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► **To cite this version:**

Aurélie Vandebrouck, Thomas Ducret, Olivier Basset, Stéphane Sebille, Guy Raymond, et al.. Regulation of store-operated calcium entries and mitochondrial uptake by minidystrophin expression in cultured myotubes.. FASEB Journal, 2006, 20 (1), pp.136-138. <10.1096/fj.04-3633fje>. <hal-02880269>

HAL Id: hal-02880269

<https://hal.science/hal-02880269v1>

Submitted on 17 Nov 2022

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The FASEB Journal express article 10.1096/fj.04-3633fje. Published online October 27, 2005.

Regulation of store-operated calcium entries and mitochondrial uptake by minidystrophin expression in cultured myotubes

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ABSTRACT

Defective expression of dystrophin in muscle cells is the primary feature of Duchenne muscular dystrophy (DMD), which is accompanied by fiber necrosis and intracellular calcium mishandling. These features led to the hypothesis that dystrophin could control calcium movements. Calcium mishandling in human DMD myotubes is dependent on contraction and/or calcium release activity, suggesting the involvement of channels being activated during these processes. Forced expression of minidystrophin at the plasma membrane of dystrophin-deficient Sol8 myotubes reactivates appropriate sarcolemmal expression of dystrophin-associated proteins and results in normal calcium homeostasis. In active dystrophic myotubes, store-operated calcium channels could be responsible for a sustained calcium influx in muscle cells. We show here that depletion of calcium stores (sarcoplasmic reticulum) by repetitive activation of calcium release and blockade of SERCA leads to a calcium influx. In myotubes expressing recombinant minidystrophin, these store-dependent influxes were reduced to a level similar to that observed in myotubes expressing native dystrophin. High store-dependent calcium influxes in dystrophin-deficient myotubes were associated with sustained cytosolic calcium transients and high intramitochondrial entries, while lower store-dependent calcium influx in myotubes expressing minidystrophin resulted in shorter calcium transients and reduced calcium uptake into mitochondria. We propose that minidystrophin negatively regulates sarcolemmal store-dependent calcium channels, which reduces store-dependent calcium influx, as well as its mitochondrial uptake. Forced expression of minidystrophin in dystrophic cells might restore the regulation of sarcolemmal store-dependent channels, which could protect against calcium mishandling.

Key words: mitochondria • skeletal muscle cells • Duchenne muscular dystrophy

In skeletal muscle, dystrophin and spectrin are two major fibrillar proteins that constitute an elastic cytoskeleton meshwork surrounding the fibers. Dystrophin, a 427 kDa cytoskeletal protein, is normally expressed at the inner face of the sarcolemma in skeletal muscle fibers (1–4). The complete function of dystrophin in skeletal muscle has not yet been fully elucidated, although it is thought to provide a crucial link between the intracellular actin-based cytoskeleton and the extracellular matrix (5, 6). In humans, defects in the dystrophin gene that prevent the production of dystrophin cause Duchenne muscular dystrophy (DMD) (7) and lead to a progressive loss of muscle mass and function and ultimately to premature death (8). Mutations of the dystrophin gene also lead to the milder Becker muscular dystrophy (BMD), and a 6.3 kb minidystrophin cDNA has been cloned from an asymptomatic BMD patient (9). These mutations lead to the expression of a truncated 229 kDa protein lacking 17 units in the central rod domain, which also accumulates at the sarcolemma of muscle fibers. The truncated yet still functional protein retains the C-terminal and N-terminal domain essential for the protein to interact with the transmembrane complex and actin-based cytoskeleton. The size of minidystrophin cDNA is sufficiently small to be accommodated by current retroviral or adenoviral vector-mediated DMD gene therapy. BMD-truncated dystrophin has been successfully expressed in vivo with accumulation of minidystrophin at the sarcolemma and functional recovery in *mdx* dystrophin-deficient mice (10, 11).

The extensive study of DMD in human and animal models has also suggested that an alteration of calcium homeostasis occurs in dystrophic cells. These alterations include increased transsarcolemmal calcium influx (12, 13), increased permeability to divalent cations that enter through channel-blocker sensitive pathways (14, 15), and entry of calcium via nonspecific cationic channels. Different observations obtained in our group suggested that dystrophin deficiency could result in an elevation of the resting $[Ca^{2+}]_c$ in human DMD myotubes (16). The high steady state levels of $[Ca^{2+}]_c$ were dependent on the activity of human DMD myotubes, that is, spontaneous calcium release and contraction. When these events were blocked by pretreatment with TTX, a blocker of voltage-dependent sodium channels, the elevation of the resting $[Ca^{2+}]_c$ characterizing human DMD myotubes was no longer observed. These studies led to the hypothesis that dystrophin may regulate calcium homeostasis in muscle and control calcium transport and/or buffering apparatus. In agreement with this idea, a previous study from McCarter and colleagues (17) reported that lipofection of full-length dystrophin in *mdx* myotubes lowered the resting $[Ca^{2+}]_c$ and the activity of calcium leak channels, which are responsible for calcium influx. Moreover, using a subline of Sol8 skeletal muscle cells, displaying alteration of calcium homeostasis and deficiency in dystrophin (18), we showed that forced expression of full-length dystrophin rapidly restores calcium handling in culture (only after two days of transfection), by reducing the abnormal resting steady-state level of calcium ions, as well as the amplitude of transient calcium release from internal stores during depolarization (19). We also showed for the first time that stable expression of the BMD minidystrophin was also able to improve the control of calcium handling in Sol8 myotubes (19). This is characterized by restoration of lower steady-state $[Ca^{2+}]_c$, and by a decrease in calcium-transient amplitudes and in the occurrence and propagation of spontaneous subcellular calcium events (19). Measurements showed that minidystrophin expressing myotubes displayed a pattern of calcium-release events and resting $[Ca^{2+}]_c$ closer to the ones observed in primary cultures expressing native full-length dystrophin.

During the activity of a skeletal muscle cell, various calcium transport system could be mobilized in addition to the channels of transverse tubules and of cisterna that are triggered by

depolarization. Contraction can lead to activation of stretch-activated cationic channels or can promote wounds on the surface membrane when dystrophin is absent. The activity of muscle cells is also mobilizing repetitive calcium release from the sarcoplasmic reticulum (SR), which can lower the level of calcium ions inside the SR lumina and, in turn, activate store-operated cationic channels at the plasma membrane. A previous study in *mdx* and C57 muscle fibers showed that plasma membrane of both fiber types contained cationic channels activated after depletion of the calcium stores with thapsigargin or caffeine (20). The activity of these store-operated channels was increased in adult skeletal muscle fibers from dystrophic mice. This prompted us to determine whether store-operated calcium entry (SOCE) could be activated in Sol8 myotubes and whether transfection of BMD minidystrophin, could change the properties of SOCE. As described previously (19), stable transfection of a dystrophin-deficient myogenic cell line by means of a retroviral vector allowed to obtain myotubes with minidystrophin targeted at the sarcolemma and partially restored the dystrophin-associated complex (19). We explored in these cells transmembrane influx after promoting depletion of calcium stores and also measured elevation of calcium activity in the cytosol. Other studies (21, 22) have shown that the mitochondria are able to rapidly capture Ca^{2+} ions that enter the cells via store-operated calcium influx, which, in turn, regulates the pattern and rate of calcium entry. Moreover, it was suggested that close contacts exist between the mitochondria and the SR, permitting Ca^{2+} exchanges during depolarization (23, 24). In addition, calcium uptake by the mitochondria during calcium influx or calcium release from SR stores may be crucial for maintaining a normal calcium homeostasis in skeletal muscle cells. Indeed, the use of a targeted calcium probe (m-aequorin) into the mitochondrion (25), has demonstrated an increase in calcium uptake in the mitochondria of *mdx* myotubes during depolarization. Using the same technique, we thus explored calcium uptake in the mitochondria during activation of SOCE in Sol8 myotubes, and we also anticipated improved regulation when minidystrophin was expressed.

MATERIALS AND METHODS

Cell culture

Experiments were performed on two cell line subclones of the Sol8 myogenic cell line (19) previously established from primary culture of normal C3H mouse soleus muscle. SolD7minidys+ or SolD6minidys+ clones of myoblasts were selected after transfection of Sol 8 with a retroviral vector encoding for minidystrophin (19), and mock-transfected SolC1dys- myoblasts were selected after transfection of an empty plasmid PcDNA₃. A control cell line, SolC57, expressing endogenous 427 KDa dystrophin was also cultured.

Cells were seeded on glass coverslips in 35 mm plastic dishes (Nunc Delta, Nunc, Roskild, Denmark). Myoblasts were grown in 50% DMEM (Cambrex, Verviers, Belgium), 50% Ham's F-12 (Cambrex), supplemented with 10% fetal calf serum (Biowest, Nuaille, France), 1% L-glutamine (Cambrex), 1% antibiotics (penicillin-G, 100 IU/ml and streptomycin, 100 µg/ml; Cambrex). After 2-3 days of proliferation, the growth medium was replaced by a differentiation medium (DMEM supplemented with 2% heat-inactivated horse serum (Invitrogen, Cergy-Pontoise, France), 10 µg/ml insulin (Sigma, St. Louis, MO), 1% L-glutamine and 1% antibiotics) to promote fusion of myoblasts into myotubes. This medium exchange was used as time zero for culture differentiation (the day after start of fusion in the text).

Measurement of Ca²⁺ influx using Mn²⁺ quenching of fura-2 fluorescence after SR depletion with thapsigargin

Myoblasts and myotubes, plated on glass coverslips were incubated with 5 μM fura-2 AM (Molecular Probes, Carlsbad, CA) and 0.02% Pluronic F127 (Molecular Probes) in Hank's balanced salt solution (HBSS) at 37°C for 60 min and then washed and maintained at room temperature in the same saline solution until fluorescence was measured. The coverslip was then mounted in a home-made microscope chamber (1 ml) continuously superfused with HBSS containing 50 μM MnCl₂, allowing quick changes of extracellular solution (1 ml/min⁻¹). Fura-2-loaded cells were alternatively excited at 340 and 360 nm, and emission fluorescence was monitored at 510 nm using a Deltascan spectrofluorimeter (Photon Technology International, Lawrenceville, NJ) coupled to a Nikon Diaphot inverted microscope ($\times 40$ oil immersion fluorescence objective; numerical aperture, 1.3). The influx of Mn²⁺ through the Ca²⁺ channels could be evaluated by the quenching of the fura-2 fluorescence excited at 360 nm, that is, at the isosbestic point. The rate of this quenching reflected the rate of Ca²⁺ entry (26), as Mn²⁺ is not taken up into the endoplasmic reticulum at the low Mn²⁺ concentrations used here (27). Under these conditions, simultaneous evaluation of [Ca²⁺]_c was obtained with 340/360 nm as the pair of excitation wavelengths.

Measurement of Ca²⁺ influx using Mn²⁺ quenching of fura-2 fluorescence after SR depletion with caffeine and CPA stimulation

Myotubes, plated on glass coverslips, were briefly rinsed with a standard external solution (1.8 Ca²⁺ solution): 130 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl₂, 0.8 mM MgCl₂, 10 mM HEPES, 5.6 mM D-glucose, pH 7.4 with NaOH, incubated for 30 min at room temperature and then for 15 min at 37°C in the same solution supplemented with 3 μM (final concentration) fura-2 AM (FluoProbes, Interchim, France). After loading, cells were washed with Ca²⁺-free solution (130 mM NaCl, 5.4 mM KCl, 0.1 mM EGTA, 0.8 mM MgCl₂, 10 mM HEPES, 5.6 mM D-glucose, pH 7.4 with NaOH), before measurement of calcium influx. Interrogated cells were perfused by means of a home-made gravity microperfusion device. Fura-2-loaded cells were excited at 360 nm with a CAIRN monochromator (Cairn Research Limited, Faversham, UK), and emission fluorescence was monitored at 510 nm using a CCD camera (Photonic Science Limited, Robertsbridge, UK) coupled to an Olympus IX70 inverted microscope ($\times 40$ water immersion fluorescence objective). The influx of Mn²⁺ through the Ca²⁺ channels could be evaluated by the quenching of the fura-2 fluorescence excited at 360 nm, that is, at the isosbestic point, as previously. The variation of fluorescence was recorded with the Imaging Workbench 4.0 (IW 4.0) software (Indec BioSystems, Mountain View, CA). The rate of the influx was estimated from the slope during the first 40 s after Mn²⁺ (50 μM final) addition. Statistical analysis was performed with Origin 5.0 software (OriginLab, Northampton, MA). The difference between the mean values of measured parameters was determined by the Student's *t* test and considered significant at $P < 0.05$ (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Recording solutions

For microspectrofluorimetric studies, the standard extracellular solution was HBSS and contained (mM): 140 NaCl, 5 KCl, 2 CaCl₂, 2 MgCl₂, 0.3 Na₂HPO₄, 0.4 KH₂PO₄, 4 NaHCO₃, 5 glucose and 10 N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES). The osmolality of the

external salt solution was adjusted to 300–310 mOsm with sucrose and pH adjusted to 7.3 with NaOH.

In the Ca^{2+} -free solution, CaCl_2 was omitted and 0.2 mM EGTA added.

Immunological staining

Dystrophin and minidystrophin labeling was performed by indirect immunofluorescence using primary monoclonal mouse antibodies. We used monoclonal antibodies (Novocastra Laboratory, Newcastle, UK) raised against the C-terminal domain of dystrophin (NCL-DYS2). These antibodies have been previously used in muscle cell lines (19).

The cultured cells were fixed in TBS/4% paraformaldehyde and permeabilized with TBS/0.5% tritonX-100. Samples were then incubated for 1 h with primary antibodies in TBS (20 mM Tris base, 154 mM NaCl, 2 mM EGTA, 2 mM MgCl_2 , pH 7.5)/1% BSA (Sigma). After washing in TBS, the cells were incubated for 30 min in TBS/1% BSA with Cy3-conjugated goat anti-mouse antibody (Jackson Immunoresearch Laboratories, West Grove, PA). Samples were mounted using Vectashield mounting medium (Vector Laboratories, Burlingame, CA).

Intracellular calcium concentration measurements

Intracellular free calcium measurements were performed by means of a ratiometric fluorescence method using an OSP100 microscopic photometry system (Olympus) and the calcium fluorescent probe Indo-1. The ratiometric method and the calibration procedure have been published elsewhere (28, 29). The ratio (R) of the dual emission fluorescence of the Ca^{2+} -free and Ca^{2+} -bound forms of Indo-1 (at 485 and 405 nm, respectively) were separated, filtered, and collected by two photomultipliers. The intracellular free calcium concentrations were calculated from the following Eq. (28): $[\text{Ca}^{2+}]_c = K_d \times \beta \times [(R - R_{\min})/(R_{\max} - R)]$. All the experiments were performed at room temperature.

Briefly, cells were rinsed with standard external solution (1.8 Ca^{2+} solution): 130 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl_2 , 0.8 mM MgCl_2 , 10 mM HEPES, 5.6 mM D-glucose, and pH 7.4 with NaOH, incubated for 45 min at room temperature in the same solution supplemented with 3 μM (final concentration) of the acetoxymethyl ester form of Indo-1 (Indo-1/AM, Sigma). After loading, cells were washed with Ca^{2+} -free solution (130 mM NaCl, 5.4 mM KCl, 0.1 mM EGTA, 0.8 mM MgCl_2 , 10 mM HEPES, 5.6 mM D-glucose, pH 7.4 with NaOH), before intracellular free calcium measurements. Solutions were perfused close to the interrogated cells by means of a home-made gravity microperfusion device.

Various parameters like amplitudes or area under curve were calculated using an analysis program developed in the lab under IDL software. Statistical analysis was performed with Origin 5.0 software (OriginLab, Northampton, MA). The difference between the mean values of measured parameters was determined by the Student's *t* test and considered as significant at $P < 0.05$ (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Mitochondrial calcium concentration measurements

To measure mitochondrial calcium concentration $[Ca^{2+}]_m$, we used the calcium-sensitive photoprotein aequorin that is specifically targeted to mitochondria via a peptide leader sequence derived from mitochondrial cytochrome *c* oxidase (mtAeq). Cells were transiently transfected with mtAeq/pcDNA1 plasmid.

Cells were seeded on gelatin-coated 13-mm diameter Thermanox coverslips (Nunc, Life Technologies, Basel, Switzerland). After 2 days of proliferation to ~60–80% confluency, the growth medium was replaced by lipofectin (Invitrogen, Life Technologies) with mtAeq/pcDNA1 plasmid and Optimem (Gibco, Invitrogen, Life Technologies). The following day, the medium was replaced by differentiation medium.

After 3 days of fusion, $[Ca^{2+}]_m$ was measured. The mtAeq was reconstituted with coelenterazine (5 μ M) (Molecular Probes, Eugene, OR) in Optimem for 2 h before the experiment, to form the Ca^{2+} -sensitive protein. Emitted luminescence was detected by a highly sensitive photomultiplier tube (Thorn EMI Electron Tubes, London, UK), installed in a refrigerator set at 4°C to reduce noise, and recorded every second using a computer photon-counting board (Thorn EMI), as described previously (23). Differentiated myotubes grown on a coverslip were placed in a perfusion chamber (Meca Test, Geneva, Switzerland) maintained at 37°C and were superfused at a rate of 1 ml/min with Ca^{2+} -free solution, followed by the experimental solution. After each recording, photon quantification under digitonin treatment (100 μ M) allowed normalization of recording.

Statistical analysis was performed with Origin 5.0 software. The difference between the mean values of measured parameters was determined by the Student's *t* test and considered significant at $P < 0.05$ (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

RESULTS

Measurement of store-operated calcium influx after stimulation by a SERCA inhibitor

Skeletal muscle cells (myoblasts and myotubes) were treated with the SERCA-inhibitor thapsigargin (1 μ M), in the absence of extracellular Ca^{2+} (0.2 mM EGTA), in order to activate store-dependent entry of calcium. Thapsigargin induced a release of calcium from the intracellular stores which was of the same amplitude in both cell types (not shown). After the stores were depleted by this procedure and $[Ca^{2+}]_c$ returned back to its resting value (not shown), calcium was readmitted in the extracellular space. This induced a large increase in $[Ca^{2+}]_c$ (not shown) due to an influx, the amplitude of which could be evaluated by measuring the rate of quenching of fura-2 with Mn^{2+} (Fig. 1A).

When measured, in SolC1dys- and SolD7minidys+ myoblasts (mb) (Fig. 1B), there was no difference in the rate of quenching ($-4.28 \pm 0.51\%/100$ s, $n=6$ in SolC1dys- cells vs. $-5.02 \pm 1.28\%/100$ s $n=7$ in SolD7minidys+ cells). Immunostaining of minidystrophin, when analyzed by fluorescence confocal microscopy, shows a cytoplasmic distribution of the protein at this stage (Fig. 1C, inset). No differences in store-dependent entries were observed when minidystrophin was absent at the inner face of the sarcolemma.

Thapsigargin also induced a similar release of calcium from the intracellular stores in SolC1dys- and SolD7minidys+ myotubes (MT). However, the subsequent slope of Mn^{2+} quenching was about twofold higher in SolC1dys- than the SolD7minidys+ myotubes ($-14.30 \pm 2.27\%/100$ s, $n=11$ vs. $-8.62 \pm 0.85\%/100$ s $n=10$, respectively), showing a significantly more important store-dependent entry of calcium in dystrophin-deficient myotubes. At the stage of F+3 (3 days after induction of fusion and differentiation) minidystrophin staining showed that the recombinant protein was correctly localized at the sarcolemma ([Fig. 1C](#), inset). Thus the presence of minidystrophin underneath the sarcolemma is able to reduce calcium influx activated by depletion of SR stores.

We applied the same protocol previously used by Kurebayashi and Ogawa (30), in order to gradually deplete the SR by repetitive activation of calcium release in the presence of a SERCA inhibitor. It was performed by repetitive stimulation with caffeine in the presence of cyclopiazonic acid (CPA) and in the absence of extracellular calcium ions. The subsequent reintroduction of calcium in the extracellular medium then induced a cationic influx measured by means of the rate of quenching of fura-2 with Mn^{2+} ([Fig. 1C](#)). As for the previous protocol the slope of Mn^{2+} quenching was about twofold higher in SolC1dys- myotubes (F+3) than in myotubes expressing recombinant minidystrophin (SolD6minidys+) or expressing wild-type 427 kDa dystrophin (SolC57dys+). No differences were observed between store-dependent calcium influx measured in myotubes expressing minidystrophin (SolD6minidys+) or full-length dystrophin (SolC57dys+).

Increase in cytosol calcium activity subsequent to depletion of SR stores by repetitive caffeine-stimulation

Changes in cytosol calcium activity were analyzed by ratiometric cytofluorimetry in myotubes subsequently to reintroduction of calcium in the extracellular medium after activation of the store-operated influx. [Fig. 2A](#) shows the procedure applied for gradual depletion of intracellular Ca^{2+} store (SR). The cells were first incubated in a Ca^{2+} -free solution until equilibrium was reached. Then the cells were repetitively and alternatively challenged with Ca^{2+} -free, 10 mM caffeine, 5 μ M CPA (a reversible SERCA inhibitor) solution or Ca^{2+} -free solution, 15 μ M CPA solution for 1 min every 1 min. During this procedure, caffeine response gradually decreased. We considered that the store was emptied when responses to caffeine were no more observed.

As previously, subsequent restoration of 1.8 mM Ca^{2+} in superfusion produced a rapid elevation of $[Ca^{2+}]_c$ due to influx through opened store-operated channels. Following a fast rising phase $[Ca^{2+}]_c$ slowly decreased and returned back to resting level. In [Fig. 2B, D](#) only the recordings during perfusion of 1.8 mM Ca^{2+} are shown.

To address the pharmacology of these transient increases in calcium after reintroduction in the extracellular space, we used 2-APB ([Fig. 2B](#)). 2-APB has been applied when the signal was at its peak. While in control conditions, this peak is followed by a slow decrease, application of 50 μ M, 2-APB abolished this phase and the $[Ca^{2+}]_c$ rapidly fell. On the contrary, the application of 10 μ M 2-APB potentiated $[Ca^{2+}]_c$ elevation, which was characterized by a sustained plateau phase or an additional calcium elevation. The short-term treatment with 2-APB has already been described to have an opposite effect on SOCE when applied at 50 μ M (antagonist) or at 10 μ M (agonist), independent of IP3 receptors (31). Although the pharmacological profile of SOCs still remains

questionable, the sensitivity to 2-APB of the calcium influx recorded in myotubes suggests that the underlying cationic channels are closely related to classical store-dependent calcium channels.

Two parameters were measured in order to analyze calcium flux during capacitative entries: the variation of initial slope and the proportion of the signal returning to half amplitude. Significant differences ([Fig. 2C](#)) were found between SolC1dys- (dystrophin deficient) and myotubes expressing recombinant minidystrophin (SolD6minidys+) or full-length native dystrophin (SolC57dys+). The myotubes expressing recombinant minidystrophin or expressing native dystrophin displayed more rapid calcium increase with a faster rising phase (initial slope: $s=6.99\pm 0.76$ nM/s, $n=27$ in SolC57dys+; $s=3.42\pm 0.19$ nM/s, $n=52$ in SolD6minidys+) than in SolC1dys- ($s=2.76\pm 0.23$ nM/s, $n=49$). More obviously, the calcium increase in SolD6minidys+ or SolC57dys+ myotubes was also less sustained: that is, the proportion of the signal returning to half amplitude was only 27% for SolC1dys- vs. 81% for SolD6minidys+ vs. 93% for SolC57dys+. It shows that when minidystrophin is present underneath the sarcolemma, the increase in cytosolic calcium related to SR store depletion is more transient. This is also observed in cells expressing native dystrophin ([Fig. 2D](#)). This may be explained by a reduced transmembrane influx as shown by measuring the rate of quenching of fura-2 with Mn^{2+} , and/or by different buffering or uptake capacity of calcium ions entering the cytoplasm.

Mitochondrial depolarization reduces SOCE

Experiments were performed in order to evaluate the regulation by calcium uptake into mitochondria. After store depletion, cells were treated with 2 μ M FCCP during 1 min (a protonophore that collapses the proton motive force across the inner mitochondrial membrane), which depolarized the mitochondria. Readmission of Ca^{2+} led to a weaker Ca^{2+} signal. The initial slope was significantly reduced by FCCP compared with control recordings from the two cell types. [Fig. 3A](#) shows values of 2.76 ± 0.23 nM/s $n = 49$ in control vs. 1.74 ± 0.32 nM/s $n = 22$ in FCCP for SolC1dys-, and 3.42 ± 0.19 nM/s $n = 52$ in control vs. 1.26 ± 0.11 nM/s $n = 19$ in FCCP for SolD6minidys+. These observations are in accordance with the idea that calcium uptake by mitochondria promotes SOCE, by preventing cytosolic calcium ions to inactivate store-operated channels (22). The proportion of the signal returning to half amplitude was increased in SolC1dys- ([Fig. 3B](#)). With FCCP, 64% of cells displayed a signal returning to half amplitude vs. only 27% for control cells. On the contrary, the proportion of SolD6minidys+ myotubes with a signal returning to half amplitude was similar in presence of FCCP (76.2%) and in control conditions (81%). The comparison of two representative normalized calcium transients in SolC1dys- ([Fig. 3C](#)) clearly showed that with FCCP treatment the signal is more transient. This suggests that the slow decrease of calcium transients in SolC1dys- dystrophin-deficient myotubes is related to mitochondrial activity. A greater calcium uptake by mitochondria could maintain SOCE activation and, in turn, increase the duration of related calcium transients. We thus explored free calcium concentration into mitochondria after SOCE activation.

Measurement of Ca^{2+} transients in mitochondria during store-operated influx

For this measurement, we used an aequorin targeted to mitochondria (mtAeq). SolC1dys- and SolD7minidys+ were transiently transfected with the mtAeq/pcDNA1 plasmid. The procedure for depletion of calcium stores was the same as previously. Restoration of 1.8 mM Ca^{2+} led to an increase in $[Ca^{2+}]_m$ ([Fig. 4A](#)). Because the increase in $[Ca^{2+}]_m$ was biphasic, the maximum

amplitude and the time-to-peak were determined for each different phase. The average time to first peak was shorter than 20 s, and this value was arbitrarily chosen to define two parts of the curve: between 0 and 20 s and between 20 s and 4 min (Fig. 4A). As shown in Fig. 4B, the mean amplitude of the first phase was similar for SolC1dys- and SolD7minidys+ ($A1=2.20\pm0.55$ μM , $n=18$ vs. $A1=1.32\pm0.35$ μM , $n=20$, respectively) but was significantly higher for the second phase in SolC1dys- than in SolD7minidys+ ($A2=1.88\pm0.38$ μM , $n=17$ vs. $A2=1.07\pm0.07$ μM , $n=27$, respectively). The mean value of time to peak for the first and second phases was similar in the both cell types actually $T1=0.19\pm0.02$ s, $n=18$ and $T2=0.62\pm0.04$ s, $n=17$ for SolC1dys- and $T1=0.15\pm0.02$ s, $n=20$ and $T2=0.65\pm0.04$ s, $n=27$ for SolD7minidys+. More importantly, the mean area under the curve corresponding to the two portions of the curve was significantly reduced in SolD7minidys+ ($\text{Area}1=10.43\pm2.03$, $n=27$ and $\text{Area}2=74.84\pm5.89$, $n=27$) in comparison to SolC1dys- ($\text{Area}1=24.09\pm5.99$, $n=18$ and $\text{Area}2=110.12\pm15.09$, $n=18$). Fig. 4C illustrates with two representative recordings that the Ca^{2+} uptake by mitochondria during SOCE was higher in SolC1dys- than in SolD7minidys+.

DISCUSSION

Depletion of intracellular stores by receptor activation or inhibition of intracellular Ca^{2+} -ATPase has been previously shown to activate capacitative calcium influx in cultured skeletal muscle cells (14) and skeletal muscle fibers (30). The activation of SOCE could be demonstrated by means of patch clamp recording, manganese quenching of fura-2, and intracellular calcium measurements. During the activity of skeletal muscle cells, the extrusion of calcium through the plasma membrane is extremely slow and the majority of calcium released during a single contraction is immediately restored in the SR. However, calcium release decreases during continuous activation of skeletal muscle in calcium-free media (32), which suggests store depletion. Moreover, the development of sustained force requires extracellular calcium influx in some conditions (33, 34). During iterative stimulation by depolarization, the progressive increase in contraction obtained at a specific frequency, is also dependent on calcium influx and store refilling (35). Our observations and those from Kurebayashi and Ogawa (30) showed that complete or partial depletion of SR stores during repetitive activation of calcium release stimulated store-dependent calcium influx that could be functional in physiological conditions.

Single-channel studies on mouse muscle fibers previously demonstrated that store-dependent calcium currents are more important in fibers from *mdx* dystrophic mice than in fibers from normal mice (20). The present study shows that when minidystrophin is reintroduced in dystrophin-deficient myotubes, store-dependent calcium influx is reduced. The attenuation of store-dependent calcium influx is not observed at the myoblast stage when minidystrophin is distributed in the cytoplasm. The store-dependent calcium influx can be attenuated by the presence of minidystrophin only when it is expressed underneath the sarcolemma. This suggests that specific interactions of dystrophin with the membrane-associated complex (Fig. 5) are necessary for the regulation of store-dependent calcium channels. The level of the calcium influx observed in myotubes expressing recombinant minidystrophin, was comparable to the one measured in myotubes expressing native dystrophin. The presence of minidystrophin at the sarcolemma is thus clearly able to restore normal store-dependent calcium influx in myotubes. The regulation of SOCE was observed at day 5 (i.e., 3 days of differentiation), preceding the culture stage where abnormalities in calcium homeostasis are observed in Sol8 dystrophin-deficient myotubes (19). Thus, the negative regulation of SOCE by minidystrophin could be one

of the key parameters explaining why this cytoskeletal protein is able to maintain normal calcium homeostasis in myotubes at latter stages (19). This regulation is observed in the early developmental stage of myotubes displaying no contractile activity. This does not exclude, however, the involvement of additive deregulation induced by membrane stretching, which occurs when the contractile activity takes place in dystrophic cells. Dystrophin could control the sarcolemmal calcium entry in muscle cells by a complex mechanism involving the protection against membrane ruptures or against overactivation of mechanosensitive channels and store-dependent channels. TRPC channels could support the store-dependent calcium influx observed in skeletal muscle cells (20). Moreover, TRPC1 have been recently shown to form stretch-activated cation channels in addition to classical store-dependent channels (36). This protein could thus also support the stretch-activated currents showing higher activity in dystrophic muscle cells (37, 38). Among the seven known isoforms, five were detected by RT-PCR (TRPC1, 2, 3, 4, and 6) in our cell models (data not shown) and in adult fibers (20). Western blot analysis and immunocytochemistry of normal and *mdx* muscle fibers demonstrated the localization of TRPC1, 4, and 6 proteins in the plasma membrane.

Our study shows that skeletal muscle cells develop a cross-talk between store-dependent transmembrane influx and calcium entry in mitochondria. The rapid uptake of store-dependent calcium influx by the mitochondria is known to prevent the calcium-dependent inactivation of store-operated Ca^{2+} -current in other preparations (21, 22, 39). These previous studies demonstrated that, under physiological conditions, mitochondrial buffering of cytosolic calcium potentiates the activation of store-operated currents and controls its extent and duration. This is coherent with our observation that in both myotube populations, depolarization of mitochondria that inhibits their ability to take Ca^{2+} (21) also reduced prominently the amplitude of store-dependent calcium transients. The prolonged duration of store-dependent calcium transients in SolC1dys- myotubes was more affected by mitochondria depolarization. Under these conditions, the proportion of SolC1dys- myotubes exhibiting a more rapid relaxation of store-dependent calcium transients was similar to the one observed in SolD6minidys + myotubes. This could be explained by the fact that a high and rapid mitochondrial uptake prevents calcium inactivation of store-dependent calcium influx (Fig. 5). This suggests that in dystrophin-deficient myotubes, the mitochondria might buffer cytosolic calcium more actively, in order to compensate for the increased store-dependent calcium influx (Fig. 5). Indeed, measurements of store-dependent calcium transients in the mitochondria by means of recombinant aequorine have shown that the amount of calcium entering the mitochondria after store depletion was higher for dystrophin-deficient myotubes. This is in complete accordance with the previous observation that in activated *mdx* dystrophic myotubes, mitochondrial calcium uptake was higher than in nondystrophic cultures. The higher uptake of cytosolic calcium by mitochondria in response to an increased store-dependent influx may potentiate prolonged activation of SOCE, which, in turn, increases the amount of calcium buffered by the mitochondria (Fig. 5). This feedback loop is likely a critical point that controls the calcium overload of mitochondria and may lead to alterations of mitochondrial metabolism that will accelerate the process of necrosis.

We speculate that the dystrophin-based cytoskeleton, by means of its scaffolding ability, negatively regulates the amount of store-dependent influx during activation of calcium release in muscle cells (Fig. 5). This could involve a mechanism of cationic channel accumulation and immobilization at specific membrane domains (accumulation machine) and/or the building of complexes, including signaling molecules modulating channel activity. The disruption of the

dystrophin-associated complex removes the negative control and leads to a compensatory response of cellular calcium buffers like the mitochondria that have the tendency to overload (Fig. 5). Minidystrophin, which lacks 17 repeats of the central rod domain, retains the ability to negatively regulate store-dependent calcium influx and to restore a global normal calcium homeostasis (19). Our observations provide additional arguments for the use of minidystrophin for functional recovery of dystrophin-deficient muscles. The c-terminal domain was shown to be involved in the interaction with the dystrophin-associated complex or in the recovery of membrane associated NO synthase. One can speculate that this functional domain and the multiple interactions with associated proteins are participating in the normal regulation of SOCE. Future investigations will be conducted to explore molecular interactions of the dystrophin-associated complex with store-dependent channels.

ACKNOWLEDGMENTS

This work was supported by the CNRS, the French Ministry of Research, “Association française contre les myopathies” (AFM), the “Association belge contre les maladies neuro-musculaires” (ABMM) and by a grant ARC 00/05-05/10 from the General Direction of Scientific Research of the French Community of Belgium. We thank Françoise Mazin for technical assistance and Elise Mok for her help in editing the manuscript.

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Received March 3, 2005; accepted September 8, 2005

Fig. 1

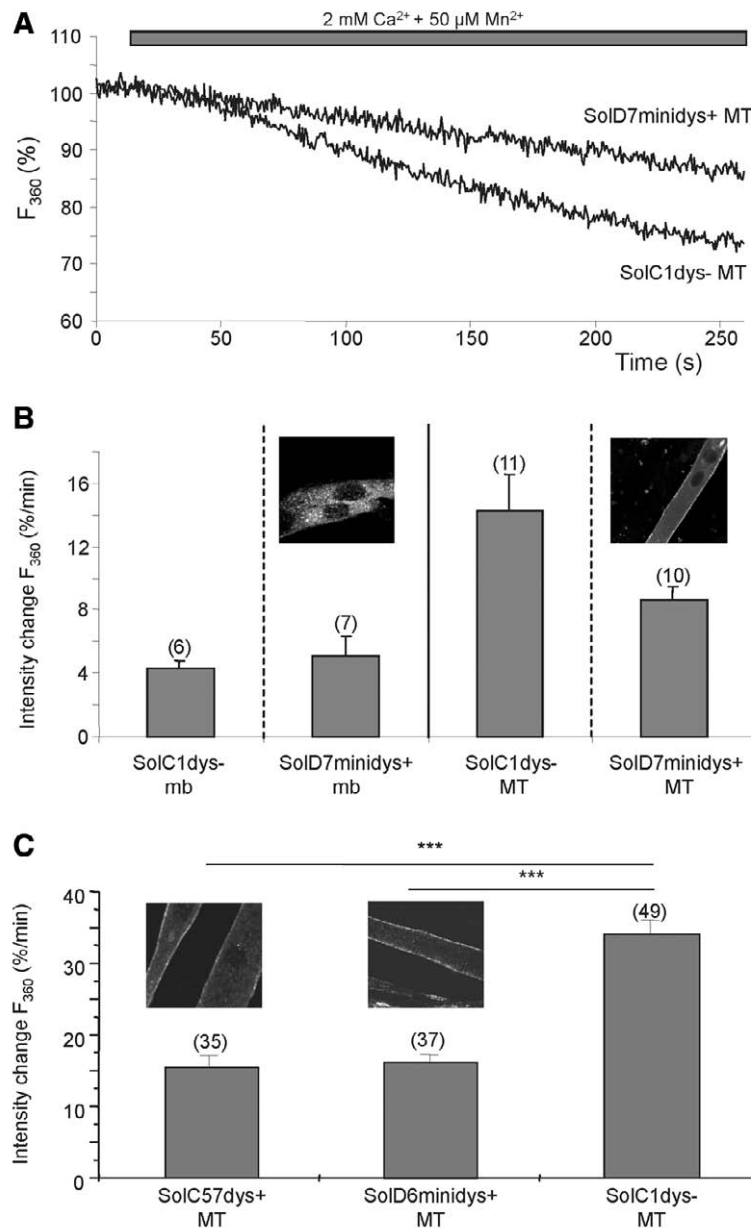


Figure 1. Effect of sarcoplasmic reticulum (SR) depletion on calcium transients in SolC1dys⁻, SolD7minidys⁺, SolD6minidys⁺ and SolC57dys⁺ myoblasts and myotubes. **A**) Measurement of the fura-2 emission intensity (with an excitation at 360 nm) in representative SolD7minidys⁺ and SolC1dys⁻ (dystrophin-deficient) myotubes. In the example illustrated here, Ca²⁺ stores had been previously depleted by application of thapsigargin (1 μM) in Ca²⁺-free recording medium. The progressive quenching of fluorescence is due to the influx of Mn²⁺ taken as a surrogate of Ca²⁺ after the reintroduction of 2 mM Ca²⁺ with 50 μM Mn²⁺ in the extracellular medium. **B**) Slope of the Mn²⁺-induced fluorescence quenching in SolC1dys⁻ (SolC1dys⁻ mb, n=6) and SolD7minidys⁺ (SolD7minidys⁺ mb, n=7) myoblasts and SolC1dys⁻ (SolC1dys⁻ MT, n=11) and SolD7minidys⁺ (SolD7minidys⁺ MT, n=10) myotubes. *B insert*) Immunostaining of minidystrophin in SolD7minidys⁺ myoblasts and myotubes at F+3 stage. Images are taken and analyzed by fluorescence confocal microscopy. **C**) Slope of the Mn²⁺-induced fluorescence quenching in SolC1dys⁻ (SolC1dys⁻ MT, n=49), SolD6minidys⁺ (SolD6minidys⁺ MT, n=37) and SolC57dys⁺ (SolC57dys⁺ MT, n=35) myotubes. Stores were emptied with three stimulations by 10 mM caffeine in presence of 5 μM CPA and in absence of Ca²⁺, spaced out by 15 μM cyclopiazonic acid (CPA) in Ca²⁺-free solution. Reintroduction of 1.8 mM Ca²⁺ with 50 μM Mn²⁺ in extracellular medium leads to a decrease of the fluorescence intensity corresponding to SOCE. *C, insert*) Immunostaining of dystrophin in SolC57dys myotubes and of minidystrophin in SolD6minidys⁺ myotubes at F+3 stage.

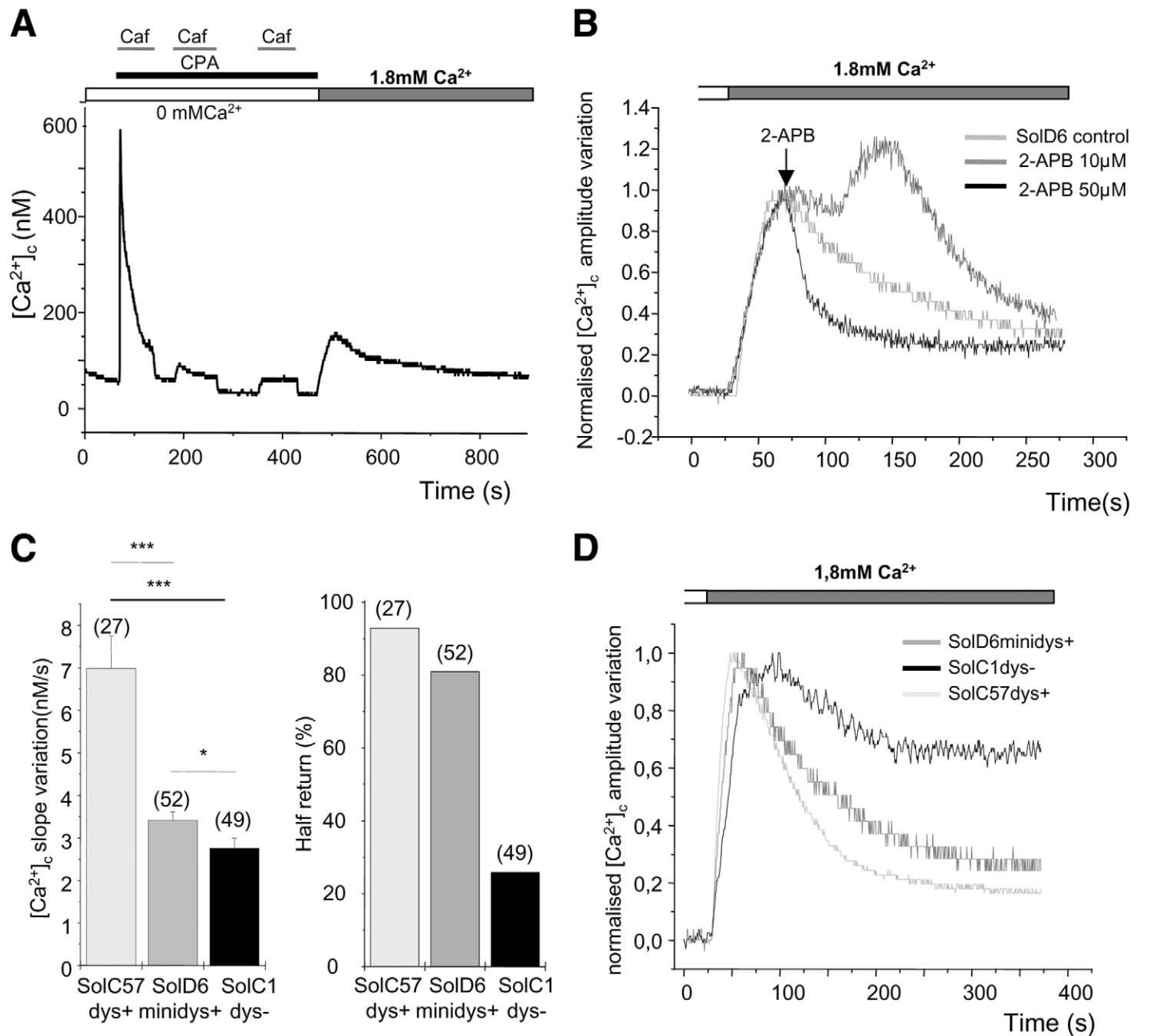
Fig. 2

Figure 2. INDO-1 ratiometric measurements of intracellular-free calcium concentration ($[Ca^{2+}]_i$) in SolC1dys- and in Sold6minidys+. Measurements were obtained 3 days after promoting fusion. **A**) Example of INDO-1 ratiometric measurements. Stores were emptied with three stimulations by 10 mM caffeine in presence of 5 μ M CPA and in the absence of Ca^{2+} . Reintroduction of 1.8 mM Ca^{2+} in extracellular medium leads to an increase of $[Ca^{2+}]_i$ corresponding to SOCE. **B**) Effect of 2-APB on SOCE in Sold6minidys+. In gray, activation of store-operated calcium entry (SOCE) by 10 μ M 2-APB and in light gray, inhibition by 50 μ M 2-APB. Solid line corresponds to the control. The arrow indicates the time of 2-APB addition. Traces were normalized vs. each peak amplitude before application of 2-APB. **C**) Bar chart comparing the initial slope of the Ca^{2+} signal upon Ca^{2+} readmission and the fraction of cells returning under half amplitude in SolC1dys- (in black), in Sold6minidys+ (in gray) and in SolC57dys+ (in light gray). Numbers of cells (n) are: SolC1dys-, 49; Sold6minidys+, 52, SolC57dys+, 27. **D**) Comparison of normalized amplitude of the entry in SolC1dys- (in black), Sold6minidys+ (in gray) and in SolC57dys+ (in light gray). Traces were normalized vs. each peak amplitude.

Fig. 3

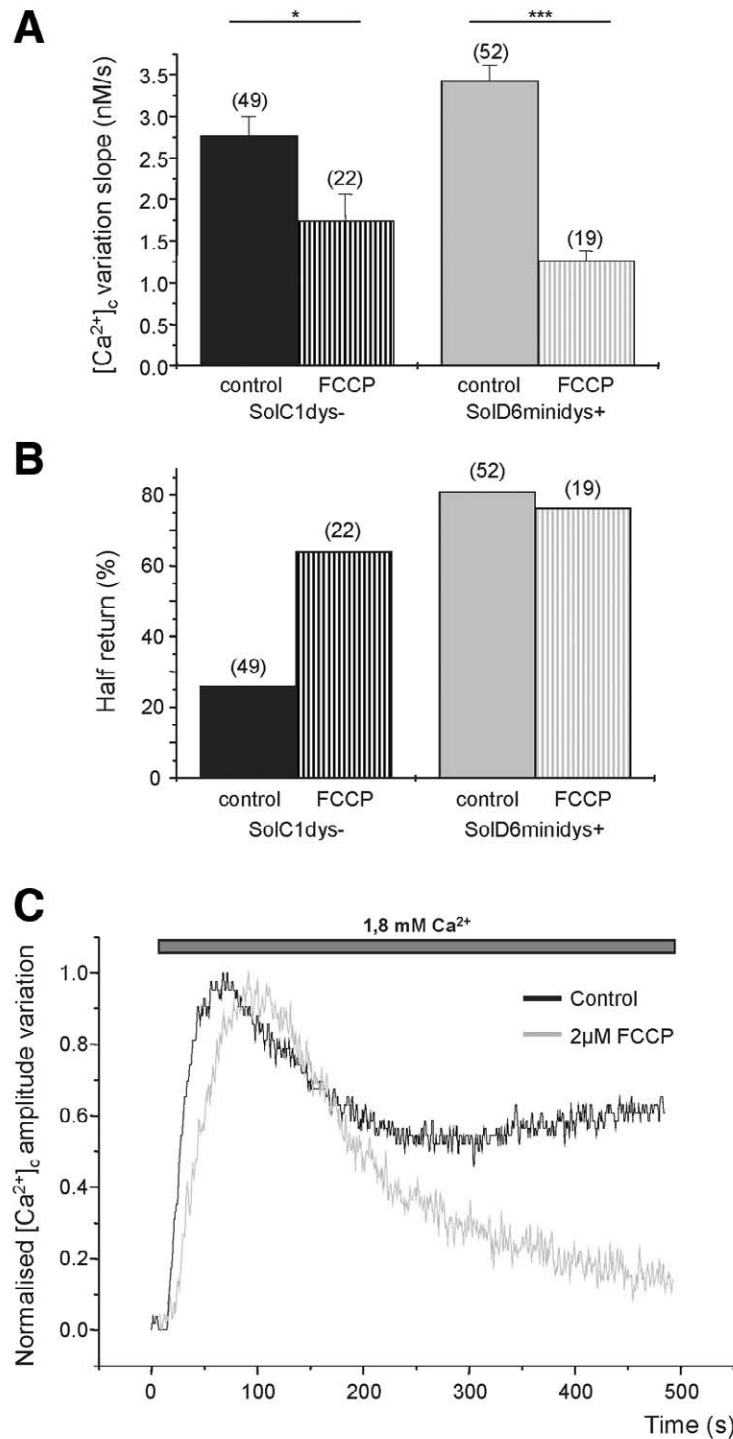


Figure 3. Mitochondrial depolarization reduces calcium entry after store depletion. Cells were treated with 2 μM FCCP + 5 μM CPA for 1 min before reintroduction of calcium in extracellular medium. INDO-1 ratiometric measurements of intracellular-free calcium concentration were obtained at 3 days after promoting fusion. *n* values are SolC1dys-, 49; SolD6minidys+, 52; SolC1dys- + FCCP, 25; SolD6minidys+ + FCCP, 21. **A**) Bar charts comparing the initial slope of [Ca²⁺]_c variation in control cells, SolC1dys- and SolD6minidys+, with those pre-exposed to FCCP. **B**) Bar charts comparing the fraction of cells returning to half amplitude in control cells, SolC1dys- (black) and SolD6minidys+ (gray), with those pre-exposed to FCCP (lined). **C**) Representative example of entry kinetics in SolC1dys- (in black) and SolC1dys- pre-exposed to FCCP (in gray). Traces were normalized vs. the maximum of each peak amplitude.

Fig. 4

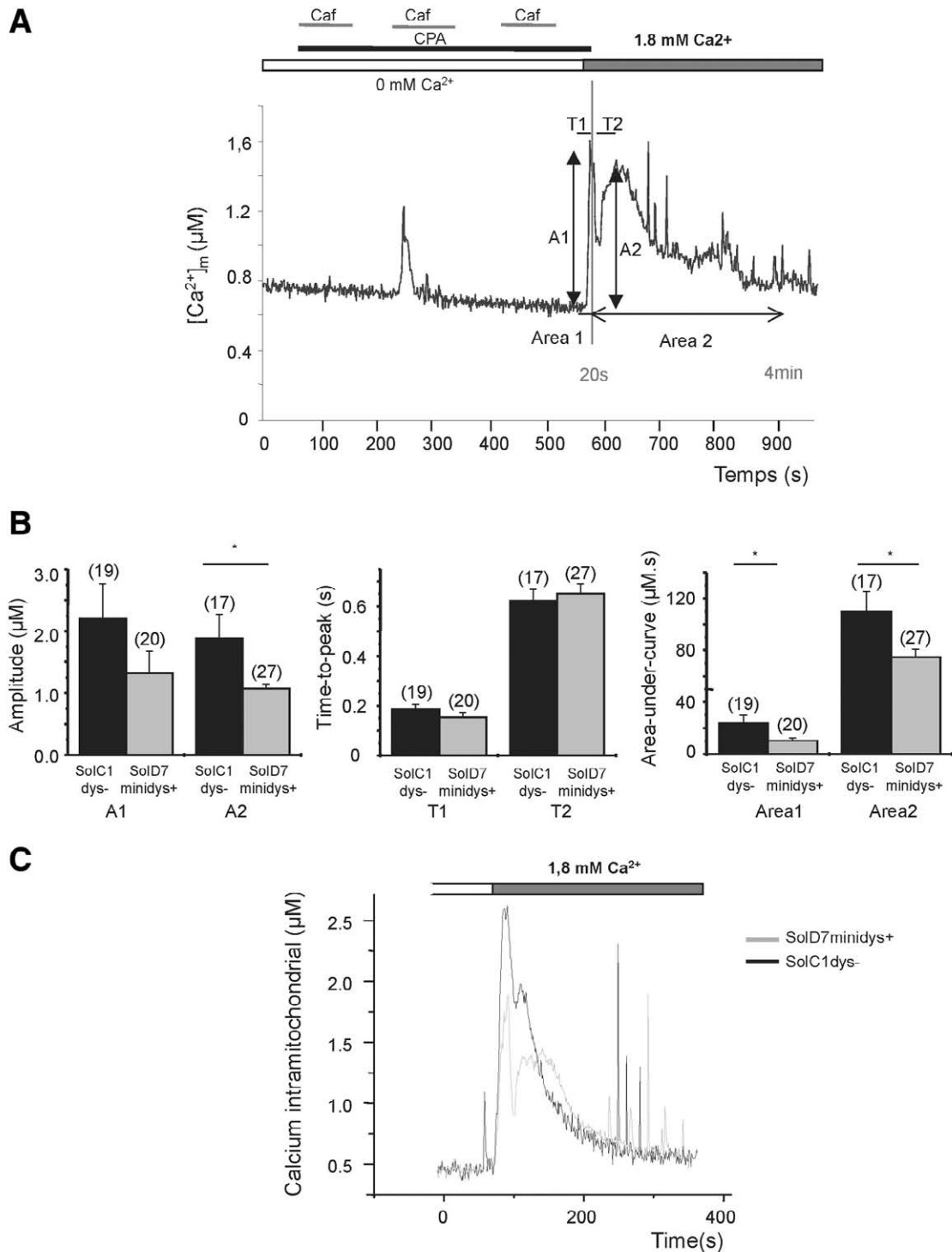


Figure 4. Measurement of intramitochondrial calcium variation during SOCE. Myotubes transiently expressed mtAEQ. **A**) Example of intramitochondrial calcium measurement. Stores were emptied with three stimulations by 10 mM caffeine in presence of 5 µM CPA and in absence of Ca²⁺. Reintroduction of 1.8 mM Ca²⁺ in extracellular medium leads to an increase of [Ca²⁺]_m, corresponding to uptake of SOCE. Six parameters were measured on biphasic Ca²⁺ increase: 2 amplitudes (A1 and A2), 2 time-to-peak (T1 and T2), and two areas under the curve (Area1 and Area2). **B**) Bar chart comparing the amplitude (µM), the time-to-peak (s), and the area under curve of the increase of Ca²⁺ signal upon readmission Ca²⁺ in SolC1dys⁻ (in black) and SolD7minidys⁺ (in gray). Numbers of cell (n) are SolC1dys⁻, 19; SolD7minidys⁺, 27. **C**) Representative example of intramitochondrial Ca²⁺ signals during SOCE in SolC1dys⁻ (in black) and SolD7minidys⁺ (in gray). Traces were normalized vs. the maximum of each peak amplitude.

Fig. 5

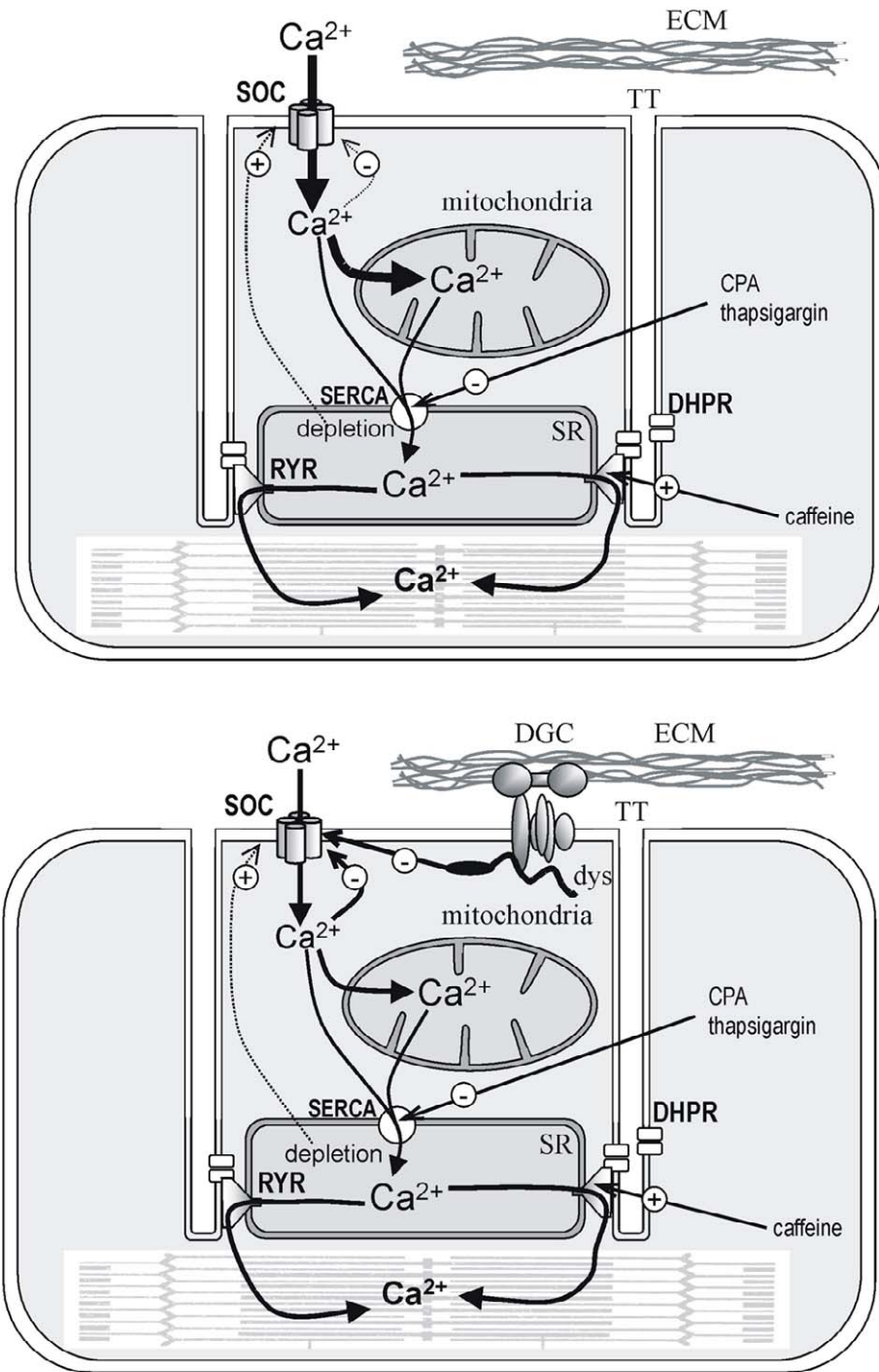


Figure 5. Model for Ca²⁺ movements in dystrophin-deficient and minidystrophin-expressing myotubes. Depolarization or caffeine activates the opening of ryanodine receptors (RYR) and calcium release from the sarcoplasmic reticulum (SR). Sustained or iterative stimulations lead to a decrease of the luminal amount in Ca²⁺, which is more pronounced by blocking the recycling in SR via the calcium ATPase SERCA. Depletion of the SR stores induces the activation of store-operated channels (SOC) generating calcium influx. The store-dependent calcium entry is pronounced in dystrophin-deficient myotubes, as well as the uptake by mitochondria, which in turn maintain the activation of SOC by reducing the inhibition of the channels by local cytosolic Ca²⁺. In minidystrophin-expressing myotubes, the store-dependent calcium entries are moderate as a result of interactions with the dystrophin-associated complex. The calcium uptake by mitochondria during SOCE is also less pronounced, and SOC are modulated by the inhibition due to local cytosolic Ca²⁺.