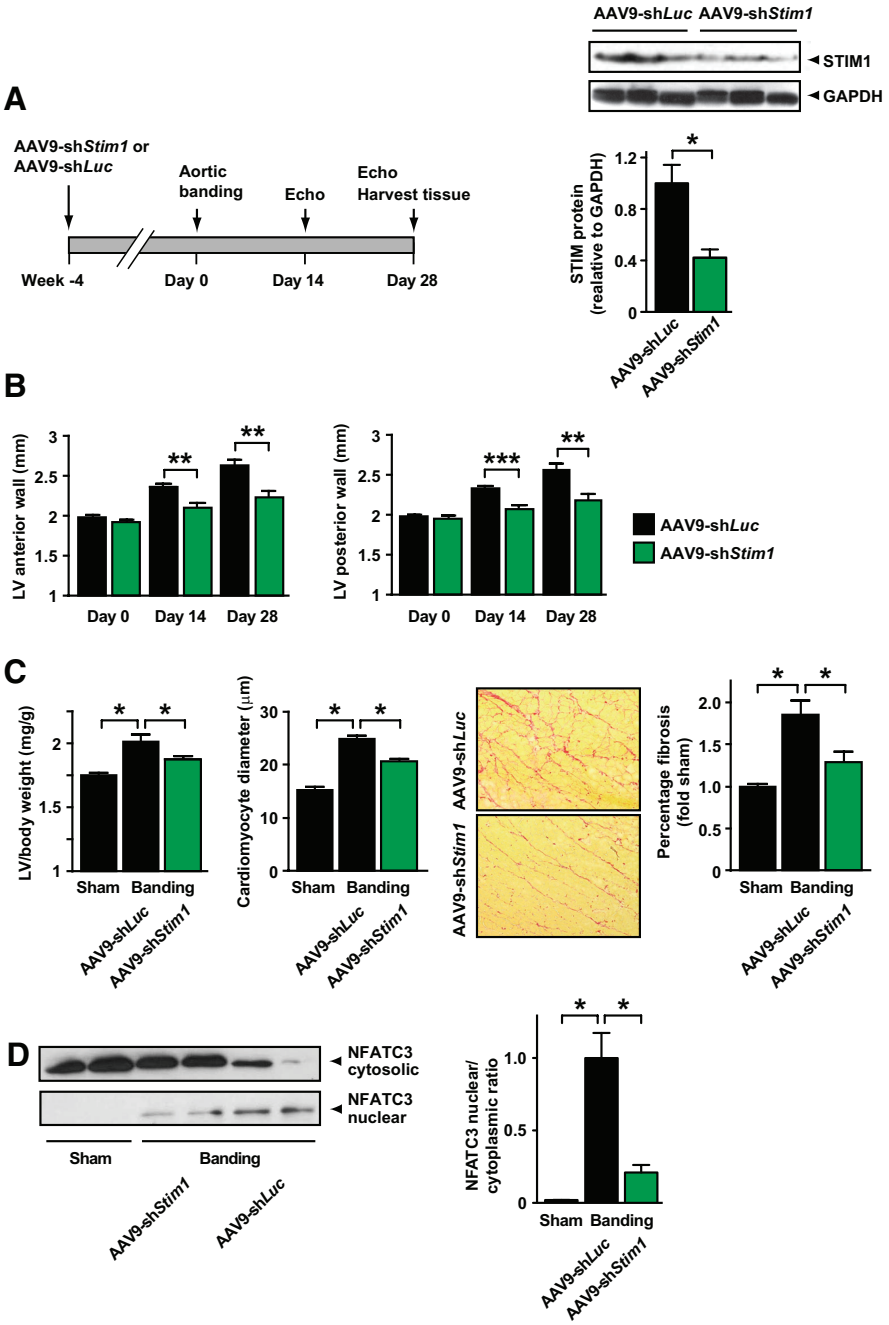

Figure 4. Silencing of stromal interaction molecule 1 (STIM1) in a model of pressure-induced cardiac hypertrophy in vivo. **A**, Left, Experimental strategy for the analysis of STIM1 silencing in living rats. Right, Immunoblot analysis of STIM1 and GAPDH in lysates from adeno-associated viruses of serotype 9 (AAV9)-sh*Luc*– and AAV9-sh*Stim1*–treated hearts. For quantification, STIM1 data were normalized to the GAPDH signal in each lane (n=8 per group). **B**, Serial echocardiographic measurements of left ventricular (LV) anterior and posterior wall dimensions in AAV9-sh*Stim1*– or AAV9-sh*Luc*–treated rats (n=8 per group). **C**, Left, Postmortem determination of the ratio of left ventricle to body weight in AAV9-sh*Stim1*– or AAV9-sh*Luc*–treated rats (n=8 per group). Middle, Average cardiomyocyte diameter in each group (>20 cells per animal from 4 animals per group). Right, Sirius red staining for interstitial fibrosis in sections of left ventricular tissue and quantitative analysis from 5 animals per group. **D**, Detection and quantification of nuclear factor of activated T cells (NFAT) in subcellular fractions by immunoblot analysis.

hypertrophy, as delineated from serial echocardiographic measurements of left ventricular wall thickness (Figure 4B and Table II in the online-only Data Supplement) and the ratio of left ventricular weight to body weight (Figure 4C). Concordantly, histological analysis showed a reduction in both cardiomyocyte size and myocardial fibrosis in AAV9-sh*Stim1*–treated rats (Figure 4C). None of these effects occurred in the control group. Moreover, *Stim1*-silenced rats exhibited a reduction in Ca^{2+} signaling; nuclear translocation of NFATC3 was reduced in cardiomyocytes from *Stim1*-silenced rats (Figure 4D).

Discussion

The cardiac muscle responds to mechanical and humoral stress by hypertrophic growth of individual myocytes.[31] Although some degree of cardiac hypertrophy serves to reduce wall stress and helps to compensate for increased load on the myocardium, sustained prohypertrophic signaling within cardiomyocytes is clearly detrimental and a major factor contributing to the progression to failure.[31,32] Cardiac hypertrophy is typically accompanied by activation of Ca^{2+}-dependent signaling pathways and reinduction of a fetal gene expression program.[31,33]

Among the Ca^{2+}-dependent signaling pathways that have been implicated in cardiac growth control, calmodulin-dependent activation of the serine-threonine phosphatase calcineurin and subsequent NFAT translocation to the nucleus are particularly important.[34] Although disturbances of cardiomyocyte SR Ca^{2+} release and SR uptake (leading to, for example, an increase in diastolic Ca^{2+}) clearly become

dominant in more advanced disease stages,[35] calcineurin activation occurs at early stages of cardiac hypertrophy, when excitation-contraction coupling and SR Ca^{2+} load are still normal and diastolic Ca^{2+} concentrations are in the physiological range.[8]

It is increasingly understood how global impairment of cardiomyocyte Ca^{2+} handling, as seen in advanced cardiac disease, elicits certain disease-related signaling pathways. In contrast, we know little about the mechanisms that drive the simultaneous activation of various Ca^{2+}-dependent signaling pathways observed at early disease stages.[1] In contrast to recent advances in understanding inositol triphosphate–mediated Ca^{2+} release from the nuclear envelope,[7] it remains unclear how activation of the CaN-NFAT axis in cardiac disease occurs.[1]

In nonexcitable cells, an important mechanism for Ca^{2+} signaling involves Ca^{2+} release from the ER and subsequent influx of extracellular Ca^{2+} into the cytosol. The key protein involved in this SOCE is STIM1, which activates ORAI1, the pore-forming subunit of a Ca^{2+} release–activated Ca^{2+} channel.[13,36,37] In contrast to nonexcitable cells, the role of STIM1 in muscle cells is barely understood. Despite the early findings by Hunton et al[9,10] that SOCE also occurs in cardiomyocytes, it remained unclear whether SOCE is causatively involved in cardiac hypertrophy and whether STIM1 ties SOCE to hypertrophy. Although 2 recent reports[25,26] could show that reduced expression of *Stim1* interferes with the response of cardiomyocytes to prohypertrophic receptor agonists, the restriction to neonatal cardiomyocytes and drug-induced SOCE in these studies left unanswered whether SOCE in adult cardiomyocytes indeed triggers disease and whether this involves a Ca^{2+}-sensing activity of STIM1.

We believe the most important aspects of our study are the identification of a sarcolemmal current in the absence of drug-induced SERCA inhibition, its stronger amplitude in hypertrophic cardiomyocytes, and its dependence on STIM1.

Remarkably, a previous study on thapsigargin-induced currents in lymphocytes and Jurkat T cells also reported a current in the absence of SERCA inhibition.[38] Although that current was not the focus of this study, its inward rectifying characteristic shares similarity with the current we found to be STIM1-dependent in cardiomyocytes. The existence of such a current in lymphocytes justifies the presumption that it may likewise depend on STIM1.

A question that remains is whether the STIM1-dependent current in the absence of SERCA inhibition mirrors a true independence from SR calcium store depletion. Opposed to this, one may envision that store depletion also occurs under physiological conditions but is obscured by STIM1-mediated refilling of the SR with calcium. The latter has similarly been proposed to occur in HeLa cells.[39] In contrast, the observation that SR-based Ca^{2+} stores remain unchanged in cardiac hypertrophy[40] and our finding that silenced STIM1 expression did not alter the SR Ca^{2+} (Figure IIIC in the online-only Data Supplement) argue against such a scenario. Evidence that STIM1 may indeed function in a store-independent manner comes from studies on arachidonic acid–stimulated Ca^{2+} signaling in HEK cells.[41] Although there are clear differences with respect to cell type (nonexcitable versus excitable) and receptor activation, STIM1 appears to function independently of ER/SR Ca^{2+} store depletion in both cases.

To the best of our knowledge, our data are the first to demonstrate a role for STIM1 in cardiomyocytes that is independent of drug-induced store depletion and suggest a critical role for STIM1 in the adult heart. Yet, several important questions remain: If STIM1 functions in the absence of Ca^{2+} depletion from the SR, what upstream regulatory mechanisms lead to its activation? Furthermore, does STIM1 in this pathway interact exclusively with the recently identified Ca^{2+} release–activated channels of the ORAI protein family, or are other channel proteins are involved?

In the years before ORAI1 was discovered, the store-operated Ca^{2+} channel was expected to be found in another protein family called TRPC. The TRPCs are less selective for cations than ORAI1,[22,23] a fact that has been cited as evidence that the STIM1-operated channel is ORAI1.[19] However, several studies have shown interactions between STIM1 and TRPC proteins,[17–20] which justifies the hypothesis that SOCE has >1 origin.[19] Indeed, aside from ORAI1, which was recently proposed to function in cardiac hypertrophy, analogous correlations were established for TRPC1, TRPC3, and TRPC6.[36,42] Given this, the currents we measured in the presence and absence of SERCA inhibitors deserve a more detailed discussion. Both currents meet criteria of I_{SOC} currents, ie, their susceptibility to the channel-blocking drug SKF or to La^{3+} ions. As in case of the thapsigargin-dependent current, its inability to discriminate between Ba^{2+} and Ca^{2+} and double rectification are consistent with currents mediated by TRPC channels, as reported by Yuan et al,[20] Stiber et al,[43] and Huang et al[16] and in studies that specifically addressed ion gating by TRPC1[42] or TRPC3 and TRPC6.[36]

On the other hand, the identity of the channel that promoted a current in the absence of thapsigargin raises a new question. Although this current appears to be mainly inward rectifying and thus compliant with ORAI gating, it appears to lack the profound ion selectivity of this channel often stated for this protein family.[44] However, the extent to which ORAI1, ORAI2, and ORAI3 discriminate between cations differs (in descending order).[29] In addition, the cardiac expression of the 3 ORAI isoforms has yet to be determined, and ORAI1 may be functionally replaced by either ORAI2 or ORAI3.[45] Although this supports the idea that the STIM1-dependent current we observe without SERCA inhibition is mediated by one or both of these channel proteins, we may also envisage the participation of heteromeric channels formed by ORAI and TRPC members, as suggested by Liao et al.[24] Future studies should identify the channel that is activated by STIM1 in the absence of SR Ca^{2+} store depletion, test whether STIM1 oligomerization within the SR and its interaction with plasma membrane ORAI is intact under conditions of cardiac hypertrophy, but also investigate the role of TRPC proteins under such conditions. Interestingly, STIM1 was recently reported to inhibit LTCC in neurons and vascular smooth muscle cells,[46,47] and the authors of both studies speculate that this mechanism promotes the decision of which Ca^{2+}-signaling pathways are specifically activated. However, LTCC blockade has been shown to exert an antihypertrophic effect[48] (as opposed to the pro-

hypertrophic role of STIM1) and our in vitro experiments to characterize the STIM-dependent current had been carried out in the presence of an LTCC blocker. This argues against a predominant role of LTCC in STIM1-dependent pro-hypertrophic signaling.

At present, it is unclear whether additional Ca^{2+}-dependent signaling mechanisms that have been implicated in cardiomyocyte hypertrophy involve STIM1. These include the direct coupling of the CaM-CaN axis to LTCC-induced Ca^{2+} entry that has been described in neurons[49] and the Ca^{2+} and integrin-binding protein 1 identified as a prohypertrophic calcineurin-interacting protein.[50] It remains to be seen whether, and how, STIM1 contributes to these pathways. The answer to these crucial questions will eventually lead to a detailed picture of how STIM1 is activated and what protein it recruits at the plasmalemma to mediate NFAT activation and thereby cardiomyocyte hypertrophy in the presence of the large fluctuations of intracellular Ca^{2+} that occur during excitation-contraction coupling. Taken together, our data demonstrate an important role for STIM1 in the progression of cardiac hypertrophy and suggest a possible role in cardiac disease.

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Disclosures

None.

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CLINICAL PERSPECTIVE

The cardiac muscle responds to mechanical and humoral stress by hypertrophic growth of individual myocytes. Although some degree of cardiac hypertrophy serves to reduce wall stress and helps to compensate for increased load on the myocardium, sustained prohypertrophic signaling within cardiomyocytes is clearly detrimental and a major factor contributing to the progression to failure. The activation of Ca^{2+}-dependent signaling pathways has been identified as critical for cardiac hypertrophy. However, it has remained largely unclear how Ca^{2+} triggers signaling in cardiac myocytes in the presence of the rapid and large Ca^{2+} fluctuations that occur during excitation contraction coupling. Here, we have studied the role of stromal interaction molecule 1 in cardiomyocytes, a molecule that has been described in several cell types as critical for Ca^{2+} entry. By manipulating its expression, we found stromal interaction molecule 1 to be both sufficient and necessary for cardiomyocyte hypertrophy in vitro and in the adult heart in vivo. *Stim1* silencing by viral gene transfer protected rats from pressure overload–induced cardiac hypertrophy. These data demonstrate an important role for stromal interaction molecule 1 in cardiac hypertrophy and may lead to the development of novel approaches to prevent cardiac dysfunction.