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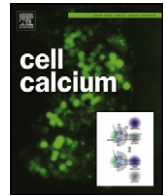
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# Dystrophin/ $\alpha$ 1-syntrophin scaffold regulated PLC/PKC-dependent store-operated calcium entry in myotubes

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### ABSTRACT

In skeletal muscles from patient suffering of Duchenne Muscular Dystrophy and from *mdx* mice, the absence of the cytoskeleton protein dystrophin has been shown to be essential for maintaining a normal calcium influx. We showed that a TRPC store-dependent cation influx is increased by loss of dystrophin or a scaffolding protein  $\alpha$ 1-syntrophin, however the mechanisms of this calcium mishandling are incompletely understood. First of all, we confirmed that TRPC1 but also STIM1 and Orai1 are supporting the store-operated cation entry which is enhanced in dystrophin-deficient myotubes. Next, we demonstrated that inhibition of PLC or PKC in dystrophin-deficient myotubes restores elevated cation entry to normal levels similarly to enforced minidystrophin expression. In addition, silencing  $\alpha$ 1-syntrophin also increased cation influx in a PLC/PKC dependent pathway. We also showed that  $\alpha$ 1-syntrophin and PLC $\beta$  are part of a same protein complex reinforcing the idea of their inter-relation in calcium influx regulation. This elevated cation entry was decreased to normal levels by chelating intracellular free calcium with BAPTA-AM. Double treatments with BAPTA-AM and PLC or PKC inhibitors suggested that the elevation of cation influx by PLC/PKC pathway is dependent on cytosolic calcium. All these results demonstrate an involvement in dystrophin-deficient myotubes of a specific calcium/PKC/PLC pathway in elevation of store-operated cation influx supported by the STIM1/Orai1/TRPC1 proteins, which is normally regulated by the  $\alpha$ 1-syntrophin/dystrophin scaffold.

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## 1. Introduction

In skeletal muscle, dystrophin, a 427 kD protein is located beneath the plasma membrane [1]. This protein binds the cytoskeleton via actin and the extracellular matrix via a complex of proteins and glycoproteins, called Dystrophin-Associated Proteins (DAPs) [2]. Because of its key localization, dystrophin provides a mechanical link from the intracellular cytoskeleton to the extracellular matrix and is thought to play a role of shock absorber [1]. Thus, dystrophin is essential for skeletal muscle and a defect of the protein due to genetic alteration in the p21 band of X chromosome causes Duchenne Muscular Dystrophy (DMD), a progressive degenerative muscle disease [3]. The absence of the dystrophin-scaffold also affects DAPs' targeting to the membrane and causes an alteration of various signaling cascades. The

disruption of dystrophin-based complex is suggested to promote aberrant Ca<sup>2+</sup> handling in muscular dystrophy and changes in MAP kinase and GTPase signaling [4]. Dystrophin-deficiency has been shown to impair Ca<sup>2+</sup> channels activity and cytosolic Ca<sup>2+</sup> concentration [5–7], which results in activation of Ca<sup>2+</sup>-sensitive proteases and fiber necrosis [8,9]. Moreover, forced minidystrophin expression was shown to restore Ca<sup>2+</sup> handling and normal Ca<sup>2+</sup> release properties in both myotubes and fibers [10–12].

Among channels which could be affected by the absence of dystrophin, a higher activity of divalent cationic channels with properties close to SOCs (Store-Operated Channels) have been recorded in dystrophic fibers from *mdx* mouse [13,14]. Measurements of store-dependent manganese entry in *mdx* flexor digitorum brevis (FDB) fibers [15] as well as in *mdx* and dystrophin-deficient myotubes [16,17] confirmed that SOCE (Store-operated Ca<sup>2+</sup> entry) is enhanced in mouse dystrophic muscle. Moreover, forced expression of minidystrophin was indeed able to restore normal SOCE in dystrophin-deficient myotubes [16,17]. In addition, a loss of the subsarcolemmal scaffolding protein  $\alpha$ 1-syntrophin alone was sufficient to induce abnormal divalent cation influx dependent on TRPC1 in myotubes [18]. In dystrophin-deficient myotubes, the

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mechanism may be dependent on the  $\alpha 1$ -syntrophin PDZ domain since increased cation influx were restored by expression of the full-length protein but not with the one lacking N-terminal segment (containing the PDZ domain) [18]. Nevertheless, the mechanism activating an elevated cation influx in dystrophin-deficient muscle, although clearly dependent on scaffolding proteins is still unknown. It was also shown that dystrophin-deficient myotubes display increased production of  $IP_3$  [19] and higher  $Ca^{2+}$  release activity dependent on  $IP_3$  receptor [20], which suggest an elevated activity of the phospholipase C (PLC) when dystrophin-associated complex is disrupted. In several cell types, store-depletion induced  $Ca^{2+}$  entry is dependent on basal PLC activity [21–23] and can be activated by a number of mediators involved in the PLC signaling cascade, including  $Ca^{2+}$  [24,25] or PKC [26–28]. In human endothelial cells, thapsigargin, used for store depletion protocols activates a PLC/PKC pathway usually triggered by agonist stimulation [29].

We recently proposed that divalent cation entry in skeletal muscle cells was regulated by the association of endogenous dystrophin or recombinant minidystrophin and  $\alpha 1$ -syntrophin with TRPC1 and TRPC4 channels [17,18]. TRPC1 was also shown to be linked to caveolin-3 [30], which raised the idea that TRPC1 is incorporated in the dystrophin-associated complex, which maintains normal cation entry through these channels. In addition, artificially increased TRPC-dependent  $Ca^{2+}$  entry was recently shown to be sufficient to induce muscular dystrophy in mouse independently on membrane fragility [31]. These studies support the idea that higher divalent cation entry supported by TRPC1 channels participates to the development of dystrophic phenotype. In skeletal myotubes, we previously showed that store-dependent cation influx activated by caffeine and Cyclic Piazonic Acid (CPA) were reduced when TRPC1 was knock-down [18]. However, a small conductance channel (13 pS) that is completely lost in adult fibers from TRPC1<sup>-/-</sup> mice was not stimulated by thapsigargin [32]. An other study revealed, in skeletal muscle, high expression of the  $Ca^{2+}$  sensor protein STIM1 [33], and its requirement in SOCE and contractile function [33]. The interaction between STIM1 and Orai1 channels functioning as highly  $Ca^{2+}$ -selective CRAC channels was shown to carry store-operated  $Ca^{2+}$  entry [34–36]. SOCE recorded in cultured myotubes were also shown to depend on STIM1 and Orai1 [37,38]. In another hand, STIM1 carboxyl-terminus binds TRPC1 which activates native SOCs [39], and heteromultimerizes TRPC channels [40]. Orai1 was also shown to interact with TRPC channels [41] and to be required in store-operated TRPC1–STIM1 channels [42]. The store-dependent cation entry elevated by the absence of dystrophin and  $\alpha 1$ -syntrophin may thus involve not only TRPC1 channels but also Orai1 channels and STIM1 activation.

In the present study, we explored the dependency of store-operated cation entry on TRPC1, Orai1 and STIM1 pathways in dystrophin-deficient myotubes. We analyzed the mechanism supporting the elevation of cation entry due to the absence of dystrophin or  $\alpha 1$ -syntrophin. The role of PLC/PC pathway and of cytosolic  $Ca^{2+}$  was investigated in the abnormal elevation of store-dependent cation entry.

## 2. Materials and methods

### 2.1. Antibodies and drugs

Antibodies against TRPC1 (Sigma–Aldrich, Saint Louis, USA), phospho-Ser/Thr PKA/PKC substrate (Cell Signaling Technology, Inc., Danvers, USA) and PLC $\gamma$  (Santa-Cruz Biotechnology, Inc., Heidelberg, Germany) were rabbit polyclonal antibodies (pAb). Antibody against PLC $\beta$  (Santa Cruz Biotechnology, Inc., Heidelberg, Germany),  $\alpha$ -tubulin (Sigma–Aldrich, Saint Louis, USA), phospho-Tyr and STIM1 (BD Biosciences, NJ,

USA) were mouse monoclonal. U73122 (1-(6-((17 beta-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1H-pyrrole-2,5-di one), U73343 (1-[6-(((17 $\beta$ )-3-Methoxyestra-1,3,5[10]-trien-17-yl)amino)hexyl]-2,5-pyrrolidinedione), BAPTA-AM (1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis-(acetoxymethyl ester)) and CPA (Cyclopiazonic acid) were from Sigma–Aldrich (Sigma–Aldrich, Saint Louis, USA). TPEN (N,N,N',N'-tetrakis-(2-pyridylmethyl)-ethylenediamine) and chelerythrine were from Calbiochem (Calbiochem, San Diego, USA).

### 2.2. Cell culture and transfections

Experiments were performed on two cell lines: SolC1, dystrophin-deficient (dys<sup>-</sup>) and Sold6 expressing minidystrophin (minidys<sup>+</sup>). These cell lines were cultured as described previously, and forced expression of minidystrophin allowed readdressing members of the dystrophin-associated complex, the recovery of  $Ca^{2+}$  handling [11] and normal cation entry in dystrophin-deficient myotubes [16]. Transfection of small interfering RNA (siRNA) for silencing of TRPC1 or  $\alpha 1$ -syntrophin and of control siRNA (sequence with no homology with any known eukaryotic gene: Eurogentec) were performed on myoblasts the day of seeding using nucleofector transfection device (Kit V, Amaxa, Lonza, Basel, Switzerland) and cultured on plastic dishes or on glass coverslips coated with gelatin (1% in sterile water, Sigma–Aldrich, Saint Louis, USA). Transfection of STIM1 siRNA was performed on myoblasts using Lullaby<sup>®</sup> siRNA Transfection Reagent (OZ Biosciences, Marseille, France). Myoblasts were transfected with STIM1 siRNAs one day after fusion initiation.

Transfection of Orai1 dominant negative E106Q MO70 (bicistronic plasmid encoding EGFP) and, as a control, pmaxGFP<sup>®</sup> (Lonza), was performed using jetPRIME<sup>™</sup> (Polyplus transfection<sup>™</sup>) transfection reagent. Cells were seeded as described above. Double transfections were performed in myotubes using the Amaxa nucleofection (Kit V, Lonza). Myoblasts was transfected with 2  $\mu$ g of TRPC1 siRNA and 2  $\mu$ g of Orai1 dominant negative E106Q MO70. The day after, cells were submitted to fusion for 72 h when the analysis was performed.

### 2.3. Small interfering RNA sequences

Mouse Sold6 myotubes with reduced  $\alpha 1$ -syntrophin expression were obtained using transient transfection of siRNA. The siRNA silencing sequence 5'-AAGGUAUGAAUGUGCCGAUCUGCGC-3' (Eurogentec, Seraing, Belgium) was designed to target mouse  $\alpha 1$ -syntrophin mRNA (NM.009228). Silencing of TRPC1 gene was performed using siRNA (Eurogentec, Liege, Belgium) against mouse TRPC1 mRNA (5'-GCAUCGUUUUACAUUCU-3').

Downregulation of STIM1 in myotubes was achieved using the following siRNA sequence against mouse STIM1 mRNA (5'-GGCCUGGACACAGUGCUGUU-3').

### 2.4. Immunoblotting and immunoprecipitation

Total cell lysates were obtained from Sold6 and SolC1 cultured myotubes. Sold6 and SolC1 myotubes were washed three times in cold TBS 1 $\times$  (20 mM Tris Base pH 7.4, 154 mM NaCl, 2 mM MgCl<sub>2</sub>, 2 mM EGTA) and then lysed in 1 ml of lysis buffer RIPA (RadioImmunoPrecipitation Assay: 50 mM Tris HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.05% NP-40 (v/v), 1% DOC (w/v), 1% Triton X-100 (v/v), 0.1% SDS (w/v)) in the presence of 1% of a protease inhibitor cocktail (1 mM PMSF, 20 mM leupeptine, 0.8 mM aprotinine and 10 mM pepstatine, Sigma–Aldrich, Saint Louis, USA). Protein lysates were homogenized by mechanical dissociation and sonication. The total protein concentration was measured by means of a Biorad DC protein assay kit. Immunoprecipitation were performed using 2  $\mu$ g of antibodies against PLC  $\beta$  PLC $\gamma$ , STIM1 and TRPC1 for an

overnight incubation at 4°C. The control was done with mouse IgG1 Isotype Control (Sigma). The day after, 30 µl of protein G-sepharose or protein A-sepharose (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) were added for 1 h at 4°C. Bead-bound complexes were washed four times and eluted in the Laëmmli buffer.

After denaturing, samples were subjected to SDS-PAGE, blotted with specific antibodies probed with horseradish peroxidase conjugated anti-rabbit, mouse or goat antibody (Interchim, Montluçon, France). Membranes were developed with an enhanced chemiluminescence kit (GE Healthcare Europe GmbH, Velizy-villacoublay, France). The molecular weight of proteins was estimated according to pre-stained protein markers (Rainbow, Sigma–Aldrich).

### 2.5. Measurement of cation influx using Mn<sup>2+</sup> quenching of fura-2 fluorescence

Myotubes of 3 or 4 days were rinsed with external 1.8 Ca<sup>2+</sup> solution (130 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl<sub>2</sub>, 0.8 mM MgCl<sub>2</sub>, 10 mM HEPES, 5.6 mM D-glucose, pH 7.4, with NaOH) and incubated for 30 min in darkness and then for 15 min at 37°C in the same solution supplemented with 3 µM (final concentration) of fura-2/AM (Interchim). Loaded cells were washed with Ca<sup>2+</sup>-free solution (130 mM NaCl, 5.4 mM KCl, 0.1 mM EGTA, 0.8 mM MgCl<sub>2</sub>, 10 mM HEPES, and 5.6 mM D-glucose, pH 7.4 with NaOH) before measurements. Fura-2 was excited at 360 nm with a CAIRN monochromator (Cairn Research Limited, Faversham, UK), and emission fluorescence was monitored at 510 nm using intensified cooled CCD camera (Photonic Science Limited, Robertsbridge, UK) coupled to an Olympus IX70 inverted microscope (40× water immersion fluorescence objective). The cation influx was evaluated by the quenching of fura-2 fluorescence when Mn<sup>2+</sup> ions enter into the cells. Fluorescence variations were recorded with the Imaging Workbench 4.0 (IW 4.0) software (Indec BioSystems, Mountain View, CA, USA). The depletion protocol was three times 1 min application of 0 Ca<sup>2+</sup> 15 µM CPA (reversible inhibitor of SERCA) alternated by three times 1 min application of 0 Ca<sup>2+</sup> 5 µM CPA + 10 mM caffeine solution (activator of ryanodine receptor). The quench rate of the fluorescence intensity was estimated using linear regression of the decaying phase (slope) during the first 40 s after Mn<sup>2+</sup> addition (50 µM final). The quench rate was expressed as percent per minute to correct for differences in the cell size or fluorophore loading. Statistical analyses were performed with Origin 5.0 software (OriginLab, Northampton, MA, USA). No photobleaching or less than 0.5%/min was observed during fluorescence decrease measurements. The difference between the mean values of measured parameters was statistically tested using the Student's *t* test and considered significant for *P* < 0.05. All values of the quenching rate were expressed in absolute value.

## 2.6. qRT PCR

### 2.6.1. Reverse transcription

Total cellular RNAs were extracted using RNABle kit (Eurobio, Courtaboeuf, France). RNAs (10 µl) were reverse transcribed in 25 µl reaction mixture consisting of first strand buffer (25 mM Tris, pH 8.3, 37.5 mM KCl, 1.5 mM MgCl<sub>2</sub>), 10 mM DTT, 1 mM each dNTP, 2.4 mg of random hexamers, 40 U RNase inhibitor (RNA guard, Amersham Biosciences), and 400 U M-MLV reverse transcriptase (GIBCO-BRL). Reverse transcription was performed at 37°C for 60 min followed by 2 min at 100°C. The solution was then diluted twice in water.

### 2.6.2. Real-time PCR

The 15 µl reaction mixture contained 7.5 µl 2× Taqman Fast Universal Master Mix (Applied Biosystems), 5 µl of RT products, and appropriate primers and probes (900 nM and 200 nM, respectively). All probes contained a 3' TAMRA (6-carboxy-tetramethylrhodamine) quencher dye and were labeled at the 5' end with a FAM (6-carboxyfluorescein) reporter fluorescent dye. Amplification was performed at 50°C for 2 min, 95°C for 10 min, followed by 40 cycles at 95°C (15 s) and 60°C (1 min). Reactions were performed in MicroAmp optical 96-well reaction plates (Applied Biosystems) using an 7500HT Real-Time PCR system (Applied Biosystems). All measurements were normalized to the mitochondrial ribosomal protein S6 (Mrps6, an endogenous control) to account for the variability in the initial concentration and quality of the total RNA.

- Forward primer for TRPC1: 5'-CTT-CTG-CGA-ACA-GCA-AAG-CA-3'; Reverse primer: 3'-GAT-GTA-CCA-GAA-CAG-AGC-AAA-GCA-5'; Probe: [5']6-FAM TGA-CAC-CTT-CCA-CTC-GTT-CAT-TGG-CA [3']TAMRA.
- Forward primer for SP6 protein control: 5'-TTT-GAT-TCT-GAA-AGC-CAT-GCG-3'; Reverse primer: 3'-CGG-TCC-ATC-AGG-GAT-TCT-ATT-G-3'; Probe: [5']6-FAM CGG-CCA-GAG-ACC-GCT-GCT-GCT [3']TAMRA.

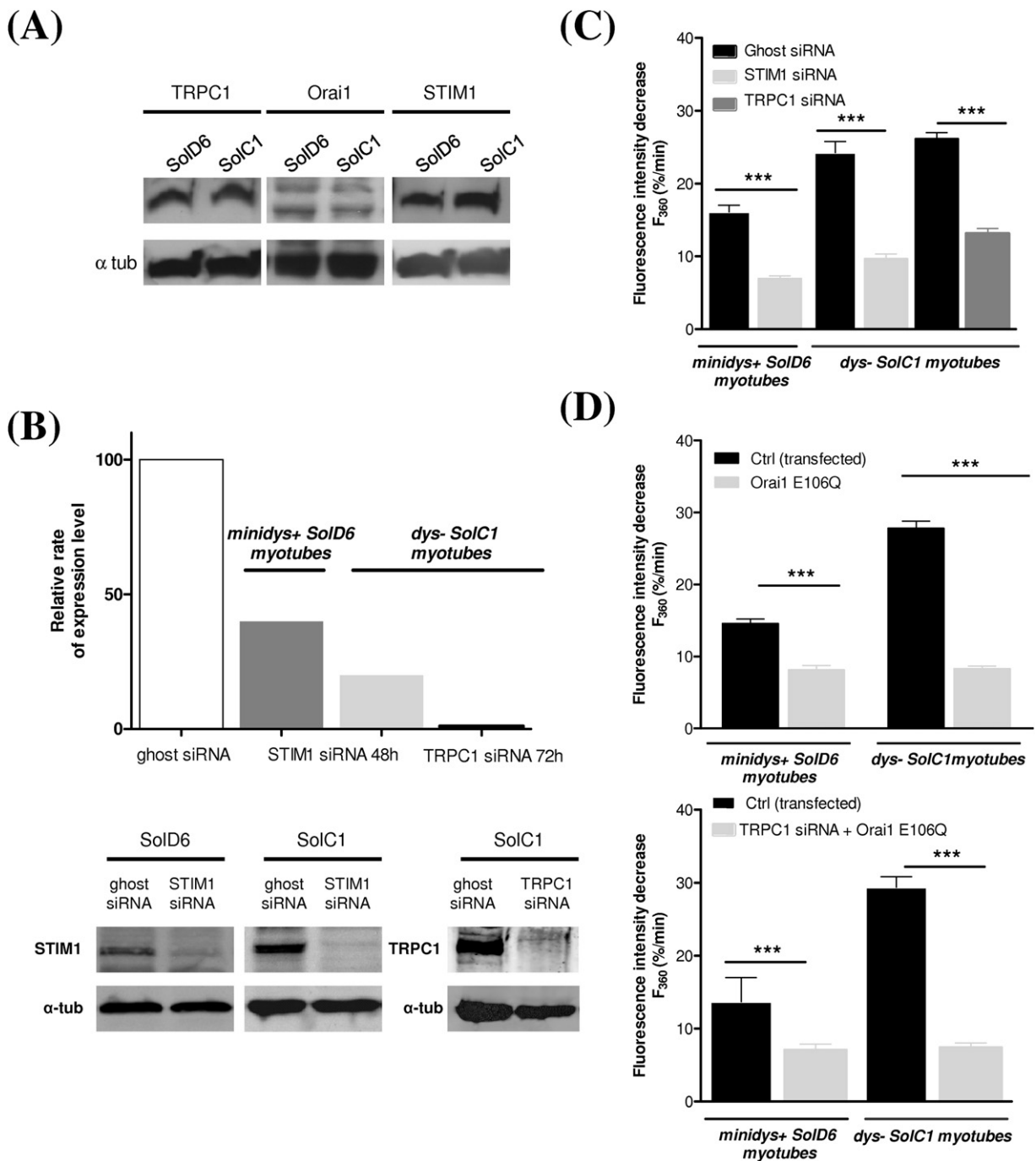
SYBR Green system (Applied Biosystems) was also used for PLCβ1 and PLCγ1. All measurements were normalized to α-tubulin as reference (endogenous control) to account for the variability in the initial concentration and quality of the total RNA. Expression was quantified with the ratio CT PLCβ or γ/CT α-tubulin.

- Forward primer for PLCβ1: 5'-CGG-AGC-TGG-AGC-AAG-AAT-AC-3'; Reverse primer: 5'-TCA-CCT-TTG-CAG-CAT-CTG-AG-3'.
- Forward primer for PLCγ1: 5'-GAC-TCA-CTG-GAG-AAC-TGG-TC-3'; Reverse primer: 5'-CCC-TTT-CTG-AGT-TCC-AGC-AG-3'.
- Forward primer for α-tubulin: 5'-ACC-TGG-AAC-CCA-CGG-TCA-T-3'; Reverse primer: 5'-AGC-TGC-TCA-GGG-TGG-AAG-AG-3'.
- Forward primer for STIM1: 5'-AAG-CTT-ATC-AGC-GTG-GAG-GAC-CT-3'; Reverse primer: 5'-CAT-CCA-CAG-TCC-AGT-TGT-ACA-CAA-CTG-3'.

## 3. Results

### 3.1. Cation entry in dystrophin-deficient myotubes is dependent on TRPC1, STIM1 and Orai1 proteins

We and other groups have already shown that elevated calcium influx in dystrophin-deficient muscle was dependent on TRPC1 activity [14,30], but a study with fibers from TRPC1 knockdown mice [32] concluded that small conductance currents dependent on TRPC1 expression were not store-operated in contradiction to previous work from Vandebrouck et al. [14]. We thus address the issue of determining whether TRPC1 is supporting the store-operated cation entry recorded in dystrophin-deficient SolC1 myotubes as already published for SolD6 [18]. We first checked the basal expression of TRPC1 in both models (Fig. 1A). No difference of basal expression was observed in SolC1 and SolD6 myotubes for TRPC1. We also looked at the proteins level of the other cation influx actor Orai1 as well as their regulatory partner STIM1 and no variation between enforced minidystrophin and dystrophic-deficient myotubes was observed. So, the basal expression of TRPC1, Orai1 and STIM1 could not explain the increase of calcium influx in SolC1 myotubes. To investigate the role of TRPC1, RNA interference was used to decrease endogenous TRPC1 mRNA level with further assessment of its impact on cation influx in SolC1 myotubes.



**Fig. 1.** TRPC1–STIM1–Orai1 proteins are involved in store-dependent cation influx in minidystrophin expressing or dystrophin-deficient myotubes. (A) Western Blot of basal expression of TRPC1, Orai1 and STIM1 in lysates from SolD6 and SolC1 myotubes.  $\alpha$ -Tubulin ( $\alpha$ -tub) was used as control of loading. (B) Differentiated *minidys+ SolD6* and *dys- SolC1* muscle cells were harvested after 72 h or 48 h of transfection with siRNAs against TRPC1 or STIM1. Bar-graphs represent the amount of TRPC1 and STIM1 mRNAs analyzed by qRT-PCR (value relative to the siRNA ghost control oligo) (upper panel). Expression level of TRPC1 and STIM1 proteins in SolC1 or SolD6 myotubes in cells transfected with ghost, TRPC1 and STIM1 siRNAs were analyzed by Western Blot using a polyclonal antibody against TRPC1 and a monoclonal antibody against STIM1 (lower panel). Levels of TRPC1 and STIM1 were compared to  $\alpha$ -tubulin ( $\alpha$ -tub) one detected with a monoclonal antibody. (C) Effect of TRPC1 and STIM1 inhibition on store-operated cation entry in SolC1 and SolD6 myotubes. Bar-graphs represent mean rates of fluorescence decrease induced by  $Mn^{2+}$  (expressed in %/min)  $\pm$  SEM in *minidys+ SolD6* or *dys- SolC1* myotubes, transfected with ghost siRNA (black column) or siRNA against TRPC1 or STIM1 (gray columns). (D) Effect of overexpression of the dominant negative mutant of Orai1 (Orai1 E106Q) alone (upper panel) or together with TRPC1 siRNA (lower panel). Bar-graphs represent mean rates of fluorescence decrease induced by  $Mn^{2+}$  (expressed in %/min)  $\pm$  SEM in *minidys+ SolD6* or *dys- SolC1* myotubes with the inhibition of Orai1 (transfection of Orai1E106Q M070; gray columns) compared to a control (transfection of pmaxGFP<sup>®</sup>; black columns). The lower panel shows the mean rates of fluorescence decrease induced by  $Mn^{2+}$  of a double transfections of Orai1E106Q M070 and TRPC1 siRNA (gray columns) compared with control transfection (pmaxGFP<sup>®</sup> and ghost siRNA; black columns) in both myotubes. The difference between mean values of measured parameters was determined by Student's *t* test and considered significant at  $P < 0.05$  (ns, non significant, \*\*\* $P < 0.001$ ).

Myoblasts were transfected by nucleofection with siRNA duplex targeting TRPC1 mRNA, and 3 days differentiated SolC1 myotubes were tested for expression level of TRPC1 mRNA (72 h after transfection). We observed a drastic extinction by 99% (TRPC1 siRNA 72 h) of the TRPC1 transcript value in SolC1 cells transfected with siRNA against TRPC1 compared to cells transfected with a non-targeting siRNA pool (ghost siRNA) (Fig. 1B). TRPC1 protein level was determined by Western Blotting. This analysis showed a large decrease of TRPC1 expression by 70% in SolC1 myotubes transfected with TRPC1 siRNAs (Fig. 1B). This result is comparable with the value obtained in SolD6 myotubes with 84% of pTRPC1 knock-down at the protein level [18]. The specificity of the TRPC1 siRNA was confirmed by the use of a different target siRNA duplex and same results were observed in mRNA and proteins levels (data not shown).

Store-depletion was achieved by repetitive activation of calcium release by caffeine (10 mM) in the presence of CPA (15  $\mu$ M), a reversible SERCA inhibitor, in order to activate store-dependent cation entry in cultured myotubes. After this procedure, 50  $\mu$ M manganese ions were perfused in the extracellular space, which led to the progressive quenching of fura-2 fluorescence by Mn<sup>2+</sup> entry in the cytosol and reflected a store-operated influx of divalent cation. We and others have previously shown that depletion of intracellular calcium stores in mouse myotubes activates store-dependent cation entry, which is more important in dystrophin-deficient myotubes [13,15,16].

SolC1 and SolD6 myotubes were transfected with siRNA against TRPC1 in order to determine the impact of the loss of this channel on cation influx after 3 days of differentiation. As previously published, TRPC1 siRNA lead to decrease of 30% of cation influx (TRPC1 siRNA 9.5%/min  $\pm$  0.7,  $n$  = 27) compared to control transfection (ghost siRNA 13.5%/min  $\pm$  0.5,  $n$  = 74) in SolD6 myotubes [18]. Knock-down of endogenous TRPC1 in dystrophin-deficient myotubes decreased the slope of store-dependent cation influx by almost 50% (TRPC1 siRNA: 13.1%/min  $\pm$  0.7,  $n$  = 51) compared to control cells value (ghost siRNA 26.1%/min  $\pm$  0.9,  $n$  = 81) (Fig. 1C). Taking into account the presence of a background non store-operated cation entry recorded in non-depleted myotubes (4.8%/min  $\pm$  0.3,  $n$  = 43 in non-stimulated SolC1 myotubes, data not shown), TRPC1 knock-down resulted in a 61% inhibition of the divalent cation influx in SolC1 myotubes. TRPC1 was also shown to contribute to elevated store-dependent cation entry in  $\alpha$ 1-syntrophin-deficient myotubes and in store-dependent cation regulated by minidystrophin [18].

Since STIM1 was demonstrated as the sensor protein triggering store-operated cation entry, silencing of STIM1 was performed in enforced minidystrophin or dystrophin-deficient myotubes. We obtained 3 days differentiated SolC1 myotubes with reduced STIM1 expression (Fig. 1B) using transient transfection (48 h transfection) of 1 day myotubes with a mixture of Lullaby<sup>®</sup> reagent and the specific siRNA. STIM1 siRNA transfection (48 h after transfection) protocol had to be adapted compared to TRPC1 siRNA transfection (72 h after transfection) in order to perform measurements on 3 days differentiation myotubes 48 h after transfection. We observed a drastic extinction by 80% and 60% of the STIM1 transcript value in SolC1 and Sol D6 cells respectively, transfected with siRNA against STIM1 (STIM1 siRNA 48 h) compared to cells transfected with a non-targeting siRNA pool (ghost siRNA) (Fig. 1B). The knock-down of STIM1 protein expression was also confirmed by immunoblotting in both myotubes (Fig. 1B). Measurements of manganese entry after store depletion showed that STIM1 silencing induced a large decrease by 60% (STIM1 siRNA 9.62  $\pm$  0.70%/min,  $n$  = 50) of cation influx in SolC1 myotubes compared to ghost siRNA (24.3  $\pm$  1.5%/min,  $n$  = 24) and by 56.6% (STIM1 siRNA 7.6  $\pm$  0.42%/min,  $n$  = 48) in SolD6 myotubes compared to control cells value (ghost siRNA 17.52  $\pm$  0.92%/min,  $n$  = 37) (Fig. 1C). The

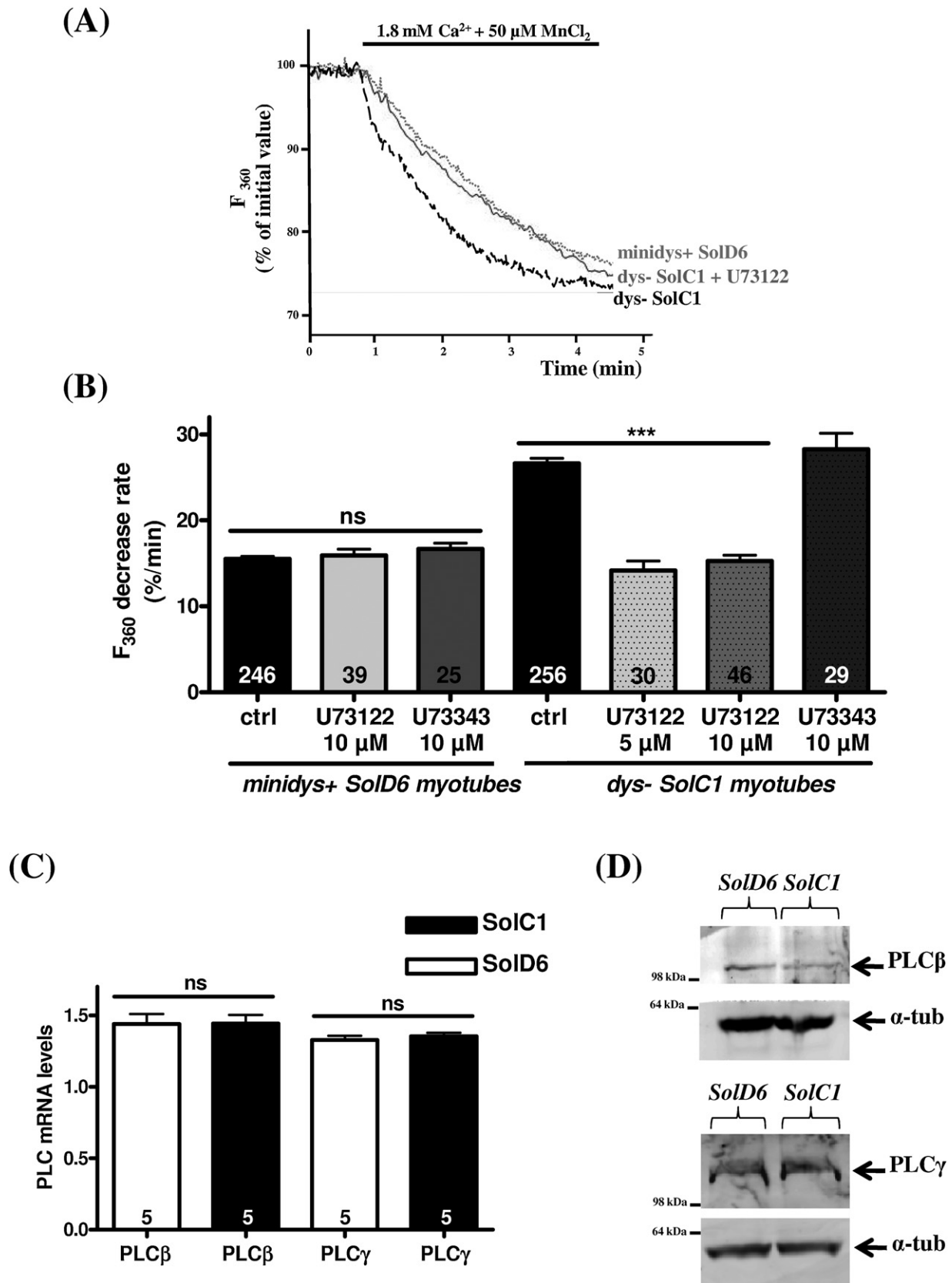
mean value was close to the one measured without application of depletion protocol (4.8  $\pm$  0.3%/min,  $n$  = 43 data not shown) in SolC1, showing that most of the store-operated cation entry was inhibited. This result showed that the cation influx activated by our protocol in dystrophin-deficient myotubes is STIM1-dependent, which was also observed in normal mouse myotubes by others [38], and in minidystrophin expressing myotubes (Fig. 1C).

Since Orai1 and TRPC1 were shown to be both STIM1-gated channels, inhibition of Orai1 activity was also performed in dystrophin-deficient and minidystrophin expressing myotubes by forced expression of Orai1 dominant negative E106Q [43]. Three days differentiated SolC1 myotubes exhibit when Orai1 dominant negative was expressed a large decrease by 70% (Orai1 E106Q 8.3  $\pm$  0.40%/min,  $n$  = 22) of cation influx after store depletion compared to control (ctrl 27.8  $\pm$  4.63%/min,  $n$  = 19) and 44.3% (8.1  $\pm$  2.16%/min,  $n$  = 10) in SolD6 compared to control (14.55  $\pm$  2.43%/min,  $n$  = 13) (Fig. 1D). This major decrease is similar to the one obtained by knocking-down STIM1 and shows the functional requirement of Orai1 in STIM1-dependent and store-operated cation entry in myotubes as previously reported [38]. We next performed a double inhibition of Orai1 and TRPC1. Indeed, we overexpressed Orai1 dominant negative E106Q and TRPC1 siRNA in order to evaluate the link between both of them in cation influx regulation. The double transfection induced a decrease of 74.8% of cation influx (7.41  $\pm$  0.62%/min,  $n$  = 10) in SolC1 and of 48.3% (7.08  $\pm$  0.83%/min,  $n$  = 10) in SolD6 compared with control cells values (ghost siRNA respectively 29.39  $\pm$  1.42%/min,  $n$  = 10; 13.7  $\pm$  3.32%/min,  $n$  = 10) (Fig. 1D). We then showed that the inhibition of TRPC1 together with Orai1 did not change the rate of decrease compared to the simple inhibition of Orai1 or STIM1. Moreover Orai1 and STIM1 inhibition reduced the cation influx of SolC1 myotubes close to the value obtained without depletion (4.8  $\pm$  0.3%/min,  $n$  = 43 vs. Orai1 E106Q 8.3  $\pm$  0.40%/min,  $n$  = 22; STIM1 siRNA 7.6  $\pm$  0.42%/min,  $n$  = 48) suggesting that Orai1 and STIM1 are essential for SOCEs. In another hand, TRPC1 siRNA (13.1%/min  $\pm$  0.7,  $n$  = 51) value is higher than the previous one. Altogether these results seem to indicate that Orai1 like STIM1 is necessary for TRPC1-dependent SOCEs activation in dystrophin-deficient myotubes.

The functional requirement of STIM1, TRPC1 and Orai1 suggests that a STIM1-activated cooperation between Orai1 and TRPC1 mediates the elevated store-operated cation entry in dystrophin-deficient myotubes as in enforced minidystrophin myotubes. In the absence of dystrophin, the same actors seem to be involved in SOCE regulation suggesting that the disorganization of the scaffolding complex is not affecting the apparatus supporting the store dependent calcium influx.

### 3.2. Store-dependent cation entry in dystrophin-deficient SolC1 myotubes is dependent on PLC and repressed by minidystrophin

As expected expression of minidystrophin in SolD6 myotubes restored normal levels of cation entry compared to dys- SolC1 myotubes (Fig. 2A and B). In order to determine the mechanisms leading to the deregulation of cation entry in dystrophin-deficient myotubes, we investigated a possible role of PLC. Activation of PLC was blocked with a widely membrane-permeable PLC inhibitor, U73122 and impact of this blockade was examined on manganese influx. Pre-treatment of cells with 5  $\mu$ M or 10  $\mu$ M U73122 has previously been documented to fully and irreversibly prevent PLC activation [44,45]. A 15 min pre-incubation of SolC1 myotubes with 5  $\mu$ M or 10  $\mu$ M U73122 inhibited cation entry by 47% and 42% respectively (control SolC1 myotubes: 26.6  $\pm$  0.6%/min,  $n$  = 256, 5  $\mu$ M U73122 treated SolC1 myotubes: 14.2  $\pm$  1%/min,  $n$  = 30, 10  $\mu$ M U73122 treated SolC1 myotubes: 15.3  $\pm$  0.7%/min,  $n$  = 46) (Fig. 2B). Incidentally, the value of cation entry after PLC



**Fig. 2.** Effect of U73122 on store-dependent cation entry in cultured myotubes. (A) Three representative recordings of fura-2 fluorescence during perfusion of 50 μM Mn<sup>2+</sup> obtained from minidys+ SolD6 myotubes (gray line), from dys- SolC1 myotubes (black line) and from dys- SolC1 myotubes treated with 10 μM U73122 (dark-gray line). (B) Slopes of the Mn<sup>2+</sup>-induced decreasing phase of fura-2 fluorescence were measured and expressed as % of decrease per min. Bar-graphs represent mean rates of fluorescence decrease induced by Mn<sup>2+</sup> (expressed in %/min) ± SEM in control SolD6 myotubes (black column), in SolD6 myotubes treated with 10 μM U73122 (gray column), in SolD6 myotubes treated with 10 μM U73343 (dark-gray column), in control SolC1 myotubes (black column), in SolC1 myotubes treated with 5 μM U73122 (dotted gray column), or with 10 μM U73122 (dotted dark-gray column) and in SolC1 myotubes treated with 10 μM U73343 (dotted black column). The difference between mean values of measured

inhibition in SolC1 myotubes was similar to the one of cation entry when regulated by minidystrophin expression in SolD6 myotubes. Therefore, the blocking effect of U73122 on PLC prevents elevated store-dependent cation entry in SolC1 dystrophin-deficient myotubes. When store-activated cation entry is already regulated by expression of minidystrophin, preincubation of SolD6 myotubes with 10  $\mu$ M U73122 had no effect ( $16 \pm 0.7\%/min$ ,  $n = 39$ ) compared to control myotubes ( $15.5 \pm 0.3\%/min$ ,  $n = 246$ ). Pre-treatment of cultured myotubes with 10  $\mu$ M U73343, an inactive analog of U73122, had no effect on cation influx ( $16.7 \pm 0.7\%/min$ ,  $n = 25$  for SolD6 myotubes and  $28.3 \pm 1.8\%/min$ ,  $n = 29$  for SolC1 myotubes) (Fig. 2B). This suggests that, in absence of the dystrophin scaffold, store-depletion protocol induces cation entries, which are elevated by a PLC-dependent pathway. On the other hand, PLC $\beta$ 1 and PLC $\gamma$ 1 (predominant isoforms in skeletal muscle) mRNA levels were analyzed by quantitative RT-PCR technique in SolD6 and SolC1 myotubes. In control conditions, PLC $\beta$ 1 and PLC $\gamma$ 1 mRNAs levels were found identical between SolD6 and SolC1 myotubes (Fig. 2C). Moreover, PLC $\beta$ 1 and PLC $\gamma$ 1 proteins levels were determined by Western Blotting vs.  $\alpha$ -tubulin ( $\alpha$ -tub), which showed no variation in PLC isoforms protein expression between SolD6 and SolC1 myotubes (Fig. 2D). This indicated that higher expression levels of PLC isoforms are not responsible in dystrophin-deficient SolC1 myotubes for the enhancement of divalent cation influx.

### 3.3. Store-dependent cation entry in dystrophin-deficient SolC1 myotubes is sensitive to inhibition of PKC by chelerythrine

Activation of a PLC-dependent pathway in dystrophin-deficient myotubes could result in stimulation of PKC. Moreover, calcium entry is known to be modulated by phosphorylation of calcium channels like TRPCs [26,46,47]. To test the possibility of a direct PKC effect on elevated divalent cation influx, we applied the widely selective PKC inhibitor 10  $\mu$ M chelerythrine [48]. Compared to untreated dystrophin-deficient cells ( $26.6 \pm 0.6\%/min$ ,  $n = 256$ ) cation influx in SolC1 myotubes is significantly decreased (40% inhibition) by 10  $\mu$ M chelerythrine ( $16.1 \pm 1.1\%/min$ ,  $n = 88$ ) (Fig. 3A). Similarly to inhibition of PLC, the blockade of PKC restored normal cation entry with mean values close to the one achieved by expression of minidystrophin. As previously, administration of 10  $\mu$ M or 50  $\mu$ M chelerythrine had no effect on regulated store-dependent cation entry in SolD6 myotubes ( $14 \pm 0.8\%/min$ ,  $n = 44$  and  $15 \pm 0.8\%/min$ ,  $n = 27$ , respectively) compared to untreated control cells ( $15.5 \pm 0.3\%/min$ ,  $n = 246$ ) (Fig. 3A). Therefore, the specific blocking effect of chelerythrine on PKC counteracts the elevated component of store-dependent cation entry in SolC1 myotubes, indicating that the elevated cation influx in dystrophin-deficient myotubes is involving a PLC and PKC-dependent pathway. Because TRPC1, STIM1 and Orai1 are known to be regulated by phosphorylation, we next checked their basal state of phosphorylation in minidystrophin expressing or dystrophin deficient myotubes. Therefore, we performed immunoprecipitation with specific antibodies for TRPC1 and STIM1 (Fig. 3B). We found that TRPC1 protein is phosphorylated in serine/threonine residues using a PKA/PKC substrate antibody but not in tyrosine amino acid. No basal difference could be noticed between SolC1 and SolD6 lysates and so the involvement of PLC/PKC pathway seems not directly link to the phosphorylation of TRPC1. No serine/threonine but a tyrosine phosphorylation of STIM1 could be observed in both myotubes.

This technical problem prevents us to evaluate the phosphorylation state of Orai1. These results could not link the activation of PLC/PKC pathway in SolC1 cation influx to a basal change in phosphorylation of TRPC1 and STIM1.

### 3.4. $\alpha$ 1-Syntrophin is involved in the abnormal increase of PLC/PKC-dependent cation influx in myotubes and formed a complex with PLC $\beta$

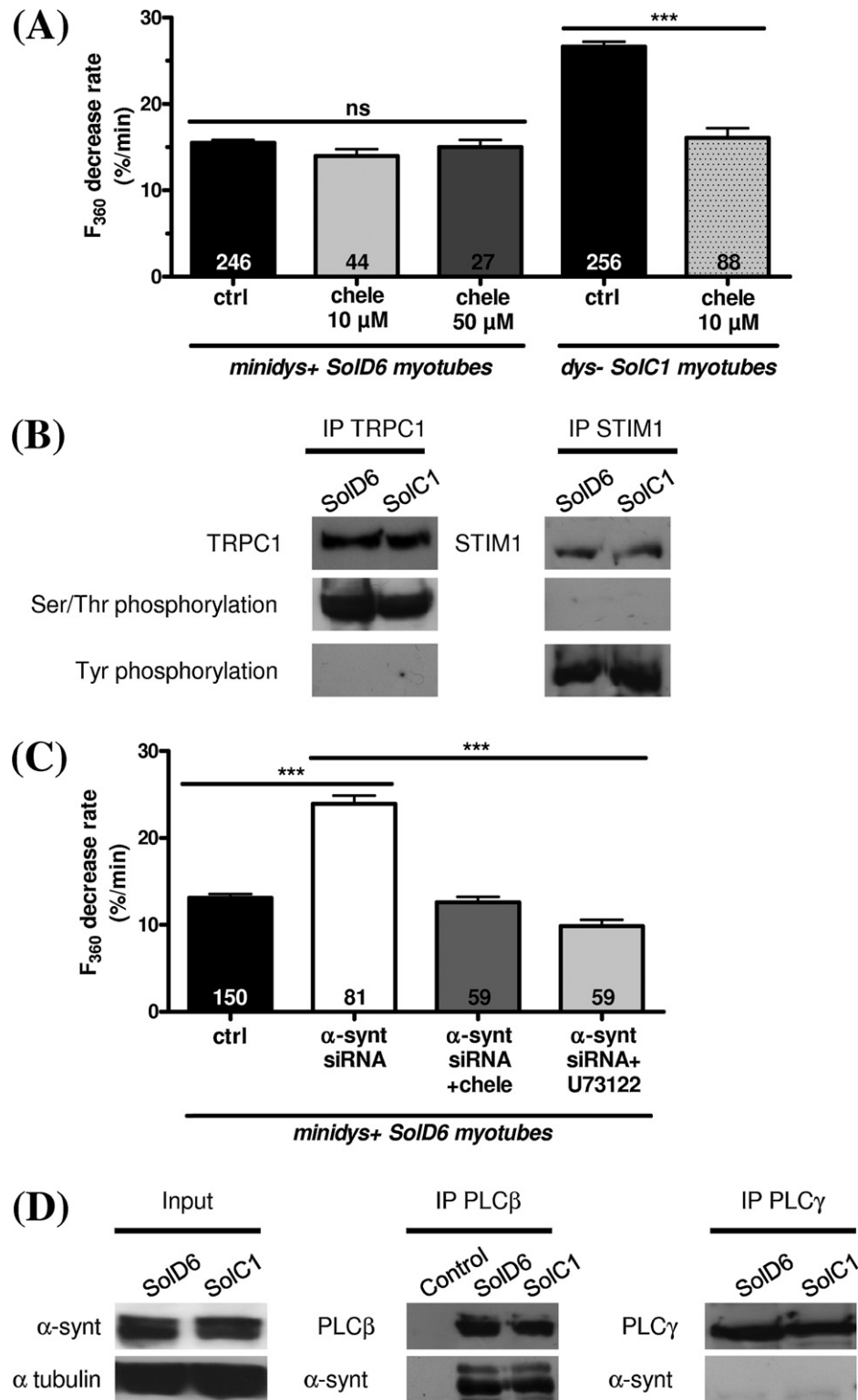
We previously demonstrated the requirement of  $\alpha$ 1-syntrophin in the dystrophin-syntrophin complex for normal regulation of TRPC-dependent cation entry in cultured myotubes [17,18]. As previously published [18], the reduction of  $\alpha$ 1-syntrophin expression with siRNAs led to abnormally increased cation influx ( $24.0\%/min \pm 0.9$ ,  $n = 81$ ) in myotubes expressing minidystrophin (Fig. 3C). The involvement of the PLC/PKC pathway in elevated cation entry of  $\alpha$ 1-syntrophin-reduced myotubes was tested by treatments with U73122 or chelerythrine. The results in Fig. 3C demonstrate that the abnormal influx measured when  $\alpha$ 1-syntrophin was knock-down ( $24.0\%/min \pm 0.9$ ,  $n = 81$ ) was reduced after U73122 pre-incubation ( $10\%/min \pm 0.7$ ,  $n = 59$ ) or after chelerythrine pre-incubation ( $12.6\%/min \pm 0.6$ ,  $n = 59$ ). The inhibition of PLC and PKC counteracted the increase of cation influx resulting from repression of  $\alpha$ 1-syntrophin. The remaining part of cation entry is similar to the one recorded in control minidys+ myotubes. Therefore, these data clearly show that when dystrophin is absent or  $\alpha$ 1-syntrophin is reduced, store-activated cation influx is elevated by a PLC/PKC pathway. To further investigate the link between  $\alpha$ 1-syntrophin and PLC, we performed co-immunoprecipitation of  $\alpha$ 1-syntrophin with specific antibodies for PLC $\beta$  and PLC $\gamma$  in SolC1 and SolD6 lysates (Fig. 3D). Like previously described in Fig. 2D, PLC $\beta$  and PLC $\gamma$  but also  $\alpha$ 1-syntrophin (Fig. 3D input) are equally expressed in both. PLC $\beta$  and PLC $\gamma$  proteins were immunoprecipitated with specific antibodies but not with an IgG isotypes control. These experiments showed that  $\alpha$ 1-syntrophin forms a complex with PLC $\beta$  but not with PLC $\gamma$ . PLC $\beta$  has already been described to be able to bind PDZ domain [49].  $\alpha$ 1-Syntrophin has a PDZ domain which was previously shown to interact with TRPC1 protein [18]. So  $\alpha$ 1-syntrophin is a molecular link with PLC $\beta$  and TRPC proteins and seems to play a key role of scaffolding protein for the regulation of store dependent cation influx in dystrophin-deficient myotubes.

### 3.5. Calcium dependency of cation entry in dystrophin-deficient SolC1 myotubes

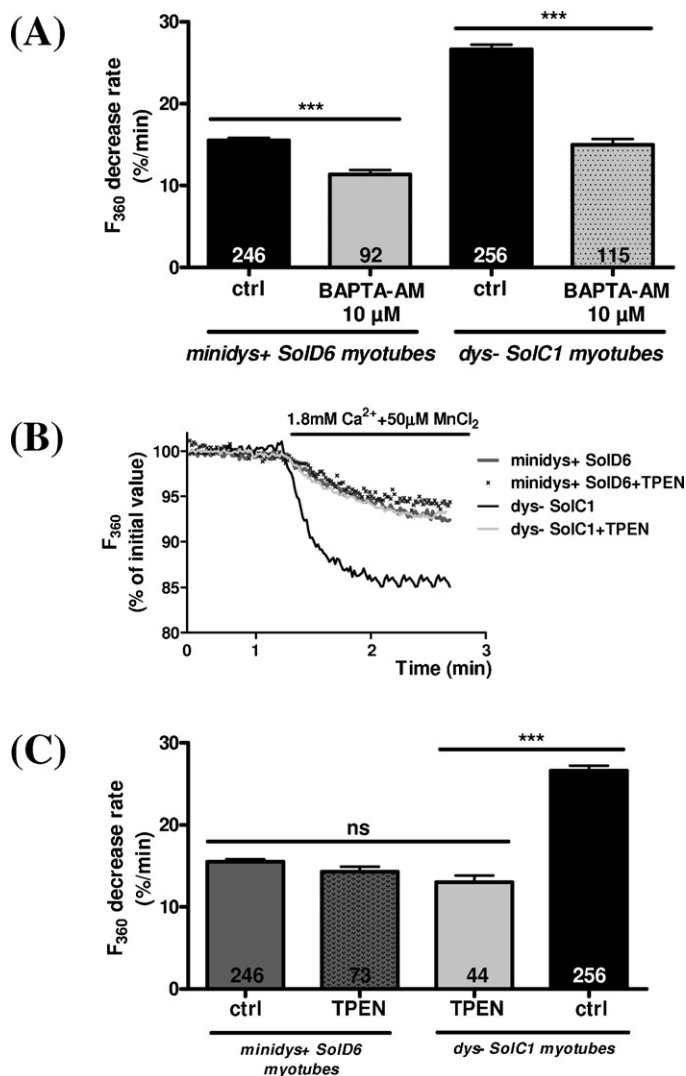
During application of the protocol, caffeine and CPA addition results in store depletion but also in elevation of the free concentration of calcium in cytoplasm. The rise of intracellular calcium was then inhibited by the presence of BAPTA-AM (10  $\mu$ M, 15 min pre-incubation), a diffusible chelator of calcium in order to explore if this is involved in the elevation of cation influx in dystrophin-deficient SolC1 myotubes. In BAPTA-loaded myotubes, store-dependent cation entry was decreased by 21.3% in minidys+ SolD6 myotubes (control cells:  $15.5\%/min \pm 0.3$ ,  $n = 246$ ; BAPTA loaded cells:  $12.2\%/min \pm 0.5$ ,  $n = 82$ ) (Fig. 4A). This slight decrease of store-operated cation entry in control myotubes may be due to competition of BAPTA with Fura-2, and allow us to evaluate the amount of decrease due to this effect. In BAPTA-pretreated

parameters was determined by Student's *t* test and considered significant at  $P < 0.05$  (ns, non significant,  $***P < 0.001$ ). (C) PLC $\beta$  and PLC $\gamma$  mRNA expression was addressed by quantitative RT-PCR-based analysis. The  $\alpha$ -tubulin mRNA was used as an internal standard and mRNA expression level of PLC $\beta$  and PLC $\gamma$  was normalized with  $\alpha$ -tubulin mRNA expression in SolD6 and SolC1 myotubes. (D) Expression levels of PLC $\beta$  and PLC $\gamma$  in SolD6 and in SolC1 myotubes were analyzed by Western Blots using monoclonal antibody against PLC $\beta$  and polyclonal antibody against PLC $\gamma$ . Levels of PLC isoforms were compared to the one of  $\alpha$ -tubulin ( $\alpha$ -tub) detected with a monoclonal antibody in both types of myotubes.





**Fig. 3.** Enhanced cation entry in dystrophin or syntrophin-deficient myotubes is dependent on PKC and PLC. (A) Effect of chelerythrine on store-dependent cation entry in cultured myotubes. Slopes of the Mn<sup>2+</sup>-induced decreasing phase of fura-2 fluorescence were measured and expressed as % of decrease per min. Bar-graphs represent mean rates of fluorescence decrease induced by Mn<sup>2+</sup> (expressed in %/min) ± SEM in control minidys+ SolD6 myotubes (black column), in minidys+ SolD6 myotubes treated with 10 μM chelerythrine (gray column), or with 50 μM chelerythrine (dark-gray column), in control dys- SolC1 myotubes (black column) and in dys- SolC1 myotubes treated with 10 μM chelerythrine (dotted gray column). The difference between mean values of measured parameters was determined by Student's *t* test and considered significant at *P* < 0.05 (ns, non significant, \*\*\**P* < 0.001). (B) Phosphorylation state of TRPC1 and STIM1 in SolD6 and SolC1 myotubes. SolD6 or SolC1 myotubes lysates were submitted to immunoprecipitation (IP) using TRPC1 or STIM1 antibodies. Their phosphorylations were revealed by phospho-serine/threonine or phospho-tyrosine antibodies. (C) Store-dependent cation influx deregulated by α1-syntrophin knock-down in minidys+ SolD6 myotubes is sensitive to chelerythrine and U73122. Effect of 10 μM chelerythrine and of 10 μM U73122 on store-dependent cation influx in minidys+ SolD6 myotubes transfected with siRNAs against α1-syntrophin. Bar-graphs represent mean rates of fluorescence decrease induced by Mn<sup>2+</sup> (expressed in %/min) ± SEM in control SolD6 myotubes (black column), in SolD6 transfected with α1-syntrophin siRNA (white column), in SolD6 transfected with α1-syntrophin siRNA after administration of 10 μM chelerythrine (dark-gray column) or after administration of 10 μM U73122 (gray column). The difference between mean values of measured



**Fig. 4.** Involvement of intracellular  $Ca^{2+}$  in elevated cation entry in SolC1 myotubes. Slopes of the  $Mn^{2+}$ -induced decreasing phase of fura-2 fluorescence were measured and expressed as % of decrease per min. (A) Bar-graphs represent mean rates of fluorescence decrease induced by  $Mn^{2+}$  (expressed in %/min)  $\pm$  SEM in control Sold6 myotubes (black column), in Sold6 myotubes loaded with 10  $\mu$ M BAPTA-AM (gray column), in control SolC1 myotubes (black column) and in SolC1 myotubes loaded with 10  $\mu$ M BAPTA-AM (dotted gray column). (B) Representative recordings of fura-2 fluorescence during perfusion of 50  $\mu$ M  $Mn^{2+}$  after caffeine + CPA or TPEN depletion obtained from *minidys+ Sold6* myotubes (light gray for control condition and dark gray for TPEN depletion), from *dys- SolC1* myotubes (black line for control condition and gray TPEN depletion). (C) Mean rates of fluorescence decrease induced by  $Mn^{2+}$  (expressed in %/min)  $\pm$  SEM in control Sold6 myotubes (gray column), in Sold6 myotubes depleted with TPEN (dotted gray column), in SolC1 myotubes depleted with TPEN (gray column) and in control SolC1 myotubes (black column). The difference between mean values of measured parameters was determined by Student's *t* test and considered significant at  $P < 0.05$  (ns, non significant, \*\*\* $P < 0.001$ ).

SolC1 myotubes, store-dependent cation entry was inhibited by 44% (control cells: 26.6%/min  $\pm$  0.5,  $n = 256$ ; BAPTA loaded cells: 15%/min  $\pm$  0.7,  $n = 115$ ) (Fig. 4A). A part of the decrease in the rate of fura-2 fluorescence quenching may be also due to chelation of manganese ions by BAPTA itself. However the decrease is low in Sold6, which suggests a slight effect of BAPTA. The more prominent effect

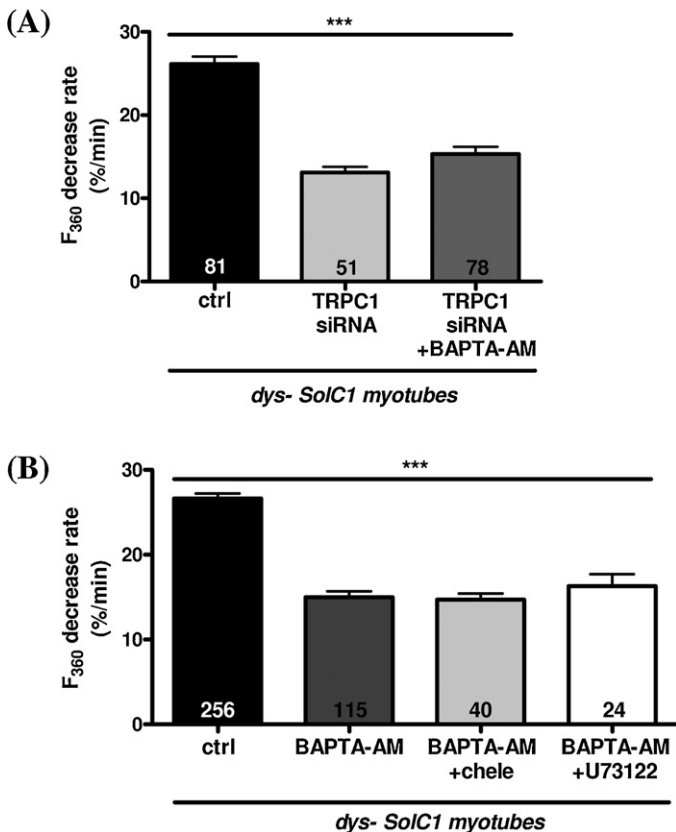
of calcium chelation in dystrophin-deficient myotubes, suggests that at least one part of cation entry was dependent on released intracellular calcium during stores depletion and/or cytosolic free calcium. This difference suggests that a calcium-sensitive pathway is increasing cation influx in dystrophin-deficient myotubes. TPEN was used to investigate the mechanism of store-operated entry due to its ability to reduce the intraluminal free  $Ca^{2+}$  concentration. The use of TPEN avoids potential confounders, such as changes in cytosolic  $Ca^{2+}$  and second messenger levels and permits us to discriminate the  $Ca^{2+}$  originating from internal stores or external sources. TPEN is a membrane-permeant multivalent cation chelator, that shows moderate affinity for calcium (Kd for  $Ca^{2+} \sim 130 \mu$ M), which has been used to rapidly and reversibly chelate  $Ca^{2+}$  within the intracellular stores [50]. In absence of extracellular  $Ca^{2+}$ , the addition of 1 mM TPEN to cultured myotubes did not modify cytosolic calcium concentration (data not shown) and efficiently chelated intraluminal calcium because calcium release by caffeine was completely blocked (data not shown). In Sold6 myotubes, the rate of  $Mn^{2+}$  entry was similar after the two different depletion protocols (ctrl: caffeine + CPA vs. TPEN) (Fig. 4B and C). This shows that both protocols induce the same kind of SOCEs and that the low affinity calcium chelator TPEN does not compete with fura-2 for  $Mn^{2+}$  binding. Subsequent addition of 50  $\mu$ M  $Mn^{2+}$  induced a store-operated cation entry, due to chelation of calcium in intracellular stores (+TPEN), which is similar between Sold6 (14.3%/min  $\pm$  0.6,  $n = 73$ ) and SolC1 myotubes (13%/min  $\pm$  0.8,  $n = 44$ ) (Fig. 4B and C). This result demonstrates clearly the presence of a similar store-operated component in both myotubes. It confirms that store-dependent cation influx is similar in both types of myotubes and that its increase in dystrophin-deficient SolC1 myotubes is dependent on the intracellular calcium released during the depletion protocol.

### 3.6. Role of cytosolic calcium level in TRPC1 cation influx activated by PLC/PKC pathway

The cation influx dependent on cytosolic calcium is chiefly supported, in part, by TRPC1 as shown in Figs. 1C and 5A by siRNA experiments in *dys- SolC1* myotubes. Indeed, pre-incubation of BAPTA-AM in myotubes transfected with siRNA TRPC1 had no additional effect on store-dependent cation entry (siRNA TRPC1 transfected SolC1 myotubes: 13.1%/min  $\pm$  0.7,  $n = 51$ ; siRNA TRPC1 transfected SolC1 myotubes treated with BAPTA-AM: 15.3%/min  $\pm$  0.8,  $n = 78$ ). Clearly, the component of cation influx affected by TRPC1 knock-down in SolC1 myotubes is dependent on cytosolic calcium level. This is in agreement with the idea that the positive modulation of SOCs mediated by TRPC1 is dependent on cytosolic calcium concentration.

The modulation of calcium ion on cation entry may be mediated by an intermediary target which can be PLC and PKC. It is possible that increased intracellular calcium may cause store-dependent channel modulation since PLC [51,52] and PKC isoforms are calcium sensitive [53]. To investigate if calcium-dependent cation entry is modulated by PLC and PKC activities in SolC1 myotubes, we performed double treatments of cells: to one side with U73122 plus BAPTA-AM and on the other side chelerythrine plus BAPTA-AM. Double treatments with U73122 and BAPTA-AM lead to store-dependent cation entry value similar to the one measured in single treatment with BAPTA-AM (BAPTA-AM treated cells: 15%/min  $\pm$  0.7,  $n = 115$  and U73122 plus BAPTA-AM treated

parameters was analyzed by Student's *t* test and considered significant at  $P < 0.05$  (\*\* $P < 0.001$ ). (D)  $\alpha$ 1-Syntrophin interacts with PLC $\beta$  but not PLC $\gamma$ . The expression of  $\alpha$ 1-syntrophin was analyzed by Western Blot in Sold6 and SolC1 (Input  $\alpha$ -synt) and compared to  $\alpha$  tubulin level (Input  $\alpha$  tubulin). IP were performed in both myotubes lysates with PLC $\beta$  and PLC $\gamma$  antibodies or with IgG1 isotypes used as a negative control. PLC $\beta$  and PLC $\gamma$  could be immunoprecipitated but  $\alpha$ 1-syntrophin ( $\alpha$ -synt) is present only in complex with PLC $\beta$ .



**Fig. 5.** Involvement of intracellular  $\text{Ca}^{2+}$  in elevation of TRPC1-dependent cation influx by PLC/PKC pathway in SolC1 myotubes. (A) Mean rates of fluorescence decrease induced by  $\text{Mn}^{2+}$  (expressed in %/min)  $\pm$  SEM in control SolC1 myotubes (black column), in SolC1 myotubes transfected with TRPC1 siRNAs (gray column) and in SolC1 myotubes transfected with TRPC1 siRNAs and loaded with  $10 \mu\text{M}$  BAPTA-AM (dark-gray column). (B) Mean rates of fluorescence decrease induced by  $\text{Mn}^{2+}$  (expressed in %/min)  $\pm$  SEM in control SolC1 myotubes (black column), in SolC1 myotubes loaded with  $10 \mu\text{M}$  BAPTA-AM (dark-gray column), in SolC1 myotubes loaded with  $10 \mu\text{M}$  BAPTA-AM and treated with  $10 \mu\text{M}$  chelerythrine (gray column) or with  $5 \mu\text{M}$  U73122 (white column). The difference between mean values of measured parameters was determined by Student's *t* test and considered significant at  $P < 0.05$  (\*\*\*)  $P < 0.001$ .

cells:  $16.3\%/ \text{min} \pm 1.4$ ,  $n = 24$ ) (Fig. 5B). Double treatments with chelerythrine and BAPTA-AM also did not have additional effect on store-dependent cation entry ( $14.7\%/ \text{min} \pm 0.7$ ,  $n = 40$ ) (Fig. 5B). The absence of additional inhibition suggests a calcium dependence of the PLC/PKC pathway increasing store-operated cation entry in dystrophin-deficient myotubes.

#### 4. Discussion

Store-dependent cation entry was shown to be regulated by the dystrophin/syntrophin scaffold in cultured myotubes, and the present study suggests new mechanisms leading to increased  $\text{Ca}^{2+}$  entry in dystrophin-deficient myotubes. This work shows that inhibition of PLC caused a substantial decrease in cation entry through Store-Operated Channels in dystrophin-deficient myotubes (Fig. 2). This could be related to higher activity of PLC in dystrophic myotubes as suggested by the first observation that  $\text{IP}_3$  levels remained high after potassium depolarization in the human dystrophic cell line [19]. Store-dependent cation influx was inhibited to the same level than PLC with chelerythrine, the usual inhibitor of PKC, in dystrophin-deficient myotubes. Inhibition of PKC and PLC decreased the cation influx in dystrophin-deficient myotubes at the same level measured in myotubes expressing minidystrophin. On the contrary, the dependency of store-dependent cation entry

on PLC and PKC activity was never observed when normal  $\text{Ca}^{2+}$  homeostasis and divalent cation entry were restored by expression of minidystrophin. Silencing  $\alpha 1$ -syntrophin in minidystrophin myotubes resulted in a PLC/PKC-dependent increase of store-operated cation influx, showing the crucial role of the scaffolding protein in inhibiting this signaling pathway mobilized during a depletion protocol (Fig. 3).

Recently, thapsigargin stimulation of human endothelial cells was shown to activate both store-dependent  $\text{Ca}^{2+}$  entry and PLC/PKC-dependent  $\text{Ca}^{2+}$  entry suggesting that thapsigargin stimulation led to activation of PLC and PKC [29]. This does not necessarily mean that the depletion of sarcoplasmic reticulum (SR)  $\text{Ca}^{2+}$  stores per se leads to PLC activation, since the consequences of the depletion protocol, for instance the cytosolic  $\text{Ca}^{2+}$  elevation may be responsible of this activation. It is well known that PLC and classical PKC isoforms ( $\text{PKC}\alpha$ ,  $\beta_1$ ,  $\beta_{II}$  and  $\gamma$ ) are sensitive to  $\text{Ca}^{2+}$  [51–54]. Moreover, in fetal skeletal muscle, Collet and Ma revealed an important mechanism of  $\text{Ca}^{2+}$ -dependent facilitation of SOCE [55]. In the present work, a dependency of elevated cation influx on cytosolic  $\text{Ca}^{2+}$  was supported by experiments using direct intracellular  $\text{Ca}^{2+}$  chelation by BAPTA-AM (Figs. 4 and 5). This led to reduction to the half of increased cation entry induced by CPA and caffeine, which was comparable to the effect of PLC or PKC inhibition. Moreover, PLC and PKC inhibitors had no additional effect to BAPTA-AM treatment, suggesting that a same cation influx was modulated by a single  $\text{Ca}^{2+}$ /PLC/PKC pathway (Fig. 5B). On the contrary,  $\text{Ca}^{2+}$  stores depletion with TPEN did not induce  $\text{Ca}^{2+}$  release, and allowed to trigger store-operated cation entry without elevation of cytosolic  $\text{Ca}^{2+}$  concentration.  $\text{Ca}^{2+}$  store depletion with TPEN stimulated divalent cation entry at similar level in dystrophin-deficient myotubes and after enforced minidystrophin expression (Fig. 4C). This is supporting the idea that the difference in divalent cation entry activated after store-depletion by caffeine/CPA perfusion is not dependent on a different sensitivity of  $\text{Ca}^{2+}$  stores.  $\text{Ca}^{2+}$  stores depletion with TPEN led to lower cation entry in dystrophin-deficient myotubes compared to influx obtained after depletion with CPA and caffeine (Fig. 4C). These results confirm that the increased store-dependent cation entry in dystrophin-deficient myotubes is dependent on other signaling pathways mobilized during the depletion protocol, such as  $\text{Ca}^{2+}$  release in the cytoplasm.

Store-dependent cation entry activated by caffeine and CPA were shown to be dependent on TRPC1 expression in dystrophin-deficient myotubes (Fig. 5A). Store-dependent cation entry was reduced by 50% after TRPC1 knockdown and chelating cytoplasmic  $\text{Ca}^{2+}$  with BAPTA-AM had no additional effect. This strongly suggests that TRPC1 channels support the  $\text{Ca}^{2+}$ -dependent elevation of cation entry stimulated by depletion protocol in dystrophin-deficient myotubes. The TRPC1 channel was shown to be implicated in abnormal divalent cation entry associated with myopathy, and shown to be regulated by scaffolding proteins,  $\alpha 1$ -syntrophin, Homer-1, and caveolin-3 [17,30,56]. Constitutively active cation influx dependent on TRPC1 was shown to be involved in dystrophy caused by homer-1 knockdown [56]. In our hands, inhibiting TRPC1 expression by siRNAs strongly attenuated the elevated divalent cation entry measured by quenching of fura-2 by manganese in  $\alpha 1$ -syntrophin-deficient myotubes [18] as well as in dystrophin-deficient SolC1 myotubes (Figs. 1 and 5). This is in accordance with the idea that TRPC1 channels participate in elevated  $\text{Ca}^{2+}$  entries activated in dystrophic muscle cells, when the dystrophin-based cytoskeletal complex is altered. Other TRPC channels could also be involved since forced TRPC3 overexpression in transgenic mice resulted in an increased  $\text{Ca}^{2+}$  entry correlated with a dystrophic phenotype [31]. It was demonstrated that TRPC1 can be a STIM1-gated channel and can bind STIM1 [39], an ER/SR  $\text{Ca}^{2+}$ -binding protein with multidomains that activates SOCs [57]. Store-operated  $\text{Ca}^{2+}$  entry was already shown to be dependent on STIM1 in skeletal

muscle [33]. We found that inhibiting STIM1 expression suppressed a major part of the cation entry recorded in dystrophin-deficient and minidystrophin expressing myotubes after store depletion (Fig. 1), which was also observed in normal mouse myotubes by others [38]. TRPC1 or STIM1 silencing induced a large decrease by almost 50% and 63% respectively of store-dependent cation influx in SolC1 myotubes (Fig. 1). This suggests that TRPC1 is participating to store-operated cation entry (SOCE) and is stimulated by STIM1. This is in contradiction with the work of Zanou et al. [32] in TRPC1 knock-out fibers suggesting that SOCE was not affected by the absence of TRPC1. This discrepancy could be explained by a different activation mode of TRPC1 in developing myotubes and adult fibers or by participation of other channels replacing TRPC1, such as TRPC4 [18] or Orai1 [37,38] in mature cells. Orai1 was proposed to be necessary for activation by STIM1 of cation entry through TRPC channels [34,41,42]. The present work shows that store-operated cation entry dependent on TRPC1 expression is drastically decreased by STIM1 knockdown and also by expression of Orai1 dominant negative (Fig. 1). These observations suggest a functional requirement for STIM1 and Orai1 in store-operated TRPC1-mediated cation entry of skeletal myotubes, which is in agreement with the model of functional interactions among STIM1, Orai1 and TRPC1 [41].

Our work shows that enhanced cation entry of dystrophin-deficient myotubes is dependent on TRPC1 channels but also on STIM1 and Orai1, which are necessary for stimulating store-operated cation influx (Fig. 1). However, in absence of dystrophin, the increase in STIM1/Orai1/TRPC1-dependent cation entry appeared to be enhanced by additional signaling pathways, which were activated during the depletion protocol. The potentiation of store-operated divalent cation entry appeared to be dependent on  $Ca^{2+}$ /PLC/PKC pathway and is regulated by the  $\alpha$ 1-syntrophin/dystrophin scaffold. This modulation may be achieved by the loss of an inhibitory mechanism due to the decrease of a  $\alpha$ 1-syntrophin-mediated regulation as suggested previously [18]. Quantification of mRNA and protein levels encoding for PLC isoforms, showing no expression changes at the transcriptional and protein levels, are in favor of a modulation of PLC activity by  $\alpha$ 1-syntrophin (Figs. 2 and 3). The  $\alpha$ 1-syntrophin is a component of dystrophin-associated complex in normal skeletal muscle and recruits signaling proteins such as nNOS [58], sodium channels Skm1 and Skm2 [59,60], the potassium channel Kir4.1 [61] via their multiples protein–protein interaction motifs. As  $\alpha$ 1-syntrophin is present in the same complex than PLC $\beta$  (Fig. 3D), it could act directly on PLC pathway as a regulatory molecule or indirectly through the modulation of trimeric G proteins, which is also shown to interact with  $\alpha$ 1-syntrophin [62,63]. As shown before [18], the N-terminal part of  $\alpha$ 1-syntrophin, containing the PDZ domain, is necessary for recovery of normal influx in dystrophin-deficient myotubes. PLC $\beta$  is also known to be able to bind PDZ domain [49] and so participated to the regulatory complex. This may reflect a critical role of this domain for recruiting regulatory components near Store-Operated Channels. Moreover, the increase in sub-plasmalemmal  $Ca^{2+}$  concentration related to higher activity of sarcolemmal cation channels or from other internal sources [16,64,65] may in turn amplify the overactivation of this STIM1, Orai1 and TRPC1-dependent cation entry.

Because all the actors of SOCE are regulated by phosphorylation, we checked the basal phosphorylation state of TRPC1, STIM1 and Orai1 in SolD6 and SolC1 (Fig. 3B). STIM1 is not phosphorylated on serine/threonine residues and seems not to be the target of PKC activation in SolC1 myotubes. Because of technical problem we could not study the phosphorylation of Orai1 but this protein is described as a phospho-protein [66]. In heterologous HEK model, Orai1 has been shown to be phosphorylated on serine residues by PKC which induced SOCE inhibition. So PKC-dependent phosphorylation of

Orai1 could not explain the increase of cation influx in SolC1. In basal condition, TRPC1 is phosphorylated on serine/threonine residues but no difference could be observed between SolC1 and SolD6 myotubes. The result is maybe due to the basal strong phosphorylation that can hide a light modulation. In another hand, Ahmed et al. have shown that PKC $\alpha$  phosphorylated TRPC1, which increases cation entries through SOCs in endothelial cells [26]. Moreover, recent works on vascular smooth muscle proved that in native heteromeric TRPC1/C5, the sensibility for PKC is mediated by TRPC1 isoform reinforcing the idea of a crucial role of TRPC1 for PKC in SOCE regulation [67]. Although SOCE was clearly dependent on Orai1 and STIM1 in myotubes, the presence of TRPC1 in the signalplex may confer the sensibility to PKC, and thus mediate the increase of cation entry in dystrophin-deficient myotubes. Further experiments would be pertinent to explore the link between PLC and TRPC1, but we have shown that  $\alpha$ 1-syntrophin both binds TRPC1 and PLC $\beta$ . In this work, we reinforced the central implication of  $\alpha$ 1-syntrophin, and suggest that its delocalization from the sarcolemma potentiates Store-Operated Channels through an mechanism dependent on calcium release and PLC/PKC pathway, which may phosphorylate TRPC1.

### Conflict of interest statement

The authors declare that there are no conflicts of interest.

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