STIM1 (stromal interaction molecule 1) mediates SOCE (store-operated Ca\(^{2+}\) entry) in skeletal muscle. However, the direct role(s) of STIM1 in skeletal muscle, such as Ca\(^{2+}\) release from the SR (sarcoplasmic reticulum) for muscle contraction, have not been identified. The times required for the maximal expression of endogenous STIM1 or Orai1, or for the appearance of puncta during the differentiation of mouse primary skeletal myoblasts to myotubes, were all different, and the formation of puncta was detected with no stimulus during differentiation, suggesting that, in skeletal muscle, the formation of puncta is a part of the differentiation. Wild-type STIM1 and two STIM1 mutants (Triple mutant, missing Ca\(^{2+}\)-sensing residues but possessing the intact C-terminus; and E136X, missing the C-terminus) were overexpressed in the myotubes. The wild-type STIM1 increased SOCE, whereas neither mutant had an effect on SOCE. It was interesting that increases in the formation of puncta were observed in the Triple mutant as well as in wild-type STIM1, suggesting that SOCE-irrelevant puncta could exist in skeletal muscle. On the other hand, overexpression of wild-type or Triple mutant, but not E136X, attenuated Ca\(^{2+}\) releases from the SR in response to KCl (evoking ECC (excitation–contraction coupling) via activating DHPR (dihydropyridine receptor)) in a dominant-negative manner. The attenuation was removed by STIM1 knockdown, and STIM1 was co-immunoprecipitated with DHPR in a Ca\(^{2+}\)-independent manner. These results suggest that STIM1 negatively regulates Ca\(^{2+}\) release from the SR through the direct interaction of the STIM1 C-terminus with DHPR, and that STIM1 is involved in both ECC and SOCE in skeletal muscle.

Key words: dihydropyridine receptor (DHPR), excitation–contraction coupling (ECC), punctum, skeletal muscle, store-operated Ca\(^{2+}\) entry (SOCE), stromal interaction molecule 1 (STIM1).

INTRODUCTION

During ECC (excitation– contraction coupling) in skeletal muscle, the DHPR (dihydropyridine receptor), a membrane voltage-sensing protein located in the t (transverse)-tubule membrane, is activated by membrane depolarization, which allows physical interactions between DHPR and RyR1 (ryanodine receptor 1), an internal Ca\(^{2+}\)-releasing channel in the SR (sarcoplasmic reticulum) membrane, the release of Ca\(^{2+}\) from the SR into the myoplasm through RyR1, and, ultimately, skeletal muscle contraction [1,2]. Additionally, the canonical-type TRPC (transient receptor potential canonical cation channel) 3, which is a Ca\(^{2+}\) entry channel located in sarcolemmal and t-tubule membranes, is required for the full gain of skeletal ECC (duration and maintenance) [3]. To obtain the functionally efficient coupling and correct spatial arrangement of the Ca\(^{2+}\) channels, the t-tubule and the SR membrane are closely juxtaposed (known as a triad junction or junctional membrane complex) [4–6].

SOCE (store-operated Ca\(^{2+}\) entry) is a ubiquitous inside-out signal with very high Ca\(^{2+}\) selectivity for replenishing the ER (endoplasmic reticulum) with Ca\(^{2+}\) and sustaining the presence of a certain concentration of Ca\(^{2+}\) in the cytoplasm. SOCE is also found in skeletal muscle [7]. The main molecular components corresponding to SOCE in skeletal muscle are the STIM1 (stromal interaction molecule 1), which is a Ca\(^{2+}\) sensor located in SR membranes, and Orai1, a Ca\(^{2+}\) entry channel located in t-tubule membranes [8,9]. In non-excitable cells, the first step in SOCE is the depletion of ER-stored Ca\(^{2+}\), which results in the dissociation of Ca\(^{2+}\) from the Ca\(^{2+}\)-sensing STIM1 (apo STIM1). The second step is the relocation of the self-oligomerized apo STIM1s in the ER membrane near the plasma membrane, and, simultaneously, the gathering of Orai1s in the plasma membrane directly opposite the oligomerized apo STIM1s, which results in the formation of ‘punctа’. Finally, physical interactions between STIM1 and Orai1 induce extracellular Ca\(^{2+}\) entry through Orai1 [10–14]. Interestingly, in skeletal myotubes, puncta by STIM1 and Orai1 is necessary but does not initiate SOCE [6], and the SOCE in the fibers of rat skeletal muscle is more rapid, occurring in the order of seconds, than in non-excitable cells [15,16].

STIM1 has a single-transmembrane domain, a short intraluminal N-terminal region, and a cytoplasmic C-terminal region. The N-terminal region contains two EF-hands (containing a pseudo EF-hand) and a SAM (sterile α-motif) domain [17,18]. The C-terminus contains three coiled-coil domains, an ezrin-radixin-moesin domain, a proline-serine-rich domain and a lysine-rich domain [18–20]. Under ER Ca\(^{2+}\) store depletion, the apo EF-SAM domain is responsible for the self-oligomerization and the relocation of apo STIM1s to ER regions near the plasma membrane [12,21]. The disruption of the EF Ca\(^{2+}\)-sensing sites by single mutations (D76A, D84G or E87A) results either in
a constitutively active Orai1 or in an exaggerated SOCE [22–24]. Either the SOAR (STIM1-Orai1-activating region; [25]) or the CAD (CRAC (Ca\(^{2+}\) release-activated Ca\(^{2+}\))-activating domain; [26]) is responsible for the activation of Orai1 via physical interactions with STIM1. The EF-hand and the CAD also participate in the self-oligomerization of STIM1s [27–30]. The roles of other STIM1 domains are controversial or have not been well studied. STIM1L is a splice variant of STIM1 in mouse and human skeletal muscle [31].

There have been reports regarding the role of STIM1 in skeletal muscle. STIM1-deficient mice die from a perinatal myopathy and show defects in muscle differentiation [8,32]. In the C2C12 skeletal myoblast cell line, the STIM1 expression level is increased during differentiation [8]. Silencing STIM1 in human skeletal myoblasts (but not during the middle of mouse skeletal myoblast differentiation) using siRNA reduces SOCE, the differentiation of the myoblasts to myotubes, myoplasmic resting C\(^{2+}\) levels and the SR C\(^{2+}\) content [33,34]. In the same manner, the overexpression of STIM1 in mouse skeletal myoblasts or the C2C12 myoblast cell line enhances the differentiation [35]. STIM1 is also related to several skeletal muscle diseases. Patients with loss-of-function STIM1 mutations (E136X or R429C) show severe combined immunodeficiency due to the lack of SOCE [36–38]. Interestingly, all patients show ‘congenital myopathies,’ such as muscular hypertonia. Muscle fibres from the mdx mouse, an animal model for Duchenne muscular dystrophy characterized by progressive muscle weakness, show increased STIM1 and Orai1 expression and SOCE [39,40]. Hypertrophy in mouse skeletal myotubes involves increased SOCE [41]. Muscle fibres from patients with malignant hyperthermia, leading to life-threatening muscle contracture and an increase in core body temperature due to the direct activation of mutated RyR1 by volatile anaesthetics, show SOCE even in the presence of the clinical range of volatile anaesthetics [42].

Although there have been reports regarding the role of STIM1 in skeletal muscle, the direct relevance of STIM1 to C\(^{2+}\) release from the SR, the early event of skeletal ECC, has not been characterized. In the present study, we examined the role of STIM1 in C\(^{2+}\) release from the SR for ECC using two STIM1 mutants in mouse primary skeletal myotubes. One is a triple mutant of the EF-hand (missing C\(^{2+}\)-sensing residues in the EF-hand but possessing the intact C-terminus) and the other is a deletion mutant (E136X, missing the C-terminus but possessing the intact EF-hand, i.e. the reverse mutant of the Triple mutant).

**EXPERIMENTAL**

**Materials**

FBS, F-10 Nutrient Mixture, low-glucose DMEM (Dulbecco’s modified Eagle’s medium), L-glutamine, penicillin, streptomycin, bFGF (basic fibroblast growth factor), fura-2, fluo-4 and fluo-5N were obtained from Invitrogen. Matriptel™ was obtained from BD Biosciences. NGS (normal goat serum) and secondary antibodies [a Cy3 (indocarbocyanine)-conjugated anti-mouse secondary antibody (1:500 dilution), and horseradish peroxidase-conjugated anti-mouse, anti-rabbit and anti-donkey antibodies (1:2000 dilution)] were obtained from Jackson ImmunoResearch. An anti-DHPR antibody (against the α1 subunit of DHPR, 1:500 dilution) was obtained from Affinity BioReagents. An anti-RyR1 antibody (1:2000 dilution) was provided by Dr J. Airye and Dr J. Sutko (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, U.S.A.). The anti-CFP and anti-SERCA1 (sarcoplasmic/endoplasmic reticulum Ca\(^{2+}\)-ATPase) antibodies (1:1000 dilution) were obtained from Cell Signaling Technology. The anti-Orai1 and anti-STIM1 antibodies (1:500 dilution in immunoblot assay, 10 μg/ml in immunocytochemistry) were obtained from Abcam. The anti-STIM2 antibody (1:1000 dilution) was obtained from Proteintech. The anti-TRPC1, anti-TRPC3, anti-TRPC4 and anti-TRPC6 antibodies (1:800 dilution) were obtained from Alomone Labs. An anti-JP (junctophilin) 1 antibody (1:1000 dilution) was obtained from Zymed. The anti-CSQ (calsequestrin), anti-JP2 and anti-α-actin antibodies (1:1000 dilution) were obtained from Santa Cruz Biotechnology. The FuGENE6 Transfection Reagent, X-tremeGENE siRNA Transfection Reagent and the protease inhibitor cocktail tablets were obtained from Roche. Reagents for total RNA isolation were obtained from Qiagen. Reagents for the RT (reverse transcription)–PCR and qPCR (quantitative real-time PCR) were obtained from Bio-Rad Laboratories and TaKaRa. The Protein G–Sepharose beads were obtained from GE Healthcare. The SuperSignal ultrasensitive chemiluminescent substrate was obtained from Pierce. Caffeine, KCl, horse serum, a FITC-conjugated anti-rabbit secondary antibody (1:500 dilution), collagen, ionomycin and other reagents were obtained from Sigma–Aldrich.

**Construction of the cDNAs for the Triple mutant and E136X**

Full-length human STIM1 cDNA in an expression vector (pM901) was obtained from Addgene (plasmid 19755). The preparation of the Triple mutant was carried out using a site-directed mutagenesis kit (Stratagene). Using the full-length STIM1 as the template DNA, the D76A mutant was synthesized by PCR in the presence of a pair of complementary synthetic oligonucleotide primers containing the desired mutation (forward, 5′-CACCACAACTGATGCGATGATGCCAATGG-3′; and reverse, 5′-CCAATTGGCATCATCGCCATCATTTGTGGATG-3′) and Pfu DNA polymerase [18 cycles at 95°C for 40 s, 60°C for 40 s, and 68°C for 15 min with a GeneAmp PCR System 2700 thermocycler (Applied Biosystems)]. Next, the template DNA was digested for 1 h with DpnI endonuclease, and the nicked vector DNA incorporating the desired mutation was then transformed into DH5α super-competent cells. The D76A DNA was subjected to a second mutation at Glu55 using a pair of complementary synthetic oligonucleotide primers containing the desired mutation (forward, 5′-GGTGTGATGTGATGGAAGCGAAGTGATGAGTTCC-3′; and reverse, 5′-GGGACTTACATCTTGCTTCCACATCCACATCCAC-3′) under the same PCR conditions followed by DpnI digestion and transformation into DH5α cells (D76A/E87A). The D76A/E87A DNA was subjected to a third mutation at Asp64 with a pair of complementary synthetic oligonucleotide primers containing the desired mutation (forward, 5′-CCATGTGTGATGGAAGCGAAGTGATGAGTTCC-3′; and reverse, 5′-GGGACTTACATCTTGCTTCCACATCCACATCCAC-3′) under the same PCR conditions followed by DpnI digestion and transformation into DH5α cells (D76A/D84G/E87A, i.e. Triple mutant). To make the cDNA for E136X, which lacks the amino acids from Glu55 to the end of STIM1, three oligonucleotide primers were designed on the basis of the nucleotide sequence of E136X obtained from the patient [38]. The forward primer was 5′-CGGCTCAGGATGAGTATGCTCCGGTCTCTGGC-3′, corresponding to nucleotides 1–23, and the reverse primer was 5′-TCCACCGGCTCAATTGTATACTCTTGATGAGCTTCC-3′, corresponding to nucleotides 371–405 with an additional A nucleotide at position 382. With these primers, PCR was performed under the following conditions: 30 cycles
Role of STIM1 in skeletal muscle

Cell culture and overexpression of wild-type STIM1, Triple mutant or E136X

Mouse primary skeletal myoblasts were derived from mouse skeletal muscle as described previously [43]. All steps for surgical intervention as well as for pre- and post-surgical animal care were provided in accordance with the Laboratory Animals Welfare Act, the Guide for the Care and Use of Laboratory Animals and the Guidelines and Policies for Rodent Survival Surgery provided by the Institutional Animal Care and Use Committee of the College of Medicine at The Catholic University of Korea. The isolated primary myoblasts were cultured on 10-cm-diameter dishes coated with collagen in a growth medium (F10 Nutrient Mixture containing 20% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and 20 nM bFGF) at 37°C in a 5% CO2 incubator. For differentiation into myotubes, the myoblasts were replated either on 10-cm-diameter dishes (for the preparation of myotube lysate) or on 96-well plates (for the single myotube Ca2+ imaging experiments or the immunocytochemistry) coated with MatrigelTM. When the myoblasts reached ~70% confluence, the growth medium was replaced with a differentiation medium (5% heat-inactivated horse serum and low-glucose DMEM without growth factors instead of 20% FBS and F-10 Nutrient Mixture in the growth medium), and the myoblasts were placed in an 18% CO2 incubator to induce differentiation. After 3 days of culture in the differentiation medium, the myoblasts were transfected with the cDNA for wild-type STIM1, the Triple mutant or E136X for 4 h (with a mixture of 30 μl of FuGENE6 and 20 μg of cDNA per 10-cm-diameter dish or the same ratio of the components in the wells of 96-well plates). On D5 (differentiation day 5; at 37°C for 45 min. The myotubes were then washed three times with 0.05 M of free Ca2+ level, the myotubes were treated with ionomycin dissolved in dimethyl sulfoxide (<0.05%). Dimethyl sulfoxide (0.05%) alone had no effect on the Ca2+ release from the SR. For the measurement of SOCE, myotubes were incubated in the imaging solution with zero Ca2+ for 5 min and were treated with caffeine. To measure the total cellular Ca2+ level, the myotubes were treated with ionomycin dissolved in dimethyl sulfoxide (<0.05%) to induce SR Ca2+ depletion. Once the myoplasmic Ca2+ level returned to the baseline, the imaging solution with 2 mM Ca2+ was added to the myotubes to measure SOCE. To analyse the Ca2+ release from the SR obtained from the Ca2+ imaging experiments, both the peak amplitude and the area under the curve, which exhibited similar increases and decreases, were considered. For relatively long-term Ca2+ releases (more than 10 min of recording), the areas under the curves were analysed.

Immunocytochemistry and width measurement of the myotubes

The myotubes were fixed in ice-cold methanol (−20°C) for 15 min and permeabilized with 0.05% Tween 20 in PBS for 1 min. After blocking with 2% NGS in PBS, the myoblasts were incubated with anti-STIM1 or anti-Orai1 antibody for 3 h, washed three times with 2% NGS in PBS for 10 min, incubated with a Cy3-conjugated anti-mouse or FITC-conjugated anti-rabbit secondary antibody for 45 min at room temperature (20°C), and visualized with an inverted fluorescence microscope using a 40× objective lens (ECLIPSE Ti) equipped with a monochrome camera (ProRes MF, JENOPTIK Optical Systems). The number of puncta on the images obtained from immunocytochemistry was counted per unit area, and the number of puncta was normalized to the mean value of the controls. For the width measurement of myotubes that were transfected with either one of STIM1 constructs or the #2 siRNA, images of fully differentiated myotubes on D5 were captured using the monochrome camera (ProgRes MF, JENOPTIK Optical Systems). The total number of puncta on the images obtained from immunocytochemistry was counted per unit area, and the number of puncta was normalized to the mean value of the controls. For the width measurement of myotubes that were transfected with either one of STIM1 constructs or the #2 siRNA, images of fully differentiated myotubes on D5 were captured using the monochrome camera, and the width of the thickest part in each myotube was measured using the ImageJ program [45].

Preparation of the myotube lysate, co-immunoprecipitation and immunoblot assay

The myotubes were solubilized in a lysis buffer (1% Triton X-100, 10 mM Tris/HCl, pH 7.4, 1 mM Na3VO4, 10% glycerol, 150 mM NaCl, 5 mM EDTA and protease inhibitor cocktail tablets) overnight at 4°C with gentle mixing (300 μl of the lysis buffer per myotubes in a 10-cm-diameter dish) [46]. To obtain 100 μM of free Ca2+ concentration in the co-immunoprecipitation, the lysis buffer was set by adding 5 mM CaCl2, and the adjusted lysis buffer was used for further procedures. The solubilized lysate was then centrifuged at 1500 g for 30 s to remove the insoluble matter. For co-immunoprecipitation, the solubilized lysate (800 μg of total

The myotubes was subjected to a second PCR with the same forward primer and a different reverse primer (5′-GGCTCGAC-GTCGAGATTCCGACGGTTCAATG-3′) under the same PCR conditions. The PCR fragments were subcloned into the pMO91 vector. The sequences of both strands were confirmed by sequencing using an ABI Prism 3700 DNA Sequencer (Applied Biosystems).
protein) was incubated with the anti-STIM1 antibody overnight at 4 °C, followed by incubation with Protein G–Sepharose beads for 4 at 4 °C. The beads (immunoprecipitates) were washed five times with the lysis buffer without Triton X-100. The bound proteins were subjected to an immunoblot assay. For the immunoblot assay, the bound proteins or the myotube lysates (30 μg of protein) boiled with a SDS sample buffer were subjected to SDS/PAGE (8 or 10 % acrylamide gel), and the proteins on the gel were transferred on to a PVDF membrane at 100 V for 2 h. The membrane was blocked with 5 % (w/v) non-fat dried skimmed milk powder dissolved in PBS for 1 h, incubated with one of the corresponding horseradish peroxidase-conjugated secondary antibodies for 45 min at room temperature. The membrane was washed three times with PBST and developed using the SuperSignal ultrachemiluminescent substrate. Band intensities on the membrane were measured using the ImageJ program [45]. The band intensities on each day were normalized to the intensities of the corresponding protein (Orai1 or STIM1) on D5 (which were considered to be 1). For STIM1L, the band intensities of STIM1 on D5 were considered to be 1.

Knockdown of STIM1 in myotubes by siRNA

The sequences of different siRNAs for STIM1 (GenBank® accession number NM_009287.4) were selected using siRNA design software, siDirect [47], and the sequences are summarized in Table 1. A scrambled siRNA was used as a negative control. Immature myotubes on D3 were transfected with one of the siRNAs in a mixture containing 600 μl of low-glucose DMEM, 60 μl of X-tremeGENE Transfection Reagent, and 200 nM of the specific synthetic siRNAs for 3 h according to the manufacturer’s protocols (Roche). After the transfection, fully differentiated myotubes on D5 were subjected to further experiments. Optimal quantities of the transfection reagent and the siRNA and transfection frequencies were defined by evaluating the strongest effect of siRNAs.

Table 1 Sequences of siRNAs used for knocking down STIM1 and qPCR primers

<table>
<thead>
<tr>
<th>siRNA</th>
<th>Sense</th>
<th>Anti-sense</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scrambled siRNA</td>
<td>5′-CGGCGGTCAGAGCTTTCG-3′</td>
<td>5′-GACGCTTCTCTTACCTGG-3′</td>
</tr>
<tr>
<td>#1 siRNA</td>
<td>5′-GGGAGGACCACAUCCACCU-3′</td>
<td>5′-UUGUGAAGGCGCUCCUCU-3′</td>
</tr>
<tr>
<td>#2 siRNA</td>
<td>5′-GCAGAGAAGGACAGUGA-3′</td>
<td>5′-AUUCAAACUUUGGCGCUU-3′</td>
</tr>
</tbody>
</table>

(a) siRNAs

(b) qPCR primers (expected size of PCR product: 192 bp)

<table>
<thead>
<tr>
<th>Direction</th>
<th>Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward</td>
<td>5′-AGAATGGAGGAGGCGGTC-3′</td>
</tr>
<tr>
<td>Reverse</td>
<td>5′-GCCCTCTCTGATTTTGCTTC-3′</td>
</tr>
</tbody>
</table>

RT–PCR and qPCR

Total RNA was isolated from the STIM1-knockdown myotubes using QIAshredder and the RNeasy Mini Kit (Qiagen) according to the manufacturer’s protocols. RT–PCR and qPCR were performed on the CFX Connect Real-Time PCR (Bio-Rad Laboratories) using a One-Step kit (TaKaRa). Briefly, the following reaction mixture was prepared on ice: 12.5 μl of 2 × One-Step SYBR RT-PCR buffer III, 2.5 units of Ex TaqHS, 0.5 μl of PrimeScript RT Enzyme Mix II, 0.2 μM of each PCR forward or reverse primer and 50 ng of total RNA (25 μl of total reaction volume). Reaction conditions were 1 cycle at 42 °C for 20 s for qPCR, and incubation at 95 °C for 10 s for RT–PCR, 50 cycles at 95 °C for 5 s and 60 °C for 20 s for qPCR, and incubation at 60 °C for 1 min for the dissociation step. After the reaction was completed, signal verifications were conducted with the amplification and melting curves using a comparative threshold cycle method from the CFX Connect Real-Time PCR. A mean quantity was calculated from four to eight qPCRs for each sample, and this quantity was normalized to the endogenous control gene, α-actinin. Primers used for qPCR are described in Table 1.

Table 2 Properties of myotubes overexpressing wild-type STIM1, the Triple mutant or E136X

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Control vector</th>
<th>Wild-type STIM1</th>
<th>Triple mutant</th>
<th>E136X</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCl response</td>
<td>1.00 ± 0.09 (109)</td>
<td>0.71 ± 0.06* (151)</td>
<td>0.77 ± 0.07* (147)</td>
<td>1.19 ± 0.11* (78)</td>
</tr>
<tr>
<td>Caffeine response</td>
<td>1.00 ± 0.08 (109)</td>
<td>0.94 ± 0.03 (151)</td>
<td>1.02 ± 0.08 (147)</td>
<td>1.28 ± 0.06* (78)</td>
</tr>
<tr>
<td>Releasable SR Ca2+</td>
<td>1.00 ± 0.15 (62)</td>
<td>1.07 ± 0.16 (71)</td>
<td>1.03 ± 0.07 (54)</td>
<td>1.10 ± 0.16 (57)</td>
</tr>
<tr>
<td>Total cellular Ca2+ level</td>
<td>1.00 ± 0.07 (45)</td>
<td>1.06 ± 0.08 (45)</td>
<td>1.02 ± 0.07 (34)</td>
<td>1.04 ± 0.05 (40)</td>
</tr>
<tr>
<td>SOCE</td>
<td>1.00 ± 0.10 (166)</td>
<td>1.32 ± 0.10* (172)</td>
<td>0.99 ± 0.13 (165)</td>
<td>1.00 ± 0.10 (146)</td>
</tr>
<tr>
<td>Resting (Ca2+ In myotube)</td>
<td>83.38 ± 8.85 (206)</td>
<td>83.14 ± 10.12 (151)</td>
<td>87.71 ± 13.98 (164)</td>
<td>88.57 ± 6.91 (191)</td>
</tr>
<tr>
<td>Width of myotubes</td>
<td>1.00 ± 0.05 (114)</td>
<td>1.02 ± 0.05 (120)</td>
<td>1.02 ± 0.04 (120)</td>
<td>0.98 ± 0.05 (123)</td>
</tr>
</tbody>
</table>

The values, except for those of resting Ca2+ level, were normalized to the mean value of those from the control vector. The values are presented as the means ± S.E.M. for the number of myotubes shown in parentheses. *Significant difference compared with the control vector (P < 0.05).

Statistical analysis

The results are presented as the means ± S.E.M. for the number of myotubes shown in parentheses in Tables 2 and 3. The values were normalized to the mean value from the corresponding controls. The significant differences were analysed using a paired Student’s t test (GraphPad InStat, v2.04, GraphPad Software). The differences were considered to be significant at P < 0.05. The graphs were prepared using Origin v7 software.
Role of STIM1 in skeletal muscle

Figure 1  The expression level of Orai1 and STIM1 and the formation of puncta during the differentiation of mouse primary skeletal myoblasts into myotubes

(A and B) Representative immunoblots of Orai1, STIM1 and STIM1L during the differentiation of myoblasts into myotubes (A) and the quantification of the expression level for each protein by differentiation date (B). α-Tubulin was used as a loading control. Three independent experiments were conducted. Band intensities on three independent experiments were measured. The band intensities on each day were normalized to the intensities of corresponding protein (Orai1 or STIM1) on D5 (which were considered to be 1) and presented as means ± S.E.M. For STIM1L, the band intensities of STIM1 on D5 were considered to be 1. (C) Cells during the differentiation of myoblasts into myotubes were double-stained with anti-Orai1 and anti-STIM1 antibodies. The boxed areas in the merged images (a and b) are enlarged in the bottom panels. DIC, differential interference contrast microscopy. The merged images show clear puncta from D4.

Table 3 Properties of STIM1-knockdown myotubes

The values, except for those of the resting Ca$^{2+}$ level, were normalized to the mean value of those from the untransfected control. The values are presented as the means ± S.E.M. for the number of myotubes shown in parentheses. *Significant difference compared with the untransfected control (P < 0.05).

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Untransfected control</th>
<th>Scrambled siRNA</th>
<th>#2 siRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCl response</td>
<td>1.00 ± 0.11 (82)</td>
<td>1.02 ± 0.13 (62)</td>
<td>1.39 ± 0.15* (64)</td>
</tr>
<tr>
<td>Releasable SR Ca$^{2+}$</td>
<td>1.00 ± 0.15 (65)</td>
<td>0.98 ± 0.11 (121)</td>
<td>0.95 ± 0.09 (113)</td>
</tr>
<tr>
<td>Total cellular Ca$^{2+}$ level</td>
<td>1.00 ± 0.11 (60)</td>
<td>0.95 ± 0.10 (57)</td>
<td>1.04 ± 0.08 (63)</td>
</tr>
<tr>
<td>Resting [Ca$^{2+}$]$_{myoplasm}$ (nM)</td>
<td>89.33 ± 3.35 (93)</td>
<td>92.13 ± 7.35 (86)</td>
<td>89.77 ± 4.60 (75)</td>
</tr>
<tr>
<td>Width of myotubes</td>
<td>1.00 ± 0.05 (111)</td>
<td>1.02 ± 0.04 (141)</td>
<td>1.03 ± 0.04 (132)</td>
</tr>
</tbody>
</table>

RESULTS

During the differentiation of mouse primary skeletal myoblasts to myotubes, the times for the maximal expressions of STIM1 and Orai1 differed, and immature myotubes showed puncta during differentiation.

The expression levels of Orai1 and STIM1 were examined during the differentiation of skeletal myoblasts to myotubes using immunoblot assays (Figure 1A). Orai1 expression was not detected in myoblasts on D0. A small amount of Orai1 was detected on D1, and a substantial expression of Orai1 appeared beginning on D2. After an additional increase on D3, Orai1 expression was maintained across further differentiation days after a small decrease. Unlike Orai1, STIM1 expression was detected in the myoblasts on D0. During differentiation, the STIM1 expression gradually increased until D2 and was maintained across further differentiation days after a small decrease. The anti-STIM1 antibody against the full-length STIM1 also detected STIM1L, a splice variant of STIM1. STIM1L expression was detected on D2 and decreased slightly on subsequent differentiation days. The relative amount of STIM1L to STIM1 in fully differentiated myotubes on D5 was approximately 1:7, suggesting that STIM1 is the major isoform in mouse primary skeletal myotubes. These expression profiles raise the possibility that STIM1 could play a role in unique skeletal muscle functions, such as Ca$^{2+}$ release from the SR for ECC, as well as for SOCE.

The formation of puncta by the oligomerization of STIM1 and Orai1 is a prerequisite for SOCE in both excitable and non-excitable cells. During the differentiation of skeletal myoblasts into myotubes, the formation of puncta was examined using immunocytochemistry (Figure 1B). During differentiation, the puncta formed naturally, without stimulus, such as SR Ca$^{2+}$ depletion. The puncta were clearly observed beginning on D4. These results suggest that STIM1 relocation into punctum
K. J. Lee and others

Figure 2  Schematic diagram showing primary sequences of STIM1, the Triple mutant and E136X, and the overexpression of these constructs in mouse primary skeletal myotubes

(A) The position of each domain in STIM1 is presented according to previous reports: Asp76 [22], Asp84 [23], Glu87 [24], OL (oligomerizing region) [28], CAD [26], SOAR and SS (SOAR-strengthening region) [25], T (transmembrane) [56], E136X [38] and the overall diagram [57]. (B) The lysate from myotubes transfected with cDNA encoding wild-type STIM1, the Triple mutant or E136X was subjected to an immunoblot assay with an anti-STIM1 antibody (for wild-type and endogenous STIM1, and the Triple mutant) or an anti-CFP antibody (for E136X, which shows no reactivity with the anti-STIM1 antibody). α-Actin was used as a loading control. Three independent experiments were conducted and a representative result for each protein was presented. Each protein was successfully expressed in the myotubes without affecting the expression level of endogenous STIM1. Molecular mass in kDa is indicated to the left-hand side.

arrangements is a part of the differentiation process. This also supports the possibility that STIM1 could play a role in unique skeletal muscle functions.

With no change in RyR1 activity itself, STIM1 reduces the Ca\textsuperscript{2+} release from the SR to the myoplasm in response to KCl in skeletal myotubes

On the basis of the facts that Ca\textsuperscript{2+} in the SR is the main Ca\textsuperscript{2+} source for skeletal muscle contraction and that STIM1 is an ER/SR Ca\textsuperscript{2+}-sensing protein, the role of STIM1 in Ca\textsuperscript{2+} release from the SR was examined using two distinct STIM1 mutants (Figure 2A): (i) the Triple mutant, missing ER/SR Ca\textsuperscript{2+}-sensing residues (Asp\textsuperscript{76}, Asp\textsuperscript{84} and Glu\textsuperscript{87}), but possessing the intact C-terminus; and (ii) E136X, a truncated mutant that contains intact ER/SR Ca\textsuperscript{2+}-sensing residues but no C-terminus, making it a reverse mutant of the Triple mutant. Indeed, E136X is found in a patient with immunodeficiency and muscular hypotonia [38]. The cDNA for each mutant was transfected into and successfully expressed in mouse primary skeletal myotubes without affecting endogenous STIM1 expression (Figure 2B). The cDNA for the empty vector and wild-type STIM1 were used as negative and positive controls respectively. To measure the Ca\textsuperscript{2+} release from the SR, KCl was applied to myotubes expressing either wild-type STIM1 or one of the mutants (Figure 3A and Table 2). KCl depolarizes t-tubule membranes, activates DHPR and induces the subsequent Ca\textsuperscript{2+} release from the SR to the myoplasm via RyR1, which ultimately results in skeletal ECC (i.e. KCl activates DHPR and the response to KCl reflects ECC). The overexpression of wild-type STIM1 induced a significant decrease in the Ca\textsuperscript{2+} release from the SR in response to KCl compared with the control vector. This suggests that STIM1 could participate in the negative regulation of the Ca\textsuperscript{2+} release from the SR for skeletal ECC, and overexpression of wild-type STIM1 could enforce negative regulation. Similar to wild-type STIM1, the Triple mutant also showed a decrease in Ca\textsuperscript{2+} release from the SR, suggesting that the Ca\textsuperscript{2+}-sensing residues of STIM1 are not responsible for the negative regulation of the Ca\textsuperscript{2+} release by STIM1. E136X did not induce a decrease in the Ca\textsuperscript{2+} release in response to KCl, and even showed an increase in Ca\textsuperscript{2+} release.

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Role of STIM1 in skeletal muscle

Figure 3 The response of myotubes overexpressing wild-type STIM1, the Triple mutant or E136X to KCl or caffeine

KCl (A) or caffeine (B) was applied to the myotubes. Histograms of the peak amplitude normalized to the mean value of those from the control vectors are shown. Myotubes expressing wild-type STIM1 or the Triple mutant showed a significant decrease in Ca\(^{2+}\) release from the SR in response to KCl compared with the control vector, but E136X-expressing myotubes showed a significant increase compared with the control vector. Following caffeine administration, only the E136X-expressing myotubes showed a significant increase in Ca\(^{2+}\) release from the SR compared with the control vector. *Significant difference compared with the control vector (P < 0.05). (C) To measure the width of the myotubes, the thickest part of the myotubes expressing either wild-type STIM1 or one of the mutants (Supplementary Figure S1 at http://www.biochemj.org/bj/453/bj4530187add.htm) was measured. Width values were normalized to the mean value of those from the control vector and presented as histograms. There was no considerable change in the width of the myotubes. (D) Lysate from the myotubes was immunoprecipitated with an anti-STIM1 antibody followed by an immunoblot assay of the immunoprecipitates with an anti-STIM1, anti-DHPR or anti-RyR1 antibody. STIM1 was co-immunoprecipitated with DHPR but not with RyR1. (E) The co-immunoprecipitation of STIM1 with DHPR was conducted in the absence or presence of Ca\(^{2+}\). The interaction between STIM1 and DHPR was independent of the presence of Ca\(^{2+}\). Three independent experiments were conducted and a representative result for each protein is presented in (D) and (E). Ab, antibody; IB, immunoblot; IP, immunoprecipitation.

STIM1 attenuates DHPR activity directly via its C-terminus in skeletal myotubes

There are two possible explanations for the decreased Ca\(^{2+}\) release in response to KCl in myotubes expressing either wild-type STIM1 or the Triple mutant. The wild-type STIM1 and the Triple mutant could attenuate either DHPR activity, as shown in Figure 3(A), or RyR1 activity without affecting DHPR activity. To test the latter scenario, RyR1 activity was assessed by applying a direct agonist of RyR1, caffeine, to myotubes expressing wild-type STIM1 or one of the mutants (Figure 3B). Wild-type STIM1 and the Triple mutant did not induce a change in the Ca\(^{2+}\) release in response to caffeine, suggesting that the decreased Ca\(^{2+}\) release in response to KCl was not due to the attenuation of RyR1 activity. To rule out the possibility that changes in the responses to either KCl or caffeine were due to considerable differences in the size of the myotubes, the widths of the myotubes were measured (Figure 3C). There was not a considerable difference between the widths of the myotubes expressing wild-type STIM1 or one of the mutants compared with that of myotubes transfected with the control vector (Table 2). To examine whether the attenuation of DHPR activity by either wild-type STIM1 or the Triple mutant is direct or via other proteins, the interaction between DHPR and STIM1 was assessed using a co-immunoprecipitation assay with the myotube lysate (Figure 3D). STIM1 was directly bound to DHPR, and the binding was independent of the presence of Ca\(^{2+}\) (Figure 3E). When considering wild-type STIM1, Triple mutant containing the intact C-terminus decreased the Ca\(^{2+}\) release from the SR, whereas E136X, which lacks the C-terminus, did not induce a decrease (Figure 3A). Therefore the C-terminus of STIM1 could be responsible for the direct interaction between STIM1 and DHPR.

In accordance with the unchanged Ca\(^{2+}\) release in response to caffeine in myotubes expressing either a wild-type STIM1 or the Triple mutant (Figure 3B), STIM1 did not directly interact with RyR1 (Figure 3D). Therefore the decreased Ca\(^{2+}\) release in response to KCl by either wild-type STIM1 or the Triple mutant could have been through a direct interaction between STIM1 and DHPR. In addition, the lack of an interaction between STIM1 and RyR1 indicated that the increased RyR1 activity observed for E136X in Figure 3(B) could not have been mediated by the direct interaction of E136X. The increase in Ca\(^{2+}\) by E136X (Figure 3A) could have resulted from the increased RyR1 activity that could have been indirectly triggered by E136X.
Figure 4 The releasable Ca\(^{2+}\) from the SR to the myoplasm, the total cellular Ca\(^{2+}\) level and the expression level of skeletal ECC-mediating proteins in myotubes overexpressing wild-type STIM1, the Triple mutant or E136X

(A) To measure the releasable Ca\(^{2+}\) from the SR to the myoplasm, caffeine (40 mM) was applied to the myotubes loaded with fluo-5N in the absence of extracellular Ca\(^{2+}\). (B) To measure the total cellular Ca\(^{2+}\) level, ionomycin (5 μM) was applied to the myotubes in the absence of extracellular Ca\(^{2+}\). Released Ca\(^{2+}\) by the applications is summarized as histograms in the right-hand panels of (A) and (B) (the area under the curve was normalized to the mean value of those from the control vector). Both the releasable Ca\(^{2+}\) from the SR to the myoplasm and the total cellular Ca\(^{2+}\) level were not changed by the overexpression of wild-type STIM1, the Triple mutant or E136X. (C) Lysate from the myotubes was subjected to an immunoblot assay with one of the antibodies against skeletal ECC-mediating proteins. α-Actin was used as a loading control. Three independent experiments were conducted and a representative result for each protein is presented. The expression level of skeletal ECC-mediating proteins was not changed by the overexpression of wild-type STIM1, the Triple mutant or E136X.

It is possible that the altered responses to KCl or caffeine shown in Figures 3(A) and 3(B) could have been induced by a simple change in the amount of releasable Ca\(^{2+}\) from the SR to the myoplasm. To rule out this possibility, the amount of releasable Ca\(^{2+}\) from the SR to the myoplasm was estimated using caffeine applied to myotubes loaded with fluo-5N (which is suitable for detecting a high level of Ca\(^{2+}\) ranging from micromolar to millimolar) in the absence of extracellular Ca\(^{2+}\) (Figure 4A). There was no significant change in the releasable Ca\(^{2+}\) from the SR to the myoplasm on the basis of either the expression of wild-type STIM1 or one of the STIM1 mutants. In addition, the total cellular Ca\(^{2+}\) level in myotubes was also examined by the administration of a Ca\(^{2+}\) ionophore, ionomycin (Figure 4B). There was also no significant change in the total cellular Ca\(^{2+}\) level by the expression of wild-type STIM1 or one of the STIM1 mutants. The expression levels of the major proteins mediating or regulating Ca\(^{2+}\) movement during skeletal ECC were examined using an immunoblot assay (Figure 4C). None of the eleven proteins that were tested had changed in terms of their expression level. Therefore the altered responses to KCl or caffeine were also not due to changes in the expression level of skeletal ECC-mediating proteins.

STIM1 knockdown in skeletal myotubes removes the attenuation of DHPR by STIM1

To confirm that the reduced response to KCl in myotubes expressing wild-type STIM1 was directly mediated by STIM1, STIM1 was knocked down in myotubes. To interfere with the mRNA of STIM1, two different siRNAs for STIM1 were designed (shown in Table 1) and subjected to transfection into immature myotubes on D3, as described in the Experimental section. A qPCR analysis of cDNA from each transfected myotube batch showed that #2 siRNA was more efficient in knocking down STIM1 (84.5 ± 7.4% reduction in mRNA and ~95% reduction in protein, Figures 5A and 5B). Therefore, #2 siRNA was subjected to further experiments. STIM1-knockdown myotubes showed no significant change in the differentiation to myotubes compared with either untransfected or scrambled siRNA-transfected myotubes (Figure 5C).

The STIM1-knockdown myotubes showed a significant increase in Ca\(^{2+}\) release from the SR in response to KCl compared with either untransfected or scrambled siRNA-transfected myotubes (Figure 5D and Table 3). To rule out the possibility that changes in the responses to KCl were due to the considerable differences in the sizes of the myotubes, the widths of the myotubes were measured. There was no considerable difference in the widths of myotubes transfected with #2 siRNA compared with those of untransfected myotubes (Figure 5E and Table 3). The expression level of DHPR was not significantly changed in the STIM1-knockdown myotubes (Figure 5F). In addition, the expression levels of STIM1-related proteins, such as the partner protein of STIM1, Orai1 or another type of STIM protein, STIM2, were not significantly changed (Figure 5F). These results suggest that STIM1 is...
Figure 5 Knockdown of STIM1 in myotubes and the response of the STIM1-knockdown myotubes to KCl

To interfere with the mRNA of STIM1, myotubes were transfected with the siRNAs for STIM1 or scrambled siRNA as described in the Experimental section. (A) qPCR analysis showed that STIM1 mRNA was reduced by 84.5 ± 7.4 % by #2 siRNA transfection compared with untransfected myotubes. The data are presented as the means ± S.E.M. for the number of myotubes shown in parentheses. *Significant difference compared with the untransfected myotubes (P < 0.05). (B) Myotube lysate from STIM1-knockdown myotubes was subjected to immunoblot analysis with an anti-STIM1 antibody. The expression of the STIM1 protein was reduced by ∼95 %. α-Actin or protein bands stained with Coomassie Brilliant Blue were used as a loading control. (C) Imaging of STIM1-knockdown myotubes. STIM1-knockdown myotubes showed no significant change in the myotube formation compared with either untransfected or scrambled siRNA-transfected myotubes. Scale bar, 300 μm. (D) KCl was applied to the STIM1-knockdown myotubes. Histograms of the peak amplitude normalized to the mean value of those from untransfected myotubes are shown. The STIM1-knockdown myotubes showed a significant increase in Ca^{2+} release from the SR in response to KCl compared with untransfected myotubes. *Significant difference compared with the untransfected myotubes (P < 0.05). (E) For the width measurement of myotubes, the thickest part of the myotubes transfected with either scrambled or #2 siRNA (indicated by white arrows in C) was measured. Images (37, 47 and 44) of myotubes (for untransfected, scrambled siRNA-transfected and #2 siRNA-transfected myotubes, respectively) were used and three random myotubes from each image were used for the measurement. Width values were normalized to the mean value of those from untransfected myotubes and presented as histograms. There was no considerable change in the width of the myotubes. (F) Myotube lysate from STIM1-knockdown myotubes was subjected to an immunoblot assay with anti-DHPR, anti-Orai1 or anti-STIM2 antibody. α-Actin was used as a loading control. Three independent experiments were conducted and a representative result for each protein was presented. The expression level of DHPR, Orai1 or STIM2 was not changed by the knockdown of STIM1.

directly responsible for the negative regulation of DHPR during skeletal ECC. There was no significant change in either the releasable Ca^{2+} from the SR to the myoplasm (by the administration of caffeine, Figure 6A), the total cellular Ca^{2+} levels (by the administration of ionomycin, Figure 6B), or the resting myoplasmic Ca^{2+} levels in STIM1-knockdown myotubes (Figure 6C and Table 3) compared with either untransfected or scrambled siRNA-transfected myotubes. Therefore, as with an overexpression of wild-type STIM1 or one of the STIM1 mutants, the participation of changes in the releasable SR Ca^{2+}, the total cellular Ca^{2+} level or the resting myoplasmic Ca^{2+} level in the increased response to KCl was ruled out.

Puncta irrelevant to SOCE could exist in skeletal myotubes

SOCE was measured in myotubes overexpressing wild-type STIM1 or one of the mutants (Figure 7A). Wild-type STIM1 increased SOCE compared with the control vector (approximately 30 %, Table 2). However, neither the Triple mutant nor E136X
Figure 6 The releasable Ca$^{2+}$ from the SR to the myoplasm, the total cellular Ca$^{2+}$ level and the resting myoplasmic Ca$^{2+}$ level in STIM1-knockdown myotubes

(A) To measure the releasable Ca$^{2+}$ from the SR to the myoplasm, caffeine was applied to the STIM1-knockdown myotubes (loaded with fluo-5N) in the absence of extracellular Ca$^{2+}$. (B) To measure the total cellular Ca$^{2+}$ level, ionomycin was applied to the STIM1-knockdown myotubes in the absence of extracellular Ca$^{2+}$. Ca$^{2+}$ released by the experiments is summarized as histograms in the right-hand panels of (A) and (B) (the area under the curve was normalized to the mean value of those from the untransfected myotubes). Both the releasable Ca$^{2+}$ from the SR to the myoplasm and the total cellular Ca$^{2+}$ level were not significantly changed by the knockdown of STIM1. (C) The resting myoplasmic Ca$^{2+}$ levels in the STIM1-knockdown myotubes was measured. The resting myoplasmic Ca$^{2+}$ level was not significantly changed by the knockdown of STIM1.

increased SOCE, suggesting that neither of these mutants has a dominant-negative effect on SOCE. The participation of the releasable SR Ca$^{2+}$, the total cellular Ca$^{2+}$ level, and the myoplasmic Ca$^{2+}$ level in the change in SOCE was ruled out by the evidence showing that they were unchanged (Figures 4A, 4B and 7B).

The correlation between SOCE and the formation of puncta was also examined via immunocytochemistry using anti-Orai1 and anti-STIM1 antibodies (Figure 7C). Wild-type STIM1 increased the formation of total puncta (by the combination of endogenous STIM1 and exogenously expressed STIM1) compared with the control vector, which agreed with the increased SOCE observed in myotubes that overexpress wild-type STIM1 (Figures 7A, 7C and 7D). Unexpectedly, the Triple mutant also increased the formation of total puncta, although SOCE was not increased by the Triple mutant (Figures 7A, 7C and 7D), suggesting the possibility that puncta irrelevant to SOCE exist in skeletal myotubes. E136X did not increase the formation of puncta compared with the control vector and also did not interfere with the formation of puncta by endogenous STIM1, which was in accordance with the observation that most E136X is not co-localized with endogenous Orai1 (right-hand panel in Figure 7C).

DISCUSSION

In this present study, we examined the role of STIM1 in the Ca$^{2+}$ dynamics of mouse primary skeletal myotubes using two STIM1 mutants (Triple mutant and E136X). On the basis of our data from immunoblot assay, immunocytochemistry, co-immunoprecipitation, various Ca$^{2+}$ imaging experiments and the knockdown of STIM1, we suggest that STIM1 negatively regulates Ca$^{2+}$ release from the SR to the myoplasm through the direct interaction of the STIM1 C-terminus with DHPR in a Ca$^{2+}$-independent manner.

STIM1 could be a housekeeping protein of skeletal muscle

Different lines of evidence support the idea that STIM1 is involved in the developmental stages of skeletal muscle. STIM1 is expressed in the embryo and is up-regulated in adult skeletal muscle, resulting in a mature SR with full SOCE capabilities [48]. During mouse oocyte maturation, STIM1 expression is up-regulated at the germinal vesicle breakdown stage and remains steady during the following maturation progress [49]. STIM1-knockout mice die from a perinatal myopathy and show defects in muscle differentiation [8,32]. STIM1 knockdown from the myoblast stage induces severe defects in the differentiation of myotubes [33]. However, STIM1 knockdown in immature myotubes (during differentiation) does not perturb the differentiation to mature myotubes (Figure 5C and [34]). STIM1 expression is found in myoblasts and is permanently up-regulated in myotubes compared with myoblasts (Figure 1A and [8]), although skeletal ECC-mediating proteins, such as RyR1 and CSQ, are expressed during differentiation [50]. Considering all of these studies, apart from the formation of puncta with Orai1, STIM1 appears to be an upstream housekeeping protein for skeletal muscle development and function.
Figure 7  SOCE, the resting myoplasmic Ca^{2+} level, and the formation of puncta in myotubes overexpressing wild-type STIM1, the Triple mutant or E136X

(A) The SR of the myotubes was depleted following treatment with CPA in the absence of extracellular Ca^{2+}. Extracellular Ca^{2+} (2 mM) was applied to the myotubes to induce SOCE. A representative trace for each group is shown. Only the myotubes overexpressing wild-type STIM1 showed a significant increase in SOCE. The SOCE is summarized in the histograms (the area under the curve was normalized to the mean value of the control vectors). *Significant difference compared with control vectors (P < 0.05). (B) The resting myoplasmic Ca^{2+} levels in the myotubes were measured. The resting myoplasmic Ca^{2+} level was not changed by the overexpression of wild-type STIM1, the Triple mutant or E136X. (C) The myotubes were double-stained with anti-Orai1 and anti-STIM1 antibodies. The boxed areas in the merged images are enlarged in the bottom panels. The merged images show more puncta in the myotubes overexpressing wild-type STIM1 or the Triple mutant, but not E136X. For E136X, the CFP image (right) and the merged CFP image with the Orai1 image (bottom right) are also presented. DIC, differential interference contrast microscopy. (D) The number of puncta per unit area in myotubes is summarized in the histograms (the number of puncta was normalized to the mean value of the control vectors). *Significant difference compared with the control vectors (P < 0.05). The number of puncta was increased only in myotubes transfected with wild-type STIM1 or Triple mutant.

On the other hand, as with the STIM1-knockdown myotubes in the present study, muscle fibres from neonatal STIM1-knockout mice show almost intact fibres compared with those from wild-type mice, although the knockout of STIM1 limits the total growth of skeletal muscle [32]. STIM2, another STIM isoform, is also expressed in mouse and human skeletal muscle [50,51], and STIM1 and STIM2 are functionally largely redundant [51]. Their redundant roles in skeletal muscle could be a possible reason why conditional deletion of STIM1 from the skeletal muscle of mice did not perturb the formation of muscle fibres [32]. STIM1L, a splice variant of STIM1, has been found in mouse and human skeletal muscle and is thought to be responsible
for the formation of SR Ca\(^{2+}\)-independent permanent puncta in skeletal muscle [31]. In the present study, STIM1L was also detected during the differentiation of mouse primary myoblasts to myotubes beginning on D2 (Figure 1A). However, STIM1L is expressed at least 7-fold lower than STIM1 in fully differentiated myotubes, suggesting that STIM1L is not the major isoform in mature skeletal myotubes. In addition, increases in the formation of puncta and SOCE in myotubes overexpressing wild-type STIM1 (Figures 7C and 7D) suggest that STIM1 could also contribute to the formation of the permanent puncta in skeletal muscles. It remains unclear what proportion of the total puncta is formed by STIM1.

In skeletal muscle, there could be a cue for the formation of puncta in the middle of differentiation, apart from the time of expression of STIM1 or Orai1

The expression pattern of STIM1 during the differentiation of the immortal myoblast cell line (C2C12) has been reported previously [8]. However, there has been no report pinpointing the correlation between Orai1 and STIM1 expression or on the correlation between the expression of Orai1/STIM1 and the formation of puncta according to differentiation date. The present study shows that the times required for the maximal expressions of STIM1 and Orai1 are different (D2 and D3 respectively, Figure 1A) and that the times for the appearances of puncta also differ from that for the maximal expression of each protein (from D4, Figure 1B). These results suggest that STIM1 expression has priority over Orai1 expression during differentiation, and more expression does not directly mean more puncta. The latter suggestion raises the possibility that there should be a cue for the formation of puncta during differentiation, apart from the time of expression of STIM1 or Orai1. We used mouse primary skeletal myoblasts and myotubes in the present study, which is a good point because most previous studies have used either immortal skeletal muscle cell lines or heterologous/ectopic expressions of STIM1 or Orai1 in cell lines.

In skeletal muscle, STIM1 in punctum arrangements could have two different conformations, one that is favourable to DHPR attenuation and one that is favourable to SOCE

Unlike other non-excitable cells [22–24], the formation of puncta in skeletal myotubes occurs before the unbinding of Ca\(^{2+}\) from the EF-hand of STIM1 under SR Ca\(^{2+}\) depletion because the puncta are already formed as a part of the differentiation process (Figure 1B and [6]). Puncta are more frequently found in myotubes expressing wild-type STIM1 (Figures 7C and 7D). Similar to wild-type STIM1, the Triple mutant also increases the formation of puncta (Figures 7C and 7D). However, the additional puncta formed in myotubes expressing the Triple mutant seem to differ from the puncta formed by endogenous STIM1 or wild-type STIM1, because the Triple mutant does not increase SOCE (Figure 7A). The SOCE-irrelevant puncta of the Triple mutant seem to favour the attenuation of DHPR activity, because the Triple mutant decreases the Ca\(^{2+}\) release in response to KCl (Figure 3A). Again, this emphasizes that, in skeletal muscle, morphological puncta do not necessarily indicate SOCE, i.e. the formation of puncta is a prerequisite for SOCE, but does not necessarily mean that SOCE will occur. On the other hand, considering the direct interaction between DHPR and STIM1 (Figures 3D and 3E), it is possible that DHPR also contributes to the pre-localization of STIM1 in punctum arrangements during differentiation.

To handle cellular Ca\(^{2+}\), skeletal muscle is equipped with a complicated but co-ordinated system

The major role of DHPR (i.e. Ca\(_{\text{v}}\), 1.1) in skeletal muscle is to ‘activate RyR1 by its conformational changes’ rather than to function as a Ca\(^{2+}\) channel in response to membrane depolarization [1]. What we found in the present study is that the negative regulation of DHPR by STIM1 reduces Ca\(^{2+}\) releases from the SR to the myoplasm through RyR1 in response to KCl. KCl depolarizes membranes and activates DHPR, and the response to KCl reflects ECC. These results suggest that STIM1 in skeletal muscle could participate in ECC as well as SOCE. On the other hand, it has been reported that STIM1 negatively regulates Ca\(_{\text{v}}\), 1.2 (an isoform of DHPR) in neuronal and smooth muscle cells [52,53]. Those studies show that the major role of Ca\(_{\text{v}}\), 1.2 in the neuronal and smooth muscle cells is to function as a Ca\(^{2+}\) channel, and that STIM1 reduced Ca\(_{\text{v}}\), 1.2 channel activity. Therefore STIM1 could negatively regulate both Ca\(_{\text{v}}\), 1.1 and Ca\(_{\text{v}}\), 1.2, but the scope of the work for negative regulation is very different for Ca\(_{\text{v}}\), 1.1 than it is for Ca\(_{\text{v}}\), 1.2.

In skeletal myotubes, ECC (excitation-coupled Ca\(^{2+}\) entry) occurs during the very initial phase of ECC [54]. ECC is activated by membrane depolarization without SR depletion, but questions remain as to the corresponding protein for ECC and how ECC is involved in the initial phase of ECC. A STIM1-knockdown study has suggested that, at least, ECCE and SOCE are two distinct Ca\(^{2+}\) entry pathways in skeletal myotubes [34]. However, considering reports that STIM1 and STIM2 are functionally largely redundant in skeletal muscle [51] and that ECC and SOCE are interrelated by STIM1 (in the present study), much more remains to be investigated about the correlations among ECC, ECCE and SOCE, and there must be a complicated, but co-ordinated, system to handle cellular Ca\(^{2+}\) in skeletal muscle.

STIM1 could be a balancing protein of Ca\(^{2+}\) movements between ECC and SOCE in skeletal muscle

The SR Ca\(^{2+}\) content during the initial phase of skeletal ECC begins to decrease gradually as a result of local Ca\(^{2+}\) movements into the myoplasm through RyR1, and the decreasing SR Ca\(^{2+}\) content could be the initial step in SR Ca\(^{2+}\) depletion followed by STIM1 activation for SOCE. The refilling of the SR Ca\(^{2+}\) store by SERCA and SOCE during skeletal muscle relaxation should precede the next ECC. Therefore these two different SR Ca\(^{2+}\) store-mediated events, SOCE and ECC, never occur simultaneously with a maximal value, but one should always follow another with ‘a regularly overlapping period,’ similar to sine and cosine waves in mathematics (\(y = \sin x\) and \(y = \cos x\), Figure 8). To achieve the sine–cosine relationship between SOCE and ECC, at least one protein (like x in the equations) should balance the two events and, until now, STIM1 was thought to be the best qualified protein for mediating both events. In this scenario, punctum arrangements of STIM1 in the triad junction of skeletal muscle are well positioned for the fast, efficient and tight regulation of the two events. Similarly, Edwards et al. [16] have suggested a tight relationship between the two events by measuring Ca\(^{2+}\) waves in the myoplasm (reflecting the Ca\(^{2+}\) release from the SR, i.e. the SR-depletion during ECC) and in sealed t-tubules (reflecting SOCE) from skinned mouse skeletal muscle fibres: one event is on when the other is off, which is different from our sine–cosine scenario showing a regularly overlapping period. Under the experimental conditions in Edwards et al. [16], 130 mM K\(^+\) in t-tubules maintains sealed
extracellular space into the myoplasm during SOCE. SOCE and ECC, two different SR Ca\(^{2+}\) movements from the SR to the myoplasm (representing ECC, indicated by a broken line) fluctuate like the cosine wave: peaks indicate maximal Ca\(^{2+}\) movement into the myoplasm for maximal contraction. The following sine-like wave (representing SOCE, indicated by a solid line) presents Ca\(^{2+}\) movements from extracellular space into the myoplasm during SOCE. SOCE and ECC, two different SR Ca\(^{2+}\)-store-mediated events; never occur simultaneously with a maximal value, but one should always follow another with ‘a regularly overlapping period’, similar to cosine and sine waves in mathematics (SOCE looking like the curve of \(y = \sin x\) and ECC looking like the curve of \(y = \cos x\)). More details of the model graph are explained in the Discussion section.

Figure 8 Relationship between SOCE and ECC: a model graph for Ca\(^{2+}\) movement into the myoplasm during repetitive skeletal ECC accompanied by SOCE

During the contraction–relaxation cycle of skeletal muscle, Ca\(^{2+}\) movements from the SR to the myoplasm (representing ECC, indicated by a broken line) fluctuate like the cosine wave: peaks indicate maximal Ca\(^{2+}\) movement into the myoplasm for maximal contraction. The following sine-like wave (representing SOCE, indicated by a solid line) presents Ca\(^{2+}\) movements from extracellular space into the myoplasm during SOCE. SOCE and ECC, two different SR Ca\(^{2+}\)-store-mediated events; never occur simultaneously with a maximal value, but one should always follow another with ‘a regularly overlapping period’, similar to cosine and sine waves in mathematics (SOCE looking like the curve of \(y = \sin x\) and ECC looking like the curve of \(y = \cos x\)). More details of the model graph are explained in the Discussion section.

t-tubules and induces constitutively active DHPRs, the results of which may have different explanations.

**STIM1 could be an amplitude-limiting protein of SOCE in skeletal muscle**

Given the observed Orai1 expression (Figure 4C), SOCE was increased by the overexpression of wild-type STIM1 (Figure 7A), suggesting that STIM1 is an amplitude-limiting protein in the SOCE of skeletal muscle. This is supported by the fact that the maximal opening of one functional Orai1 channel (four Orai1 monomers) requires an octameric STIM1 oligomer, and SOCE is not an all-or-nothing event; rather, it undergoes a graded process via the binding of different numbers of STIM1 [55]. In terms of the oligomerization of STIM1, there are two possible explanations for the increased SOCE in myotubes overexpressing wild-type STIM1 (Figure 7A). One is the maximization of the activity of each Orai1. The overexpression of wild-type STIM1 increases the number of octameric STIM1 oligomers enough to induce the maximal activation of each Orai1. The other is by activating previously unactivated spare Orai1s. The formation of more STIM1 oligomers due to the overexpression of wild-type STIM1 activates more Orai1s. In either case, STIM1 could be an amplitude-limiting protein for SOCE.

**Skeletal myopathy in a patient with the E136X mutation could be due to the mislocalization of the truncated STIM1**

The E136X mutation, which lacks the C-terminus, did not increase SOCE or interfere with the formation of puncta by endogenous STIM1 (Figures 7A, 7C and 7D). Accordingly, E136X was not co-localized with endogenous Orai1 (Figure 7C), suggesting that E136X is not located in the triadic junction. Therefore the myopathy due to the lack of SOCE found in the patient with the E136X mutation [38] could have been due to the mislocalization of the truncated STIM1 as well as to the absence of the C-terminal Orai1-activating SOAR or CAD. With regard to ECC, the E136X-expressing myotubes in the presence of endogenous STIM1 is dominant-negative in overcoming the attenuation of DHPR activity by endogenous STIM1 without affecting the myoplasmic Ca\(^{2+}\) level, the releasable SR Ca\(^{2+}\) or the total cellular Ca\(^{2+}\) level (Figures 3A, 4A, 4B and 7B), suggesting that the presence of E136X (either transiently or permanently) with endogenous STIM1 could act as a muscle contractor by increasing RyR1 sensitivity to agonists, as shown in Figures 3(A) and 3(B).

**AUTHOR CONTRIBUTION**

Keon Jin Lee and Eun Hui Lee designed the experiments and wrote the paper. Keon Jin Lee, Jin Seok Woo, Ji-Hye Hwang and Changdo Hyun conducted the experiments. Keon Jin Lee, Chung-Hyun Cho, Do Han Kim and Eun Hui Lee contributed to the data analysis. Chung-Hyun Cho, Do Han Kim and Eun Hui Lee contributed to the discussion of the results.

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SUPPLEMENTARY ONLINE DATA

STIM1 negatively regulates Ca\(^{2+}\) release from the sarcoplasmic reticulum in skeletal myotubes

Keon Jin LEE*, Jin Seok WOO*, Ji-Hye HWANG†, Changdo HYUN*, Chung-Hyun CHO†, Do Han KIM‡ and Eun Hui LEE*1

*Department of Physiology, College of Medicine, The Catholic University of Korea, Seoul 137-701, Korea, †Department of Pharmacology and Ischemic/Hypoxic Disease Institute, College of Medicine, Seoul National University, Seoul 110-799, Korea, and ‡Department of Life Science, Gwangju Institute of Science and Technology, Gwangju 500-712, Korea

Figure S1 Width measurement of myotubes expressing wild-type STIM1 or one of its mutants

To rule out the possibility that changes in the responses to KCl or caffeine were owing to the considerable differences in the size of the myotubes, the widths of the thickest part in each myotube expressing wild-type STIM1 or one of the mutants (indicated by white arrows) was measured using the ImageJ program (NIH). Images of the myotubes expressing wild-type STIM1 or one of the mutants, shown in Figure 7(C) of the main text, were used to measure the width, and representative images are presented in this Figure. Images (38, 40, 40 and 41) of myotubes (for control vector, wild-type STIM1, Triple mutant and E136X respectively) were used and three random myotubes from each image were used for measurement. DIC (differential interference contrast microscopy) images are shown in the first row, images stained with an anti-STIM1 antibody are shown in the second row, and a CFP image of E136X is shown in the bottom row. The results are summarized as histograms in Figure 3(C) and Table 2 of the main text. There was no considerable difference in the width of myotubes expressing wild-type STIM1 or one of the mutants compared with that of myotubes transfected with the control vector.

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1 To whom correspondence should be addressed (email ehui@catholic.ac.kr).