



Overexpression of transient receptor potential canonical type 1 (TRPC1) alters both store operated calcium entry and depolarization-evoked calcium signals in C2C12 cells

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ABSTRACT

When the intracellular calcium stores are depleted, a Ca^{2+} influx is activated to refill these stores. This store-operated Ca^{2+} entry (SOCE) depends on the cooperation of several proteins as STIM1, Orai1, and, possibly, TRPC1. To elucidate this role of TRPC1 in skeletal muscle, TRPC1 was overexpressed in C2C12 cells and SOCE was studied by measuring the changes in intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$). TRPC1 overexpression significantly increased both the amplitude and the maximal rate-of-rise of SOCE. When YM-58483, an inhibitor of TRPC1 was used, these differences were eliminated, moreover, SOCE was slightly suppressed. A decrease in the expression of STIM1 together with the downregulation of SERCA was confirmed by Western-blot. As a consequence, a reduction in maximal Ca^{2+} uptake rate and a higher resting $[\text{Ca}^{2+}]_i$ following the Ca^{2+} transients evoked by 120 mM KCl were detected. Morphological changes also accompanied the overexpression of TRPC1. Differentiation of the myoblasts started later, and the myotubes were thinner in TRPC1-overexpressing cultures. For these changes the observed decrease in the nuclear expression of NFAT1 could be responsible. Our results suggest that enhanced expression of TRPC1 increases SOCE and has a negative effect on the STIM1-Orai1 system, indicating an interaction between these proteins.

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1. Introduction

In eukaryotic cells the endoplasmic reticulum (ER) serves as an intracellular store for and enables the rapid release of calcium ions upon activating stimuli. The consequent increase in intracellular calcium concentration ($[\text{Ca}^{2+}]_i$) is the key regulator in a number of different processes. As recognized more than two decades ago [1] the depletion of the ER initiates an influx of calcium ions through the surface membrane to replenish these intracellular calcium stores. The process termed store operated calcium entry (SOCE) and its importance has since been described in a wide variety of cell types (for recent reviews see e.g. [2,3]), including both excitable and non-excitable cells, and been shown to play a fundamental role in the calcium homeostasis of these cells.

Although the basic properties of SOCE have been characterized in detail, the molecular make-up of the calcium entry pathway has

remained elusive. Experiments have first brought the attention to members of the transient receptor potential (TRP) family of surface membrane channels. In particular, members of the canonical sub-family, especially TRPC type 1 (TRPC1) was suggested to be a store operated cationic channel [4]. Recently, the stromal interacting molecule 1 (STIM1) and its surface membrane interactive partner Orai1 were suggested, respectively, to form the ER calcium sensor and the entry pathway [5–9]. However, dissecting the interacting site(s) between STIM1 and Orai1 revealed that STIM1 can also interact with TRPC1 [10] and, furthermore, TRPC1 might also be a member of the molecular complex formed by STIM1 and Orai1 [11,12].

Skeletal muscle is very special in its calcium handling since extracellular calcium entry is not required for its major calcium-dependent process, the contraction. Upon depolarization calcium ions are released from the sarcoplasmic reticulum (SR) via specialized calcium release channels (Ryanodine receptor type 1; RyR1) following its interaction with the voltage-sensor dihydropyridine receptors (DHPR) of the surface and transverse-tubular membranes (for review see e.g. [13]). After repolarization calcium ions are effectively transported back to the SR by the calcium pump (sarcoplasmic reticulum calcium ATPase, SERCA).

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Until very recently the presence and importance of SOCE in skeletal muscle has thus remained elusive. Experiments in the past years have, however, revealed that not only is SOCE important in the alterations accompanying aging [14] but are rapidly activated and deactivated during normal excitation–contraction coupling (ECC) [15,16]. Together with the demonstration of the presence of both STIM1 and Orai1 (e.g. [17]) and in independent studies of TRPC1 in skeletal muscle (e.g. [18]), this led to the conclusion that SOCE, albeit not involved directly in ECC, is functional and important in skeletal muscle, too.

As a calcium entry pathway, the activation of SOCE and of TRPC1 channels in particular, were suggested to regulate muscle differentiation. Reduced TRPC1 expression by RNA interference was shown to suppress myoblast fusion and migration [19]. TRPC1 channels have, to date, been also associated with a number of different pathologies in skeletal muscle, which involve the alteration of calcium homeostasis. Since TRPC1 can also be activated by mechanical stress [20,21] its involvement in Duchenne muscular dystrophy (DMD) [18,22,23] and in the myogenesis in C2C12 cells [21] has also been proposed. This idea was strengthened by the findings that in skeletal muscles from *mdx* mice, an animal model of DMD, TRPC1 is overexpressed [24], while suppressing its expression in *mdx* mice lowers both store-operated and stretch-activated calcium entry as well as the opening of channels carrying inward Ca^{2+} currents in cell-attached patches [18]. The same group recently showed that a small-conductance channel was lost in fibers from TRPC1 knock-out mice [25]. Voltage-clamp studies, on the other hand, failed to demonstrate an inward current that could be associated with the activation of this influx pathway [26,27], while recently, Berbey et al. [28] reported that TRPC1 was not expressed in the sarcolemma in adult skeletal muscle fibers rather in the longitudinal SR where it operates as a Ca^{2+} leak channel.

In the current study we demonstrate an increased SOCE associated with the overexpression of TRPC1 suggesting that TRPC1 behaves differently in adult muscle than in differentiating muscle cells. We present, for the first time, evidence that this overexpression not only manifested as an increased influx following pharmacological store depletion but also as an elevated resting $[\text{Ca}^{2+}]_i$ after depolarization-evoked calcium signals. Moreover, TRPC1 overexpression also suppressed the expression level of STIM1 and SERCA but not of RyR1, Calsequestrin or Orai1 suggesting an interaction of TRPC1 with the former molecules in the SR membrane.

2. Materials and methods

2.1. Cell cultures and transfection

C2C12 mouse skeletal muscle cell line were obtained from the European Collection of Cell Cultures (ECACC) and were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Sigma, Budapest, Hungary) supplemented with 10% fetal bovine serum (FBS), 50 U/ml penicillin, and 50 $\mu\text{g}/\text{ml}$ streptomycin and were incubated at 37 °C in a humidified incubator with 5% CO_2 and 95% O_2 (according to the instructions of the supplier). Full length coding sequence of human TRPC1 was cloned into pcDNA3.1 expression vector. Stable transfection was performed in Opti-MEM reduced serum content medium using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) for 2.5 h at 37 °C. Cells were allowed to express the introduced genes for 48 h in growth medium then were selected in DMEM containing 1 mg/ml geneticin. After 14–15 days, single colonies were isolated and experiments were carried out on separated clones of TRPC1 transfected cells. To obtain controls for the transfection, cells were mock-transfected by the empty pcDNA 3.1 vector using the same protocol, and

experiments were carried out on pools of transfected cells. Differentiation of non-transfected, referred to as parental hereon, and transfected cells was induced at 80% confluency by exchanging the culture medium for DMEM supplemented with 5% horse serum (HS), 2% FBS and penicillin–streptomycin. The efficiency of TRPC1 overexpression was monitored at mRNA level by RT-PCR and at protein level by immunostaining and Western-blot using a specific anti-TRPC1 antibody corresponding to the C-terminal end of the peptide. Functional experiments were carried out on 5–6-day-old differentiated myotubes of parental and transfected cultures.

2.2. Immunostaining

Cultured cells were washed with ice-cold phosphate-buffered saline (PBS; 0.02 M NaH_2PO_4 , 0.1 M NaCl), fixed with -20°C 100% methanol for 15 min, permeabilized with 0.1% Triton X-100 in PBS for 10 min, and blocked with 1% bovine serum albumin (BSA) diluted in PBS (blocking solution) for 30 min at room temperature. The cells were then incubated overnight at 4 °C with the anti-TRPC1 primary antibody (dilution was 1:100 in blocking solution), corresponding to intracellular amino acid residues 557–571 of TRPC1 produced in rabbit (Alomone Labs Ltd., Jerusalem, Israel), the anti-STIM1 primary antibody (dilution was 1:50 in blocking solution), corresponding to amino acid residues 56–69 of STIM1 produced in rabbit (Alomone Labs Ltd.), and the anti-Orai1 primary antibody (dilution was 1:50 in blocking solution) corresponding to amino acid 288–301 of Orai1 produced in rabbit (Sigma). Then fluorescein (FITC) or Cy3 labeled anti-rabbit secondary antibody was applied for 1 h at room temperature. Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA, USA) was used to make the nuclei visible. Images were taken using LSM 510 META confocal microscope (Zeiss, Oberkochen, Germany).

2.3. Preparation of cell extracts and nuclei

Cells in cultures were washed with ice-cold PBS, harvested in homogenization buffer (20 mM Tris–Cl, 5 mM EGTA, 1 mM 4-(2-aminoethyl) benzenesulphonyl fluoride, 20 μM leupeptin, pH 7.4; all from Sigma) and disrupted by sonication on ice. Nucleus rich preparation of the cultures was obtained as described earlier [29]. Protein content of the samples was measured by a modified bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA) using BSA as a standard.

2.4. Western-blot analysis

Total cell lysates were examined by Western-blot analysis. Samples for SDS–PAGE were prepared by the addition of 1/5 volume of 5-fold concentrated electrophoresis sample buffer (310 mM Tris–HCl, pH 6.8; 10% SDS, 50% glycerol, 100 mM DTT, 0.01% bromophenol blue) to cell lysates and boiled for 5 min at 80 °C. About 50 μg of protein was separated by 7.5% SDS–PAGE gel for immunological detection of examined proteins. Samples were transferred electrophoretically to nitrocellulose membranes (BioRad, Wien, Austria). After blocking with 5% non-fat dry milk in PBS, membranes were incubated with the following primary antibodies overnight at 4 °C: anti-TRPC1 primary antibody (dilution: 1:200), anti-STIM1 primary antibody (dilution: 1:500), anti-Orai1 primary antibody (dilution: 1:500), anti-Calsequestrin primary antibody produced in mouse (dilution: 1:200, BD Transduction Laboratories, USA), anti-Ryanodine receptor primary antibody produced in mouse (dilution: 1:2500, Affinity BioReagents, USA), anti-SERCA primary antibody (dilution: 1:1000), and anti-NFAT1 (nuclear factor of activated T-cells) primary antibody (dilution: 1:1000, Abcam,

Cambridge, UK), anti-actin primary antibody produced in rabbit (dilution 1:200, Santa-Cruz Biotechnology Inc., Heidelberg, Germany). After washing three times for 10 min with PBST (PBS supplemented with 0.1% Tween 20), membranes were incubated with a secondary antibody, peroxidase-conjugated goat anti-rabbit, and anti-mouse IgG (BioRad) in 1:1000 dilution in PBS containing 5% non-fat dry milk for 1 h. Signals were detected by enhanced chemiluminescence (ECL) reaction (Thermo Scientific, Budapest, Hungary).

2.5. RT-PCR analysis

For RT-PCR analysis, colonies were washed three times with ice cold PBS, snap-frozen in liquid nitrogen and stored at -70°C . Total RNA was isolated from myotubes using Qiagen RNeasy® Mini Kit according to the instructions of the manufacturer (Qiagen, Budapest, Hungary). The assay mixture (20 μl) for reverse transcriptase reaction (Omniscript, Qiagen) contained 500 ng RNA, 0.25 μl RNase inhibitor, 0.25 μl oligo (dT), 2 μl dNTP (200 μM), 1 μl M-MLV RT in $1 \times$ RT buffer. Amplifications of specific cDNA sequences were performed with specific primers (Integrated DNA Technologies, Coralville, IA, USA) that were designed based on published human and mouse TRPC1 nucleotide sequences (GenBank accession no.: NM.003304, forward primer: 5'-GATGTGCTTGGGAGAAATGC-3', reverse primer: 5'-GTCCAAACCAACCGTGTC-3'). PCR reactions were allowed to proceed in a final volume of 50 μl (containing 2 μl forward and reverse primers, 1 μl dNTP (200 μM), and 0.5 μl Promega GoTaq® DNA polymerase in $1 \times$ reaction buffer) in a programmable thermocycler (Eppendorf Mastercycler, Netheler, Hinz GmbH, Hamburg, Germany) with the following settings: 2 min at 95°C for initial denaturation followed by repeated cycles of denaturation at 94°C for 1 min, primer annealing for 60 s at an optimized temperature, and extension at 72°C for 1 min 30 s. After the final cycle, further extension was allowed to proceed for another 10 min at 72°C . PCR products were analyzed using a 2% ethidium bromide-stained agarose gel.

2.6. Examination of apoptosis

Abolishment of mitochondrial membrane potential is one of the earliest markers of apoptosis [30]. To assess the process, mitochondrial membrane potential of parental and TRPC1-overexpressing C2C12 myotubes was determined using a MitoProbe DiI $\text{C}_1(5)$ Assay Kit (Invitrogen). 9–9 wells of parental and TRPC1-overexpressing myotubes were cultured in 96-well black-well/clear-bottom plates (Greiner Bio One), and additional 3–3 wells of each cell type was pre-treated with the protonophore carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) to obtain positive control for apoptosis. After removal of supernatants, cells were incubated with DiI $\text{C}_1(5)$ working solution (100 μl /well), and the fluorescence of DiI $\text{C}_1(5)$ was measured at 630-nm excitation and 670-nm emission wavelengths using FLIPR [31,32].

2.7. Determination of viable cell number

The viability of the cells was determined by measuring the conversion of the tetrazolium salt MTT (Sigma-Aldrich) to formazan by mitochondrial dehydrogenases. Parental and TRPC1-overexpressing cells were plated in 8–8 wells of 24-well plates and were differentiated into myotubes. Cells were then incubated with 0.5 mg/ml MTT for 2 h, and the concentration of formazan crystals (as an indicator of the viable cell number) was determined colorimetrically (A570) according to the manufacturer's protocol.

2.8. Whole cell calcium measurements

Changes in $[\text{Ca}^{2+}]_i$ were measured using the calcium sensitive fluorescent dye Fura-2 as reported earlier [33]. Myotubes were placed on a coverslip and incubated with Fura-2 AM (acetoxymethyl ester; 10 μM) for 1 h (37°C , 5% CO_2) in DMEM supplemented with 10% FBS and neostigmin to inhibit acetylcholinesterase. Furthermore, cells were equilibrated in normal Tyrode's solution (NTY; in mM, 137 NaCl, 5.4 KCl, 0.5 MgCl_2 , 1.8 CaCl_2 , 11.8 HEPES, 1 g/l glucose, pH 7.4, all from Sigma) for 30 min at room temperature. Coverslips with the Fura-2 loaded cells were then placed on the stage of an inverted fluorescent microscope (Diaphot; Nikon, Tokyo, Japan). Measurements were performed in normal or calcium-free Tyrode's solution. To obtain a calcium-free Tyrode's solution no CaCl_2 was added in the presence of 5 mM EGTA (Sigma). The SERCA inhibitor thapsigargin (Sigma) was used at a final concentration of 4 μM in calcium-free Tyrode's solution. The SOCE blocker, non-specific TRPC antagonist YM-58483 (Sigma) was used in 1 μM concentration. KCl was applied in 120 mM final concentration in NTY by replacing equal amount of NaCl. Cells were continuously washed with NTY using a background perfusion system. Test solutions were directly applied onto the cells through a perfusion capillary tube (Perfusion Pencil™; AutoMate Scientific, San Francisco, CA, USA) with an internal diameter of 250 μm at a 0.35 ml/min rate, using a local perfusion system (Valve Bank™ 8 version 2.0, AutoMate Scientific). All measurements were performed at room temperature. Excitation wavelength was alternated between 340 and 380 nm by a dual wavelength monochromator (Deltascan, Photon Technology International, New Brunswick, NJ, USA), while the emission was monitored at 510 nm using a photomultiplier.

2.9. Calculation of $[\text{Ca}^{2+}]_i$ and the Ca^{2+} flux

$[\text{Ca}^{2+}]_i$ was calculated from the ratio of fluorescence intensities ($R = F_{340}/F_{380}$) using an *in vivo* calibration ($R_{\min} = 0.2045$, $R_{\max} = 8.315$, $K_d \times \beta = 1183 \text{ nM}$). To determine the activity of the Ca^{2+} pump and the Ca^{2+} flux entering the myoplasmic space, the Ca^{2+} binding to intracellular binding sites and the removal of Ca^{2+} from the intracellular space was modeled as presented in earlier reports [33,34]. In brief, Ca^{2+} binding to intracellular sites—to the SERCA pump, to troponin C, to parvalbumin and to the dye—and the removal were considered. The computer routine determined, as a single best fit parameter, the maximal transport rate of the Ca^{2+} pump ($PV_{\max}$) from the declining phase of the Ca^{2+} transient following the stimulation. All other parameters in the model were held constant at values taken from the literature [35].

All values were expressed as mean $\pm$ standard error (SE) of the mean, statistical significance was calculated using Student's *t*-test assuming significance for $p < 0.05$.

2.10. Analysis of the rate of proliferation and differentiation

Parental and TRPC1-overexpressing myoblasts and myotubes were cultured under the same conditions in Petri-dishes. 10–10 pictures of randomly selected visual fields were taken on each day of culturing both in parental and transfected cells. The number of cells at the beginning of the experiment was the same ($p > 0.2$). To calculate the rate of proliferation the number of nuclei were determined and the value was divided by the number of nuclei counted on the previous day. To obtain the fusion index the number of nuclei in multinucleated myotubes were compared to the total number of nuclei after induction of differentiation. Experiments were carried out in triplicates.

3. Results

3.1. Cell culturing and transfection

The effect of TRPC1 overexpression was examined in a mouse skeletal muscle cell line. Endogenous expression of this non-specific cation channel could be demonstrated in C2C12 myotubes (Fig. 1A, left panel). TRPC1 protein synthesis has been amplified using a pcDNA3.1 plasmid-vector system, and the stable overexpression of the protein in the selected clones was confirmed with immunocytochemistry at the myotube stage (Fig. 1A, right panel). A membrane-bound localization was clearly visible mostly in the TRPC1-overexpressing myotubes but a cytoplasmic appearance was also detectable in both parental and transfected cells. The efficiency of overexpression was confirmed both at mRNA (Fig. 1B) and protein (Fig. 1C) levels in order to select the clone showing the most significant TRPC1 expression from the plasmid. RT-PCR reactions were performed using mRNA sequences of human and mouse TRPC1. Amplimers of the expected sizes were identified and mRNA overexpression from the plasmid was clearly observable in clone number 8, TRPC1-overexpressing myotubes were differentiated from these cells. Western-blot analysis also confirmed the overexpression of the TRPC1 protein in the clone mentioned above. Quantitative analysis of the results revealed a significant 344% ($p < 0.01$) increase in TRPC1 expression in the selected clone as compared to non-transfected parental C2C12 cells (Fig. 1D). TRPC1 expression in two other clones which overexpress the protein to a lesser extent (referred to as clones 11 and 10) is demonstrated in Supplementary Fig. 1A. Parental and TRPC1-transfected, differentiated, 5 days old C2C12 myotubes were used for functional experiments.

3.2. Apoptosis and the viability of cells

To examine whether the TRPC1 overexpression induces apoptosis in the cell cultures, mitochondrial membrane potential of parental and TRPC1-overexpressing C2C12 myotubes was determined in 9–9 wells of a 96-well plate, using a MitoProbe DiI_{C1}(5) Assay Kit (Fig. 1E). Additional 3–3 wells of parental and transfected cultures were pre-treated with the protonophore CCCP to obtain positive controls for apoptosis. In TRPC1-overexpressing myotubes the mitochondrial membrane potential did not decrease significantly compared to parental, which suggests that the overexpression of TRPC1 did not increase the extent of apoptosis in these cultures. To determine the viability of parental and transfected myotubes, MTT assay was performed (Fig. 1F). The difference in the absorbance at 570 nm was not significant between the two cell types. These results suggest that the overexpression of TRPC1 did not alter the viability of the C2C12 myotubes.

3.3. Effect of TRPC1 overexpression on SOCE and Ca^{2+} -homeostasis

We first examined whether or not the overexpression of the protein alters the SOCE mechanism of the myotubes. To this end the changes in $[\text{Ca}^{2+}]_i$ evoked by re-establishing the normal 1.8 mM extracellular calcium concentration ($[\text{Ca}^{2+}]_o$) were measured in parental (Fig. 2A) and in TRPC1-overexpressing C2C12 myotubes (Fig. 2B). Beforehand the internal calcium-stores were emptied by the application of 4 μM thapsigargin (TG) in the absence of $[\text{Ca}^{2+}]_o$. As a result of TRPC1 overexpression, the amplitude and the maximal rate of rise of SOCE was significantly increased in TRPC1-overexpressing C2C12 myotubes. In accordance with the above, pooled data showed that TRPC1 overexpression resulted in a significantly higher maximal rate-of-rise ($3.7 \pm 0.6 \text{ nM/s}$, $n = 20$ in parental and $6.1 \pm 0.7 \text{ nM/s}$, $n = 24$ in TRPC1-overexpressing C2C12

myotubes) and amplitude ($129 \pm 6 \text{ nM}$, $n = 14$, and $210 \pm 19 \text{ nM}$, $n = 24$, respectively) of SOCE indicating the possibility that more TRPC1 Ca^{2+} -channels were available as a Ca^{2+} -influx pathway. Importantly, SOCE in mock-transfected myotubes proved to be similar ($3.0 \pm 0.3 \text{ nM/s}$, $n = 12$ for the maximal rate-of-rise and $155 \pm 10 \text{ nM}$, $n = 13$ for the amplitude of SOCE; Supplementary Fig. 1D and E; $p > 0.3$) to parental cells. If other clones overexpressing TRPC1 to a lesser extent (clones 11 and 10; Supplementary Fig. 1B and C) were examined the magnitude of their SOCE seemed to correlate with the level of TRPC1 expression.

On the other hand, the resting intracellular calcium concentration was similar ($p > 0.2$) in both groups ($73.0 \pm 1.6 \text{ nM}$, $n = 40$ in parental and $76.8 \pm 3.1 \text{ nM}$, $n = 32$ in TRPC1-overexpressing myotubes; Fig. 3A). In addition, no significant difference ($p > 0.5$) could be detected in the amplitude of thapsigargin-evoked calcium transients in the absence of external calcium between parental and TRPC1-overexpressing myotubes (96.4 ± 15.9 and $126.8 \pm 15.8 \text{ nM}$ respectively; Fig. 3B). This suggests that the Ca^{2+} -content of the SR did not change as a result of TRPC1 overexpression.

When parental (Fig. 2C) and transfected (Fig. 2D) C2C12 myotubes were pre-treated with the non-specific TRPC inhibitor YM-58483 (4-methyl-40-[3,5-bis(trifluoromethyl)-1Hpyrazol-1-yl]-1,2,3-thiadiazole-5-carboxanilide) applied together with TG before measuring SOCE, these differences were not only eliminated, rather, the development of SOCE was slightly slower in TRPC1-overexpressing myotubes. The drug significantly decreased the maximal rate of rise of SOCE in parental and transfected ($1.8 \pm 0.3 \text{ nM/s}$, $n = 19$, and $1.1 \pm 0.2 \text{ nM/s}$, $n = 16$ respectively), and the amplitude in transfected ($88.5 \pm 7.5 \text{ nM}$, $n = 16$) C2C12 myotubes as compared to the untreated cells. In the presence of the drug SOCE was slightly but not significantly lower in TRPC1-overexpressing myotubes than in parental, suggesting the downregulation of the STIM1-Orai1 system (Fig. 3C and D).

3.4. Altered expression and functional activity of other proteins involved in calcium signaling

To confirm this hypothesis the significant decrease in the expression level of STIM1 was confirmed by Western-blot together with the downregulation of SERCA. In contrast the expression of Actin, Orai1, RyR, and Calsequestrin remained unchanged (Fig. 4A). Quantitative analysis of the results also confirmed the significant alterations in STIM1 and SERCA expression in TRPC1 in overexpressing as compared parental cultures (Fig. 4B). The expression of STIM1 and Orai1 (see Supplementary Fig. 2), and the aggregation of STIM1 following store depletion (Supplementary Fig. 3) was also detected by immunocytochemistry.

To examine the functional consequences of the overexpression of TRPC1, and of those above, Ca^{2+} -transients were evoked by individual (Fig. 5A and B) or repeated (Fig. 5C and D) applications of 120 mM KCl. The amplitude of these transients, the underlying Ca^{2+} flux, the changes in the resting $[\text{Ca}^{2+}]_i$ following the transients and the maximal transport rate of the Ca^{2+} pump (PV_{max}) were compared in parental (Fig. 5A and C) and TRPC1-overexpressing myotubes (Fig. 5B and D). Neither the amplitude of the transients ($235 \pm 30 \text{ nM}$, $n = 16$ and 230 ± 23 , $n = 26$ respectively) nor the underlying flux ($188 \pm 21 \mu\text{M/s}$ and $193 \pm 14 \mu\text{M/s}$ respectively) were altered significantly ($p > 0.8$) in TRPC1-overexpressing myotubes (Fig. 5E), suggesting that the Ca^{2+} release via RyRs and the function of the voltage-dependent Ca^{2+} -channels were not altered. On the other hand, following the transients $[\text{Ca}^{2+}]_i$ declined slower—especially evident in the traces with repeated application using a longer time scale (Fig. 5D)—and returned to a significantly higher level in the transfected myotubes (the difference in the resting $[\text{Ca}^{2+}]_i$ before and after the transients was $3.5 \pm 0.6 \text{ nM}$ in parental and $11.0 \pm 1.2 \text{ nM}$ in TRPC1-overexpressing

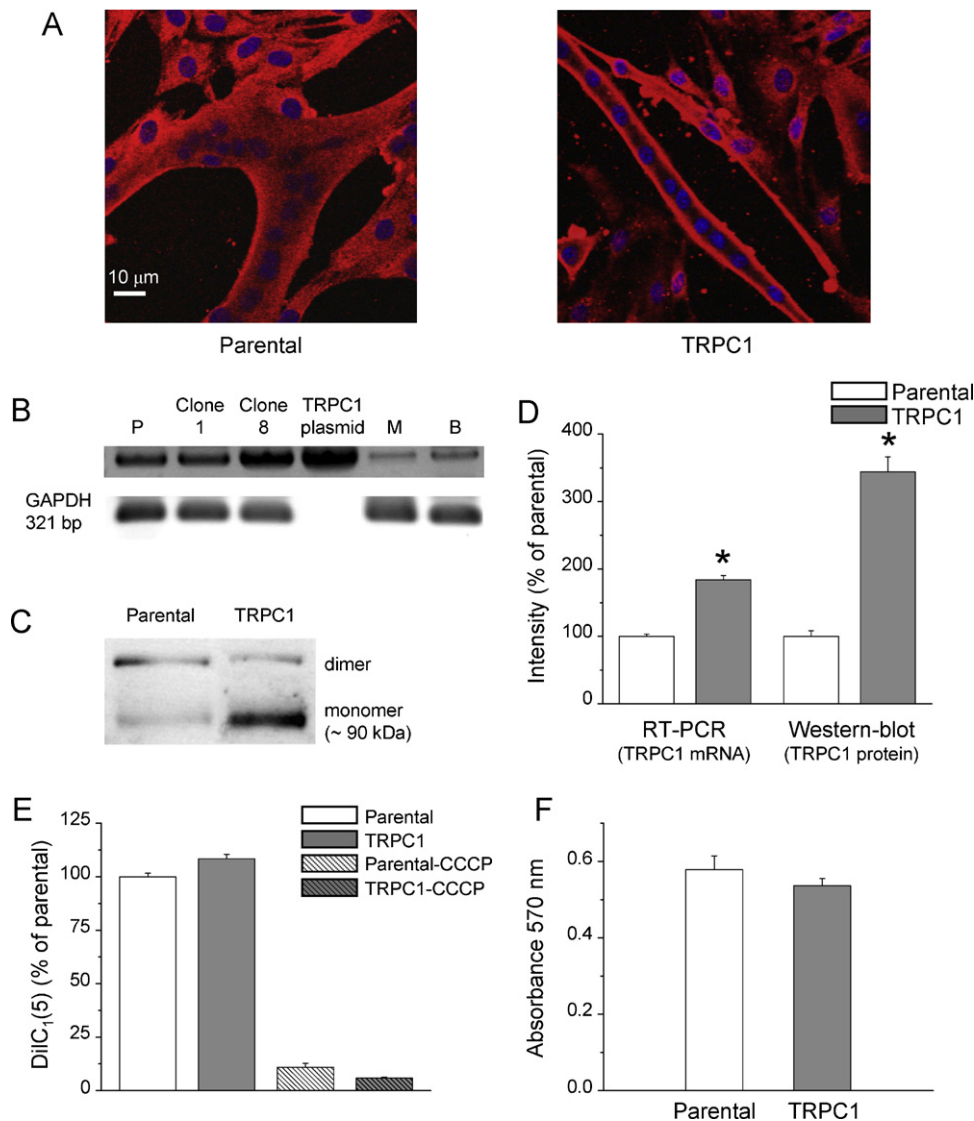


Fig. 1. Examination of TRPC1 overexpression in C2C12 myotubes. (A) Immunocytochemical staining of parental and transfected C2C12 skeletal muscle cultures demonstrating the presence of TRPC1 protein. Images were recorded from 1 μ m thick optical slices. Original magnification was 40 $\times$. (B) mRNA expression of TRPC1 was detected by RT-PCR. Samples from different transfected clones and tissues (P—parental C2C12 cells; B—brain; M—muscle) were compared. GAPDH was used as a control. (C) Western-blot analysis of TRPC1 was performed to prove the overexpression at protein level. The 87 kDa human isoform was expressed from the plasmid used for stable transfection. (D) Quantitative analysis of both RT-PCR and Western-blot results obtained from parental and transfected myotubes. Here, and in all subsequent figures asterisks (*) mark significant difference between parental and TRPC1 overexpressing cells and data represent mean $\pm$ standard error (SE) of the mean. (E) Examination of apoptosis by measuring the abolishment of mitochondrial membrane potential. CCCP was used as a positive control. (F) Determination of cell viability. Parental and TRPC1-overexpressing cells were compared using MTT-assay.

myotubes) indicating a decreased Ca^{2+} uptake capability of the SERCA pumps (Fig. 5F). This was quantified by extracting (see Section 2) PV_{max} , which was clearly ($p < 0.01$) decreased in TRPC1-overexpressing myotubes ($342 \pm 20 \mu\text{M/s}$) as compared to parental ($465 \pm 39 \mu\text{M/s}$) in line with the decreased expression of SERCA.

To examine the possible role of TRPC1 channels as a leak pathway in the higher resting $[\text{Ca}^{2+}]_i$ after the transients, TRPC1-overexpressing myotubes were pre-treated with YM-58483 to inhibit TRPC channels. Although $[\text{Ca}^{2+}]_i$ after the transients was higher than in parental myotubes, it was significantly lower ($7.3 \pm 1.1 \text{ nM}$, $n = 26$) than in the untreated TRPC1-overexpressing myotubes which could indicate a partial role of the Ca^{2+} leak via the TRPC1 channels. The idea that the leak through the TRPC1 channels rather than the downregulation of SERCA was responsible for this increase in resting $[\text{Ca}^{2+}]_i$ was strengthened by the finding that PV_{max} was similar to that in parental cells ($496 \pm 24 \mu\text{M/s}$).

3.5. Comparisons of differentiation of parental and TRPC1-overexpressing cells

Morphological alterations of cells in TRPC1-overexpressing cultures were observed during the differentiation and compared to those of parental cells (Fig. 6A and B). Cultured under the same conditions multinucleated myotubes showed a delayed appearance (day 4) and thinner diameter (day 6, arrows) in TRPC1-overexpressing cultures. To confirm these alterations, the rate of proliferation (Fig. 6C) and the fusion index (Fig. 6D) were analyzed on each day of culturing. When the cells were plated, the number of parental and TRPC1-overexpressing myoblasts did not differ significantly ($p > 0.2$). On the 2nd day of culturing the rate of proliferation was not significantly higher in the transfected cultures, as compared to parental (2.78 ± 0.16 and 2.44 ± 0.13 , respectively). After exchanging the proliferating medium to differentiating medium, the proliferation ended in parental cultures

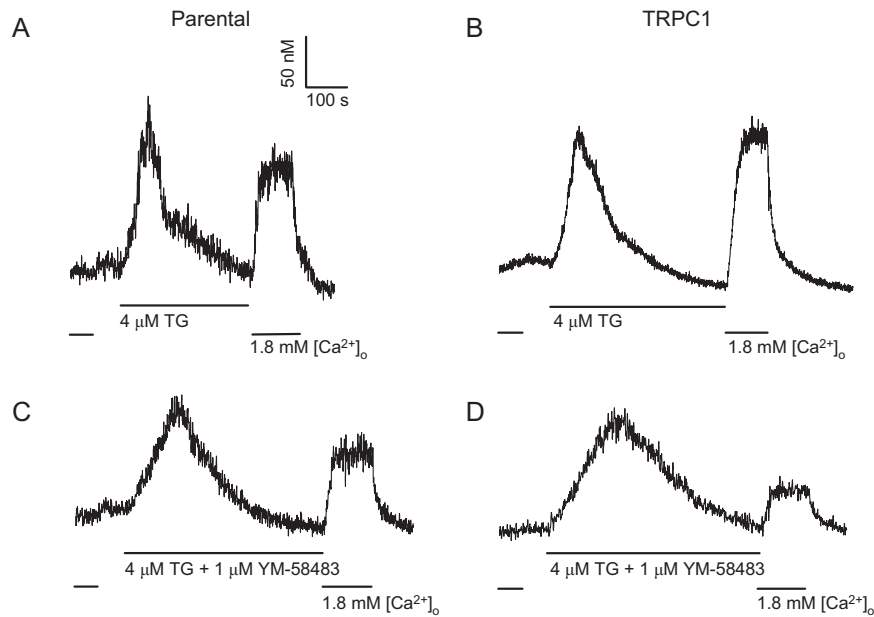


Fig. 2. Functional examination of store operated calcium entry (SOCE) in terminally differentiated C2C12 myotubes. Changes in calcium concentration demonstrating SOCE were recorded following the re-establishing of the normal (1.8 mM) external calcium concentration ($[Ca^{2+}]_o$) in parental (A) and in TRPC1 overexpressing (B) C2C12 myotubes. The internal calcium-stores were emptied by 4 μ M thapsigargin (TG) in the absence of $[Ca^{2+}]_o$. Representative records of 5 independent cultures. Parental (C) and transfected (D) C2C12 myotubes were pre-treated with the SOCE inhibitor YM-58483 applied together with TG before measuring SOCE. Representative records of 5–5 independent cultures.

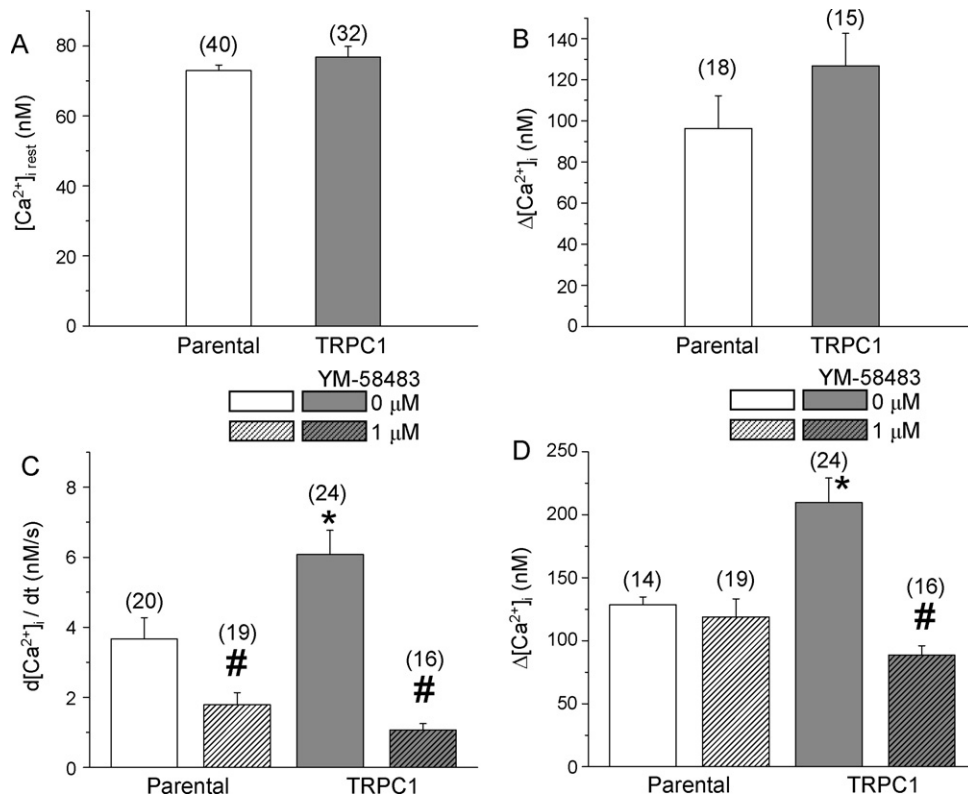


Fig. 3. Characteristic parameters of $[Ca^{2+}]_i$ changes in parental and TRPC1 overexpressing C2C12 myotubes. (A) Values of resting calcium concentration ($[Ca^{2+}]_{i,rest}$). (B) Pooled data for the amplitude of thapsigargin-evoked calcium transients in the absence of external calcium. (C) Mean values of the maximal rate of rise of SOCE (calculated as $d[Ca^{2+}]_i/dt$) after the re-administration of external calcium. Measurements were performed after the application of either thapsigargin alone or thapsigargin together with 1 μ M YM-58483. (D) Pooled data for the amplitude of SOCE measured following the re-establishing the normal (1.8 mM) external calcium concentration. Measurements were performed after the application of either thapsigargin alone or thapsigargin together with 1 μ M YM-58483. Hashmarks (#) indicate significant ($p < 0.01$) change between data obtained in the absence and presence of YM-58483. Numbers in parentheses indicate the number of cells measured.

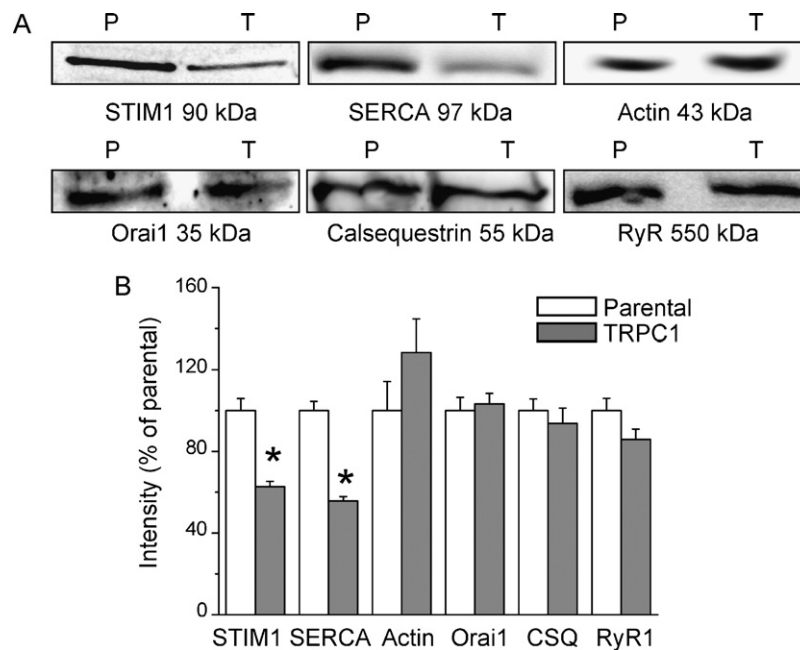


Fig. 4. (A) Western-blot analysis showing the altered expression of other proteins involved in calcium signaling. Total protein samples (P—parental C2C12 myotubes, T—TRPC1-overexpressing C2C12 myotubes) were used (50 μ g in each lane) to examine the expression level. (B) Quantitative analysis of Western-blot results (mean $\pm$ SE) obtained from parental and transfected myotubes.

and the cells started to form multinucleated myotubes (day 3). In contrast, the transfected cells showed a prolonged proliferation until day 4. The ratio of differentiated myotubes was higher in the parental cultures as compared to transfected on each day of differentiation. These results confirmed the observations obtained visually, that is, TRPC1-overexpressing cultures start to differentiate later and form thinner myotubes. This comparison was also made with mock-transfected myotubes, but these morphological changes could be observed only for the TRPC1-overexpressing myotubes (Supplementary Fig. 4C).

To find an explanation for these morphological changes and the decreased ability of differentiation of the TRPC1-overexpressing cultures, NFAT1 expression was examined in nuclear fractions and total cell lysates of parental and transfected cultures by Western-blot analysis (Fig. 6E). While NFAT1 expression did not show significant difference in total cell lysates, its expression was significantly reduced in the nuclear fractions of TRPC1-overexpressing myotubes as compared to parental (Fig. 6F). These results were confirmed by the application of an NFAT-reporter plasmid developed by Rosenberg and co-workers [36] (Supplementary Fig. 4A and B).

4. Discussion

SOCE was described in non-excitable cells several decades ago, but its existence and function in skeletal muscle was revealed only in the past few years [15,37,38]. This Ca^{2+} entry process is required not only for refilling the intracellular Ca^{2+} stores, but it also has a considerable role in regulating the differentiation of myotubes (e.g. [17,19]). Although originally SOCE has been suggested to involve TRPC1 channels in skeletal muscle, the role of these channels in the Ca^{2+} entry mechanism became controversial (e.g. [39]) after STIM1, the Ca^{2+} sensor of the SR [5,6], and Orai1 the pore-forming subunit [7–9] of the SOC channel in the plasma membrane were discovered, and recent data enlightened the connection between these molecules. In addition, transgenic mice overexpressing another member of the TRPC family, TRPC3, exhibit increased SOCE [40]. STIM1, nonetheless, proved to be regulating not only Orai1 but also TRPC1 [10–12,41], and TRPC1 is likely to be involved in the forma-

tion of a complex responsible for SOCE together with [42–44] or independently from STIM1 and Orai1 [45].

In our study we provide evidence that the overexpression of TRPC1 increases SOCE and, furthermore, it alters the expression of other proteins involved in the Ca^{2+} homeostasis of skeletal muscle cells in culture and has an effect on the differentiation of multinucleated myotubes. The overexpression of TRPC1, on the other hand, neither affected the viability nor did it result in the apoptosis of the cells.

Following the depletion of internal Ca^{2+} -stores the amplitude and the maximal rate of rise of SOCE was significantly increased in TRPC1-overexpressing C2C12 myotubes suggesting the possible role of TRPC1 in Ca^{2+} -influx. This elevated SOCE could either be due to the fact that the TRPC1 channel was directly involved in the entry mechanism or that its overexpression somehow augmented the STIM1–Orai1 pathway. While the latter cannot be excluded completely, the former seems more likely based on the finding that STIM1 expression was down- rather than up-regulated, suggesting a reduced STIM1–Orai1 interaction. Indeed, the functional consequence of altered STIM1 expression could be observed following the application of YM-58483 which blocked the TRPC1 channels and supposedly only the Orai1 channels were available for SOCE. Under these conditions the Ca^{2+} -influx was reduced suggesting a lesser involvement of the STIM1–Orai1 pathway. On the other hand, despite of the reduced STIM1 expression STIM1 puncta could still be observed following store depletion (see Supplementary Fig. 3) suggesting that STIM1 aggregation to puncta was not impaired. One can speculate, based on the above findings, that if TRPC1 channels are indeed part of the SOCE complex activated by STIM1 following store depletion, the reduced STIM1 expression in TRPC1-overexpressing cells might be a compensatory response from the cells to maintain the tightly regulated Ca^{2+} -homeostasis.

In this framework one would expect that different levels of TRPC1 expression should result in different magnitudes of SOCE. To confirm it experimentally with precise quantitative measurements is, however, hard. On the one hand, the overall expression level of TRPC1 does not necessarily correlate directly with the amount of the protein found in the surface membrane and, there-

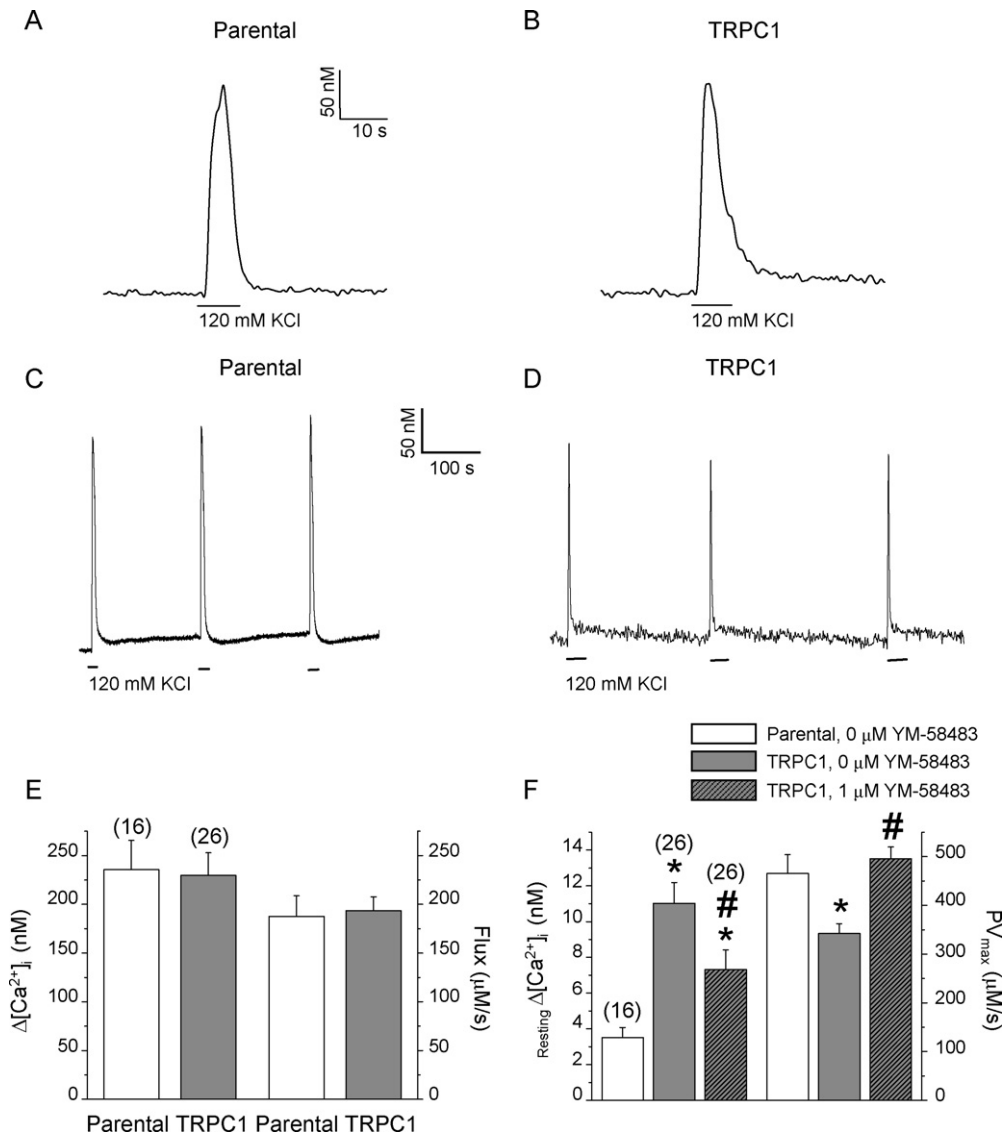


Fig. 5. Functional effects of TRPC1 overexpression on depolarization-evoked calcium transients. Representative records of calcium transients evoked by single (A and B) or repetitive (C and D) 120 mM KCl in parental (A and C) and in TRPC1 overexpressing (B and D) C2C12 myotubes. (E) The amplitude of the calcium transients and the maximal calcium flux (the amount of calcium entering the cell in one second) of parental and TRPC1 overexpressing myotubes. KCl-evoked calcium transients of TRPC1-overexpressing myotubes treated by 1 μM YM-58483 were also examined. Numbers in parentheses indicate the number of cells measured.

fore, with the number of putative Ca^{2+} entry channels. In addition, even if the above correlation would be confirmed, the amplitude and rate-of-rise of SOCE transients are greatly influenced by the buffering capacity of the intracellular space. Nevertheless, different TRPC1 overexpressing clones with different levels of expression were compared and an acceptable degree of correlation was found between the level of TRPC1 expression and the magnitude of SOCE (see [Supplementary Fig. 1](#)). In particular, both the maximal rate-of-rise and the amplitude of SOCE in these clones increased with increasing level of TRPC1 expression. In clone 11 the maximal rate-of-rise of SOCE was similar (3.3 ± 0.4 nM/s, $n = 14$) to that of the mock-transfected cells and significantly lower than in TRPC1-overexpressing cells (clone 8). In clone 10, on the other hand, it did not differ significantly ($p > 0.05$) if compared to TRPC1-overexpressing cells (5.4 ± 0.3 nM/s, $n = 7$), but it was significantly higher than in the mock-transfected cells ([Supplementary Fig. 1D](#)).

It should be noted that a reduced SERCA expression was also clearly observable in TRPC1-overexpressing cells. The effect was especially pronounced following the Ca^{2+} -transients evoked by the

addition of 120 mM KCl. Both a slowed removal of Ca^{2+} from the myoplasmic space and the elevated resting $[Ca^{2+}]_i$ following the Ca^{2+} -transients were clearly detectable in TRPC1-overexpressing cells. The former seems to be a direct consequence of the reduced SERCA expression as confirmed by the lower maximal transport rate of the calcium pump (PV_{max}) estimated from the removal model. While the latter could also be explained by the reduced rate of elimination of Ca^{2+} from the myoplasm, it is intriguing to speculate that it is the consequence of an elevated and prolonged calcium influx through TRPC1 channels in response to store depletion. This idea is strengthened by the finding that this elevation in resting $[Ca^{2+}]_i$ following the Ca^{2+} -transients could be eliminated by blocking the TRPC1 channels.

It is intriguing to speculate what could be the importance of the reduced SERCA expression. It was clearly established in our earlier reports (e.g. [33]) that in cultured skeletal muscle cells the second and subsequent calcium transients of repeated KCl stimulation are greater than the first (see also [Fig. 5C](#)). This is due to the influx of Ca^{2+} from the external solution and the consequent

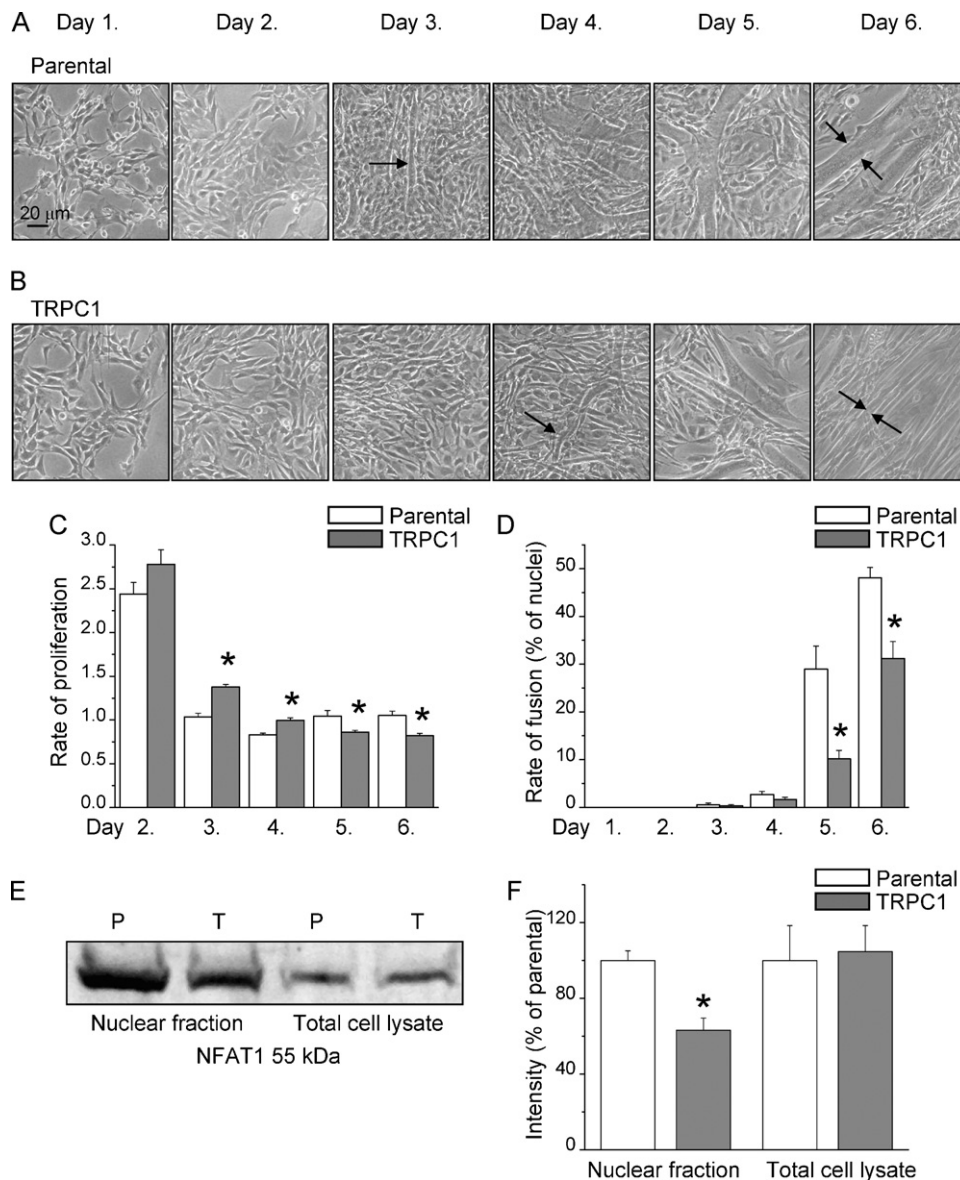


Fig. 6. Representative images of differentiating C2C12 cells on different days of culturing. Parental (A) and TRPC1 overexpressing (B) C2C12 myotubes were cultured under the same conditions. The proliferating solution was exchanged to differentiating solution on the 2nd day of culturing. Images were recorded at a magnification of 20 $\times$. Note the delayed appearance (4th day) and thinner diameter (6th day, arrows) of the multinucleated myotubes in TRPC1 overexpressing cultures. (C) Rate of proliferation calculated as the ratio of the number of nuclei of the given and of the previous day of culturing in parental and TRPC1-overexpressing cells. (D) Fusion index, expressed as percentage of nuclei in multinucleated myotubes as compared to all nuclei. (E) Western-blot analysis showing the expression of NFAT1 in nuclear fractions and in total cell lysates of parental (P) and TRPC1-overexpressing (T) myotubes. (F) Quantitative analysis of Western-blot results (mean $\pm$ SE) obtained from parental and transfected myotubes.

increase in SR Ca^{2+} load. In TRPC1 overexpressing cells there is an even more pronounced elevation in $[\text{Ca}^{2+}]_i$ after each and every KCl-evoked Ca^{2+} transient (Fig. 5F). One would, therefore, expect an even more pronounced elevation in SR load and in the amplitude of the calcium transients. Fig. 5D clearly demonstrates that there is no such increase in the amplitude of these transients in TRPC1 overexpressing cells. We believe that this is the direct consequence of the reduced SERCA activity. That is, a lesser amount of the Ca^{2+} that enters the cell during the depolarization is transported into the SR and, consequently, the SR content is not augmented after each transient, rather, the calcium is slowly transported out through the surface membrane.

In spite of the fact that neither the resting $[\text{Ca}^{2+}]_i$ nor the Ca^{2+} -content of the SR was changed significantly, alterations in the differentiation process were detectable. The proliferation was

prolonged after changing to differentiating medium and thinner myotubes developed. It cannot be excluded, however, that the above described elevation in $[\text{Ca}^{2+}]_i$ following stimulation is responsible for these alterations. Indeed, the essential role of TRPC1 in skeletal muscle differentiation was described previously [19], whereas as a consequence of the silencing of TRPC1 expression the migration of myoblasts became slower and differentiation did not occur. Similar observation, reduced fiber cross-sectional area, was founded in mice lacking the Homer 1 protein and associated to the altered scaffolding of TRPC1 [46]. In addition, another TRPC channel, TRPC3, was suggested to be crucial in dendritic growth and development in cerebellar Purkinje cells further linking this channel family to differentiation in excitable cells [47]. Taken these results together, we can propose that both the downregulation and the overexpression of TRPC1 alters the Ca^{2+} homeostasis of the

cells and decreases the ability of the myoblasts to differentiate. Supporting these observations, the expression of the key regulator of skeletal muscle differentiation, NFAT1 [48,49] decreased in the nuclei of TRPC1-overexpressing myotubes in our experiments which could be responsible for the decreased ability to differentiate in these cells.

Interestingly though, no overall changes in resting $[Ca^{2+}]_i$ was observed in TRPC1 overexpressing cells. One could, therefore, speculate that the activation of calcineurin, the crucial step of NFAT translocation should not be altered in these cells. We, on the other hand, believe that the decisive step is the transient but prolonged elevation of $[Ca^{2+}]_i$ observed in TRPC1 overexpressing cells following stimulation. This transient rise in $[Ca^{2+}]_i$ initiates a transient activation of calcineurin and a temporal nuclear translocation of NFAT. It should be mentioned that a parallel increase in the expression of RCAN1-4, a regulator protein enabling a negative feedback mechanism that inhibits calcineurin and NFAT translocation [50] could also explain the observations. Although at this point the exact mechanism is not clear, the altered calcium homeostasis and the consequent NFAT translocation seem to be the underlying steps in the altered differentiation of TRPC1 overexpressing cells.

Recent results in adult fibers have demonstrated that TRPC1 operates in the SR as a Ca^{2+} leak channel [28]. Although our data obtained in C2C12 myotubes cannot exclude that overexpressed TRPC1 contributes at least in part to the increased SOCE by generating an increased SR Ca^{2+} leak, our immunolabeling and functional data strongly suggest that TRPC1 behaves differently in cultured myotubes as compared to adult muscle. To conclude, we suggest that enhancing the expression level of TRPC1 increases SOCE and has a negative feedback effect on both the STIM1–Orai1 system and SERCA activation, suggesting a cooperation between these proteins.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ceca.2011.03.012.

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