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UltraRapid Communication

Interactions, Functions, and Independence of Plasma Membrane STIM1 and TRPC1 in Vascular Smooth Muscle Cells

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Abstract—Stromal interaction molecule 1 (STIM1) is a predicted single membrane–spanning protein involved in store-operated calcium entry and interacting with ion channels including TRPC1. Here, we focus on endogenous STIM1 of modulated vascular smooth muscle cells, which exhibited a nonselective cationic current in response to store depletion despite strong buffering of intracellular calcium at the physiological concentration. STIM1 mRNA and protein were detected and suppressed by specific short interfering RNA. Calcium entry evoked by store depletion was partially inhibited by STIM1 short interfering RNA, whereas calcium release was unaffected. STIM1 short interfering RNA suppressed cell migration but not proliferation. Antibody that specifically bound STIM1 revealed constitutive extracellular N terminus of STIM1 and extracellular application of the antibody caused fast inhibition of the current evoked by store depletion. The antibody also inhibited calcium entry and cell migration but not proliferation. STIM1 interacted with TRPC1, and TRPC1 contributed partially to calcium entry and cationic current. However, the underlying processes could not be explained only by a STIM1-TRPC1 partnership because extracellular TRPC1 antibody suppressed cationic current only in a fraction of cells, TRPC1-containing channels were important for cell proliferation as well as migration, and cell surface localization studies revealed TRPC1 alone, as well as with STIM1. The data suggest a complex situation in which there is not only plasma membrane–spanning STIM1 that is important for cell migration and TRPC1-independent store-operated cationic current but also TRPC1-STIM1 interaction, a TRPC1-dependent component of store-operated current, and STIM1-independent TRPC1 linked to cell proliferation. (***Circ Res*****. 2008;103:e97-e104.)**

Key Words: vascular smooth muscle ■ calcium channel ■ stromal interaction molecule 1 ■ transient receptor potential canonical 1

Vascular smooth muscle cells (VSMCs) in their contractile phenotype determine the caliber of most blood vessels and thus regulate blood pressure and local tissue perfusion. Throughout life, however, VSMCs also retain capacity for plasticity, which enables switching to a noncontractile modulated phenotype that is important for blood vessel formation, vascular adaptation, and response to injury, as well as contributing substantially to the vascular diseases of atherosclerosis, neointimal hyperplasia and in-stent restenosis.[1,2] Critical events regulating these VSMC properties are plasma membrane ion fluxes, such as Ca^{2+} entry through Ca^{2+}-permeable ion channels.[3–8] Voltage-gated Ca^{2+} channels, especially the dihydropyridine-sensitive L-type channel ($Ca_V1.2$), have established pharmacological importance and roles in contractile VSMCs. There are, however, other types of Ca^{2+}-permeable channels that are not voltage-gated and have importance in both VSMC phenotypes.[4–8] One type is coupled to Ca^{2+} store depletion: the store-operated channels, which are Ca^{2+}- and Na^{+}-permeable (nonselective cationic) channels in VSMCs.[9–12] Several studies have suggested that the ion-permeation pathway of these channels is formed at least partially by TRPC1 combining with other TRPC proteins (eg, TRPC5).[9,13–20] Such TRPC1-containing channels are important for VSMC hyperplasia, hypertrophy, migration, and proliferation.[4–9,17] However, TRPC1-independent store-operated channels have also been suggested,[12] and studies of other cell types have shown a different, Ca^{2+}-selective, store-operated channel referred to as the CRAC channel.[21] Seminal studies of the CRAC channel have revealed additional protein components, among which is stromal interaction molecule (STIM)1.[21,22]

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STIM1 was first identified as a plasma membrane protein not related to Ca^{2+} handling,[23] but subsequent studies have shown major roles in Ca^{2+} entry coupled to store depletion.[21,22,24] STIM1 is not thought to be an ion channel itself but to regulate other proteins that determine ion permeation.[21] Although plasma membrane STIM1 has been linked to CRAC channels,[25] attention has been focused on STIM1 as an endoplasmic reticulum protein that senses luminal Ca^{2+}, oligomerizes, and locates to sub–plasma membrane space in response to store depletion.[21,22,24] One target of STIM1 is the novel CRAC channel subunit Orai1,[21,26] but STIM1 is also suggested to interact with TRPC1.[27–29] Although STIM1 is expressed and functional in VSMCs,[12,30,31] there is still much to understand about it in this context. Here, we investigated the presence and potential importance of endogenous plasma membrane STIM1 and the relationships of STIM1 to TRPC1, Ca^{2+} entry, and store-operated nonselective cationic current. We used noncontractile, modulated VSMCs of human saphenous vein, which commonly exhibits neointimal hyperplasia when used as a coronary artery bypass graft.[6]

Materials and Methods

Human Saphenous Vein and Its VSMCs

Freshly discarded human saphenous vein segments were obtained anonymously and with informed consent from patients undergoing open heart surgery in the General Infirmary at Leeds. Approval was granted by the Leeds Teaching Hospitals Local Research Ethics Committee. Migratory VSMCs were prepared using an explant technique[7] and grown in DMEM supplemented with 10% FCS, penicillin/streptomycin, and L-glutamine at 37°C in a 5% CO_2 incubator. Experiments were performed on cells passaged 2 to 5 times. Cells stained positively for smooth muscle α-actin. Growth of neointima has been described.[7]

Transfection with Short Interfering RNAs or Dominant Negative Mutant TRPC5

Cells (0.5 to 2×10^6) were centrifuged (100*g*) for 10 minutes, resuspended in Basic Nucleofector solution (Amaxa GmbH), mixed with 1 μmol/L short interfering (si)RNA (Ambion Europe Ltd; Table I in the online data supplement), and transferred into a cuvette for electroporation (Amaxa). With this method, the short interfering (si)RNA concentration is higher than used with lipid transfection methods, but the exposure time to the siRNA is considerably shorter. The scrambled control siRNA was Silencer Negative Control #1, which is a 19-bp scrambled sequence with no significant homology to human gene sequences (Ambion). Cells were transferred from cuvettes to prewarmed culture medium and incubated in a 5% CO_2 incubator at 37°C. Culture medium was changed after 24 hours and recordings were made after a further 24 hours. When 3 siRNA probes were used as a cocktail, each probe was used at one-third of its usual concentration, so the total siRNA concentration was 1 μmol/L. Dominant negative mutant TRPC5 (DN-TRPC5) and its use have been described.[7]

Intracellular Ca^{2+} Measurements

Cells were incubated with fura-2/acetoxymethylester for 1 hour at 37°C, followed by a 0.5-hour wash at room temperature. Measurements were made at room temperature on a 96-well plate reader (FlexStation, Molecular Devices). The change (Δ) in intracellular calcium (Ca^{2+}_i) concentration is indicated as the ratio of fura-2 emission intensities for 340 and 380 nm excitation (*F* ratio). Wells within columns of the 96-well plate were loaded alternately for test and control conditions. The standard extracellular recording solution contained (mmol/L): 130 NaCl, 5 KCl, 8 D-glucose, 10 HEPES, and 1.2 $MgCl_2$, titrated to pH 7.4 with NaOH. When indicated, 0.2 mmol/L $CaCl_2$ was added.

Patch-Clamp Recordings

Recordings were made using the Patchliner planar patch-clamp system (Nanion) in whole-cell mode. Before recordings, cells were detached from culture flasks with 0.05% Trypsin/EDTA (Sigma) or Detachin (Gelantis Inc) and resuspended at a density of 1×10^6 to 5×10^7 per milliliter in extracellular solution that was the same as the recording solution for Ca^{2+} measurements except it contained 0.2 mmol/L $CaCl_2$ and 100 μmol/L niflumic acid. During seal formation, the external solution contained (mmol/L): 160 NaCl, 4.5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 5 D-glucose, 10 HEPES (pH 7.4 with NaOH); and the internal solution (mmol/L): 75 CsCl, 10 NaCl, 70 CsF, 2 $MgCl_2$, 10 EGTA, 10 HEPES (pH 7.2 with CsOH). During whole-cell recordings, the extracellular solution was the standard extracellular recording solution containing 0.2 mmol/L $CaCl_2$, and 100 μmol/L niflumic acid, and intracellular recording solution contained (mmol/L): 40 EGTA, 17 $CaCl_2$, 2 $MgCl_2$, 8 NaCl, 1 Na_2ATP, 10 HEPES, 66 L-glutamic acid, 66 CsOH, titrated to pH 7.2 with CsOH (calculated unbound Ca^{2+}, 100 nmol/L). Voltage ramps were applied from −100 mV to +100 mV for 1 second every 10 seconds from a holding potential of 0 mV. Currents were filtered at 1 kHz and sampled at 3 kHz. General salts, HEPES, niflumic acid, 2-aminoethoxydiphenyl borate, and adenosine 5′-triphosphate were purchased from Sigma.

Cell Assays

Invasion assays were performed using a modified Boyden chamber containing membranes occluded by Matrigel basement membrane matrix (8-μm pores; BD Biosciences). Cells (1×10^5) were loaded in the upper chamber in DMEM supplemented with 0.4% FCS. The lower chamber contained 0.4% FCS supplemented with the chemoattractants platelet-derived growth factor-BB (10 ng/mL) and interleukin-1α (10 ng/mL) (Invitrogen). After incubation for 24 hours at 37°C in a 5% CO_2 incubator, cells were scraped from the upper surface, duplicate membranes were fixed, and the migrating cells were stained with hematoxylin/eosin and evaluated by counting cells in 10 randomly chosen fields under light microscopy. Migration assays were performed similarly except the polycarbonate inserts were not coated with Matrigel and incubation was for 8 hours. To measure cell proliferation, equal numbers of cells from the same patient were transfected and seeded in parallel into 6-well tissue culture plates in DMEM culture medium plus 10% FCS. Medium was changed after 24 hour and cells incubated for a further 24 hour. Cells were collected after trypsinization, stained with trypan blue and the cell number determined in duplicate wells and counted at least twice with a hemocytometer. Trypsinized wells were observed microscopically to confirm that all cells had been released.

Immunostaining, Western Blotting, and Coimmunoprecipitation

For functional experiments, anti-STIM1 (anti-GOK; targeting the N terminus) and mouse IgG2a control antibodies (BD Biosciences) were dialyzed in Ca^{2+}-free recording solution before use at 10 or 20 μg/mL. Before use, the dialysis membrane (Scientific Laboratory Supplies Ltd) was boiled in 2% $NaHCO_3$ with 1 mmol/L EDTA for 10 minutes and rinsed with distilled water. The same anti-STIM1 antibody was used for immunostaining at 5 μg/mL. Anti-TRPC1 antiserum (T1E3, 1:500 dilution) has been described.[6,13] Antibody labeling of cells and intact vein sections was as described,[6,7] except fixation of cells was for 4 minutes in 3% paraformaldehyde and incubation with primary antibody was overnight at 4°C. Images were collected using a laser confocal microscope (Zeiss) and analyzed using Image J software (NIH). Images shown are representative of 4 independent experiments, each carried out in duplicate.

For Western blotting, cells were collected using 1 mL of ice-cold PBS and pelleted at 4°C and then lysed in Laemmli sample buffer supplemented with complete protease inhibitor cocktail (Roche). The lysate was centrifuged to remove particulate matter, and equal

amounts of protein were separated by 8% SDS-PAGE and transferred onto nitrocellulose membrane. Membranes were incubated with blocking buffer (containing 0.1% Tween 20 and 5% nonfat dry milk) for 2 hours at room temperature, followed by incubation in anti-STIM1 antibody (1 μg/mL) or anti-TRPC1 antiserum (T1E3, 1:500) overnight at 4°C. Anti–β-actin antibody (1:1000) was from Santa Cruz Biotechnology. Membranes were then washed and incubated with secondary antibody. Labeling was detected using SuperSignal West Pico chemiluminescent substrates (Pierce).

For coimmunoprecipitation (co-IP), cells were harvested and lysed in 300 μL of lysis buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 2 mmol/L EGTA, pH 8, plus 1% Triton X-100, and 5% glycerol) supplemented with complete protease inhibitor cocktail (Roche). After sonication and clearing by centrifugation, cell lysates were mixed with 3 μL of anti-FLAG (Sigma), anti-STIM1, anti-TRPC1, or anti-EE (Bethyl) antibodies and incubated at 4°C for 2 hours. On addition of 20 μL of protein A/G-agarose bead suspension (Santa Cruz Biotechnology), incubation was continued for 3 hours at 4°C. Following extensive washing with the lysis buffer, agarose beads were resuspended in 40 μL of electrophoresis buffer (50 mmol/L Tris-HCl, 100 mmol/L dithiothreitol, 2% SDS, pH 7.4, plus 10% glycerol and 0.05% bromophenol blue). Protein in the cell lysates or immunoprecipitated samples were separated on 10% SDS-PAGE gels, transferred onto nitrocellulose membrane, and detected using anti-FLAG (1:1000), anti-STIM1, anti-TRPC1 (T1E3), anti-EE (1:2000), or anti–β-actin primary antibodies, and horseradish peroxidase conjugated antimouse (1:4000), anti-rabbit (1:5000) or anti-goat (1:5000) IgG secondary antibodies. When indicated, cells were first transfected with 2 μg cDNA encoding Flag-TRPC1 in pcDNA3 (from C. Montell, Baltimore, Md; J. Liou, Stanford, Calif) or YFP-STIM1 in pDS SP (from J. Liou) 24 hours before co-IP.

Data Analysis

Averaged data are presented as means±SEM. Data were produced in pairs (test and control) and compared using *t* tests, where statistical significance is indicated by * ($P<0.05$) and no significant difference by NS ($P>0.05$).

Results

Messenger RNA encoding STIM1 was detected in human saphenous vein VSMCs (Figure 1a) and Western blotting with anti-STIM1 antibody revealed STIM1 protein that was reduced in abundance by STIM1-specific siRNA (Figure 1b and supplemental Figure I). Cross-sections of vein showed STIM1 in VSMCs of the medial layer and neointimal formation (Figure 1c). STIM1 siRNA reduced Ca^{2+} entry in store-depleted cells (Figure 1d and supplemental Figure II) but lacked effect on agonist-evoked Ca^{2+} release (supplemental Figure III). STIM1 siRNA inhibited cell migration or invasion but not cell proliferation (Figure 2a and 2b). Therefore, endogenous STIM1 is expressed and relevant to VSMC function.

STIM1 in the plasma membrane is predicted to have an extracellular N terminus, which is the target of anti-STIM1 antibody (Figure 1b). Nonpermeabilized VSMCs (supplemental Figure IV) were labeled specifically and in a punctuate manner by the antibody (Figure 3a), suggesting the presence of clusters of plasma membrane–spanning STIM1. The clusters were not altered by store depletion (Figure 3b and 3c) and were, therefore, considered to be constitutive. The antibody also inhibited Ca^{2+} entry (Figure 3d) and cell migration but not proliferation (Figure 3e), suggesting importance of plasma membrane–spanning STIM1 specifically to migration.

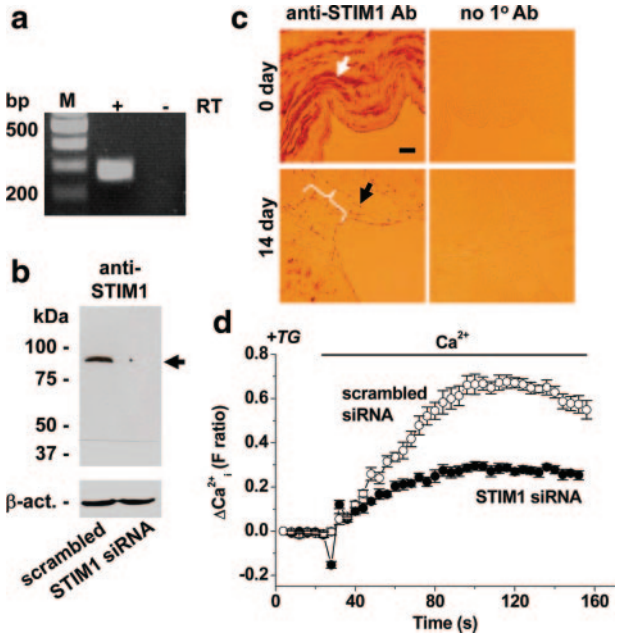

Figure 1. Expression and function of STIM1 in human saphenous vein VSMCs. a, Gel electrophoresis of PCR products with (+) and without (−) prior reverse transcriptase (RT) reaction and using STIM1 primers (see supplemental Table I). M indicates the marker DNA ladder. b, Western blotting of lysates from saphenous vein cells transfected with control (scrambled) siRNA or STIM1 siRNA. The blot was probed with anti-STIM and anti–β-actin antibodies. The predicted mass of full-length human STIM1 is 78 kDa. c, Images show cross-sections of vein labeled with anti-STIM1 antibody (left) or using the same protocol in the absence of primary antibody (right). Upper images show vein fixed with formalin within 15 minutes of removal from the patient (0 day). The white arrow points to a medial layer VSMC. Lower images focus on neointimal VSMCs grown over 14 days. The black arrow points to a smooth muscle cell in the neointimal layer (labeled by a white bracket). Positive labeling for STIM1 is brown. The scale bar applies to all images and is 50 μm. d, Example paired experiment comparing Ca^{2+} entry in cells transfected either with STIM1 siRNA or scrambled siRNA (24 wells per data point). Shown are changes in Ca^{2+} in response to readdition of extracellular Ca^{2+} (0.2 mmol/L) after pretreatment with 1 μmol/L thapsigargin (+TG) in Ca^{2+}-free solution for 30 minutes.

The anti-STIM1 antibody was also investigated using a planar patch-clamp system that enabled fast microfluidic extracellular solution exchange. Whole-cell recordings were made with intracellular Ca^{2+} buffered strongly at the physiological resting Ca^{2+} concentration. Basal current occurred, but there were large additional and lanthanum-sensitive currents in response to store depletion evoked by thapsigargin (Figure 4a). Similar currents were observed in conventional patch-clamp recordings under the same ionic solutions (data not shown). Currents were suppressed within a few minutes by extracellular anti-STIM1 antibody (Figure 4b). Antibody was effective in 70% of cells (n=27). Current–voltage relationships for thapsigargin-evoked and antibody-inhibited currents were similar, showing mild outward rectification and reversing polarity close to 0 mV (Figure 4c). Control IgG or boiled anti-STIM1 antibody had no effect (Figure 4d).

Co-IP assays were used to investigate if STIM1 and TRPC1 form a complex. Firstly, VSMCs were transfected so

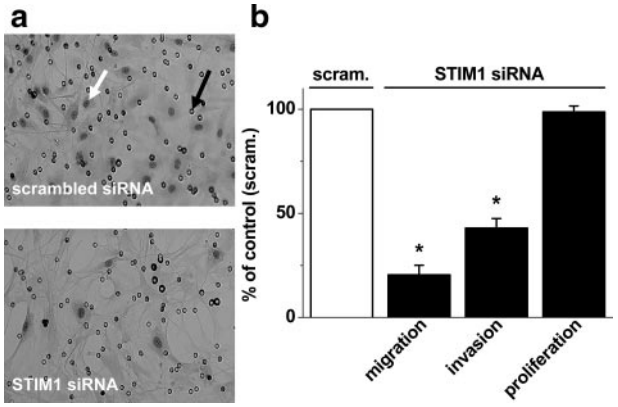

Figure 2. Specific role of STIM1 in cell migration and invasion. a, Typical bright-field images of VSMCs that had moved through pores in polycarbonate membranes. An example cell (nucleus) is indicated by a white arrow and a pore by a black arrow. b, Summary for all cell migration, invasion, and proliferation data where test data (STIM1 siRNA) are given as percentages of their own control data (scrambled siRNA). Experiments were in duplicate for cells from at least 4 patients.

that they expressed exogenous STIM1 tagged with yellow fluorescent protein (YFP) and TRPC1 tagged with the Flag epitope. Anti-STIM1 antibody precipitated Flag-tagged TRPC1 (Figure 5a) and the reverse co-IP was also positive (Figure 5b). To study endogenous TRPC1, we used anti-TRPC1 antibody[6,13] validated using TRPC1 siRNA (Figure 5c and supplemental Figure I). Results were similar to those obtained with expressed tagged proteins, with anti-STIM1 antibody precipitating TRPC1 (Figure 5d) and the reverse co-IP also positive (Figure 5e). Co-IPs are, however, prone to a lack of specificity, even when control and peptide-preadsorbed antibodies are ineffective (Figure 5a, 5b, 5d, and 5e). To investigate if there was nonspecific precipitation of other membrane proteins, we overexpressed P2X7 receptor containing an EE tag. Anti-EE antibody precipitated P2X7 receptor (Figure 5f) but failed to precipitate STIM1 (Figure 5g). Therefore, the co-IP conditions were specific even in the face of overexpressed P2X7 receptor.

We previously reported that anti-TRPC1 antibody (Figure 5c), which targets an extracellular epitope,[6,13] caused modest but statistically significant inhibition of Ca^{2+} entry in store-depleted saphenous vein VSMCs,[6] indicating that TRPC1 and STIM1 may contribute to the same type of Ca^{2+}-entry pathway. To independently investigate the contribution of TRPC1, we developed siRNA probes to suppress expression of TRPC1 and the associated TRPC4 and TRPC5 (Figure 5c and supplemental Figure I). A cocktail of siRNAs targeting TRPC1, 4 and 5 partially suppressed Ca^{2+} entry (Figure 6a and 6b). The effect was slightly, but not significantly, greater than that caused by the single siRNAs targeting TRPC1 or TRPC5 alone (Figure 6b). As a further independent test, we expressed a dominant negative ion-pore mutant of TRPC5 (DN-TRPC5), which has functional effects against TRPC5 and endogenous TRPC1-TRPC5 heteromultimeric channels.[7] Again, Ca^{2+} entry was partially suppressed (Figure 6b). The siRNA effect was less than that of the DN-TRPC5, possibly because the protein components were not sufficiently suppressed by the siRNA cocktail. Anti-TRPC1 antibody was investigated in planar patch-clamp recordings and found, in some cells, to inhibit current by an amount greater than the basal current (eg, Figure 6c). However, a substantial fraction of cells (75%, n=28) failed to respond, even though they subsequently responded to lanthanum (eg, Figure 6d) or 75 μmol/L 2-aminoethoxydiphenyl borate.

siRNAs targeting TRPC1, -4, and -5 and DN-TRPC5 were used to investigate cellular effects of TRPC1-containing channels. Cell migration and proliferation were both inhibited (Figure 7a and 7b), contrasting with observations for STIM1, which was only related to cell migration (Figure 2), suggesting that TRPC1 has STIM1-independent functions. We therefore investigated the cell surface localization of TRPC1 using anti-TRPC1 antibody (Figure 5c),[6,13] first validating that the staining reflected TRPC1 by using TRPC1 siRNA (Figure 7c). TRPC1 appeared in punctae like those for STIM1 except denser and more frequent. Partial colocalization of TRPC1 and STIM1 occurred but independent TRPC1 and STIM1

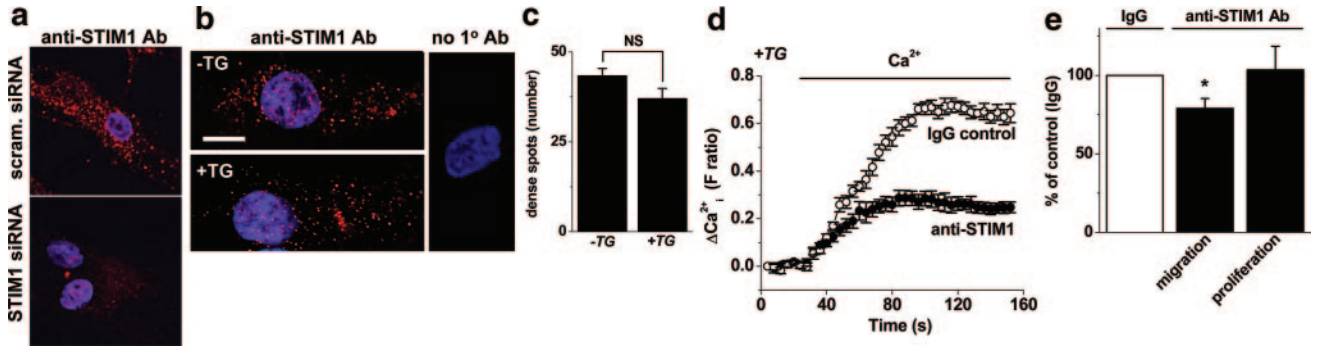

Figure 3. Plasma membrane–spanning STIM1. a and b, Representative images of nonpermeabilized VSMC labeled by anti-STIM1 antibody (Cy3, red) or in the absence of this primary antibody (no 1° Ab). a, Paired comparison of staining after transfection with STIM1 siRNA or scrambled (control) siRNA. Nuclei were stained with DAPI (blue). Quantitative analysis of images confirmed that STIM1 siRNA significantly reduced the immunofluorescence (>10 cells per treatment and 3 independent experiments). b, Staining after exposure of live cells for 30 minutes to recording solution (−TG) or Ca^{2+}-free solution containing 1 μmol/L thapsigargin (+TG). Scale bar, 10 μm. c, For the type of experiment illustrated in b but showing the mean number of punctae with intensity above a defined threshold (123 cells were analyzed for +TG and 169 cells for −TG). d, Example of block of Ca^{2+} entry by extracellularly applied anti-STIM1 antibody (20 μg/mL). Cells were pretreated for 30 minutes at room temperature with anti-STIM1 antibody or IgG2a isotope control (16 wells per data point). In 4 independent experiments, the antibody inhibited Ca^{2+} entry to 41±23.7% of the control. e, Effect of 10 μg/mL anti-STIM antibody on cell migration but not proliferation (4 independent experiments).

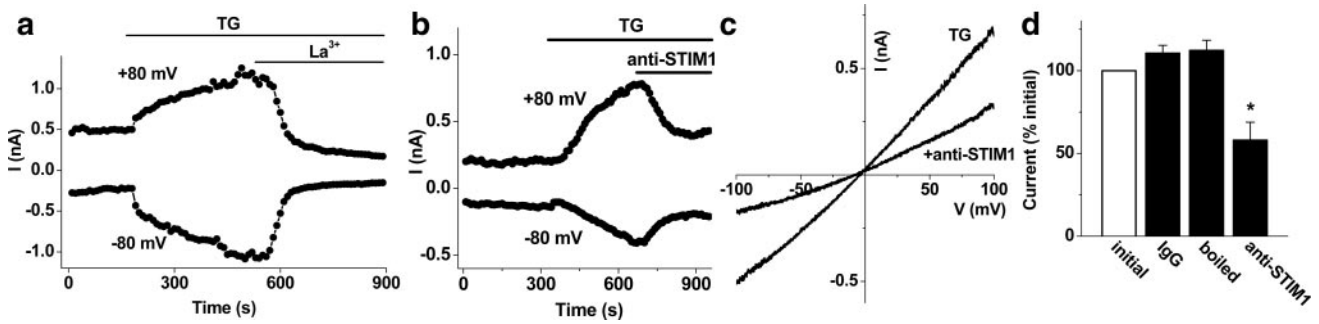

Figure 4. Rapid inhibition of current by anti-STIM1 antibody. a, Example whole-cell recording showing current evoked by extracellular application of 2 μmol/L thapsigargin (TG) and subsequent inhibition by 10 μmol/L lanthanum (La^{3+}). b, As for a but showing the effect of extracellular 20 μg/mL anti-STIM1 antibody. c, For the experiment shown in b, current–voltage relationships (I-V) for the current evoked by TG and blocked by anti-STIM1 antibody. d, Mean currents normalized to preantibody values showing the effect of 20 μg/mL anti-STIM1 antibody (n=6) and lack of effects of IgG (n=13) or anti-STIM1 antibody denatured by boiling (n=10).

were also apparent (Figure 7d). Store depletion with thapsigargin did not alter these relationships (data not shown).

Discussion

The data suggest that modulated human VSMCs express STIM1 as a constitutive plasma membrane–spanning protein. The STIM1 made positive contributions to Ca^{2+} entry and cell migration, while having little or no impact on cell proliferation. It associated with TRPC1 but STIM1 also interacted functionally with at least 1 other type of unidentified nonselective cationic channel, and there was STIM1-independent TRPC1 of importance to cell proliferation (Figure 8).

Existence of STIM1 as a constitutive trans–plasma membrane protein is consistent with early literature on STIM1[23] and a study of CRAC channels.[25] Our experiments did not address whether STIM1 also exists in intracellular membranes.[21,24] Trans–plasma membrane localization is also evident for TRPC subunits because extracellular anti-TRPC1 or anti-TRPC5 antibodies affected Ca^{2+} entry and cell functions.[6,7,13,15,18] Studies of other cell types and overexpressed tagged proteins have suggested physical associations between STIM1 and TRPC1, TRPC4, or TRPC5,[27–29] and we previously showed association of TRPC1 with TRPC5 in VSMCs.[7]

STIM1 siRNA that strongly reduced Ca^{2+} entry in VSMCs also suppressed leucine incorporation by ≈18%, indicating a modest effect of STIM1 on translation.[30] Our experiments did not directly address this parameter but, if there was such an effect, it was not sufficient to affect the overall phenomenon of cell proliferation because STIM1 did not have significant impact on proliferation under conditions when it had major influence over cell migration (Figures 2b and 3e).

The molecular basis of store-operated channels is incompletely understood. A complicating factor is that there is more than 1 type of store-operated channel. One is the CRAC-type, which confers an extremely small, Ca^{2+}-selective, and inwardly rectifying whole-cell current[21,26]: it is normally measured under conditions of high concentrations of extracellular Ca^{2+} and buffering of intracellular Ca^{2+} to low levels; it is prominent in lymphocytes and related cells; it has not been unambiguously described in VSMCs, and we have not observed it in saphenous vein VSMCs (J.L. and D.J.B., unpublished findings, 2008), although we cannot be certain it does not exist. A reason why CRAC channel currents have not been clearly seen in VSMCs could be because of overriding store-operated channels of a nonselective cationic type.[9–12] Because of concern that such a nonselective current might not be a bona fide store-operated current (but instead a Ca^{2+}-activated current), we chose strong intracellular Ca^{2+}-buffering conditions. Notably, even after 10 minutes of intracellular dialysis with 40 mmol/L EGTA-containing so-

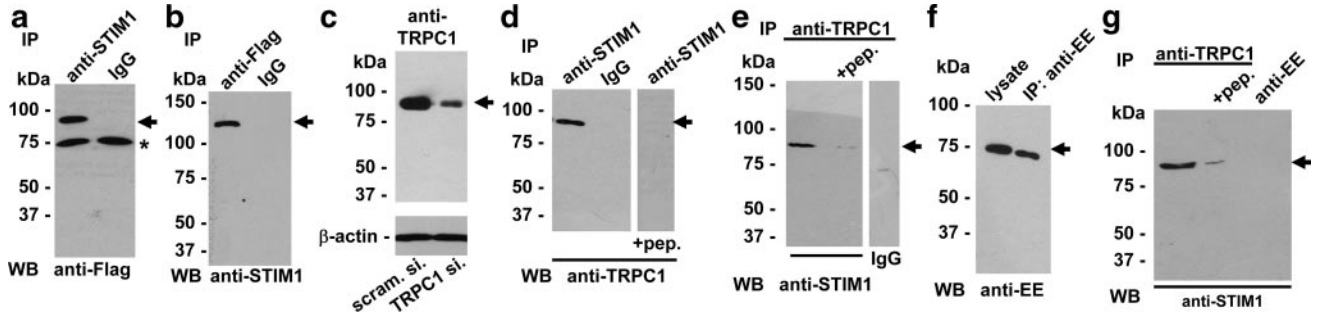

Figure 5. Physical association between STIM1 and TRPC1. Shown are Western blots (WB) with or without prior IP or transfection. The black arrows indicate the expected positions of the proteins targeted by the indicated antibodies. In all cases, human saphenous vein VSMCs were used as the source of proteins. +pep. indicates preadsorption to the antigenic peptide used to generate antiserum. a, *Nonspecific antibody fragments from the IP. a and b, Cells were transfected with cDNA encoding YFP-STIM1 and Flag-TRPC1. The mass of STIM1 appears larger than its predicted mass because of the YFP tag, whereas the Flag tag is small relative to the mass of TRPC1. c, Western blot of lysates from cells transfected with control (scrambled) siRNA or TRPC1 siRNA. The blot was probed with anti-TRPC1 and anti–β-actin antibodies. The predicted mass of full-length human TRPC1 is 91 kDa. d and e, Endogenous proteins from nontransfected cells. f and g, Cells were transfected with cDNA encoding EE-tagged P2X7 receptor.

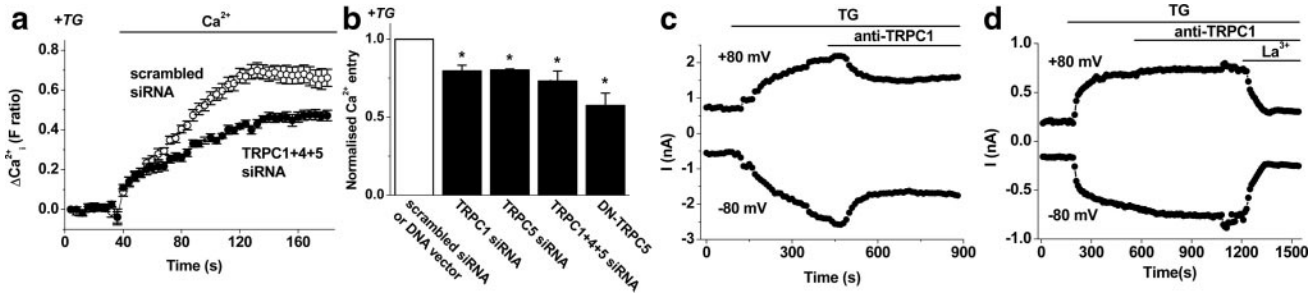

Figure 6. Partial contribution of TRPC1-containing channels to Ca^{2+} entry and ionic current. a, Inhibition of Ca^{2+} entry by the cocktail of 3 siRNA probes targeting TRPC1, TRPC4, and TRPC5. The protocol was the same as described for Figure 1d. b, Summary data for experiments like the example of a and for the transfections indicated. Each test data set (black bars) was compared with its own paired control and was from ≥4 independent experiments on cells from different patients, each using ≥14 wells. c, As for Figure 4b but an example of a cell that showed an inhibitory effect of extracellular application of anti-TRPC1 antibody (1:500 dilution). d, As for c but an example of a cell that showed no effect of anti-TRPC1 antibody.

lution, we consistently observed large, lanthanum-sensitive currents in response to store depletion evoked by thapsigargin, which reversed polarity at 0 mV and, thus, not at the chloride equilibrium potential. Therefore, this current would seem to satisfy the definition of being store operated.

The CRAC type of channel is suggested to be explained by Orai1,[21,26] but the described properties of Orai1 are not consistent with it explaining the store-operated cationic current of VSMCs. Some combinations of TRPC channels do have suitable electrophysiological characteristics and many studies have provided direct evidence for the contribution of TRPC channels, including TRPC1 and TRPC5.[9,13–20] Also, Smani et al[32] suggested that the final messenger linking depleted stores to nonselective store-operated channels in VSMCs is a lysophospholipid such as lysophosphatidylcholine. Importantly, TRPC channels are activated by this phospholipid.[33,34] Nevertheless, studies of VSMCs from a *Trpc1*$^{-/-}$ mouse have suggested that nonselective store-operated current can exist despite the absence of TRPC1.[12] The latter study did not exclude possible complications attributable to redundancy or compensatory regulation in heteromultimeric TRPC (or other) channels and may have lacked sufficient sensitivity to detect a relatively small contribution from TRPC1 channels. Nonetheless, the result is similar to ours because we found that TRPC1-containing channels contributed only partially to Ca^{2+} entry or ionic current evoked by store depletion and made no contribution to current in a significant fraction of cells. We conclude from this that TRPC1 is stimulated by store depletion[9,13–20,35] but that there is also another TRPC1-independent store-operated nonselective cationic channel. In some types of VSMC, or under some experimental conditions, this other type of channel may explain most or all of the store-operated current. That we observed sensitivity to anti-TRPC1 antibody in some cells but not others suggests there was heterogeneity in the VSMCs, within a vein or between patients.

Although there is much interest in store-operated mechanisms, it is important to consider how such phenomena might relate to vascular physiology or disease. Our results show that STIM1 and TRPC1 contributed strongly despite the absence of store depletion; ie, data for cell migration and proliferation were not obtained from cells exposed to a store-depleting agent such as thapsigargin, and releasable Ca^{2+} stores were detected (supplemental Figure III). Therefore, STIM1 and TRPC1 are either constitutively active or stimulated by factors that do not require store depletion. Other activators of

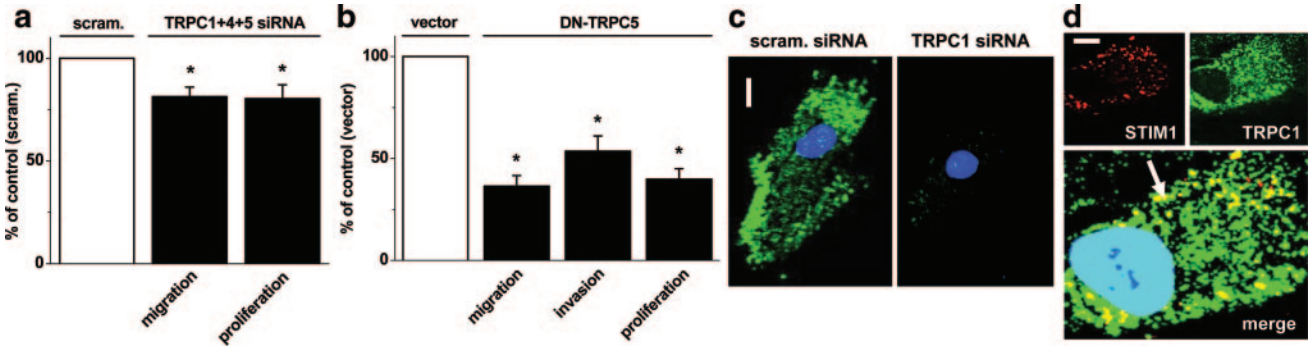

Figure 7. Cellular effects of TRPC1-containing channels and localization compared with STIM1. a, Inhibition of cell migration and proliferation by the cocktail of 3 siRNA probes targeting TRPC1, TRPC4, and TRPC5. The control was the scrambled (scram.) siRNA probe. b, Inhibition of cell migration, invasion, and proliferation by dominant negative mutant TRPC5 (DN-TRPC5). The control was the DNA vector without DN-TRPC5 insert. Each set of experiments was performed in duplicate for cells from at least 4 patients. c, Representative images of cells labeled by anti-TRPC1 antibody (FITC, green) after transfection with TRPC1 siRNA or scrambled (control) siRNA. Quantitative analysis confirmed that TRPC1 siRNA significantly reduced the immunofluorescence (>10 cells per treatment and 2 independent experiments). d, Top, Two representative images of the same cell dual-labeled with anti-STIM1 (red) and anti-TRPC1 (green) antibodies. The lower, larger image is a merge of the 2 upper images. STIM1 and TRPC1 colocalization is indicated by yellow, with 1 example highlighted by the white arrow. c and d, Nuclei were stained with DAPI (blue) and the scale bars are 10 μm.

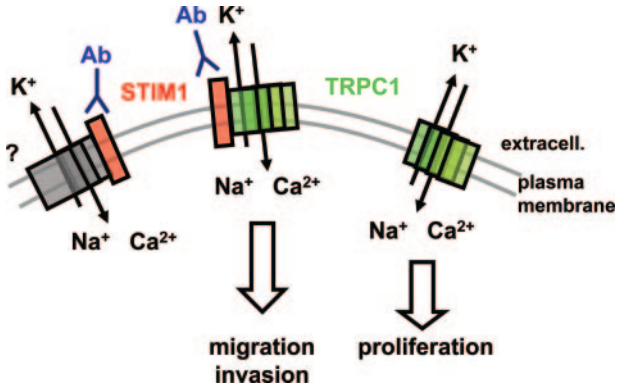

Figure 8. Different types of plasma membrane nonselective cationic channel suggested by the data obtained in this study. Ab indicates antibody targeting the N terminus of STIM1. STIM1 is represented in red and TRPC1 in green to align with the immunofluorescence data of Figure 7. Other colors are associated with TRPC1 to indicate that this protein is likely to exist as a heteromultimer. The gray channel subunits on the left suggest that STIM1 associates with another unknown nonselective channel because the ionic current evoked by thapsigargin was commonly blocked by anti-STIM1 antibody but only inhibited by anti-TRPC1 antibody in 25% of cells. Intracellular STIM1 has been suggested but is not shown.

TRPC1-containing channels include receptor agonists (eg, sphingosine-1-phosphate),[7] protein kinase C,[11] and extracellular thioredoxin.[36] Whether extracellular agonists act at plasma membrane STIM1 is unknown.

Our experiments have focused on the human VSMC in its modulated phenotype and are, therefore, likely to have most relevance to adaptive vascular processes occurring in development, after injury, and in response to ageing and disease. Contributions of constitutive plasma membrane–spanning STIM1 have been observed and accessibility of this STIM1 to extracellular antibody, the inhibitory effect of the antibody and localization of STIM1 to neointimal formations raise the possibility that extracellular modulators of plasma membrane STIM1 might be useful as specific inhibitors of cell migration. The data suggest that TRPC1 and STIM1 interact and both contribute to store-operated Ca^{2+} entry and ionic current but that TRPC1 also functions independently of STIM1 and that STIM1 interacts with a TRPC1-independent ion channel that is not yet identified but makes a major contribution to nonselective cationic current evoked by store depletion.

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Disclosures

None.

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SUPPLEMENTARY INFORMATION

Interactions, functions and independence of plasma membrane STIM1 and TRPC1 in vascular smooth muscle cells

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SUPPLEMENTARY MATERIALS AND METHODS

RNA isolation and quantitative RT-PCR

Total RNA was extracted from saphenous vein smooth muscle cells using a Tri-reagent protocol followed by DNase I (Ambion) treatment[1]. 1 μg of total RNA was used for reverse transcription (RT) based on oligo-dT primers and AMV RT enzyme. The specificity of PCR was verified by reactions without RT (-RT) and by melt-curve analysis of PCR products. Sequences of PCR primers are in Supplementary Table I. PCR products were electrophoresed on 2 % agarose gels

containing ethidium bromide. No PCR products occurred in the absence of RT. With RT there was only a single product of the correct size, giving a single peak in the melt-curve. All PCR products were sequenced to confirm identity (Lark, UK). Real-time PCR was carried out using a Lightcycler (Roche) largely as described previously[1]. Relative abundance of target RNA was normalized to β-actin RNA, which showed no difference between samples. PCR efficiency (E) was $10^{(-1/slope)}$. Relative abundance of target RNA was calculated from $(E_{\beta\text{-actin}}{}^{Cp}) / (E_{target}{}^{Cp})$, where PCR cycle crossing-points (C_P) were determined by fit-points methodology. PCR reactions were at least in duplicate.

Anti-TRPC5 antibody

Antibody targeted to the C-terminus of TRPC5 (T5C3) has been described[2]. Permeabilisation of cells was achieved with 0.1 % Triton X-100 in 1 % bovine serum albumin for 2 h.

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure I. Effectiveness and specificity of siRNA probes. (**a-c**) siRNA knock-down of STIM1 mRNA. (**a**) Using STIM1 PCR primers, a melt-curve analysis of PCR product showing change in SYBR Green I fluorescence (F) with temperature (t). When there was reverse transcriptase (+RT) reaction a single peak occurred, corresponding to the single band on the gel of Fig 1a. (**b**) PCR amplification curves following treatment with scrambled siRNA (control) or siRNA targeted to STIM1 mRNA (black circles). The rightward shift shows reduced abundance of STIM1 mRNA caused by STIM1 siRNA. (**c**) Relative abundance of mRNA species after STIM1 siRNA treatment and normalization to paired control data (only one bar is indicated as

'scrambled' but each data set was normalized to its own scrambled siRNA control data; $n \geq 3$ in each case). (**d**) Similar to the data of (**c**) but showing the effectiveness of each TRPC siRNA against its own mRNA species. (**e**) Lack of effect of the TRPC1+4+5 siRNA cocktail on STIM1 mRNA ($n \geq 3$).

Supplementary Figure II. Background Ca^{2+}-entry was small compared with Ca^{2+}-entry after store-depletion. Shown is a typical example of the Ca^{2+}-add back response with (+) or without (-) store-depletion evoked by 1 μmole/L thapsigargin (TG) (16 wells per data point).

Supplementary Figure III. Lack of effect of STIM1 siRNA on agonist-evoked Ca^{2+}-release. (**a**) Summary data for paired experiments exploring peak agonist-evoked Ca^{2+}-release after treatment with STIM1 siRNA normalized to control. The agonists tested were adenosine triphosphate (ATP 10 μmole/L: 20 wells) and angiotensin II (Ang II 0.1 μmole/L: 24 wells); data are for two independent experiments. (b) Agonist responses were not lost in Ca^{2+}-free solution. Responses were compared in paired experiments: in the presence of 1.5 mmole/L extracellular Ca^{2+}; in the absence of Ca^{2+} and presence of 0.4 mmole/L EGTA to chelate residual Ca^{2+}. ATP, n=12 wells per column; Ang II, n=24 wells per column.

Supplementary Figure IV. Confirmation that intracellular targets were inaccessible in non-permeabilised cells. Antibody to the TRPC5 C-terminus (intracellular) labeled protein (FITC, green) in permeabilised but not non-permeabilised cells. Labeling with anti-TRPC5 antibody was absent when it was preadsorbed to its antigenic peptide (+peptide). Where FITC fluorescence was not apparent, the presence of cells was detected by DAPI fluorescence (not shown). Scale bar, 20 μm.

SUPPLEMENTARY TABLE

Supplementary Table I: PCR primer pairs (F, forward direction; R, reverse direction), PCR amplicon sizes, and siRNA probes sequences.

Gene	Primer 5'-3'	Predicted amplicon (bp)	siRNA sequence (5'-3')
STIM1	F CTCTCTTGACTCGCCA R GCTTAGCAAGGTTGATCT	276	CAAUUCGGCAAAACUCUGCtg GCAGAGUUUUGCCGAAUUGtt
TRPC1	F TTAGCGCATGTGGCAA R CCACTTACTGAGGCTACTAAT	303	GCCCGGAAUUCUCGUGAAUtt AUUCACGAGAAUUCCGGGCtt
TRPC4	F ATTAGCTTCACGGGGT R CTTCGTGGGTGACTGT	241	GGAGGUACUCUGCCUACUCtt GAGUAGGCAGAGUACCUCCtt
TRPC5	F ACATTTTAAGTTCGTTGCG R ACATCGGATCCCCTTG	218	GCAACCUUGGGCUGUUCAUtt AUGAACAGCCCAAGGUUGCtc

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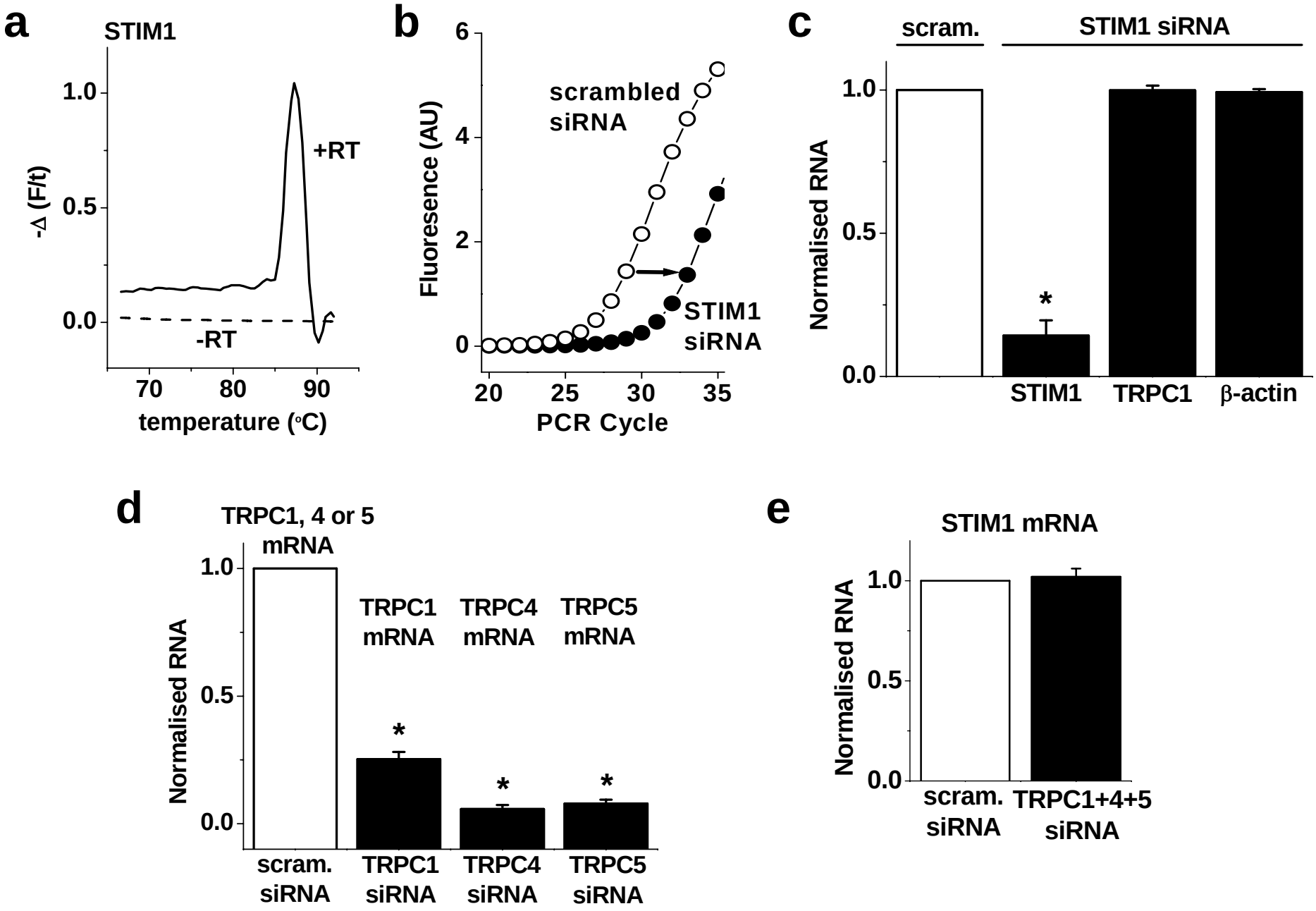
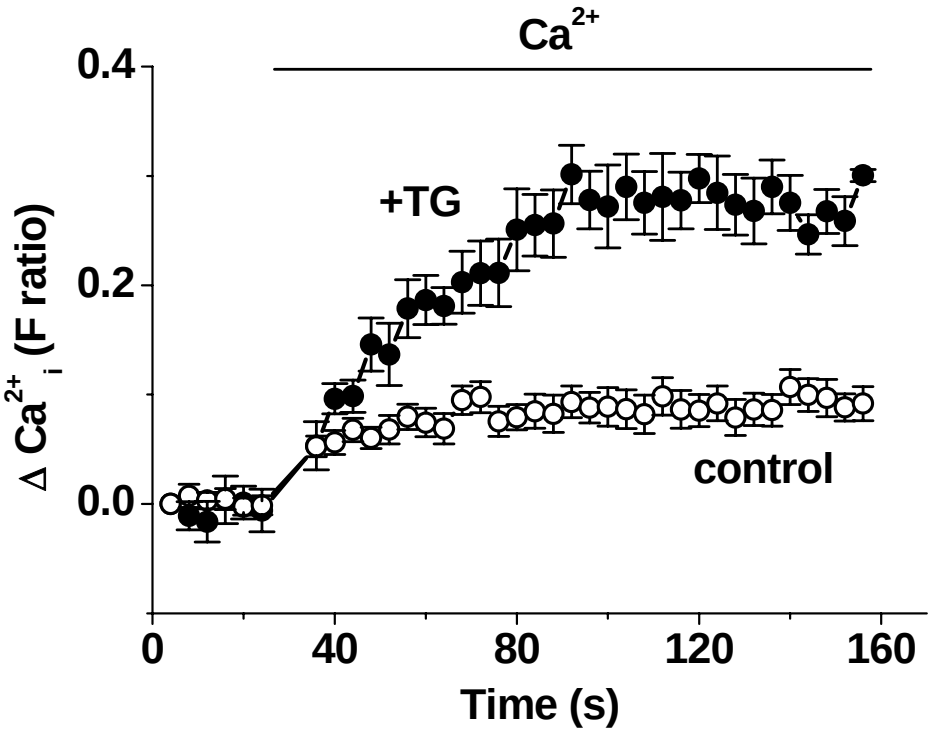
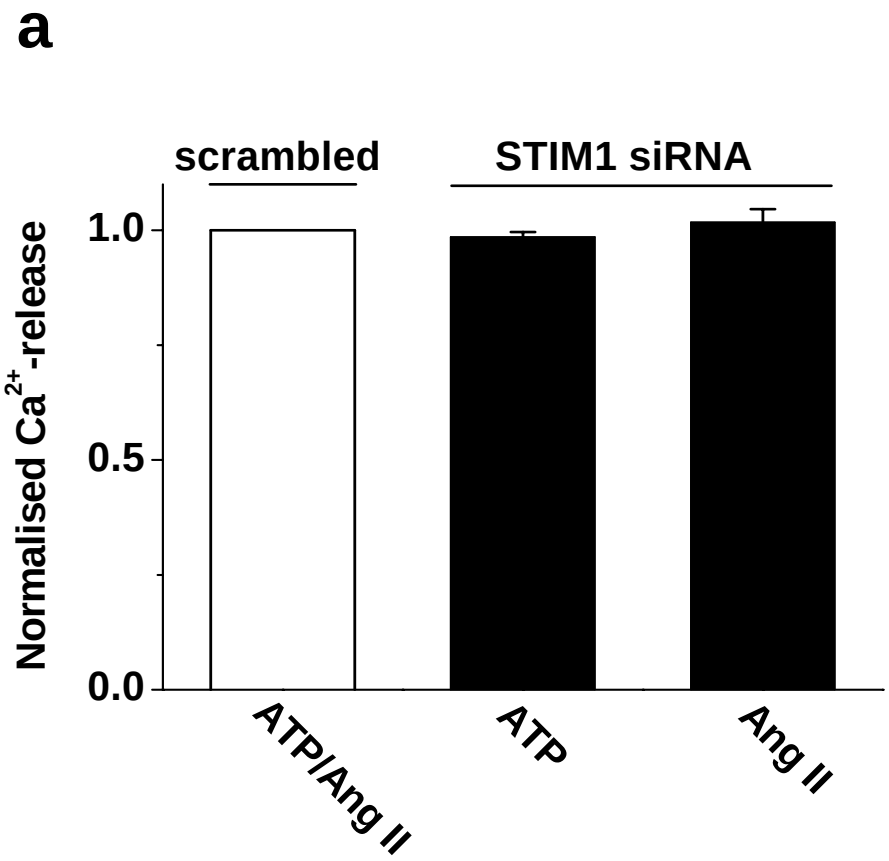
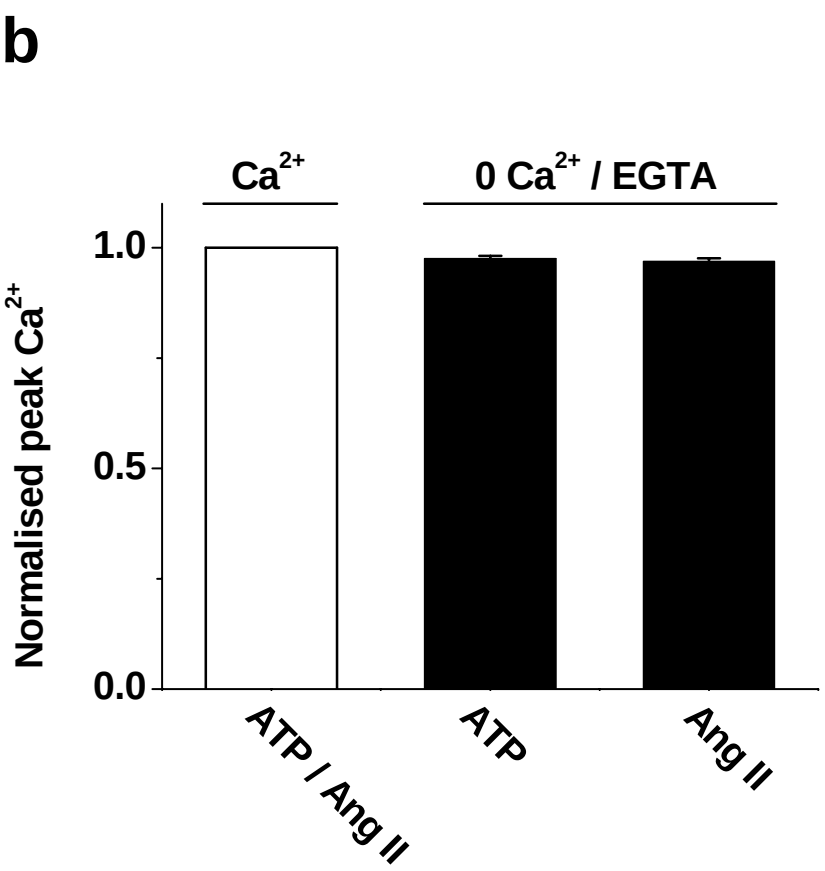


Li *et al* Supplementary Figure I

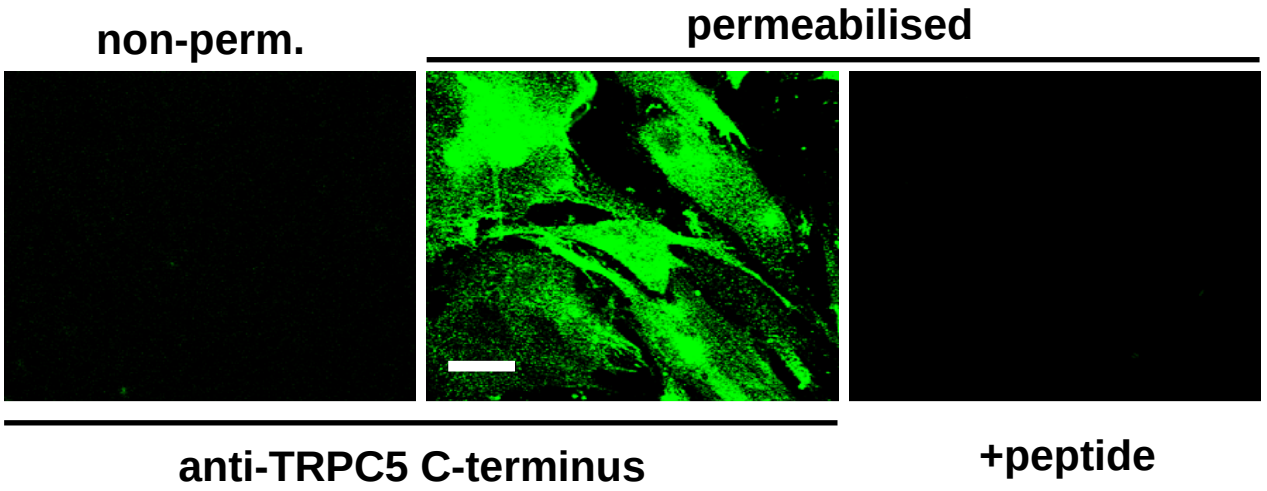


Li *et al* Supplementary Figure II





Li *et al* Supplementary Figure III



Li *et al* **Supplementary Figure IV**