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TRPC channels function independently of STIM1 and Orai1

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Recent studies have defined roles for STIM1 and Orai1 as calcium sensor and calcium channel, respectively, for Ca^{2+}-release activated Ca^{2+} (CRAC) channels, channels underlying store-operated Ca^{2+} entry (SOCE). In addition, these proteins have been suggested to function in signalling and constructing other channels with biophysical properties distinct from the CRAC channels. Using the human kidney cell line, HEK293, we examined the hypothesis that STIM1 can interact with and regulate members of a family of non-selective cation channels (TRPC) which have been suggested to also function in SOCE pathways under certain conditions. Our data reveal no role for either STIM1 or Orai1 in signalling of TRPC channels. Specifically, Ca^{2+} entry seen after carbachol treatment in cells transiently expressing TRPC1, TRPC3, TRPC5 or TRPC6 was not enhanced by the co-expression of STIM1. Further, knockdown of STIM1 in cells expressing TRPC5 did not reduce TRPC5 activity, in contrast to one published report. We previously reported in stable TRPC7 cells a Ca^{2+} entry which was dependent on TRPC7 and appeared store-operated. However, we show here that this TRPC7-mediated entry was also not dependent on either STIM1 or Orai1, as determined by RNA interference (RNAi) and expression of a constitutively active mutant of STIM1. Further, we determined that this entry was not actually store-operated, but instead TRPC7 activity which appears to be regulated by SERCA. Importantly, endogenous TRPC activity was also not regulated by STIM1. In vascular smooth muscle cells, arginine-vasopressin (AVP) activated non-selective cation currents associated with TRPC6 activity were not affected by RNAi knockdown of STIM1, while SOCE was largely inhibited. Finally, disruption of lipid rafts significantly attenuated TRPC3 activity, while having no effect on STIM1 localization or the development of I_{CRAC}. Also, STIM1 punctae were found to localize in regions distinct from lipid rafts. This suggests that TRPC signalling and STIM1/Orai1 signalling occur in distinct plasma membrane domains. Thus, TRPC channels appear to be activated by mechanisms dependent on phospholipase C which do not involve the Ca^{2+} sensor, STIM1.

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Abbreviations DMEM, Dulbecco's modified Eagle's medium; ER, endoplasmic reticulum; eYFP, enhanced yellow fluorescent protein; I_{crac}, calcium release activated Ca^{2+} current; IP_3, inositol trisphosphate; OAG, oleyl acetyl glycerol; PLC, phospholipase C; SERCA, sarcoplasmic endoplasmic reticulum Ca^{2+} ATPase; siRNA, small inhibitory RNA; SOCE, store-operated Ca^{2+} entry; STIM1, stromal interacting molecule 1; TRPC, cannonical transient receptor potential channel.

Elevations in cytoplasmic Ca^{2+} concentration are used by all cells as a signalling mechanism for a variety of distinct physiological processes (Berridge, 1993; Carafoli, 2002). Therefore, it is important to understand the mechanisms by which cells regulate Ca^{2+} concentrations. Cytoplasmic Ca^{2+} concentrations are tightly regulated by pumps (such as the sarcoplasmic–endoplasmic reticulum Ca^{2+} ATPase (SERCA) and plasma membrane Ca^{2+} ATPase (PMCA)) and high-affinity Ca^{2+} binding proteins (Milner *et al.* 1992), and Ca^{2+} signals (rises in intracellular Ca^{2+} concentrations) can occur through Ca^{2+} release from internal Ca^{2+} stores or flux through plasma membrane Ca^{2+} channels. One ubiquitous Ca^{2+} signalling pathway involved in a variety of physiological processes is known as store-operated Ca^{2+} entry (SOCE) or capacitative Ca^{2+}

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entry (Putney, 1986; Berridge, 1995). SOCE is a process in which the depletion of Ca^{2+} stores located in the endoplasmic reticulum (ER) activates Ca^{2+} channels in the plasma membrane. Physiologically, this occurs in many cell types by the receptor-mediated activation of phospholipase C (PLC) and generation of the second messenger inositol 1,4,5-trisphosphate (IP_3). IP_3 binds to and activates specific ion channels located in the ER known as IP_3 receptors, thus allowing Ca^{2+} ions to move out of the lumen of the ER and into the cytoplasm. This depletion of luminal ER Ca^{2+} then signals in a retrograde fashion to the plasma membrane and leads to the activation of SOCE. This process has proved critical not only in the maintenance of ER Ca^{2+} pools, but also in providing signals for numerous physiological functions, especially in haematopoietic cells (Gwack *et al.* 2007; Luik & Lewis, 2007).

While advances in the basic understandings of SOCE have been made over the past 20 years, it wasn't until recently that the molecular components of SOCE were described. We now know that STIM1 functions as the ER Ca^{2+} sensor (Liou *et al.* 2005; Roos *et al.* 2005), and Orai (also known as CRACM) family proteins function as the pore-forming subunits of SOCE channels known as the Ca^{2+} release-activated Ca^{2+} (CRAC) channels (Feske *et al.* 2006; Vig *et al.* 2006*b*; Zhang *et al.* 2006). Orai-formed CRAC channels exhibit low conductance, strong inward rectification and high Ca^{2+} selectivity. However, evidence exists for SOCE channels that have biophysical properties distinct from CRAC currents (I_{CRAC}) (Parekh & Putney, 2005). One such class of channels which has been suggested to function as SOCE channels under certain conditions are the canonical transient receptor potential, or TRPC, channels.

Like SOCE channels, TRPC channels are activated downstream of PLC. Therefore, these channels were first suggested to be the channels underlying I_{CRAC} (Hardie & Minke, 1992, 1993). However, electrophysiological analysis of cloned mammalian TRPCs revealed ion channels which are non-selective, conducting Na^+ and K^+ as well as Ca^{2+} (Hurst *et al.* 1998; Hofmann *et al.* 1999). Nonetheless, there are numerous reports of apparent store-operated channels which are much less selective for Ca^{2+} than the prototypical CRAC channels (Parekh & Putney, 2005). Further, there are a number of reports of reduced SOCE when TRPC expression is knocked down or knocked out (Parekh & Putney, 2005; Liu *et al.* 2007), and in some instances, TRPC channels can display apparent SOCE activity when exogenously expressed (Liu *et al.* 2000; Vazquez *et al.* 2001; Lievremont *et al.* 2004). However, there is also extensive literature indicating that TRPC channels do not operate as SOCE channels (Zitt *et al.* 1997; McKay *et al.* 2000; Trebak *et al.* 2003*a*; Dietrich *et al.* 2007; Varga-Szabo *et al.* 2008). In spite of this controversy, we have learned a great deal about store-independent TRPC channel activation and regulation. While TRPC3, TRPC6 and TRPC7 appear to be activated by the second messenger diacylglycerol (DAG), a product of PLC hydrolysis of phosphatidylinositol-4,5-bisphosphate, TRPC1, TRPC4 and TRPC5 activation is less defined, albeit still downstream of PLC activity (Schaefer *et al.* 2000; Ma *et al.* 2001; Hofmann *et al.* 2002; Venkatachalam & Montell, 2007). Interestingly, recent reports suggest that certain TRPC channels are also regulated by STIM1, and that TRPC activity (at least for TRPC1, TRPC4 and TRPC5) requires both PLC activity and STIM1 interaction (Huang *et al.* 2006; Lopez *et al.* 2006; Yuan *et al.* 2007). In one study, the gating of TRPC channels by STIM1 was seen to occur through electrostatic interactions between the polybasic domain of STIM1 and conserved aspartate residues in TRPCs, in this case TRPC1 and TRPC3 (Zeng *et al.* 2008). Further, at least in the case for TRPC1, it has also been suggested that TRPC gating by STIM1 is mediated somehow through interactions with Orai proteins (Cheng *et al.* 2008; Jardin *et al.* 2008*a*) and requires lipid raft clustering of signalling proteins (Alicia *et al.* 2008; Pani *et al.* 2008).

In this laboratory, we have published results showing that TRPC channels are not generally activated by store depletion (Trebak *et al.* 2003*a*), but under certain conditions it appears they may be (Vazquez *et al.* 2003; Lievremont *et al.* 2004). However, in contrast to previous studies by ourselves and others on Orai channels, we have not investigated the function of TRPC channels in the context of STIM1 signalling. Thus, in the current study, we have sought to determine whether TRPC channels are functionally regulated by the ER Ca^{2+} sensor, STIM1, regardless of whether or not they are activated by Ca^{2+} store depletion. Using both imaging and electrophysiological techniques on expressed and native proteins, we have concluded that TRPC1, TRPC3, TRPC5, TRPC6 and TRPC7 function independently of STIM1 when activated by agonist. Further, we report that while TRPC3 activity was attenuated by the disruption of lipid rafts, I_{CRAC} and STIM1 movements in response to store depletion remained impervious to the loss of cholesterol from the lipid bilayer. Taken together, we believe these data strongly suggest that TRPC channels are activated by mechanisms dependent on phospholipase C and do not involve interactions with the Ca^{2+} sensor, STIM1.

Methods

Ethical information

No animals or human subjects were used in these studies.

Cell culture

Wild-type (WT) HEK293, A7r5 and A10 cells were obtained from the American Type Culture

Collection (ATCC). HEK293 cells stably expressing TRPC3, TRPC6 and TRPC7 protein were described previously (Lievremont *et al.* 2004). Briefly, both WT and TRPC expressing HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 2 mM glutamine. Continuous selection of TRPC-expressing cells was maintained by the application of 500 μg ml^{-1} geneticin (Invitrogen). A7r5 and A10 cells were also cultured using DMEM (ATCC) supplemented with 10% FBS. Cells were passed by treating with trypsin–EDTA and maintained at 37°C in a humidified incubator set at 5% CO_2. All experiments were carried out on cells plated onto 30 mm round glass coverslips mounted in a Teflon chamber.

Plasmids

Full-length STIM1, STIM2 and Orai1 cDNA plasmids were purchased from Origene in the pCMV6-XL5 and pCMV-XL4 vectors, respectively. An eYFP-STIM1 plasmid was obtained from Dr Tobias Meyer, Stanford University. A multi-amino acid mutation to the putative EF-hand of eYFP-STIM1 (D76N/D78N) was made by site-directed mutagenesis with the QuikChange Site-Directed Mutagenesis Kit (Stratagene), and was sequence confirmed. M5-muscarinic receptor, TRPC1, TRPC3, and TRPC5 plasmids were obtained from Dr Lutz Birnbaumer at the NIEHS (Research Triangle Park, NC, USA), TRPC6 was obtained from Dr Thomas Gudermann (University of Marburg; Marburg, Germany), and TRPC7 was jointly supplied by Christine Murphy and Adrian Wolstenholm of University of Bath, UK, and John Westwick of Novartis, UK.

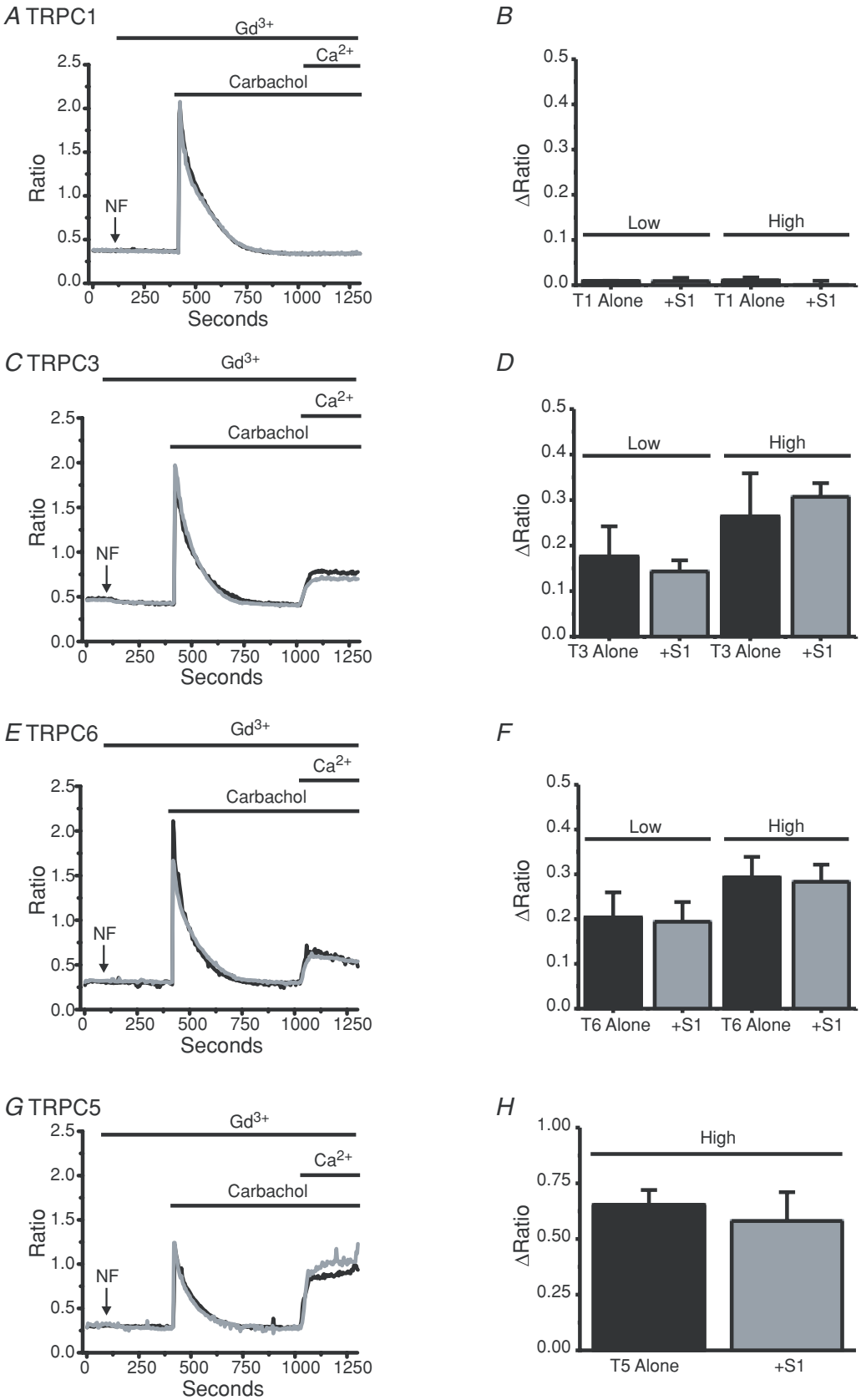
Transfections

For experiments carried out on A10 vascular smooth muscle cells or stable TRPC-expressing cells (and their WT controls), cells were transfected with the Amaxa electroporation system (Amaxa Inc., a Lonza Cologne company) following the guidelines set forth by the company for each cell line. A10 cells were electroporated using program D-030 and the nucleofector kit L buffer. HEK293 cells stably expressing TRPC channels were electroporated using the HEK293 (ATCC) cell line setting and the nucleofector kit V buffer. All other experiments utilized cells transfected with Lipofectamine 2000 (Invitrogen; 2 μl per well). For studies of muscarinic receptor activation of TRPCs, HEK293 cells were transfected with 1 μg per well of M5-muscarinic receptor cDNA together with 0.2 μg per well of eYFP cDNA, cDNA encoding one of several TRPCs (see below), with or without 0.32 μg per well of STIM1 cDNA. In order to permit comparison with previously published results, amounts of TRPC1, TRPC3, TRPC5, TRPC6 and STIM1 cDNA for experiments carried out in Figs 1 and 2 (as well as Supplemental Fig. 1) corresponded to those used by Yuan and co-workers for both high and low expression levels of the TRPC channels, adjusted only for different final transfection volumes (see Fig. 1) (Yuan *et al.* 2007). For all other experiments, the amounts of cDNA were as follows: eYFP cDNA (0.5 μg per well), eYFP-STIM1 cDNA (0.5 μg per well), eYFP-STIM1 D76N/D78N cDNA (0.5 μg per well), STIM2 cDNA (2.0 μg per well), and Orai1 cDNA (0.5 μg per well). After a 6–8 h incubation period, the medium bathing the cells was replaced with complete DMEM and maintained in culture. The following day, cDNA-treated cells were transferred to 30 mm glass coverslips in preparation for imaging or electrophysiological studies as described below.

siRNA knockdown

The RNAi experiments in Fig. 2 were carried out as previously described (Wedel *et al.* 2007). Briefly, HEK293 cells were plated on 6-well plates on day 1. On day 2, cells were transfected with siRNA (100 nM) against STIM1 (Dharmacon, Lafayette, CO, USA) using Metafectene (Biontex Laboratories GmbH, Martinsried/Planegg, Germany; 7 μl per well), and including siGLO (Dharmacon) as a marker. After an 8 h incubation period, the medium bathing the cells was replaced by DMEM and maintained in culture. On day 3, siRNA-treated cells were split into additional 6-well plates. On day 4, control and STIM1 siRNA-treated cells were transfected with TRPC5 cDNA using Lipofectamine 2000 (as described above). On day 5, cells were seeded onto coverslips for imaging experiments carried out on day 6.

For the remaining RNAi experiments on stable TRPC6 and TRPC7 cells, as well as on A10 cells, the Amaxa electroporation system was used. Once again, kit V was used for the stable TRPC HEK293 cells (and their WT controls), and kit L was used for the A10 vascular smooth muscle cells. All knock-down experiments were carried out 72 h post-electroporation. Preliminary dosing experiments revealed higher concentrations of siRNA were required for consistent knockdowns compared to the metafectene experiments (data not shown), probably because of the large number of cells (around 1 million cells per reaction) suspended in a small volume of electroporation buffer (100 μl). Therefore, we used 1 μM siRNA for STIM1, Orai1 and TRPC6 when using the Amaxa system. At this concentration, all cells show significant reduction in the expected responses, compared to lower concentrations in which a few cells still respond normally. These levels of siRNA are similar to the recommended concentrations



by Amaxa. The sequences of the siRNAs used were: human Orai1, cccuucggccugaucuuuaucgucu (Mercer *et al.* 2006); human STIM1, agaaggagcuagaaucucac (Mercer *et al.* 2006); rat STIM1, gugauacaguggcugauua; and rat and human TRPC6, ugauaugggcugaauguaa. All knockdowns using Amaxa were compared to cells electroporated with siControl (1 μM) 72 h prior to the experiments being carried out.

Calcium measurements

Fluorescence measurements of intracellular Ca^{2+} were made in all experiments by loading the cells with either fura-2 AM or fura-5f AM (indicated in the figure legends), as previously described (Lievremont *et al.* 2004; Mercer *et al.* 2006). Fura-2 was used rather than fura-5f in experiments in which Ba^{2+} was used as a surrogate for Ca^{2+} (stable TRPC7 studies) or in the A10 vascular smooth muscle cell studies because of the different affinities of the indicators for the divalent cations and in order for comparison with previously published results, respectively. The fluorescent indicator dye was loaded in FBS-supplemented DMEM at 37°C in the dark for 25 min. For $[Ca^{2+}]_i$ measurements, cells were bathed in room temperature Hepes-buffered salt solution (HBSS) containing (in mM): 140 NaCl, 3 KCl, 2 $MgCl_2$, 2.0 $CaCl_2$, 10 glucose and 10 Hepes (pH to 7.4 with NaOH). Nominally Ca^{2+}-free solutions were HBSS with no added $CaCl_2$. Fluorescence images of the cells were recorded and analysed with a digital fluorescence imaging system (InCyt2, Intracellular Imaging Inc.). Cells were alternately excited at 340 nm and 380 nm, and the 530 nm emission was captured using a CCD camera. Typically, greater than 50 cells for HEK293 and greater than 25 cells for A10 were monitored per experiment. In all cases, ratio of fluorescence with excitation at 340 nm over that at 380 nm is reported, after correction for contributions by autofluorescence, measured after treating cells with 10 μM ionomycin and 20 mM $MnCl_2$.

Electrophysiology

Whole-cell currents were investigated at room temperature using the patch-clamp technique in the whole-cell configuration, as previously described (DeHaven *et al.* 2007). The standard Hepes-buffered saline solution was the same as that used in the Ca^{2+} imaging experiments, except the Ca^{2+} concentration varied depending on the experiment (see figure legends). In A10 vascular smooth muscle cells, 5 μM nimodipine was present throughout all experiments to block voltage-gated Ca^{2+} channels, and 10 mM $CsCl_2$ was also added to block inwardly rectifying K^+ channels. The standard divalent-free solution (DVF) was prepared by removing $CaCl_2$ and $MgCl_2$ from HBSS and adding 0.1 mM EGTA. Fire-polished pipettes fabricated from borosilicate glass capillaries (WPI, Sarasota, FL, USA) with 3–5 MΩ resistance were filled with (in mM): 145 caesium methanesulfonate, 20 BAPTA, 10 Hepes and 8 $MgCl_2$ (pH to 7.2 with CsOH) for Ca^{2+}- and Na^+-I_{CRAC} measurements, while experiments using clamped Ca^{2+} (TRPC currents) contained (in mM): 145 caesium methanesulfonate, 10 BAPTA, 10 Hepes, 1 $MgCl_2$ and 3.2 $CaCl_2$ (pH to 7.2 with CsOH) (free $Ca^{2+} \approx 100$ nM). Voltage ramps were recorded immediately after gaining access to the cell, and the currents were normalized to cell capacitance. In all Na^+-I_{CRAC} measurements, ramps were applied from positive to negative potentials (to avoid activation of voltage-gated Na^+ channels) every 2 s, while all TRPC recordings were made from negative to positive potentials. For I_{CRAC} measurements, leak currents were subtracted by taking an initial ramp current before I_{crac} developed and subtracting this from all subsequent ramp currents. Leak currents were not subtracted in the TRPC studies. Access resistance was typically between 5–10 MΩ. Cell-attached patch-clamp experiments were carried out using a bath and pipette solution containing (In mM): 140 KCl, 1 $MgCl_2$, 10 glucose, 2 $CaCl_2$ and 10 Hepes (pH 7.3 with KOH), as previously described by our laboratory (Vazquez *et al.* 2006; Lemonnier *et al.* 2008). For these cell-attached experiments, data were low-pass filtered at 2 kHz and digitized at 20 kHz. For all electrophysiological experiments, the currents were acquired with pCLAMP-10 (Axon Instruments) and analysed with Clampfit (Axon Instruments) and Origin 6 (Microcal) software. All solutions were applied by means of a gravity-based multi-barrel local perfusion system with an extremely low dead volume common delivery

Figure 1. STIM1 does not increase transiently expressed TRPC1, TRPC3, TRPC5 or TRPC6 activity
Ca^{2+} imaging experiments (fura-5f) were carried out on HEK293 cells expressing TRPC1 (*A*), TRPC3 (*C*), TRPC6 (*E*) and TRPC5 (*G*) with (grey traces) and without (black traces) the co-expression of STIM1. Carbachol (200 μM) was applied under nominally Ca^{2+}-free (NF) external conditions and 1.8 mM Ca^{2+} was re-administered after 10 min in order to assess TRPC activities. Gd^{3+} was present as indicated in order to block endogenous SOCE. Bar graphs show no significant increase (unpaired *t* test) in TRPC-evoked Ca^{2+} entries when STIM1 is co-expressed with TRPC1 (*B*), TRPC3 (*D*), TRPC6 (*F*) or TRPC5 (*H*). Except for TRPC5, bar graphs also summarize experiments carried out following transfection with two different TRPC cDNA plasmid concentrations (Low: 0.32 μg per well or High: 1.0 μg per well using a 6-well plate) on TRPC-mediated Ca^{2+} influx. The data represent means ± S.E.M. from three to four coverslips with at least 25 cells for each coverslip.

port (Perfusion pencil, Automate Scientific, Inc.) with flow rates set at around 0.25 ml min^{-1}.

Live cell confocal and TIRFM imaging

Live cell confocal and total internal reflection fluorescence (TIRF) microscopy were carried out as previously described (Smyth *et al.* 2005; DeHaven *et al.* 2008). Briefly, cells were maintained in HBSS at room temperature. Confocal imaging was carried out with a Zeiss LSM 510 laser scanning system with a 63× oil-immersion (NA 1.4) objective. All confocal images were collected with the pinhole set at 1 Airy Unit. For eYFP-STIM1, 514 nm excitation was provided by an argon laser and emission was selected with a 530–600 nm bandpass filter. For fluorescently labelled GM1, cells were excited by 543 nm wavelength and emission was selected with a 560 nm long-pass filter. Appropriate controls were established to verify separation between the signals carried out in the experiments shown in Fig. 10 (data not shown). GM1 labelling, with and without cholera toxin subunit B-specific antibody crosslinking, was carried out following the guidelines provided with the Vybrant AlexaFluor Lipid raft labelling kit (Invitrogen). TIRFM measurements of eYFP-STIM1 was carried out essentially as previously described (Smyth *et al.* 2005; DeHaven *et al.* 2008). For fluorescence intensity profiles, data are represented as the fluorescence intensity at each time point divided by the fluorescence intensity at the start of the experiment (F/F_0). Fluorescence intensities were collected from regions of interest encompassing the visible footprints of single cells and were background subtracted.

Western blotting

Western blot analysis was performed as previously described (Smyth *et al.* 2008) Briefly, normalized cell lysates were electrophoresed into 10% polyacrylamide gels. Proteins were then transferred to PVDF membranes, followed by blocking with 3% bovine serum albumin (BSA) in TBS-T (24.7 mM Tris base, 137 mM NaCl, 2.7 mM KCl, 0.1% Tween-20, pH 7.4). Next, the membranes were incubated in primary antibody directed against STIM1 (rabbit polyclonal; ProSci) in TBS-T with BSA overnight at 4°C. For TRPC1, the primary antibody was a rabbit monoclonal from AbCam applied at 1 : 10000 dilution, and incubated overnight at 4°C. The blocking agent was 5% milk. Following consecutive washes, the secondary antibody was applied (horseradish peroxidase-conjugated anti-rabbit IgG; Pierce) in TBS-T with BSA for 1 h at room temperature. After three 10 min washes in TBS-T, the membranes were developed with ECL *plus* reagent (GE Life Sciences). Lastly, the membranes were stripped with ReStore (Invitrogen) and re-probed with an actin primary antibody (AbCam). Band intensities were analysed using ImageJ software and normalized to actin.

FLIPR-based membrane potential dye assays

Experiments shown in Supplemental Fig. 6 were carried out on cells plated onto poly-lysine-coated 96-well plates, and relative membrane potential effects were measured using a membrane potential assay kit (Molecular Devices) and a fluorometric imaging plate reader ($FLIPR^{384}$; Molecular Devices), following a similar protocol suggested by the vendor.

Results

STIM1 does not increase activity of transiently expressed TRPC1, TRPC3, TRPC5 or TRPC6

It is well established that STIM1 interacts with Orai proteins, either directly or indirectly, to form store-operated CRAC channels (Frischauf *et al.* 2008). A key and general finding is the striking synergism between transiently expressed STIM1 and Orai producing extremely large currents and Ca^{2+} entry signals (Mercer *et al.* 2006; Peinelt *et al.* 2006). To assess the potential for STIM1 to similarly regulate TRPC channels, we co-transfected HEK293 cells with STIM1 and various TRPC channel subunits. Of the six TRPCs functioning in human cells, we examined effects of STIM1 on TRPC1, 3, 5 and 6. TRPC4 was not investigated as in our hands, in HEK293 cells it appears to act only constitutively (McKay *et al.* 2000), while others have reported that it acts similarly to TRPC5 (Schaefer *et al.* 2000). TRPC7 is examined in depth in another context as a subsequent part of this study.

We utilized a standard method for examining TRPC function independently of endogenous store-operated channel activity. We activated the transfected HEK293 cells through PLC-linked muscarinic receptors with carbachol in the presence of Gd^{3+}, which blocks the endogenous store-operated channels (Broad *et al.* 1999*b*). We monitored agonist-activated Ca^{2+} entry with a protocol of agonist addition in the absence of Ca^{2+} followed by re-addition of extracellular Ca^{2+}. In cells that had not been transfected with TRPCs, we confirmed that no Gd^{3+}-insensitive carbachol-activated Ca^{2+} entry was observed (not shown). Figure 1 shows the results from TRPC1 (panels *A* and *B*), TRPC3 (panels *C* and *D*), TRPC6 (panels *E* and *F*) and TRPC5 (panels *G* and *H*) expressing HEK293 cells with (black traces) and without (grey traces) the co-expression of STIM1. In TRPC3-, TRPC5- and TRPC6-expressing cells, but not in TRPC1 cells, the re-addition of Ca^{2+} revealed significant Gd^{3+}-insensitive, and therefore TRPC-mediated, Ca^{2+} entry. However, the expression of STIM1 did not increase TRPC-mediated

Ca^{2+} influx under these conditions. Calcium entry under these conditions is well below saturation as shown by the substantial increase in signal observed in cells transfected with STIM1 and Orai1 (Mercer *et al.* 2006).

We considered the possibility that expression level of the TRPC channels may affect the outcome of these results, as previously reported by this laboratory in experiments with DT40 B-lymphocytes (Vazquez *et al.* 2003), and recently reported for TRPCs in HEK293 cells (Yuan *et al.* 2007). Therefore, we carried out the experiments under both high and low plasmid concentrations for TRPC1 (Fig. 1, panel *B*), TRPC3 (panel *D*) and TRPC6 (panel *F*), concentrations similar to those previously described (Yuan *et al.* 2007). With the lower plasmid concentrations, for TRPC3 and 6 there was diminished Ca^{2+} entry, and a greater number of cells showing no entry at all, confirming that protein expression was decreased to a limiting level. However, there still was no difference between the Gd^{3+}-insensitive entry in cells co-expressing STIM1 *versus* controls (Fig. 1). TRPC5 (panel *H*) studies were carried out using only the higher plasmid concentration, because TRPC5 was previously reported to interact with STIM1 independently of expression level (Yuan *et al.* 2007). We also carried out the above experiments in the absence of extracellular Gd^{3+}, and the results are summarized in Supplemental Fig. 1. Note that TRPC1 did not produce any entry in the presence of Gd^{3+}, in agreement with Hofmann *et al.* (2002) who concluded that TRPC1 does not traffic to the plasma membrane correctly in the absence of TRPC4 or TRPC5. Co-expression of STIM1 did not remedy the failure of TRPC1 to give an agonist-activated Gd^{3+}-insensitive Ca^{2+} entry, nor did low levels of TRPC1, in the presence or absence of STIM1, influence thapsigargin-activated Ca^{2+} entry in the absence of Gd^{3+} (data not shown). We confirmed by Western analysis that our plasmid and transfection protocol successfully increased TRPC1 protein expression several-fold (not shown). For TRPC5 we also examined the effects of STIM1 and TRPC5 transfections on carbachol-activated TRPC5 currents and on CRAC currents. There was no increase in TRPC5 current associated with STIM1 transfection, nor was there any increase in I_{crac} in cells trasfected with STIM1 plus TRPC5 compared to STIM1 alone (Supplemental Fig. 2).

It has previously been suggested that STIM1 is obligatory for the activation of TRPC5 by agonist (Yuan *et al.* 2007). Thus, we carried out similar experiments and examined the effects of STIM1 knockdown on TRPC5 activation by agonist. Our laboratory has previously published similar approaches using siRNA directed against STIM1 (Mercer *et al.* 2006; DeHaven *et al.* 2007; Wedel *et al.* 2007). Figure 2*A* and *B* shows that endogenous SOCE in cells treated with STIM1 siRNA was significantly reduced by 80% compared to control cells when thapsigargin was used to deplete stores, confirming that STIM1 knockdown was successful.

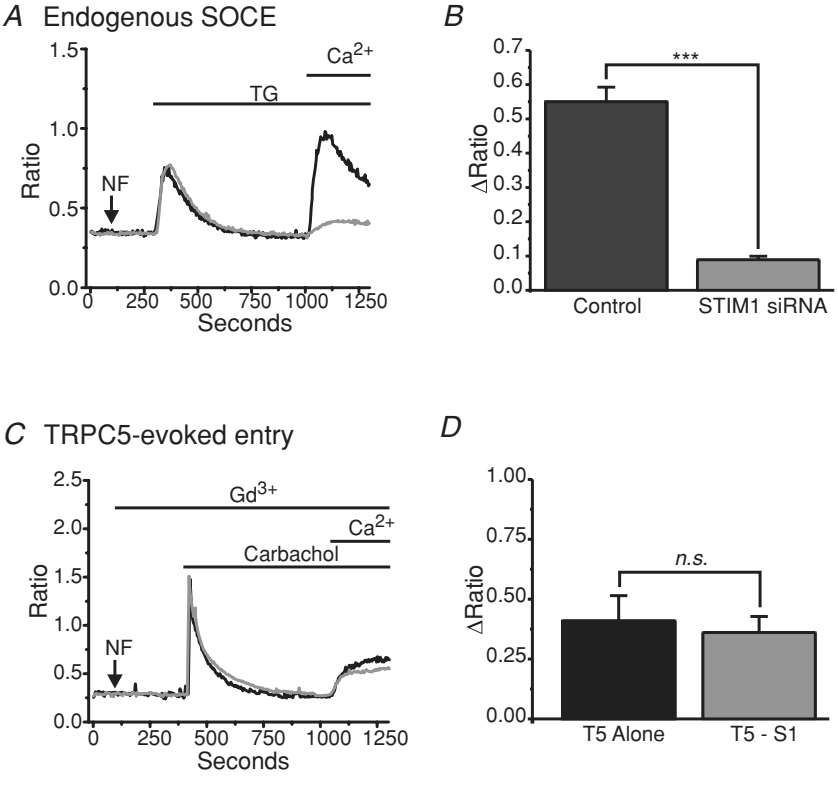


Figure 2. Knocking down STIM1 does not reduce agonist-activated TRPC5 activity
A, representative Ca^{2+} imaging experiment (fura-5f) showing the effects of RNAi against STIM1 on endogenous SOCE evoked by store depletion with the SERCA pump inhibitor, thapsigargin (TG; 2 μM). *B,* bar graph showing STIM1 siRNA treatment significantly reduces (unpaired *t* test; $P = 0.00047$) endogenous SOCE in HEK293 cells. *C,* experiments carried out as in *A*; however, TRPC5 was transiently expressed in these cells (see Methods) and 200 μM carbachol was used instead of thapsigargin in order to activate TRPC5. Note also the presence of 5 μM Gd^{3+} in these experiments to inhibit endogenous SOCE. *D,* bar graph showing no effect (unpaired *t* test) of STIM1 knockdown on TRPC5-evoked Ca^{2+} entry after stimulation with carbachol. Three to four coverslips with at least 25 cells for each coverslip were carried out for each condition.

However, in the presence of Gd^{3+}, carbachol-evoked activation of TRPC5-mediated Ca^{2+} entry was unaffected by knockdown of STIM1 (Fig. 2*C* and *D*).

TRPC7, previously reported to function in a store-dependent manner, is not regulated by STIM1 or Orai1

To this point, we have examined a potential role for STIM1 in agonist-induced Ca^{2+} entry through TRPC channels with the view that this protocol would reveal effects on either store-operated or non-store-operated entry. In the past, we have consistently failed to see activation of these channels by store depletion alone when transiently expressed in HEK293 cells (Trebak *et al.* 2003*a*; Lievremont *et al.* 2004; and Supplemental Fig. 1). However, our laboratory previously described increased TRPC7-mediated Ba^{2+} influx after store depletion with thapsigargin in HEK293 cells stably (but not transiently) expressing TRPC7 (Lievremont *et al.* 2004). Ba^{2+} was used as a surrogate for Ca^{2+} to avoid complications that might arise from alterations in Ca^{2+} transport and Ca^{2+} regulation of the channels themselves. The use of Ba^{2+} avoids these problems since it is a poor substrate for Ca^{2+}-sensitive regulatory sites and transport mechanisms (Vanderkooi & Martonosi, 1971; Uvelius *et al.* 1974). We hypothesized that this might be an example of STIM1/Orai1 interaction with a TRPC channel, conferring store dependency to the TRPC7 channels (for example, Liao *et al.* 2007). To test this, RNAi experiments were carried out to determine whether the proteins Orai1 and STIM1 play a role in this apparent TRPC7 store-dependent entry. Figure 3*A* shows single cell fura-2 imaging results in HEK293 cells stably expressing TRPC7. ER Ca^{2+} stores were depleted using thapsigargin under nominally Ca^{2+}-free conditions, and Ba^{2+} was added after the cytoplasmic Ca^{2+} levels returned to baseline (15 min). In all experiments, 5 μM Gd^{3+} was present to block endogenous store-operated channels. In these cells, thapsigargin treatment caused a significant increase in the rate of Ba^{2+} flux compared to the leak controls (Fig. 3*A* and *B*), as previously reported (Lievremont *et al.* 2004). However, the knockdown of neither STIM1 nor Orai1 reduced TRPC7-mediated Ba^{2+} entry (Fig. 3*B*).

The RNAi findings were supported by experiments carried out using a constitutively active construct of STIM1 in which aspartic acids 76 and 78 are mutated to asparagines (D76N/D78N). STIM1 D76N/D78N presumably no longer binds Ca^{2+} in its EF-hand domain, and therefore oligomerizes with other STIM1 proteins independently of store depletion, and constitutively activates store-dependent plasma membrane Orai channels (Liou *et al.* 2005; Mercer *et al.* 2006). We hypothesized that if STIM1 interacts functionally with TRPC7, then the constitutively active mutant of STIM1 should increase TRPC7 activity. However, while the expression of STIM1 D76N/D78N did significantly increase basal Ca^{2+} levels in these cells, presumably due to constitutive activation of endogenous Orai1 channels, it did not increase the rate of Gd^{3+}-insensitive Ba^{2+} entry which occurs through TRPC7 (Fig. 3*C* and *D*).

We also examined the effects of over-expressing STIM1, STIM2 and Orai1 on the thapsigargin-evoked Gd^{3+}-insensitive Ba^{3+} entry seen in these TRPC7 expressing cells. We and others have previously reported that the expression of STIM2 or Orai1 alone causes a suppression of endogenous SOCE (Mercer *et al.* 2006; Soboloff *et al.* 2006). While the cause of this suppression is not completely understood, we hypothesized that the TRPC7-dependent entry might also be suppressed by the expression of STIM2 and Orai1, or augmented by STIM1 if they functionally interact. However, the expression of neither STIM1, STIM2 nor Orai1 had any effect on the TRPC7-mediated Ba^{2+} entry seen after thapsigargin treatment, compared to eYFP controls (Fig. 3*E* and *F*). Thus, the increase in TRPC7 activity after thapsigargin treatment in these cells is completely independent of STIM1, STIM2 and Orai1. Unlike Orail, transient expression of TRPC7 did not suppress SOCE in HEK293 cells (Supp. Fig. 4). It is important to note that all of the experiments carried out in Fig. 3 were carried out in parallel with wild-type HEK293 cells, verifying that STIM1 and Orai1 were indeed knocked down as previously described (panels *A* and *B*), that STIM1 D76N/D78N caused constitutive activity that was sensitive to Gd^{3+} (panels *C* and *D*), and that STIM2 and Orai1 caused a suppression of endogenous SOCE (panels *E* and *F*) (Supplemental Fig. 3).

We next examined the effects of knocking down STIM1 and Orai1 on the second messenger-activated currents in these TRPC7 stable cells. In order to by-pass signalling and the production of other second messengers that might play a role in regulation of these channels, the synthetic diacylglycerol, OAG, was used as an agonist. Figure 4*A* shows whole-cell voltage-clamp experiments in TRPC7 cells treated with either siGLO (control; black trace), STIM1 (dark grey trace), or Orai1 (light grey trace) siRNA, 72 h post-transfection. One minute after break-in with an intracellular pipette solution containing 100 nM clamped Ca^{2+}, 50 μM OAG was focally applied to the cells to activate the TRPC7 channels. Neither the knockdown of STIM1 nor Orai1 altered the peak TRPC7 currents after the application of OAG. The kinetics of activation also were unaffected by STIM1 and Orai1 RNAi. Figure 4*B* shows representative current–voltage relationships for the conditions shown in panel *A*. Similar results were seen in fura-2 imaging assays, in which knockdown of STIM1 or Orai1 did not affect the TRPC7-mediated Ba^{2+} entries in response to OAG application (Fig. 4*C* and *D*). Similar to the RNAi experiments in Fig. 4*A* and *B*, the transient expression of STIM and Orai1 proteins also

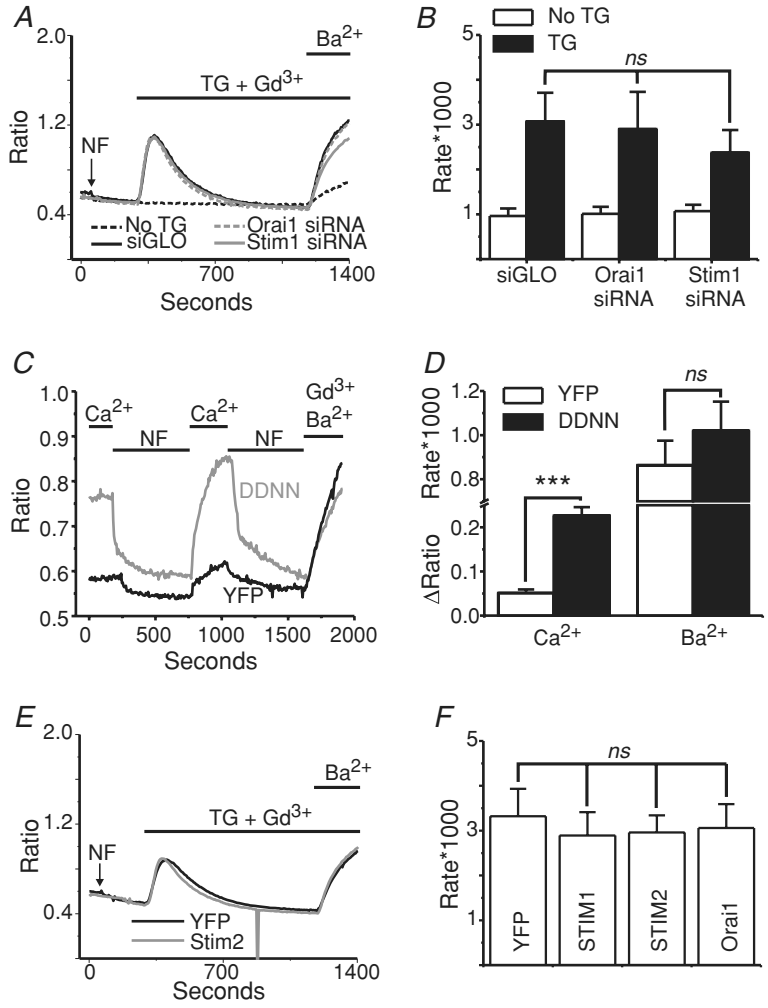


Figure 3. TRPC7 previously reported to function in a store-dependent manner is not regulated by STIM or Orai

A, Gd^{3+} (5 μM) was present throughout the TRPC7 experiments to block endogenous store-operated Ca^{2+} entry. Shown are mean traces (fura-2 AM) from stable TRPC7 HEK293 cells confirming increased TRPC7-dependent (Gd^{3+}-insensitive) Ba^{2+} entry in response to 2 μM thapsigargin (leak control: dashed black trace *vs.* siGLO control: continuous black trace), and showing no significant affect of knocking down STIM1 (STIM1 siRNA: continuous grey trace) or Orai1 (Orai1 siRNA: dashed grey trace). Ba^{2+} was used as a surrogate for Ca^{2+} to avoid Ca^{2+} buffering problems, as previously described for TRPC7 cells (Lievremont *et al.* 2004). *B,* bar graph depicting no significant difference (ANOVA) between the rates of Ba^{2+} entry (under the presence of Gd^{3+}) in TRPC7 cells transfected with Orai1 ($n = 5$ coverslips) or STIM1 ($n = 7$ coverslips) siRNA when compared to control cells ($n = 7$ coverslips) (siGLO). *C,* imaging experiments demonstrating that stable TRPC7 cells transfected with the constitutively active mutant of STIM1 (D76N/D78N; grey trace) show a significant increase in basal Ca^{2+} levels when compared to eYFP control cells (black trace), but do not show any increase in the basal activity of TRPC7 as seen by the influx of Ba^{2+} in the presence of 5 μM Gd^{3+}. Nominally Ca^{2+}-free (NF) and 2 mM external Ca^{2+} conditions, as well as the addition of Ba^{2+} and Gd^{3+} are indicated by the lines above the graph. *D,* bar graph depicting a significant increase in basal Ca^{2+} concentrations (unpaired *t* test, $P = 0.00013$) in TRPC7 cells transiently expressing STIM1 D76N/D78N; however, there was no difference between the rates of Ba^{2+} entry (under the presence of Gd^{3+}; unpaired *t* test, $P = 0.42387$) in stable TRPC7 cells expressing either eYFP alone ($n = 4$ coverslips) or in conjunction with STIM1 D76N/D78N ($n = 6$ coverslips). *E,* ratiometric measurements showing stable TRPC7 cells transfected with STIM2 (grey trace) have no change in the rate of Ba^{2+} entry compared to eYFP control cells (black trace). *F,* bar graph depicting no significant difference (ANOVA) between the rates of Ba^{2+} entry in TRPC7 cells expressing either eYFP ($n = 5$ coverslips), or in conjunction with STIM1 ($n = 5$ coverslips), STIM2 ($n = 5$ coverslips) or Orai1 ($n = 5$ coverslips). Leak entry rates between conditions were also not significantly different (not shown). Each coverslip (each *n*) was taken as the mean of at least 25 individual cells.

did not influence TRPC7 channel activity when activated by OAG (Fig. 4*E* and *F*).

We were puzzled by the finding that an apparent store-operated activity of TRPC7 was so clearly independent of the Ca^{2+} store sensor, STIM1. In the Ca^{2+} imaging studies, Ca^{2+} store depletion was always accomplished by treatment with the Ca^{2+} pump inhibitor, thapsigargin. However, store-operated channels should become active no matter how stores are depleted. Therefore, we decided to deplete the stores using IP_3 in the patch pipette in a similar approach described by Hoth and Penner in the original description of I_{CRAC} (Hoth & Penner, 1992). If in these cells TRPC7 is indeed store operated, then IP_3-mediated store depletion should also activate the TRPC7 channels in a store-dependent manner. As the experiments were carried out in parallel with wild-type HEK293 cells, and because endogenous I_{CRAC} is difficult at best to record in these cells, STIM1 was expressed in order to amplify I_{CRAC} through endogenous Orai1 channels in the control and TRPC7 conditions. We previously reported a 2- to 3-fold increase in Na^+-I_{CRAC} in HEK293 cells expressing STIM1 compared to their wild-type counterparts (DeHaven *et al.* 2007). Figure 5*A* and *B* shows representative recordings (−100 mV and +100 mV taken every 2 s) taken from WT HEK293 and stable TRPC7 cells, respectively, both transiently expressing eYFP-STIM1, in which IP_3 was dialysed into the cell to deplete ER Ca^{2+} stores. Importantly, these results indicate that no Gd^{3+}-insensitive non-selective TRPC7 currents were ever activated by ER Ca^{2+} store depletion with BAPTA and IP_3 in the patch pipette in the TRPC7 cells (Fig. 5*B*). Further, the only currents that were detected had the same properties as the endogenous store-operated CRAC currents seen in the wild-type cells expressing STIM1 (panel *A*). Switching to a divalent cation-free solution (DVF) only amplified an inwardly rectifying current that was strongly suppressed by the lanthanide, Gd^{3+} (panels *C* and *D*).

TRPC channels may be regulated by cytoplasmic Ca^{2+} in a concentration-dependent manner, in which too little or too much cytoplasmic Ca^{2+} suppresses channel activity (Shi *et al.* 2004). Experiments shown in Fig. 5 were carried out with millimolar concentrations of BAPTA in the patch pipette; therefore, it is feasible

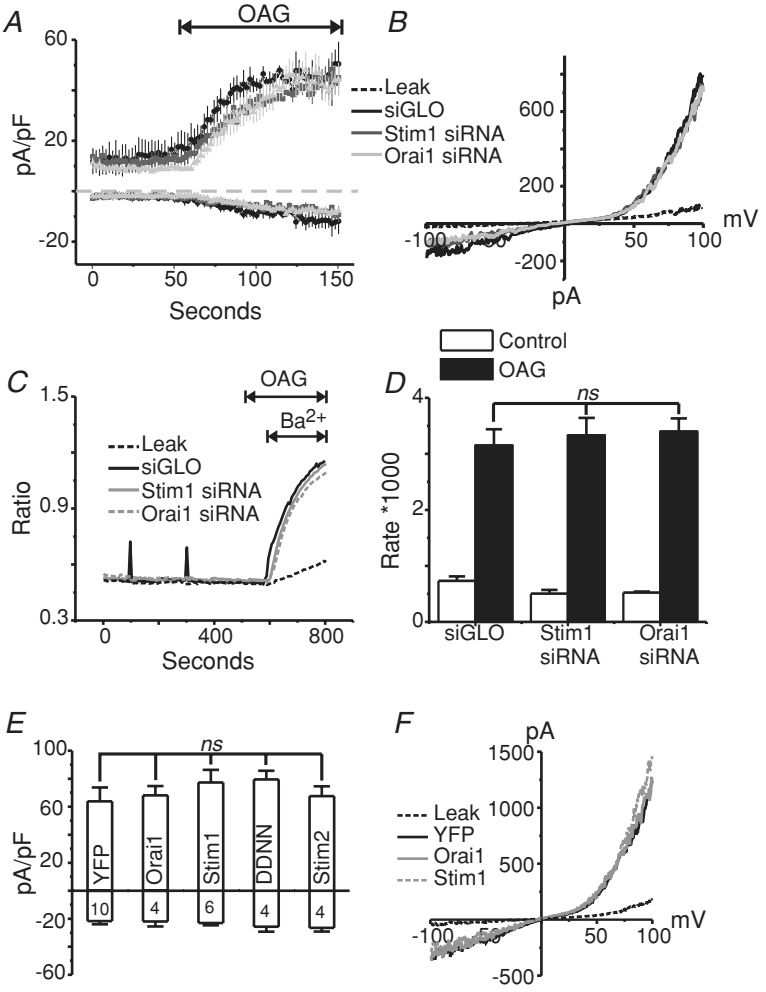


Figure 4. TRPC7 currents activated by OAG are not altered by the knockdown of STIM1 or Orai1, or by the over-expression of STIM1, STIM1 D76N/D78N, STIM2 or Orai1

A, whole-cell OAG-activated currents (means ± S.E.M.) taken at −100 and +100 mV from stable TRPC7 cells transfected with siGLO ($n = 3$), STIM1 siRNA ($n = 4$) or Orai1 siRNA ($n = 3$) 72 h prior to the experiments. The external HBSS contained 2 mM Ca^{2+}, and OAG (50 μM) was externally applied as indicated by the line above the graph. *B,* current–voltage (*I–V*) relationships taken from stable TRPC7 cells before (leak; broken black trace) and after (siGLO, STIM1 siRNA, Orai1 siRNA) focal OAG application. In all conditions, the outwardly rectifying *I–V* relationships were nearly identical. *C,* mean Ba^{2+} entries (fura-2 AM) in response to OAG (50 μM) application in stable TRPC7 expressing HEK293 cells 72 h after transfection with siGLO (Control), STIM1 or Orai1 siRNA. *D,* summary of data in *C*, showing no significant difference (ANOVA) of OAG-mediated Ba^{2+} influx in stable TRPC7 expressing cells expressing siGLO ($n = 7$ coverslips), STIM1 siRNA ($n = 8$ coverslips) or Orai1 siRNA ($n = 4$ coverslips). *E,* summary of OAG-mediated whole-cell currents (−100 mV and +100 mV) in stable TRPC7 cells transfected with eYFP (control) ($n = 10$), Orai1 ($n = 4$), STIM1 ($n = 6$), STIM1 D76N/D78N ($n = 4$) or STIM2 ($n = 4$). Nominally Ca^{2+}-free HBSS was used as the external bathing solution in these studies. *F*, *I–V* relationship showing the currents recorded under the same conditions as *E*.

that the cytoplasmic Ca^{2+} concentrations were below that which is required for TRPC7 activation. Evidence for this can be seen in Fig. 5*B* and *C*, in which the basal TRPC7 activity seen just after break-in is reduced over time, presumably due to BAPTA diffusing into the cell and chelating cytoplasmic Ca^{2+}. Therefore, under these conditions, we could not definitively conclude whether we did not see store-operated TRPC7 currents because TRPC7 is actually not store operated, or because our internal conditions were too stringent to allow for activation of the channels in a store depletion-dependent manner. To address this uncertainty, we carried out experiments on stably expressing TRPC7 cells in which the internal Ca^{2+} concentration was clamped to physiological concentrations (~100 nM). Further, we decided to deplete the stores with other store-depleting agents (thapsigargin, cyclopiazonic acid (CPA) and ionomycin) in order to avoid any complications which might occur with the IP_3 receptors which are also sensitive to cytoplasmic Ca^{2+} concentrations. Figure 6*A* shows the mean ± S.E.M. of independent voltage-clamp experiments in which thapsigargin (grey trace) was focally applied to stably expressing TRPC7 cells under nominally Ca^{2+}-free (NF) external conditions, followed by the application of 50 μM OAG. As a control, experiments were carried out with DMSO, the vehicle for thapsigargin (black trace). Consistent with the results from the Ba^{2+} entry imaging experiments, in all cells tested, the focal application caused a small, but significant increase in a whole-cell current, which had a current–voltage relationship as expected for TRPC7 (Fig. 6*B*). This increase in current was not seen in the DMSO-treated cells. When thapsigargin was replaced with CPA, a competitive SERCA pump inhibitor (Fig. 6C, grey trace), small increases in current densities were also seen similar to those when thapsigargin was applied; however, depletion of Ca^{2+} stores with the ionophore ionomycin (black trace) was ineffective at activating TRPC7 (Fig. 6*C* and *D*), even though it did activate I_{CRAC}

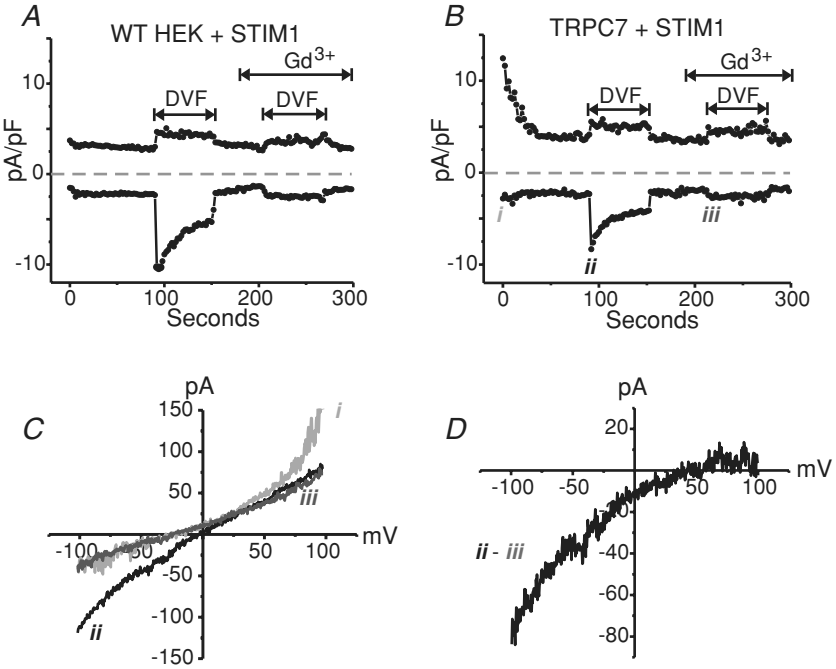


Figure 5. Whole-cell patch clamp experiments using high BAPTA and IP_3 to deplete internal Ca^{2+} stores activates only I_{CRAC} in TRPC7 cells

A, representative whole-cell CRAC current in a HEK293 cell expressing STIM1. A divalent-free (DVF) solution exchange protocol was used, as previously described (DeHaven *et al.* 2007), in order to amplify the extremely small endogenous CRAC current in these cells. STIM1 was expressed to further amplify the small CRAC currents in HEK293 cells. Current was recorded at −100 and +100 mV and stores were depleted with 20 μM IP_3 and 20 mM BAPTA in the pipette. Lines above trace indicate where different extracellular solutions were focally applied. Gd^{3+} (10 μM) was used to block the Na^+ I_{CRAC}, revealing a linear increase in leak currents under these external conditions in which all Ca^{2+} and Mg^{2+} is removed and 10 μM Gd^{3+} is the only cation present. *B,* same as *A*, except in a HEK293 cell stably expressing TRPC7. Note that there is constitutive TRPC7 activity at break in, but this activity diminishes over time. In stable TRPC7-expressing HEK293 cells, normally functioning inwardly rectifying Na^+-CRAC currents are detectable with similar current densities to control cells (*A*). *C,* current–voltage (*I–V*) relationships taken at specific time points indicated as *i*, *ii* and *iii* for current trace shown in *B*. *D*, *I–V* for the Gd^{3+}-sensitive current shown in *B* and *C*.

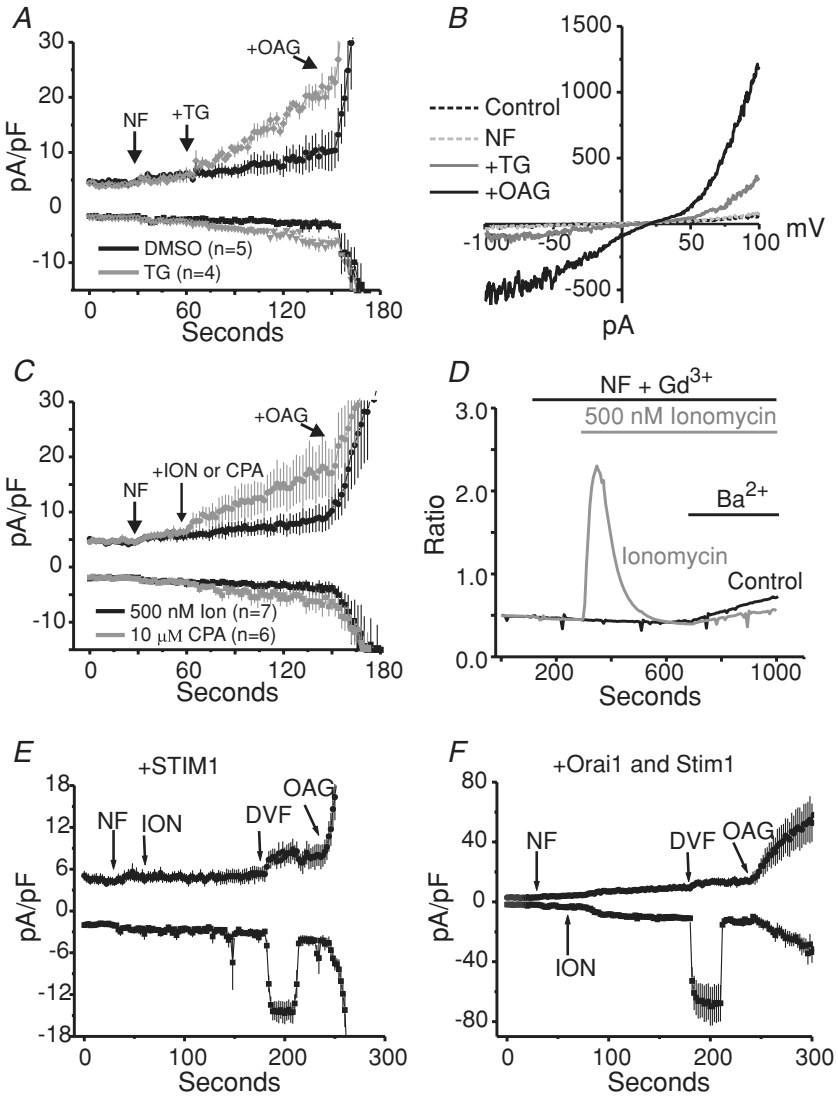


Figure 6. Thapsigargin-evoked increases in TRPC7 current
A, whole-cell currents in HEK293 cells stably expressing TRPC7. Focally applied thapsigargin ($n = 4$, grey trace) increases outwardly rectifying currents in these cells, while DMSO does not ($n = 5$, black trace). Internal Ca^{2+} was clamped to around 100 nM free Ca^{2+}, and external solutions were added as indicated by the arrows. Voltage ramps were applied from −100 mV to +100 mV (250 ms), every 2 s to record the currents which developed over time. *B,* representative current–voltage relationships showing the peak currents recorded after break-in (in the presence of 2 mM Ca^{2+}, Control, dashed black trace), after switching to nominally Ca^{2+}-free external solution (NF, grey dashed trace), after 1.5 min of focally applied thapsigargin (+TG, grey continuous trace), and after the application of 50 μM OAG (+OAG, black trace). *C,* experiments similar to *A*; however, using the ionophore ionomycin ($n = 7$, black trace) or the SERCA pump antagonist CPA ($n = 6$, grey trace), instead of thapsigargin. *D,* fura-2 AM imaging experiment showing the effects of ionomycin application (grey trace) compared to leak control (black trace). Ionomycin did not increase the rate of Ba^{2+} entry compared to the control. Shown are the means of single coverslips which are representative of three similar experiments for each condition. *E,* experiments ($n = 7$) similar to those in*C* (100 nM clamped Ca^{2+}), in which ionomycin was used to deplete internal stores. However, in these cells, eYFP-STIM1 was transiently expressed and divalent cation-free (DVF) external solution was focally applied in order to demonstrate that ionomycin did indeed deplete the stores critical for I_{CRAC} development. The inward currents seen after the application of DVF solution represent I_{CRAC}. Note there is no depotentiation of these currents because the starting solution was devoid of Ca^{2+} (NF). *F,* same as in *E*; however, in conjunction with STIM1, Orai1 was also transiently expressed to further amplify the Na^+-I_{CRAC} currents recorded ($n = 4$). All whole-cell currents shown in this figure are represented as means ± S.E.M.

in these TRPC7 cells (Fig. 6*E* and *F*). We conclude that the activation of TRPC7 by thapsigargin results in some manner from inhibition of SERCA *per se*, rather than from depletion of Ca^{2+} stores. Therefore, TRPC7 would not be considered a store-operated channel, a conclusion consistent with the lack of involvement of STIM1 or Orai1.

Knockdown of STIM1 inhibits SOCE but not endogenous TRPC6 currents in smooth muscle cells

All of the findings to this point suggested no interaction between STIM1 and exogenously expressed TRPC channels. However, it is possible that regulation might be lost or less obvious with overexpressed channels. Thus, we next investigated the role of STIM1 on the activation of arginine–vasopressin (AVP)-evoked non-selective cation currents (NSCC) in A10 vascular smooth muscle cells. In a related cell line (A7r5), a NSCC has been molecularly identified using RNAi as TRPC6 (Soboloff *et al.* 2005; Takahashi *et al.* 2008). We therefore first determined whether the AVP-activated NSCC in A10 cells is similarly mediated by TRPC6. Figure 7*A* shows a representative whole-cell patch-clamp experiment in which the focal application of 500 nM AVP activated non-selective cation channels in A10 vascular smooth muscle cells. Similar results were seen in the A7r5 cells (data not shown). Figure 7*B* shows that the mean current densities of the AVP-activated currents recorded at −100 and +100 mV were larger when the extracellular Ca^{2+} was reduced from 2 mM (black bar) to around 50 μM Ca^{2+} (grey bar). This increase in current density when extracellular Ca^{2+} is reduced is presumably from electrostatic interaction of Ca^{2+} in the pore of the channels reducing monovalent permeation under the presence of millimolar external Ca^{2+}. These effects mirror what is commonly seen in many TRPC channels (Kamouchi *et al.* 1999; Okada *et al.* 1999; McKay *et al.* 2000; Lemonnier *et al.* 2006). Cell-attached experiments revealed in some patches the presence of single channel events as shown in Fig. 7*C*, which were dependent on the presence of extracellular AVP. At +60 mV, the single channel current amplitude was 2.1 pA (Fig. 7*D*), and the slope conductance for the recording shown in panel *C* was calculated as 33.65 pS, similar to that published for TRPC6 (panel *E*) (*cf* Trebak *et al.* 2007). Further, RT-PCR analysis of TRPC transcripts in A10 cells revealed the predominant expression of TRPC1 and TRPC6 mRNA, with very low copies of

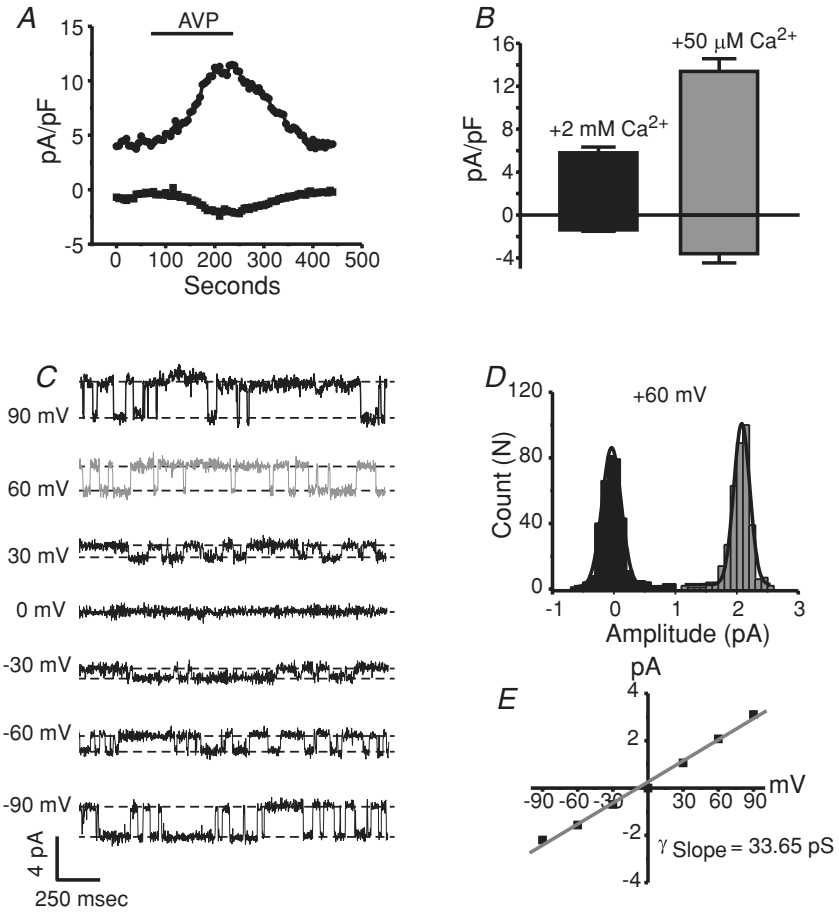


Figure 7. AVP-activated non-selective cation currents in A10 vascular smooth muscle cells
A, a representative whole-cell current (−100 mV and +100 mV) recorded from an A10 cell before, during and after the focal application of 500 nM arginine–vasopressin (AVP). External solution contained 2 mM Ca^{2+} and the patch pipette contained 100 nM clamped Ca^{2+}. AVP was applied as indicated by the line above the graph. Nimodipine (5 μM) was present throughout. *B,* bar graph depicting the effects of extracellular Ca^{2+} (HBSS + 2 mM Ca^{2+} or HBSS + 50 μM Ca^{2+}) on the non-selective cation currents recorded in A10 cells after the application of AVP. *C,* single channel events recorded in the cell-attached mode from A10 cells bathed in 500 nM AVP application at the indicated holding potentials. The single channel events were not detected in experiments in which AVP was not applied (not shown). *D,* all points histogram showing the amplitude (pA) of the single channel events recorded after AVP treatment at a holding potential of +60 mV. *E,* current–voltage relationship of the single channel events and calculated slope conductance of the AVP-activated current.

TRPC4 and TRPC7 detected, and virtually no TRPC3 and TRPC5 (data not shown). Finally, RNAi directed towards TRPC6 significantly reduced the AVP-activated current densities in these cells compared to control cells (Fig. 8*E* and *F*). RNAi knockdown of TRPC6 was verified by quantitative RT-PCR, which showed greater than 70% reduction of TRPC6 message (Supplemental Fig. 5*F*). Further, because the siRNA sequence used was compatible

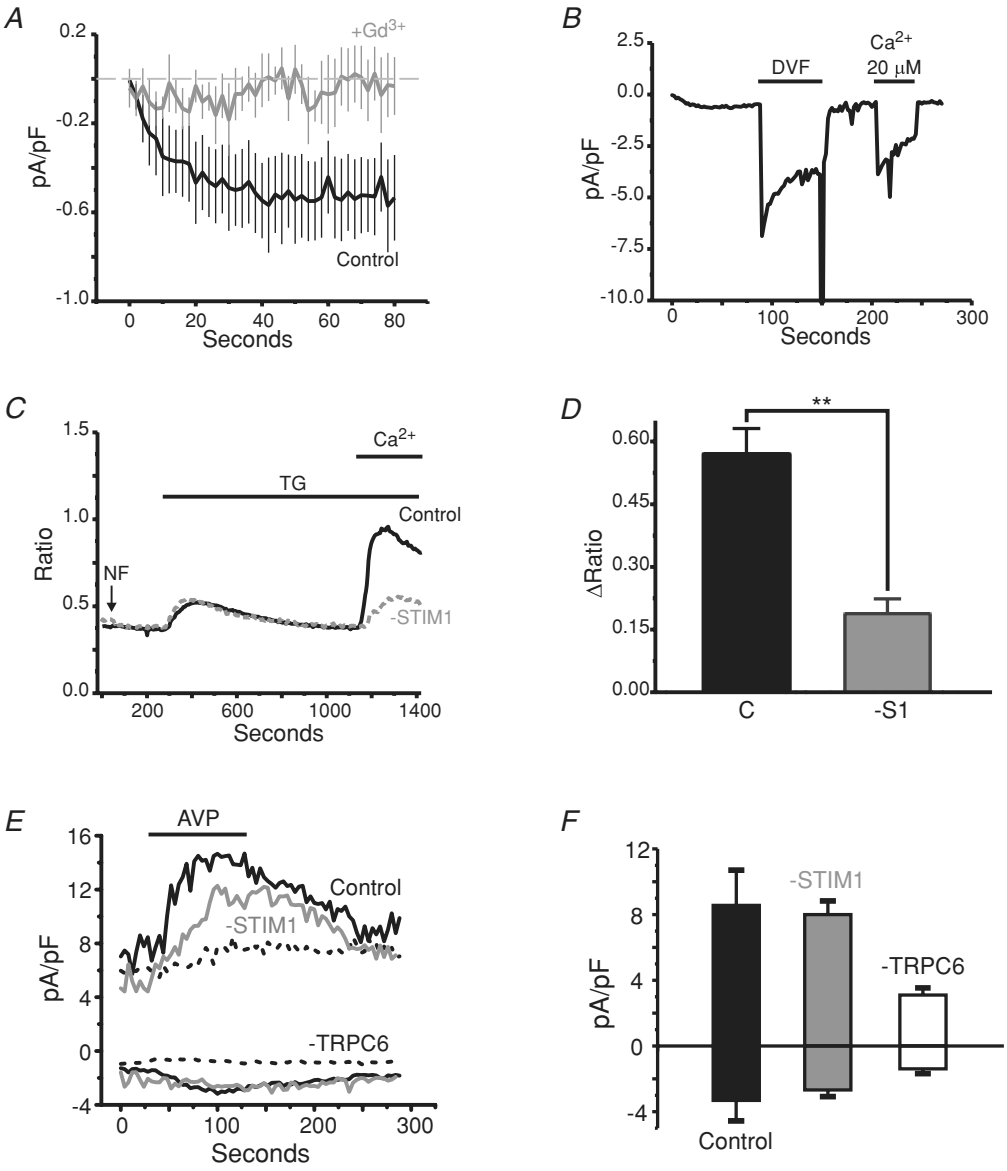


Figure 8. Knockdown of STIM1 does not alter endogenous TRPC6 currents, while significantly suppressing SOCE in smooth muscle cells

A, whole-cell I_{CRAC} measurements taken at −120 mV from A10 smooth muscle cells with ($n = 6$; grey trace) or without ($n = 7$; black trace) the presence of 3 μM Gd^{3+}. Stores were actively depleted with IP_3 and BAPTA in the patch pipette, and 5 μM nimodipine was present in the external HBSS containing 10 mM Ca^{2+}. *B*, I_{CRAC} experiment showing in smooth muscle cells the Na^+ currents are half-maximally blocked by 20 μM external Ca^{2+} at −120 mV. Ramps were applied from positive to negative potentials (see Methods) to avoid voltage-gated channel contamination of the CRAC currents. *C*, Ca^{2+} imaging experiment (fura-2 AM) showing the effects of RNAi targeted against STIM1 on thapsigargin-evoked SOCE in A10 cells. External solutions were exchanged as indicated by the lines above the graph. *D,* bar graph showing STIM1 siRNA-treated cells (-S1, $n = 4$ coverslips, grey bar) have significantly less (unpaired *t* test; $P = 0.00152$) SOCE compared to siControl-treated control (C, $n = 5$ coverslips, black bar) cells. *E,* representative whole-cell currents obtained as in Fig. 7, in which AVP-activated currents were reduced in cells treated with siRNA against TRPC6 ($n = 9$; broken black trace), but not in cells treated with STIM1 siRNA ($n = 5$; continuous grey trace), compared to siControl-treated cells ($n = 7$; continuous black trace). *F,* bar graph showing the summary of the data collected in A10 cells for the AVP-activated non-selective cation currents in which STIM1 siRNA had little effect, but TRPC6 siRNA reduced the overall current densities.

with both rat and human message, cells stably expressing human TRPC6 were tested for functional knockdown of TRPC6. Supplemental Fig. 5*C–E* shows the effects of TRPC6 siRNA treatment on these TRPC6 stable cells, in which the whole-cell currents were reduced by 80% compared to siControl cells. Taken together, these results suggest the presence of AVP-activated TRPC6 currents in A10 vascular smooth muscle cells.

We next verified the presence of store-operated Ca^{2+} entry and I_{CRAC} in these smooth muscle cells, and determined that STIM1 was a critical mediator of this pathway. Figure 8*A* shows voltage-clamp experiments in which BAPTA and IP_3 were included in the patch pipette to deplete internal Ca^{2+} stores and 10 mM Ca^{2+} was in the bathing solution. Just after breaking in, a small but detectable I_{CRAC} developed in these smooth muscle cells. Similar currents have been measured from A7R5 cells (Brueggemann *et al.* 2006). Importantly, this Ca^{2+} current is not detected in cells treated with low micromolar concentrations of the lanthanide, Gd^{3+}. The identity of these currents was further assessed by examining Na^+ currents under divalent-free conditions and the half-maximal blocking concentration of external Ca^{2+}. Figure 8*B* shows an experiment in which 20 μM external Ca^{2+} was required to half-maximally inhibit Na^+ permeation through these store-dependent channels, a concentration similar to that seen for both endogenous I_{CRAC} in non-excitable cells and over-expressed Orai (Prakriya & Lewis, 2003; Prakriya *et al.* 2006; DeHaven *et al.* 2007). The current–voltage relationships for the Ca^{2+} and Na^+ CRAC currents in these cells were taken from voltage ramps run from positive to negative direction in order to avoid contamination with depolarization activated currents (see Methods) and were strongly inwardly rectifying, indicative of I_{CRAC}.

Next, we evaluated the dependence of SOCE on STIM1 by using RNAi directed against STIM1. Figure 8*C* shows representative Ca^{2+} imaging experiments (means of single coverslips) using thapsigargin to deplete internal Ca^{2+} stores and activate SOCE. Seventy-two hours post-Amaxa electroporation with siRNAs, the STIM1 siRNA-treated cells (grey dashed trace) showed a much smaller SOCE phase compared to siControl-treated cells (black continuous trace). The bar graph in Fig. 8*D* shows that knocking down STIM1 reduces SOCE in these A10 smooth muscle cells to around 30% of the control SOCE. The knockdown of STIM1 was verified by Western blot analysis (Supplemental Fig. 5*A–B*) in which the majority of the protein was knocked down in three independent experiments. Further, similar, but less effective results were obtained in these cells when an shRNA directed against a different site on STIM1 was used (data not shown). We take these data to strongly suggest that I_{CRAC} underlies SOCE in A10 smooth muscle cells, and that STIM1 is required for activation of SOCE in these cells.

In order to determine whether STIM1 regulates endogenous TRPC6 in these A10 smooth muscle cells, whole-cell patch-clamp experiments were carried out similar to those shown in Fig. 7. AVP was focally applied to cells under voltage clamp, and voltage ramps (−100 to +100 mV) were applied every few seconds to measure the AVP-activated currents that developed over time. Figure 8*E* shows representative experiments for a siControl cell (black trace) and a STIM1 siRNA-treated cell (grey trace), in which the focal application of AVP activated TRPC6 currents which were unaffected by the loss of STIM1 protein. Figure 8*F* shows the summary of the data collected, in which the current densities activated by AVP were not altered by the knockdown of STIM1. Thus, we believe endogenous TRPC6 is not regulated by STIM1.

TRPC and STIM1/Orai1 signalling occur in distinct plasma membrane domains

A functional interaction between STIM1 and TRPC channels would suggest that STIM1 and TRPC localize to similar domains within the cell. Several studies have reported that TRPC channels are localized to lipid raft domains of the plasma membrane, and that this localization is necessary for proper channel function. However, whether STIM1 similarly localizes to lipid rafts has not been determined. We therefore tested whether TRPC and/or STIM1 and CRAC signalling is associated with lipid rafts by performing experiments using the cholesterol sequestering agent methyl β cyclodextrin (MβCD) (Christian *et al.* 1997; Graziani *et al.* 2006) in HEK293 cells stably expressing TRPC3. TRPC3 cells were pre-treated with 1, 5 or 10 mM MβCD for 1 h, and then the drug was washed out three times with normal HBSS. Whole-cell patch-clamp experiments were then carried out to determine the effects of lipid raft disruption on TRPC3 channel activity. OAG was used to activate the TRPC3 channels in order to bypass any effects MβCD might have on signalling upstream of channel activity. Figure 9*A–C* shows that disruption of lipid rafts by MβCD completely suppresses TRPC3 activity, compared to control cells. One millimolar MβCD significantly reduced the OAG-activated TRPC3 current, and 10 mM MβCD completely abolished it. Further, 10 mM MβCD also completely suppressed constitutive TRPC3 activity, as revealed by the reduction in the initial current density just after break-in seen in panel *A*.

In stark contrast to the effects of MβCD on TRPC3 activity, I_{CRAC} measurements in HEK293 cells expressing eYFP-STIM1 were completely insensitive to lipid raft disruption by MβCD pre-treatment. Figure 9*D* shows representative I_{CRAC} measurements with (grey trace) and without (black trace) the pre-treatment of 5 mM MβCD.

Experiments were carried out using IP_3 to actively deplete the stores, and divalent-free external solutions were focally applied in order to amplify the small I_{CRAC} currents in these cells, as previously described (DeHaven *et al.* 2007) (similar to Fig. 5). Figure 9*E* and *F* show the current–voltage relationships for the recordings shown in panel *D*, and the means ± S.E.M. for all cells tested, respectively.

MβCD effects on TRPC3 and CRAC channel activity and SOCE were also investigated using fura-5f imaging. In these assays, TRPC3 activity was also reduced after MβCD pre-treatment. In contrast to the results with current measurement, Ca^{2+} imaging experiments on WT HEK293 cells with thapsigargin treatment and re-addition of Ca^{2+} revealed SOCE was partially reduced in the MβCD pre-treated cells (data not shown). However, in experiments utilizing membrane potential-sensitive dyes, MβCD caused apparent depolarization when added acutely, and pre-treatment with MβCD almost completely inhibited the depolarizing effects of high KCl. This may indicate that MβCD treatment causes substantial plasma membrane depolarization (α-cyclodextrin did not depolarize these cells, Supplemental Fig. 6). Since measurement of I_{crac} under voltage clamp did not detect any effects of MβCD, we conclude that the inhibitory effect on SOCE in imaging experiments most probably reflect a decrease in driving force. Therefore, caution should be used when working with MβCD and Ca^{2+} indicator dyes.

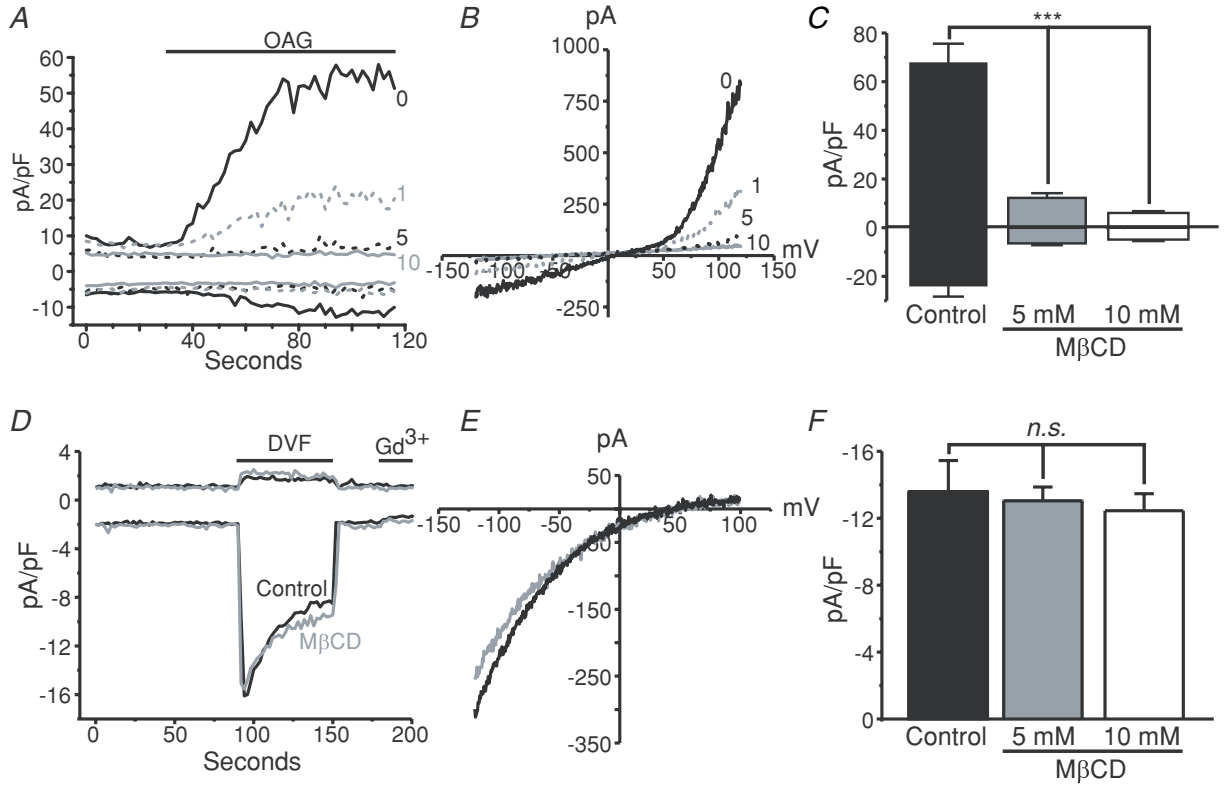


Figure 9. TRPC and STIM1/Orai1 signalling occur in distinct plasma membrane domains
A, whole-cell voltage clamp experiments (−120 to +120 mV voltage ramp applied every 2 s) taken from HEK293 cells stably expressing TRPC3 and pre-treated with vehicle (0, black continuous trace), 1 mM (1, grey dashed trace), 5 mM (5, black dashed trace) or 10 mM (10, grey continuous trace) MβCD for 30 min. Whole-cell TRPC3 currents were activated by focal application of 30 μM OAG as indicated by the line above the graph. *B,* current–voltage relationships taken from the recordings shown in *A* after OAG application. Currents were not leak subtracted. *C,* bar graph showing significant inhibition of TRPC3 currents in cells treated with millimolar concentrations of MβCD (Control: $n = 7$; 5 mM MβCD: $n = 8$; 10 mM MβCD: $n = 6$). *D*, Na^+-I_{CRAC} measurements taken from HEK293 cells expressing eYFP-STIM1 (+100 to −120 mV every 2 s) with (grey trace) or without (black trace) 10 mM MβCD treatment prior to break-in. Stores were depleted using 25 μM IP_3 in the patch pipette and all external divalent cations were removed in order to amplify CRAC currents. External solution exchanges were focally applied as indicated by the lines above the graph. *E,* leak subtracted *I–V* plots taken from the peak Na^+ CRAC currents shown in *D*. *F,* bar graph showing in eYFP-STIM1-expressing cells that sequestration of cholesterol from the lipid bilayer by pre-treating the cells with MβCD has no effect on I_{CRAC} (Control: $n = 7$; 5 mM MβCD: $n = 10$; 10 mM MβCD: $n = 5$). Plotted are peak Na^+ CRAC currents. Error bars represent means ± S.E.M. and data analyses were carried out using ANOVA followed by Tukey's test for pairwise comparisons.

The functional experiments showing that I_{CRAC} is not dependent upon lipid rafts was supported by confocal experiments looking at the localization of eYFP-STIM1 and the raft constituent ganglioside, GM1, which is used extensively for imaging lipid rafts (Galbiati *et al.* 2001). Figure 10 shows images of live HEK293 cells expressing eYFP-STIM1 (panel *A*) and labelled with a fluorophore conjugated to the cholera toxin β-subunit, which binds to GM1 (panel *B*), before (*I,* Control) and after (*ii.* +TG) 15 min treatment with the SERCA pump inhibitor, thapsigargin. Images were taken at the cell–coverslip interface in order to see near-plasma membrane events. After store depletion with thapsigargin, eYFP-STIM1 rearranged into punctate structures (Fig. 10*A*). However, store depletion had little or no effect on the arrangement of the GM1 in the plasmalemma (Fig. 10*B*). More importantly, there was essentially no co-localization between STIM1 (green) and GM1 (red) (lipid rafts) after store depletion (Fig. 10*C*). Accordingly, there was no effect of MβCD pre-treatment on STIM1 movements to near-plasma membrane areas, as assessed by TIRF microscopy. Similar to the experiments carried out on TRPC3-expressing cells, eYFP-STIM1-expressing HEK293 cells were pre-treated with MβCD for 1 h, followed by three washes in HBSS. Then, TIRF microscopy experiments were carried out before, during and after store depletion with thapsigargin. Figure 11*A* and *B* shows by TIRF microscopy that lipid raft disruption by 10 mM MβCD had no effect on the ability of STIM1 to re-arrange into punctate-like structures after store depletion. In control cells (panel *A*), the application of thapsigargin caused significant rearrangement of STIM1, causing the molecule to become punctate and approach the plasma membrane, as previously described (Liou *et al.* 2005). Notably, identical results were seen in the STIM1-expressing cells pre-treated with 10 mM MβCD. Thus, our data suggest TRPC3 channel function depends on the lipid make-up of the plasma membrane; however, STIM1 signalling and activation of Orai channels apparently does not require or involve lipid raft micro-domains.

Discussion

Since the first description of store-operated Ca^{2+} entry (SOCE) over 20 years ago, investigators have focused on determining the molecular make-up of the signal within the ER and the ion channels in the plasma membrane which form SOCE (Putney, 1997; Parekh & Putney, 2005). Within those 20 years, many hypotheses have developed for the signal; however, the recent discovery of STIM1 has clarified this issue. We now know that STIM1 functions as an ER Ca^{2+} sensor that can signal to the plasma membrane when the stores have been depleted of their Ca^{2+} and

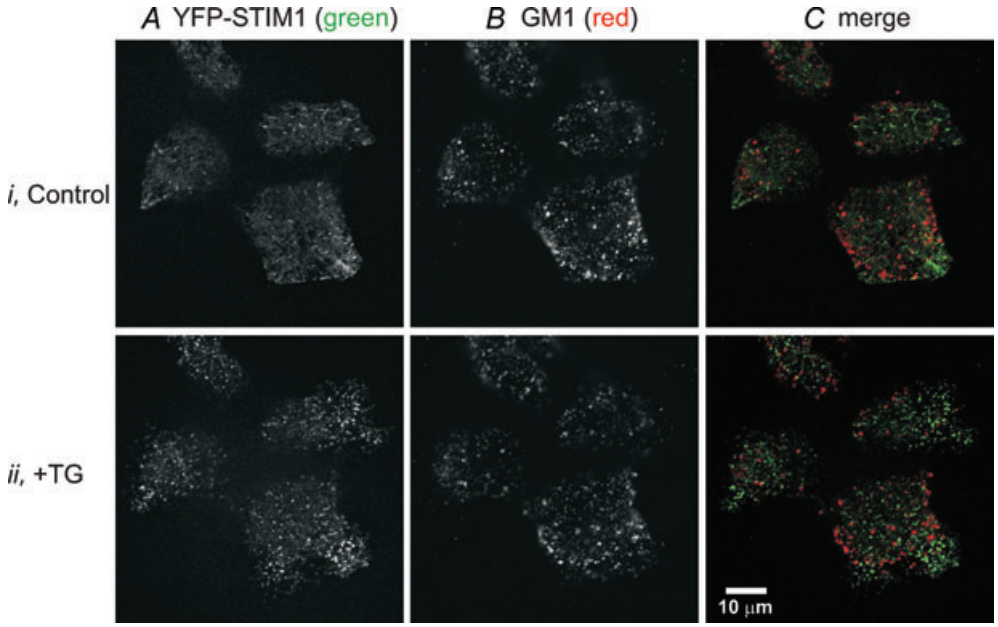


Figure 10. eYFP-STIM1 does not co-localize with the lipid raft marker, GM1
Representative confocal images of live HEK293 cells expressing eYFP-STIM1 (*A*) and labelled for the lipid raft marker, GM1 (*B*) before (Control) and after (+TG) 15 min treatment with the SERCA pump inhibitor, thapsigargin. *C,* merge of the eYFP-STIM1 (green) and GM1 (red) images before and after TG treatment showing the lack of co-localization between STIM1 and GM1. Results are shown from an experiment utilizing a cross-linking antibody directed against cholera toxin; similar results were obtained in the absence of the cross-linking antibody (not shown).

activate, either directly or indirectly, SOC channels (Luik & Lewis, 2007; Csutora *et al.* 2008). The inception of whole genome RNAi screens led to the discovery of the SOC channels, Orai1, (also known as CRACM1), which is now believed to be the pore-forming subunits of the SOCE channels known as the Ca^{2+} release-activated Ca^{2+} (CRAC) channels (Prakriya *et al.* 2006; Vig *et al.* 2006*a*). Two homologues of Orai1, Orai2 and 3, have been shown to be similarly activated by store depletion in transfection experiments but their function in native SOC channels is yet to be established. While CRAC channels are the conventional highly Ca^{2+} selective SOC channels, evidence exists for SOC channels which are much less Ca^{2+} selective (Parekh & Putney, 2005). The canonical transient receptor potential class of channels (TRPC), which are non-selective cation channels, are the leading candidates for alternative SOC channels functioning in a store dependent manner under certain conditions (Albert *et al.* 2007). Like CRAC channels, TRPC channels are activated downstream of PLC activity, and it is has been suggested that SOC currents, which are less Ca^{2+} selective, may involve channels composed of TRPC subunits.

However, to date STIM1 (and to some extent the closely related STIM2) is the only known ER Ca^{2+}-sensing molecule. Thus, it could function as a regulatory protein capable of universally regulating specific ion channels, including the archetypical Orai, but also possibly TRPC channels, in a store-dependent manner. It has been proposed that store-operated channels should be redefined as any channels regulated by STIM1 (Yuan *et al.* 2007). It is also possible that STIM1 could be a structural protein required for channel activity (in this case, TRPC), but which may not necessarily confer store dependence to the channels. Indeed, there is evidence for STIM1 regulation of one non-store-operated channel, apparently gated by arachidonic acid (Mignen *et al.* 2007). While the hypothesis that STIM1 functionally regulates TRPC channels has been investigated by many groups (Huang *et al.* 2006; Liao *et al.* 2007; Ong *et al.* 2007; Alicia *et al.* 2008; Cheng *et al.* 2008; Jardin *et al.* 2008*a*,*b*; Li *et al.* 2008; Liao *et al.* 2008; Ma *et al.* 2008), the experiments were in many cases carried out with agonists which activate both the second-messenger and store-operated pathways. Thus, there are two key questions: one, are TRPC channels activated by depletion of Ca^{2+} stores; and two, is STIM1 involved in the activation mechanism of TRPC channels, whether by store depletion or by products of phospholipase C. The interaction of STIM1 with Orai channel subunits is readily demonstrated by co-expression of STIM1 with Orai, resulting in profound synergistic increases in agonist- or thapsigargin-activated Ca^{2+} entry (Mercer *et al.* 2006; Peinelt *et al.* 2006; Zhang *et al.* 2006). Thus, in the current study, we examined the activation of Ca^{2+} entry through the PLC-linked muscarinic receptor in HEK293 cells co-transfected with selected TRPCs and STIM1. We carried out experiments in the presence and absence of the I_{crac} inhibitor, Gd^{3+}. Thus any positive interaction of STIM1 with either store-operated or PLC-dependent TRPC channels should have been revealed in these experiments. However, no significant

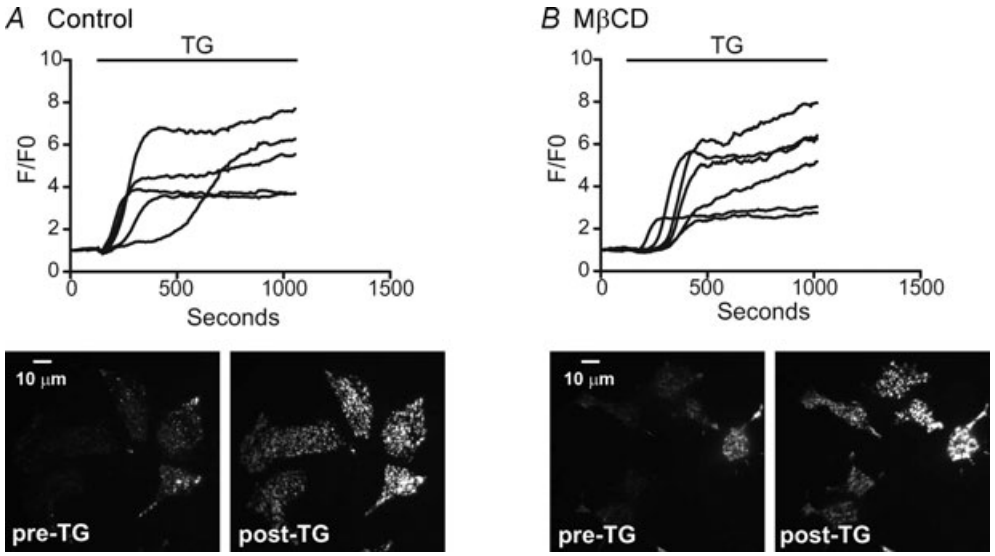


Figure 11. Disruption of lipid rafts by MβCD does not prevent eYFP-STIM1 from rearranging into puncta in response to store depletion by thapsigargin
A, time-controlled experiments showing the changes in near-plasma membrane fluorescence intensities measured by TIRF microscopy in HEK293 cells expressing eYFP-STIM1 and treated with 2 μM thapsigargin to deplete internal Ca^{2+} stores. Each trace represents an individual cell taken from a single coverslip. *B,* same as in *A*, however, in cells pre-treated with 10 mM MβCD. Lines above graphs indicate where thapsigargin was applied.

augmentation by STIM1 of agonist-induced entry was observed with any of the TRPCs, regardless of the presence or absence of Gd^{3+}, and regardless of expression level. Thus, in our hands, STIM1 appears incapable of interacting with these TRPC channels in any functional way.

In our past experiences, we have generally found that store depletion protocols do not activate TRPC channels (McKay *et al.* 2000; Trebak *et al.* 2003*a*; Lievremont *et al.* 2004, 2005; Lemonnier *et al.* 2006). However, we published two instances in which store depletion apparently activated TRPCs. In one instance, TRPC3 could be activated by thapsigargin but only when expressed at low levels in DT40 B-cells (Vazquez *et al.* 2003). In a second case, TRPC7 could be activated by thapsigargin, but only in HEK293 cells stably transfected with TRPC7 (Lievremont *et al.* 2004). In both cases, no currents were reported, and the only means of activating the cells was by application of the SERCA inhibitor, thapsigargin. In the current study, we examined in detail the activation of TRPC7 in stably transfected HEK293 cells by thapsigargin. Surprisingly, we found that only thapsigargin, and no other means of store depletion, was capable of activating TRPC7 in these cells. In addition, the activation of TRPC7 current began almost immediately upon addition of thapsigargin or another SERCA inhibitor, CPA. This is not expected for a store-operated channel which generally requires much longer times before passive depletion protocols begin to activate (Broad *et al.* 1999*a*). We conclude that TRPC7 is activated as a result of SERCA inhibition *per se* rather than by depletion of Ca^{2+} stores. Interestingly, in a previous study, we showed that in HEK293 cells transiently transfected with TRPC7, SERCA inhibitors inhibited channel activation by diacylglycerols (Lemonnier *et al.* 2006). The mechanisms underlying these interesting but complex interactions between SERCA pumps and TRPC7 channels merits further investigation. Nonetheless, since it is clear that TRPC7 is not being activated by Ca^{2+} store depletion, it is perhaps not surprising, and consistent with the findings with transiently transfected TRPCs, that TRPC7 activity is not affected by STIM1 expression, regardless of the means of its activation.

The results on stably expressing TRPC7 HEK293 cells also underscore the importance of utilizing multiple approaches to depleting internal Ca^{2+} stores in determining whether an apparent store-operated entry pathway is truly store operated. In this instance, we misinterpreted our previous work using thapsigargin-activated entry as sufficient evidence for SOCE, when clearly that is not the case.

All of the findings discussed to this point involved studies of TRPC channels transfected into cells. It is for this reason that we also investigated STIM1 function in a vascular smooth muscle cell line, A10, which appears to express a native TRPC6 channel. A similar and widely investigated vascular smooth muscle cell line, A7r5, has previously been shown to express a non-selective cation current which is activated by arginine–vasopressin (AVP) signalling through a G-protein receptor, and has many of the biophysical properties of TRPC6 (*cf* Trebak *et al.* 2007). Recently, RNAi studies have molecularly identified that TRPC6 is indeed essential in making up these channels (Soboloff *et al.* 2005; Takahashi *et al.* 2008). We detected a similar current in A7r5 cells (data not shown), as well as a related smooth muscle cell line (A10 cells). Both cells are very similar morphologically, and respond identically to AVP (Van *et al.* 1988; Korbmacher *et al.* 1989). TRPC6 siRNA attenuated the whole-cell currents in these A10 vascular smooth muscle cells, as well as TRPC6-expressing HEK293 control cells. Importantly, we also detected small but reproducible I_{CRAC}, and we found that thapsigargin-evoked SOCE in these cells was dependent on the proteins STIM1 (Fig. 8) and Orai1 (data not shown). Thus, these cells proved useful in examining possible regulation by STIM1 of an endogenous TRPC. RNAi directed against STIM1 had little to no inhibitory effect on the AVP-activated non-selective currents, while it significantly reduced SOCE. Therefore, studies on endogenous proteins recapitulate what was observed for expressed proteins: no detectable functional interaction between STIM1 and TRPC channels after the activation of PLC.

Along with the receptors that activate them, TRPC channels have long been suggested to be associated with and regulated by lipids in the plasma membrane (PM), especially with respect to the role of cholesterol-rich lipid rafts (Bergdahl *et al.* 2003; Brownlow *et al.* 2004; Groschner *et al.* 2004; Graziani *et al.* 2006; Alicia *et al.* 2008; Jardin *et al.* 2008*b*; Pani *et al.* 2008). Less has been published on SOCE and membrane lipids, and nothing has been published suggesting STIM1/Orai1 signalling requires lipid raft domains in order to function properly. However, recent reports have described clustering of STIM1 and TRPC1 complexes (Alicia *et al.* 2008; Pani *et al.* 2008) or STIM1–Orai1–TRPC1 complexes (Jardin *et al.* 2008*b*). In order to examine the possible involvement of cholesterol-rich lipid rafts, experiments were carried out using the agent MβCD, which disrupts lipid rafts by sequestering cholesterol from the plasma membrane (Christian *et al.* 1997). Using stable TRPC3 HEK293 cells, we showed that MβCD pre-treatment completely blocked OAG-activated TRPC3 activity, as assessed by whole-cell patch-clamp experiments. However, MβCD pre-treatment had no effect on I_{CRAC} measured from HEK293 cells expressing STIM1. Accordingly, there was no effect of MβCD pre-treatment on STIM1 movements in response to store depletion. However, our co-localization experiments using GM1 as a marker for lipid rafts suggested that STIM1 puncta do not associate with lipid raft domains in the plasma membrane.

Therefore, we feel it is unlikely that STIM1 could be specifically regulating an ion channel associated with lipid rafts.

All of the findings in this multi-faceted study lead one to the same conclusion: store-activated channels, composed specifically of Orai subunits, require STIM1 as an endoplasmic reticulum Ca^{2+} sensor, while TRPC channels, regardless of their mechanism of activation or expression level, apparently do not depend upon or interact with STIM1 in any detectable functional manner. We are aware that there are numerous studies that have come to a different conclusion, generally based on somewhat alternative strategies and often different cell backgrounds (Zhu *et al.* 1996; Kiselyov *et al.* 1998; Liu *et al.* 2000; Rosado & Sage, 2000; Beech *et al.* 2003; Liao *et al.* 2007; and others; reviewed in Parekh & Putney, 2005). Some studies have utilized more complex combinations of STIM1, Orai1 and TRPCs (Cheng *et al.* 2008; Jardin *et al.* 2008*a*; Liao *et al.* 2008) which are not addressed in this study. It is, of course, not possible to attempt to reproduce every experimental protocol that has utilized either overexpressed TRPCs or TRPC knockdown to evaluate their function. Rather, in the current study, we have focused on what we believe to be a simple approach to studying TRPC function, and specifically its potential interaction with STIM1. Following the logic of Popper's black swan problem (Taleb, 2007) we have tested the idea that TRPC channels do not functionally interact with STIM1 by attempting falsification, and in every instance we have failed. We feel that if such an interaction were to occur, it would readily have been detected in our experiments. We cannot speculate on the many variables that may affect the experimental findings from other laboratories. We have illustrated one example of a misleading result that led us in the past to wrongly attribute store-dependent activation to a TRPC channel. It has also been recently shown that TRPC1 can appear to contribute to a store-operated current when, in fact, it is being activated by IP_3, independently of intracellular stores (Zarayskiy *et al.* 2007).

TRPC channels support a number of important physiological functions, including smooth muscle activity (Dietrich *et al.* 2005), exocrine gland secretion (Liu *et al.* 2007), neuronal migration (Greka *et al.* 2003; Hui *et al.* 2006) to name a few. At the cellular level, they have been shown to be regulated by a myriad of signals, including IP_3 receptors (Kiselyov *et al.* 1998; Rosado & Sage, 2000; Vazquez *et al.* 2006), diacylglycerols (Hofmann *et al.* 1999), inositol lipids (Kwon *et al.* 2007; Lemonnier *et al.* 2008), src (Vazquez *et al.* 2004; Kawasaki *et al.* 2006), myosin light chain kinase (Shimizu *et al.* 2006), and by Ca^{2+} in complex ways (Trebak *et al.* 2003*b*; Lemonnier *et al.* 2006). Continued research is needed to establish the physiological pathways that regulate and activate this important ion channel family so that useful pharmacological interventions can be developed to aid in the treatment of associated diseases.

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