

RAPID REPORT

Differential dependence of store-operated and excitation-coupled Ca²⁺ entry in skeletal muscle on STIM1 and Orai1

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In non-excitabile cells, agonist-induced depletion of intracellular Ca²⁺ stores triggers Ca²⁺ influx via a process termed store-operated Ca²⁺ entry (SOCE). In T-lymphocytes, stromal interaction molecule 1 (STIM1) acts as the intra-store Ca²⁺ sensor and Orai1 functions as the Ca²⁺-permeable SOCE channel activated by STIM1 following store depletion. Two functionally distinct Ca²⁺ entry pathways exist in skeletal muscle; one activated by store depletion (SOCE) and a second by sustained/repetitive depolarization that does not require store depletion (excitation-coupled Ca²⁺ entry, ECCE). However, the role of STIM1 and Orai1 in coordinating SOCE and ECCE activity in skeletal muscle and whether these two Ca²⁺ entry pathways represent distinct molecular entities or two different activation mechanisms of the same channel complex is unknown. Here we address these issues using siRNA-mediated STIM1 knockdown, dominant-negative Orai1, and permeation-defective Orai1 to determine the role of STIM1 and Orai1 in store-operated and excitation-coupled Ca²⁺ entry in skeletal myotubes. SOCE and ECCE activity were quantified from both intracellular Ca²⁺ measurements and Mn²⁺ quench assays. We found that STIM1 siRNA reduced STIM1 protein by more than 90% and abolished SOCE activity, while expression of siRNA-resistant hSTIM1 fully restored SOCE. SOCE was also abolished by dominant-negative Orai1 (E106Q) and markedly reduced by expression of a permeation-defective Orai1 (E190Q). In contrast, ECCE was unaffected by STIM1 knockdown, E106Q expression or E190Q expression. These results are the first to demonstrate that SOCE in skeletal muscle requires both STIM1 and Orai1 and that SOCE and ECCE represent two distinct molecular entities.

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Ca²⁺ influx through store-operated Ca²⁺ entry (SOCE) channels following intracellular store depletion provides a critical mechanism to replenish internal Ca²⁺ stores in non-excitabile cells. In T lymphocytes, SOCE channels (termed Ca²⁺ release activated Ca²⁺ or CRAC channels) mediate Ca²⁺-dependent gene transcription by triggering calcineurin-dependent nuclear factor of activated T-cells (NFAT) dephosphorylation and nuclear translocation (Feske, 2007). However, the functional importance of SOCE in most cell types remains an open question. A major breakthrough in the field was the recent discovery of two molecular players in the SOCE pathway – STIM1 proteins located primarily in the

membrane of the intracellular compartment (Liou *et al.* 2005; Roos *et al.* 2005) and Orai proteins located in the plasma membrane (Feske *et al.* 2006; Vig *et al.* 2006b). There are two mammalian STIM1 homologues (STIM1 and STIM2) and three mammalian Orai homologues (Orai1–3). STIM1 serves as the Ca²⁺ sensor for store depletion through Ca²⁺ binding to an N-terminal EF-hand motif situated in the store lumen (Liou *et al.* 2005), while Orai1 channels function as highly Ca²⁺-selective CRAC channels in the plasma membrane of T lymphocytes (Feske *et al.* 2006; Prakriya *et al.* 2006; Vig *et al.* 2006b). CRAC channel activation results from store depletion causing STIM1 aggregation and reorganization into discrete regions of the endoplasmic reticulum that subsequently interact and activate Orai1 channels in the adjacent plasma membrane (Feske, 2007). However,

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little information is available with regard to the roles of STIM1 and Orai1 in mediating SOCE in other cell types, particularly excitable cells, and SOCE in some cells involves STIM1 activation of C-type transient receptor potential (TRPC) channels or heteromultimers of Orai and TRPC proteins (Worley *et al.* 2007; Cheng *et al.* 2008; Liao *et al.* 2008).

SOCE is not restricted to non-excitabile cells, but is also observed in excitable cells including neurones, cardiac myocytes and smooth muscle cells (Parekh & Putney, 2005). SOCE has also been documented in skeletal muscle fibres (Kurebayashi & Ogawa, 2001; Pan *et al.* 2002; Launikonis *et al.* 2003; Launikonis & Rios, 2007), although the electrophysiological correlate of SOCE in adult muscle remains elusive (Allard *et al.* 2006). While STIM1 and Orai1 coordinate SOCE in T lymphocytes, the importance of these proteins in mediating store-dependent Ca^{2+} influx in excitable cells remains obscure. For example, both TRPC family members (Albert *et al.* 2007) and Orai proteins (Peel *et al.* 2008) have been suggested to underlie SOCE in smooth muscle cells. Similarly, early work suggested that SOCE in skeletal muscle involves either IP_3 (Launikonis *et al.* 2003) or ryanodine (Kiselyov *et al.* 2000; Pan *et al.* 2002) receptors as the sarcoplasmic reticulum (SR) Ca^{2+} sensor and sarcolemmal TRPC proteins as the SOCE channel (Kiselyov *et al.* 2000; Rosenberg *et al.* 2004; Sampieri *et al.* 2005). More recently, STIM1 and Orai1 were shown to be expressed at high levels in skeletal muscle and skeletal myopathy results from a deficiency in either protein (Vig *et al.* 2008; Stiber *et al.* 2008). Importantly, SOCE is absent in skeletal myotubes that lack functional STIM1 proteins and STIM1 haplo-insufficiency leads to defects in force generation and fatigue resistance (Stiber *et al.* 2008). Finally, an alternate store-independent Ca^{2+} entry mechanism triggered by sustained or repetitive depolarization (excitation-coupled Ca^{2+} entry or ECCE) is found in skeletal myotubes (Cherednichenko *et al.* 2004; Yang *et al.* 2007) and adult muscle fibres (Cherednichenko *et al.* 2008). While the precise mechanism for ECCE activation in muscle remains elusive, the skeletal muscle isoforms of the ryanodine (RyR1) and dihydropyridine (DHPR) receptors are required (Cherednichenko *et al.* 2004).

In skeletal muscle, electrically evoked twitch contractions persist in the absence of extracellular Ca^{2+} , demonstrating the exquisite fidelity and efficiency of Ca^{2+} release and reuptake in this tissue. Indeed, recent estimates indicate that Ca^{2+} release during a single twitch results in only ~10% reduction of releasable Ca^{2+} in the SR (Launikonis & Rios, 2007). Together, these results indicate that SOCE and ECCE are unlikely to contribute significantly to muscle contractility during a single twitch. However, recent work indicates that SOCE limits contractile decline during trains of high-frequency fatiguing stimulation (Pan *et al.* 2002) and that extracellular Ca^{2+}

entry limits contractile fatigue in aged skeletal muscle fibres (Payne *et al.* 2007). More recently, Stiber *et al.* (2008) reported that EDL muscle from heterozygous STIM1 genotrap mice fatigue faster than wild-type muscle and that STIM1-deficient myotubes exhibit defects in refilling of internal Ca^{2+} stores. Together, these findings suggests that extracellular Ca^{2+} entry promotes store refilling and limits the development of high-frequency fatigue. Moreover, increased SOCE (Zhao *et al.* 2006) and ECCE (Yang *et al.* 2007; Cherednichenko *et al.* 2008) contribute to Ca^{2+} dysregulation in malignant hyperthermia (MH), a pharmacogenetic characterized by episodes of uncontrolled muscle contracture triggered by halogenated anaesthetics and depolarizing muscle relaxants. However, the relative contribution of SOCE and ECCE in protection from contractile decline during fatigue and uncontrolled muscle contractures during MH crises are unknown. While STIM1 is clearly required for SOCE in skeletal muscle (Stiber *et al.* 2008), the molecular identity of the STIM1-activated influx channel (e.g. Orai and/or TRPC channels) in muscle is unknown. Finally, the role of STIM1 and Orai1 in coordinating ECCE activity in skeletal muscle and whether SOCE and ECCE are mediated by single or distinct molecular entities has also not been determined. Here we show that both STIM1 and Orai1 are required for SOCE, but not ECCE, and demonstrate that these two Ca^{2+} influx pathways reflect two distinct molecular entities. Our results support the presence of two distinct molecular complexes in mediating Ca^{2+} influx across the transverse tubule (t-tubule) membrane that serve to limit contractile decline during high-frequency fatigue.

Methods

Ethical approval

All animals were housed in a pathogen-free area at the University of Rochester and all experiments were carried out in accordance with procedures reviewed and approved by the local University Committee on Animal Resources. Mice were killed by a CO_2 overdose by regulated delivery of compressed CO_2 followed by decapitation.

Primary culture, siRNA transfection and nuclear injection of Orai1 mutant cDNAs

Primary cultures of skeletal myotubes derived from newborn wild-type and RyR1-null (dyspedic) mice were prepared as previously described (Avila & Dirksen, 2001). Transfection of control and murine STIM1 siRNAs (Dharmacon, Chicago, IL, USA; Supplemental Table 1) was conducted 2 days after initial plating of myoblasts using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) and did not produce a detectable change in myotube formation or differentiation.

For STIM1 rescue experiments, a cDNA encoding hSTIM1 ($0.3 \mu\text{g } \mu\text{l}^{-1}$) was injected into nuclei 1–2 days later. All Ca^{2+} measurements and Mn^{2+} quench experiments were conducted in myotubes on the fifth day after siRNA transfection. cDNAs encoding either dominant-negative (E106Q) and pore mutant (E190Q) Orai1 were injected 5 days after initial plating of myoblasts with myotube experiments conducted 2–3 days later. Nuclear injections also included a cDNA encoding CD8 ($0.05 \mu\text{g } \mu\text{l}^{-1}$) to allow for subsequent identification with CD8 antibody-coated beads.

Western blot analysis

Cultured cells were harvested 7–8 day after initial plating. STIM1 protein was separated by SDS-PAGE on a 10% gel and then electrophoretically transferred to nitrocellulose membrane. Non-specific binding was blocked for 2 h at room temperature with 3% bovine serum albumin in a Tris-buffered saline containing 0.15% Tween 20 (TBST). Membranes were incubated with a mouse monoclonal GOK/STIM1 primary antibody (1 : 350; BD Bioscience, Sparks, MD, USA) for 2 h in 3% BSA. Membranes were washed 3 times with TBST and then incubated with a secondary anti-mouse horseradish peroxidase-conjugated antibody (1 : 1000; Bio-Rad, Hercules, CA, USA) for 1 h in TBST containing 5% milk. Enhanced chemiluminescence (Super Signal Substrate; Pierce, Rockford, IL, USA) was used to detect bound antibody. Western blot quantification of STIM1 knock-down was determined by densitometry using a UVP Bioimaging system and LabWorks™ software (UVP, Inc., Upland, CA, USA).

RNA extraction and reverse transcriptase PCR

Total RNA was isolated using TRIZOL reagent following the manufacturer's protocol. Prior to reverse transcription, total RNA was treated with DNAase I for 15 min at room temperature. RNA was then reverse-transcribed using Superscript III reverse transcriptase and oligo dT. PCR was performed using specific primers designed against STIM1 or Orai1–3 (Supplemental Table 2). In order to prevent amplification of genomic DNA, forward and reverse primers used spanned neighbouring exons. PCR products were visualized by ethidium bromide staining and confirmed by direct sequencing.

Intracellular Ca^{2+} measurements

Intracellular Ca^{2+} levels were measured in intact indo-1 AM-loaded myotubes as previously described (Avila & Dirksen, 2001). Briefly, myotubes grown on glass coverslips were incubated with $6 \mu\text{M}$ indo-1 AM for 1 h at 37°C in a Ca^{2+} -free rodent Ringer solution consisting of (mM): 145 NaCl, 5 KCl, 1 MgCl_2 , 10

Hepes, 0.2 Cs-EGTA, pH 7.4. For SOCE measurements, SR Ca^{2+} stores were depleted by exposing normal or dyspedic myotubes to $1 \mu\text{M}$ thapsigargin in Ca^{2+} -free Ringer solution for 15 min. SOCE was not significantly different following a longer exposure (60 min) with a higher concentration of thapsigargin ($3 \mu\text{M}$). For ECCE measurements, RyR-mediated Ca^{2+} release was blocked by prior exposure of myotubes to $500 \mu\text{M}$ ryanodine for 1 h. Myotubes were examined using an inverted microscope with indo-1 excited at 350 nm using a DeltaRam illumination system (Photon Technology Inc., Princeton, NJ, USA). Fluorescence emission at 405 and 485 nm was collected (100 Hz) from a small rectangular region of the myotube using a dual photomultiplier detection system. Results are presented as the ratio ($R = F_{405}/F_{485}$) of fluorescence emission at 405 nm (F_{405}) divided by the emission at 485 nm (F_{485}). Relative changes in intracellular Ca^{2+} were monitored following rapid perfusion of either 2 mM CaCl_2 Ringer solution (SOCE) or 50 mM KCl (ECCE). Results were acquired/analysed using Felix software (Photon Technology Inc., Princeton, NJ, USA).

Mn^{2+} quench measurements

Mn^{2+} quench of fura-2 emission was measured in myotubes loaded with $5 \mu\text{M}$ fura-2 AM for 1 h at 37°C in a normal rodent Ringer solution consisting of (mM): 145 NaCl, 5 KCl, 2 CaCl_2 , 1 MgCl_2 , 10 Hepes, pH 7.4. Prior to Mn^{2+} quench measurements, myotubes were treated with either $1 \mu\text{M}$ thapsigargin in Ca^{2+} -free Ringer solution for 15 min to fully deplete SR Ca^{2+} stores (SOCE measurements) or $500 \mu\text{M}$ ryanodine for 1 h to block RyR-mediated Ca^{2+} release (ECCE experiments). Fura-2-loaded myotubes were excited at the experimentally determined fura-2 isosbestic point (366 nm) and emission monitored at 510 nm during perfusion of either 0.5 mM Mn^{2+} alone (SOCE) or 50 mM KCl in the presence of 0.5 mM Mn^{2+} (ECCE).

Data analysis

Maximum rates of change in Ca^{2+} entry and Mn^{2+} quench were obtained from the peak differential of the indo-1 ratio and fura-2 emission traces, respectively. Results are given as mean \pm S.E.M. with statistical significance (** $P < 0.001$) determined using a one-way analysis of variance (ANOVA) and *post hoc* Fisher LSD test for multiple comparisons.

Results

STIM1 and Orai1–3 expression in primary skeletal myotubes

The purpose of this study was to determine the role of STIM1 and Orai1 in coordinating SOCE and ECCE

in skeletal muscle cells. RT-PCR analysis confirmed expression of mRNA transcripts for STIM1 and all three Orai isoforms in primary mouse myotubes (Supplemental Fig. 1A, upper panel) and C2C12 myotubes (Supplemental Fig. 1A, lower panel). Western blot analysis confirmed STIM1 protein expression in normal and dyspedic myotubes (Supplemental Fig. 1B).

STIM1 and Orai1 are required for SOCE

We determined SOCE activity from the magnitude and maximal rate of Ca^{2+} influx following reintroduction of extracellular Ca^{2+} to indo-1 AM-loaded myotubes in which SR Ca^{2+} stores had been depleted by prior exposure to $1 \mu\text{M}$ thapsigargin (Fig. 1). For all conditions,

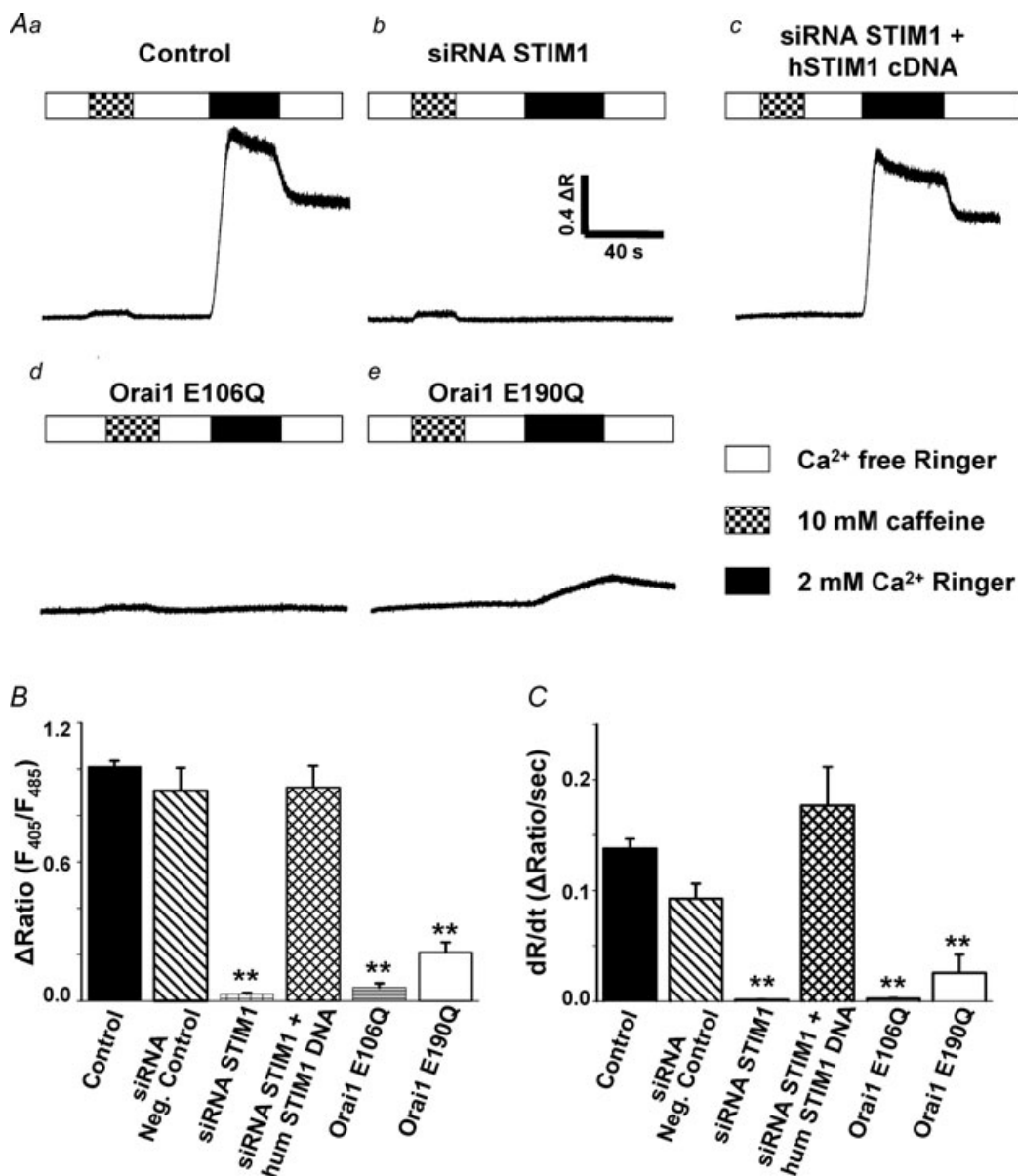


Figure 1. STIM1 and Orai1 are required for store-operated Ca^{2+} entry (SOCE) in myotubes

A, Ca^{2+} stores were depleted by incubating myotubes for 15 min with $1 \mu\text{M}$ thapsigargin in the absence of external Ca^{2+} . SR Ca^{2+} depletion was confirmed by the absence of Ca^{2+} mobilization following addition of 10 mM caffeine (hatched bars). Re-introduction of 2 mM external Ca^{2+} (black bars) elicited robust Ca^{2+} influx through SOCE channels in naïve myotubes (*a*, $n = 50$). SOCE was eliminated in myotubes transfected with a siRNA mixture directed against murine STIM1 (*b*, $n = 33$) and entry was fully rescued by expression of human STIM1 (*c*, $n = 6$). SOCE was also eliminated following expression of dominant-negative E106Q Orai1 (*d*, $n = 6$) and markedly reduced following expression of permeation-defective E190Q Orai1 (*e*, $n = 14$). In the absence of store depletion, 10 mM caffeine triggers robust SR Ca^{2+} release ($\Delta R = 0.50 \pm 0.02$, $n = 16$). B and C, summary of maximum amplitude and Ca^{2+} entry rate, respectively, for the conditions in A and for siRNA negative control ($n = 8$). $^{**}P < 0.01$.

store depletion was confirmed by the absence of robust Ca^{2+} mobilization following the addition of 10 mM caffeine (Fig. 1A, checkered bars). Subsequent perfusion of naïve myotubes with a normal 2 mM Ca^{2+} Ringer solution (Fig. 1A, black bars) elicited robust and rapid Ca^{2+} entry (Fig. 1A). We quantified both the peak of the Ca^{2+} transient (Fig. 1B; $\Delta R = R_{\text{peak}} - R_{\text{baseline}}$, where $R = F_{405}/F_{485}$) and the maximal rate of Ca^{2+} influx from the peak differential of the indo-1 ratio (Fig. 1C; dR/dt). Consistent with the results of Stiber *et al.* (2008), SOCE following reintroduction of Ca^{2+} was eliminated following siRNA-mediated STIM1 knockdown (Fig. 1A*b*, B and C), but not following transfection with negative control siRNAs (Fig. 1B and C). In our experiments, siRNA transfection efficiency was nearly 100% (Supplementary Fig. 2) and transfection of a mixture of four different murine-specific STIM1 siRNAs resulted in > 90% reduction in STIM1 protein (Supplementary Fig. 1C). Expression of human STIM1 (hSTIM1) fully restored SOCE in siRNA-treated myotubes (Fig. 1A*c*, B and C), demonstrating the specificity of the murine STIM1 siRNAs. SOCE was also abolished by expression of a dominant-negative Orai1 construct (E106Q) that eliminates ion permeation (Vig *et al.* 2006*a*) (Fig. 1A*d*, B and C). In addition, SOCE was markedly

reduced following expression of E190Q, an Orai1 pore mutant that exhibits reduced Ca^{2+} selectivity/permeation (Prakriya *et al.* 2006) (Fig. 1A*e*, B and C). Average values for the peak magnitude and maximal rate of change in indo-1 ratio in control, siRNA negative control, STIM1 knockdown, hSTIM1 rescue, E106Q-expressing, and E190Q-expressing myotubes are summarized in Fig. 1B and C, respectively.

We also used Mn^{2+} quench of fura-2 fluorescence as an alternate assay to confirm the requirement of STIM1 and Orai1 for SOCE in myotubes (Fig. 2). The relative level of SOCE activity was determined from the maximum rate of fura-2 quench (decrease in fura-2 emission when excited at the isosbestic wavelength – dF/dt) following the rapid addition of 0.5 mM extracellular Mn^{2+} . In SR Ca^{2+} -depleted naïve myotubes, basal fura-2 emission is stable under control conditions and the addition of extracellular Mn^{2+} initiates rapid fura-2 quench (Fig. 2A and F). The maximum rate of Mn^{2+} quench was not significantly different for myotubes transfected with a negative control siRNA (Fig. 2B and F). However, transfection of murine STIM1 siRNAs (Fig. 2C and F) and expression of E106Q Orai1 (Fig. 2D and F) eliminated fura-2 quench during the addition of extracellular Mn^{2+} . Moreover, the maximum rate of Mn^{2+} quench was

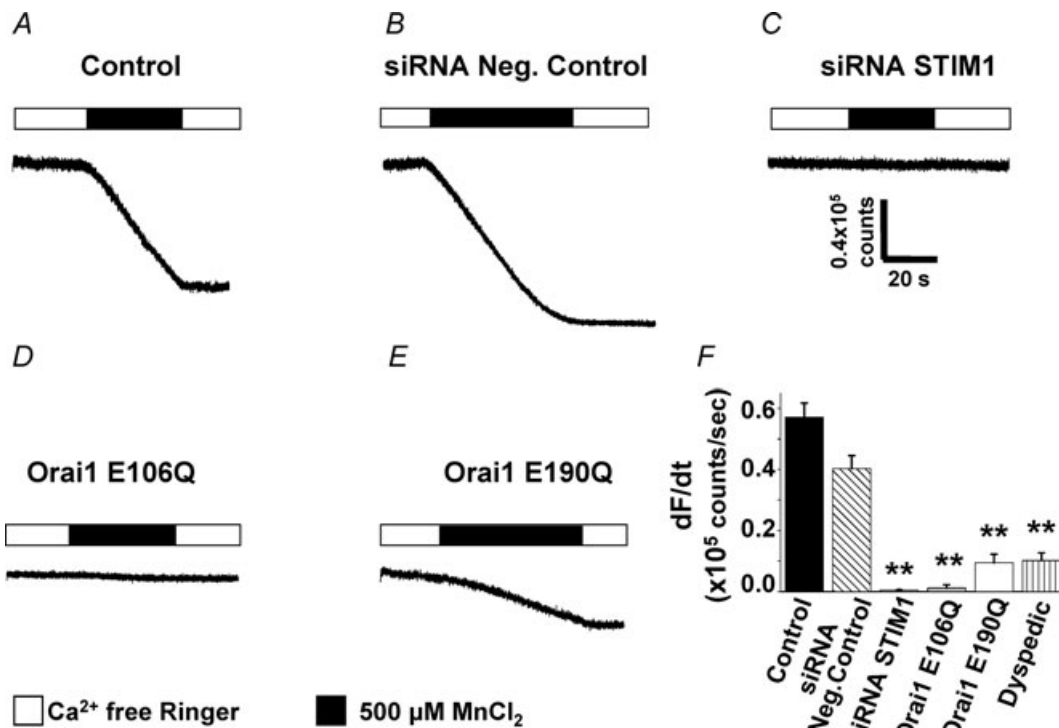


Figure 2. Rate of store-dependent Mn^{2+} quench in myotubes depends on STIM1 and Orai1

A, following store depletion with 1 μM thapsigargin, perfusion of 0.5 mM Mn^{2+} (filled bars) caused rapid quench of fura-2 emission in control myotubes ($n = 56$). B, Mn^{2+} quench was not different in myotubes transfected with negative control siRNAs ($n = 23$). C–E, fura-2 quench during Mn^{2+} addition was eliminated in myotubes transfected with either STIM1 siRNA (C, $n = 13$) or following expression of E106Q (D, $n = 13$) and markedly reduced in myotubes expressing E190Q (E, $n = 10$). F, summary of maximum rate of store-dependent Mn^{2+} quench for the conditions shown in A–E and for RyR1-null (dyspedic) myotubes ($n = 13$). ** $P < 0.01$.

markedly reduced in dyspedic myotubes and following expression of permeation-defective E190Q Orai1 (Fig. 2*E* and *F*). In Western blot experiments, we found that STIM1 protein levels are similar in normal and dyspedic myotubes (data not shown). Thus, the observed decrease in SOCE in dyspedic myotubes could reflect either a regulatory role of RyR1 on SOCE (Kiselyov *et al.* 2000; Pan *et al.* 2002) or a deficiency in Orai1 expression. Together, the Ca^{2+}

measurements and Mn^{2+} quench results demonstrate that STIM1 and Orai1 are both required for SOCE in skeletal muscle cells.

Neither STIM1 nor Orai1 are required for ECCE

The potential role of STIM1 and Orai1 in ECCE activation and whether SOCE and ECCE reflect distinct

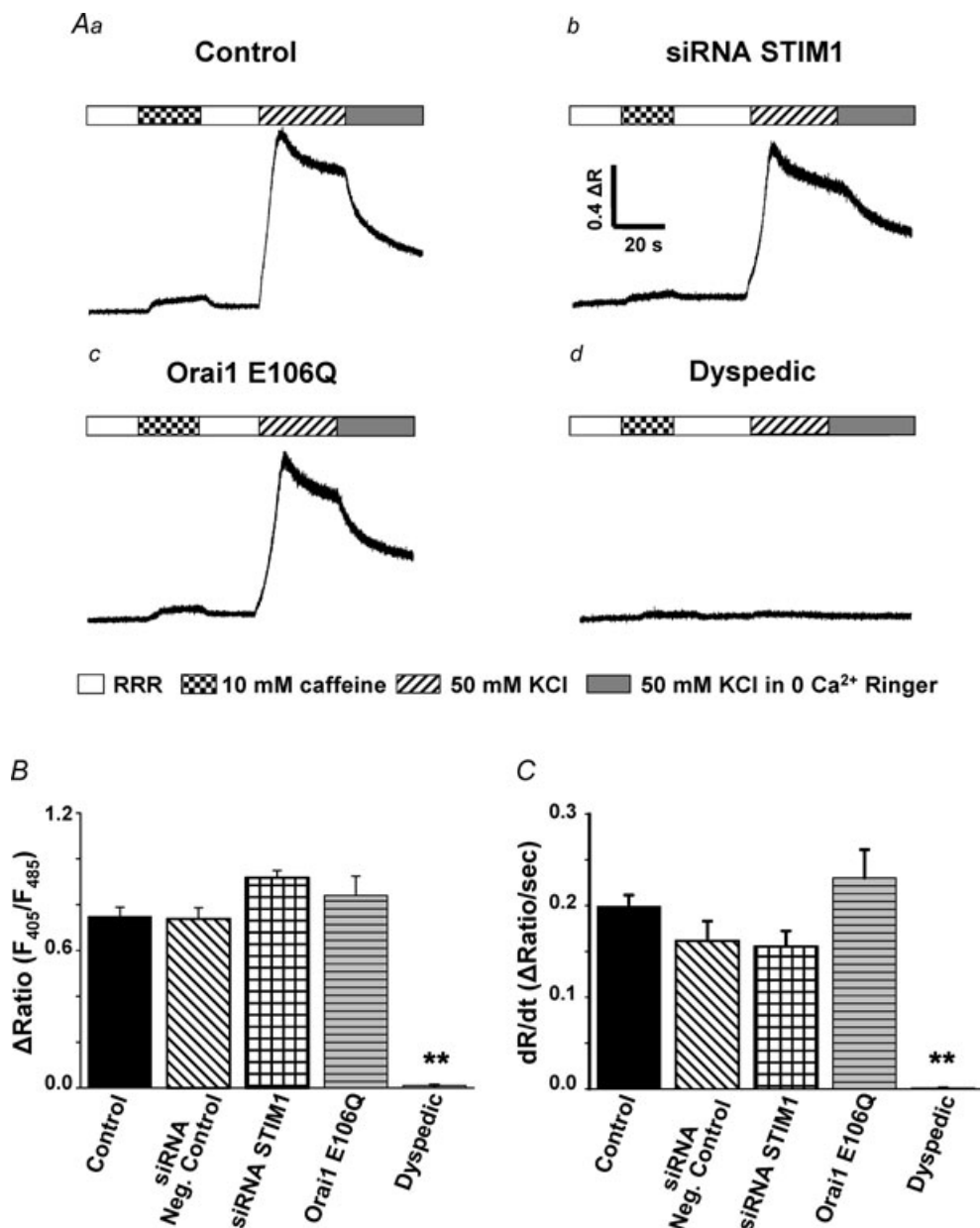


Figure 3. STIM1 and Orai1 are not required for excitation-coupled Ca^{2+} entry (ECCE) in myotubes

A, RyR-mediated Ca^{2+} release was blocked by pre-treating myotubes with $500 \mu\text{M}$ ryanodine for 1 h in Ringer solution. RyR1 blockade was confirmed by the absence of robust Ca^{2+} mobilization following the addition of 10 mM caffeine (checkered bars). Rapid perfusion of 50 mM KCl (hatched bars) elicited depolarization-induced Ca^{2+} entry in control myotubes (*a*, $n = 46$), STIM1 siRNA knock-down myotubes (*b*, $n = 6$), and Orai1 E106Q-expressing myotubes (*c*, $n = 10$). Dyspedic myotubes (*d*, $n = 13$) completely lacked KCl-induced Ca^{2+} entry. *B* and *C*, summary of maximum amplitude and Ca^{2+} entry rate, respectively, for the conditions shown in *A* and for siRNA negative control ($n = 8$). ** $P < 0.01$.

molecular entities or result from different mechanisms for activation of the same channel is unknown. Therefore, we determined the STIM1 and Orai1 dependence of Ca^{2+} entry via the ECCE pathway in myotubes in which depolarization-induced Ca^{2+} release was eliminated by pre-incubation with $500 \mu\text{M}$ ryanodine (Fig. 3). Blockade of RyR channels was confirmed by the absence of Ca^{2+} release during 10 mM caffeine exposure (Fig. 3A, checkered bars). In naïve myotubes, robust Ca^{2+} influx through ECCE channels was elicited during sustained depolarization induced by 50 mM KCl (Fig. 3A, hatched bars). Figure 3Aa–d shows representative Ca^{2+} measurements of KCl-induced ECCE in control myotubes (Fig. 3Aa), STIM1 siRNA-treated myotubes (Fig. 3Ab), E106Q-expressing myotubes (Fig. 3Ac), and dyspedic myotubes (Fig. 3Ad). Average values for the peak magnitude and maximal rate of change in indo-1 ratio for each condition are summarized in Fig. 3B and C, respectively. The results indicate that Ca^{2+} entry through the ECCE pathway is $\sim 2\times$ faster than via the SOCE pathway (compare Figs 1C and 3C). In addition, ECCE was unaltered by either STIM1 knockdown or E106Q Orai1, conditions that abolished SOCE (Figs 1 and 2). Consistent with prior findings (Cherednichenko *et al.* 2004), KCl-induced Ca^{2+} influx was absent in dyspedic myotubes.

Mn^{2+} quench experiments were also conducted to confirm the independence of ECCE from STIM1 and Orai1 (Fig. 4). Consistent with the Ca^{2+} measurements, maximal rates of KCl-induced Mn^{2+} quench (i.e. ECCE activity) were not significantly different between control myotubes (Fig. 4A and F), myotubes transfected with negative control STIM1 siRNAs (Fig. 4B and F), myotubes transfected with murine STIM1 siRNAs (Fig. 4C and F), or E106Q-expressing myotubes (Fig. 4D and F). In addition, KCl depolarization failed to elicit Mn^{2+} quench in dyspedic myotubes (Fig. 4E and F). In addition, the maximum rate of ECCE-induced Mn^{2+} quench was also $\sim 2\times$ greater than that mediated via the SOCE pathway (compare Figs 2F and 4F).

Discussion

The fundamental new finding of this study is that a STIM1–Orai1 complex is required for SOCE in skeletal muscle cells, but is not involved in coordinating depolarization-dependent Ca^{2+} influx through the ECCE pathway. A critical extension of this observation is that the differential requirement of SOCE and ECCE on STIM1 and Orai1 indicates that these two functionally different Ca^{2+} influx pathways arise from distinct molecular

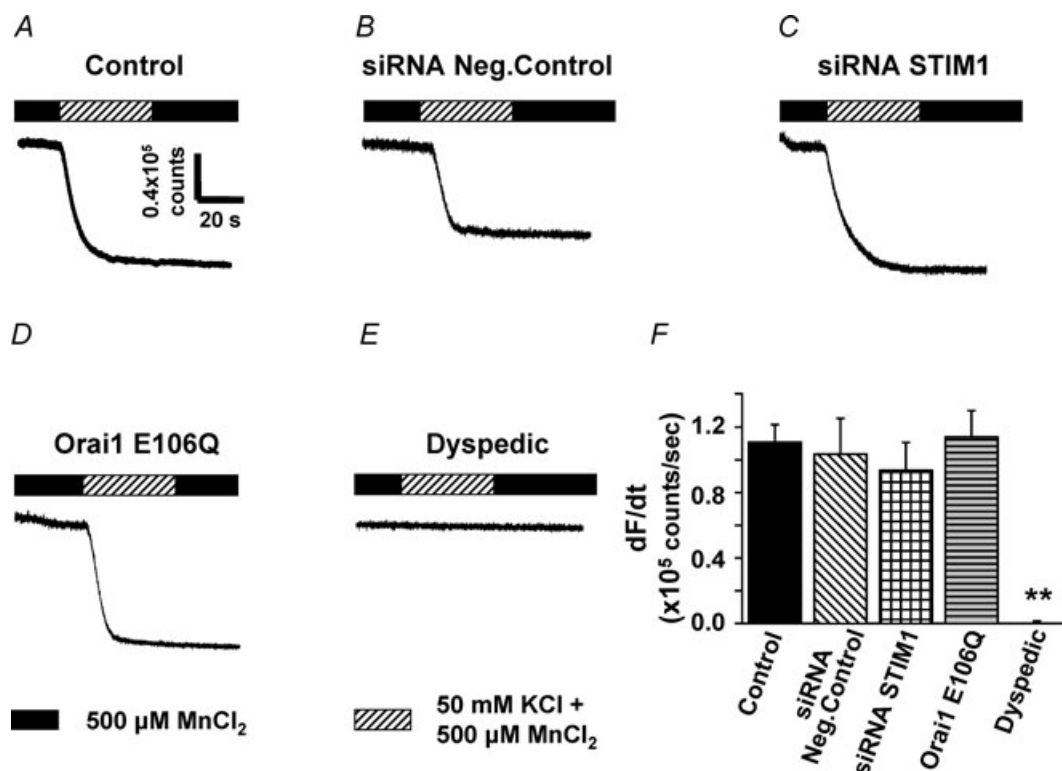


Figure 4. Rate of excitation-coupled Mn^{2+} quench in myotubes does not depend on STIM1 or Orai1
A–D, Mn^{2+} quench observed during perfusion of 50 mM KCl + $500 \mu\text{M}$ Mn^{2+} (hatched bar) in ryanodine-treated control myotubes (A, $n = 23$), siRNA negative control myotubes (B, $n = 5$), STIM1 siRNA myotubes (C, $n = 5$), and Orai1 E106Q-expressing myotubes (D, $n = 12$). E, dyspedic myotubes ($n = 6$) lacked KCl-induced Mn^{2+} quench. F, summary of maximum rate of Mn^{2+} quench for the conditions shown in A–E. ** $P < 0.01$.

entities. Proposed minimal molecular models for SOCE and ECCE complexes in skeletal muscle are depicted in Fig. 5. Our findings are consistent with the recent results of Stiber *et al.* (2008) who used STIM1 genetrapped mice to demonstrate that STIM1 is required for SOCE in skeletal muscle. Our results extend these findings by being the first to demonstrate that SOCE in muscle reflects Ca^{2+} permeation through Orai1-containing channels (Fig. 5A). Additional support for this mechanism in muscle comes from the fact that both STIM1 (Stiber *et al.* 2008) and Orai1 (Vig *et al.* 2008) are expressed at high levels in skeletal muscle, STIM1 and Orai1 deficiency results in reduced muscle mass (Vig *et al.* 2008; Stiber *et al.* 2008), and one form of severe combined immunodeficiency results from an Orai1 loss-of-function mutation (R91W) that presents with a non-progressive myopathy (Feske *et al.* 2006). However, our results do not exclude potential direct and/or regulatory contributions to SOCE and/or ECCE function in skeletal muscle of other proteins including STIM2, Orai2/3, IP_3 receptors (Launikonis *et al.* 2003), ryanodine receptors (Kiselyov *et al.* 2000; Pan *et al.* 2002), and TRPC proteins (Kiselyov *et al.* 2000; Rosenberg *et al.* 2004; Sampieri *et al.* 2005). It will also be important for future work to determine the impact of STIM1 knockdown and/or introduction of dominant-negative Orai1 constructs on skeletal muscle EC coupling, global and local Ca^{2+} release, and Ca^{2+} signalling triggered by activation of G protein-coupled receptors.

While several studies have documented SOCE in neurones and muscle cells, ours is the first single

study to unequivocally demonstrate that both STIM1 and Orai1 are required for SOCE in an excitable cell. Prior studies in skeletal muscle demonstrate that store depletion activates SOCE across the re-sealed t-tubule membrane of mechanically skinned skeletal muscle fibres (Launikonis *et al.* 2003; Zhao *et al.* 2005; Zhao *et al.* 2006; Launikonis & Rios, 2007). Moreover, activation of SOCE across the t-tubule membrane is remarkably rapid, occurring as fast as 1 s following initiation of SR Ca^{2+} release (Launikonis & Rios, 2007). Together with our findings, these results indicate that unlike T lymphocytes, STIM1 and Orai1 proteins required for SOCE activation in skeletal muscle must be co-localized within the t-tubule membrane even prior to store depletion. The pre-formation of STIM1–Orai1 complexes strategically within the triad junction (Fig. 5B) would provide efficient SR Ca^{2+} content sensing and responsiveness to store depletion required to promote SR store refilling and limit contractile decline during high-frequency fatigue.

Our results are also the first to demonstrate that ECCE in skeletal muscle requires neither STIM1 nor Orai1. While the complete molecular composition of the ECCE complex remains elusive, at a minimum it includes the DHPR, RyR1 and an as yet unidentified Ca^{2+} -permeable channel. The molecular identity of the Ca^{2+} -permeable ECCE channel will require further study, but may involve TRPC-related proteins. Nevertheless, since ECCE activity requires junctional DHPR and RyR1 proteins (Cherednichenko *et al.* 2004), the ECCE complex

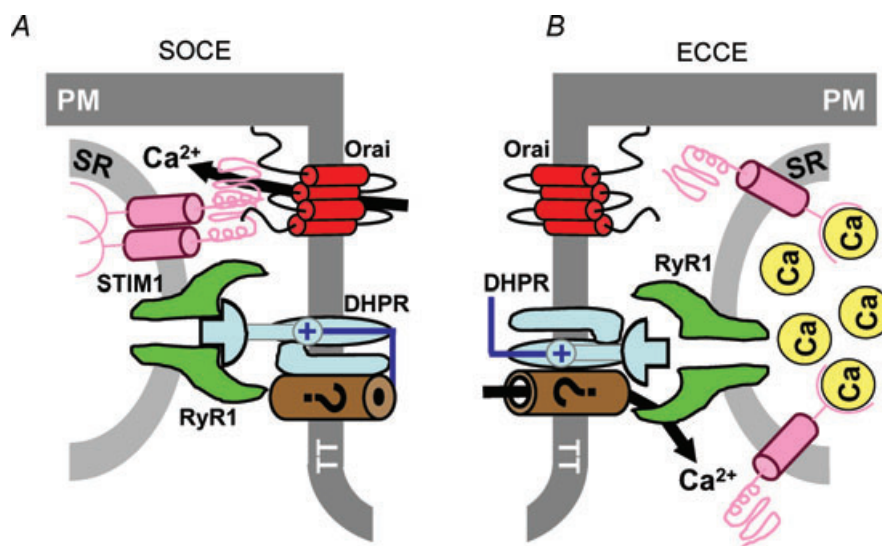


Figure 5. SOCE and ECCE are coordinated by distinct molecular complexes within the skeletal muscle triad

A, proposed minimum molecular model for SOCE in skeletal muscle. SOCE activation requires an interaction between STIM1, which serves as the Ca^{2+} sensor for changes in SR Ca^{2+} content within the terminal cisternae, and Ca^{2+} -permeable Orai1 channels in the t-tubule membrane. *B*, proposed minimum molecular model for ECCE in skeletal muscle. Functional ECCE requires the skeletal muscle DHPR and RyR1 isoforms, but neither STIM1 nor Orai1. The requirement for the DHPR (voltage sensor for activation) and RyR1 places this Ca^{2+} influx complex within the triad junction. The molecular identity of the Ca^{2+} -permeation pathway for ECCE remains to be determined (see Discussion).

must also be located within the triad. Thus, the more rapid activation and store-independent properties of the ECCE pathway would permit junctional Ca^{2+} influx to limit contractile decline at early times during high-frequency skeletal muscle activation, even prior to significant store depletion or SOCE activation. The apparent redundant positioning of two functionally and molecularly distinct Ca^{2+} influx pathways within the triadic region underscores the potential physiological importance of Ca^{2+} influx in maintaining contractile function during strenuous muscle activity.

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Supplemental material

Online supplemental material for this paper can be accessed at: <http://jp.physoc.org/cgi/content/full/jphysiol.2008.160481/DC1>