Negative modulation of inositol 1,4,5-trisphosphate type 1 receptor expression prevents dystrophin-deficient muscle cells death

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Mondin L, Balghi H, Constant B, Cognard C, Sebille S. Negative modulation of inositol 1,4,5-trisphosphate type 1 receptor expression prevents dystrophin-deficient muscle cells death. Am J Physiol Cell Physiol 297: C1133–C1145, 2009. First published August 19, 2009; doi:10.1152/ajpcell.00048.2009.—Evidence for a modulatory effect of cyclosporin A (CsA) on calcium signaling and cell survival in dystrophin-deficient cells is presented. Our previous works strongly supported the hypothesis of an overactivation of Ca2+ release via inositol 1,4,5-trisphosphate (IP3) receptors (IP3R) in dystrophin-deficient cells, both during membrane depolarization and at rest, through spontaneous Ca2+ release events. Forced expression of mini-dystrophin in these cells contributed, during stimulation and in resting condition, to the recovery of a controlled calcium homeostasis. In the present work, we demonstrate that CsA exposure displayed a dual-modulator effect on calcium signaling in dystrophin-deficient cells. Short-time incubation induced a decrease of IP3-dependent calcium release, leading to patterns of release similar to those observed in myotubes expressing mini-dystrophin, whereas long-time incubation reduced the expression of the type I of IP3 receptors (IP3R-1) RNA levels. Moreover, both IP3R-1 knockdown and blockade through 2-aminoethoxydiphenyle borate or CsA induced improved survival of dystrophin-deficient myotubes, demonstrating the cell death dependence on the IP3-dependent calcium signaling as well as the protective effect of CsA. Inhibition of the IP3 pathway could be a very interesting approach for reducing the natural cell death of dystrophin-deficient cells in development.

calci um regulation; cyclosporin A; muscular dystrophy

DUCHENNE MUSCULAR DYSTROPHY (DMD) is a severe X-linked recessive, progressive muscle-wasting disease affecting 1/3,500 male births. A milder form of the disease, Becker muscular dystrophy (BMD), has a later onset and a much longer survival. Both disorders are caused by mutations in the DMD gene that encodes a 427-kDa cytoskeletal protein dystrophin expressed at the inner surface of the sarcolemma of muscle fibers (26). The vast majority of DMD mutations results in the absence of dystrophin in all muscle fibers, apart from the sporadic “revertant” fibers (49), whereas the presence of a low level of a 229-kDa truncated protein mini-dystrophin is seen in BMD patients (20).

Perturbations in intracellular calcium homeostasis are thought to come with DMD. Indications suggesting such calcium alterations were discovered early before dystrophin protein was identified (16), and it was then proposed that the absence of dystrophin leads to increased levels of intracellular calcium (11), contributing to muscle degeneration through activation of calcium-dependent proteases. Further data progressively reinforced such a hypothesis. Elevated subsarcolemmal calcium levels were found in mdx muscle cells using direct measurements (6) and as well as the exploration of calcium-activated K+ channels activities (32). It has been also hypothesized that calcium entry due to increased microdisruptions in dystrophic muscle cells results in an alteration of calcium leak channels. First, a local exocytotic response induced by calcium influx from a membrane wound could increase the amount of calcium leak channels (2), and second, these channels could be activated by calcium-dependent proteolysis (calpain) (1). But the sustained calcium levels could also alter the activity of intracellular calcium channels as inositol 1,4,5-trisphosphate (IP3) receptors (IP3Rs) (25) or ryanodine receptors (RyRs) (8).

Important progress in the understanding of muscular dystrophies in human and animal models have been gained from studies showing that the linkage of the dystrophin-related cytoskeleton to the extracellular matrix, mediated by the dystrophin-associated proteins (DAP) complex, is involved in membrane stability for transduction and membrane proteins functions (13, 21). Many studies were focused on dystrophin, and the DAP complex was viewed as a structural scaffold protecting the sarcolemma from the mechanical constraint of the intracellular contracting machinery, but it is also clear that this complex plays an important role in the regulation of intracellular signaling (7, 15). Despite the present view in which the development of DMD pathological phenotype could result from multiple contributing factors such as calcium overload or deregulation, mechanical effect, and disturbed signaling pathway (7), the question remains: how the absence of dystrophin can lead to such a calcium disturbance at the origin of the extensive muscle degeneration is still poorly understood.

Among key elements of Ca2+ signaling, there are specialized intracellular Ca2+-released channels located at the sarcoplasmic reticulum membrane. Two types are found in skeletal muscle cells in development: the RyR and the IP3R. RyR channels play a pivotal role in muscle excitation-contraction coupling, their opening, and the concomitant release of calcium being triggered during the rapid transmembrane electrical potential variation of the action potential (AP), by a conformational change of the voltage sensors of the cell membrane dihydropyridine receptors (41, 43). IP3R channels, which play a key role in Ca2+ signaling in many other mammalian cells (9, 22), are also thought to be involved in skeletal muscle. Skeletal muscle fibers possess the basic molecular machinery for a working IP3 messenger system (14). Recent works demonstrated the involvement of IP3-dependent calcium release in skeletal muscle cells in development, and this release pathway was thought to participate particularly in...
the signaling pathways that may modulate long-term adaptative responses (44).

Our previous works strongly supported the hypothesis of an overactivation of Ca\(^{2+}\) release in dystrophin-deficient cells, both during membrane depolarization (4) and, at rest, through spontaneous Ca\(^{2+}\) release events (5). Previous studies from other groups have provided evidence for at least two phases in the calcium rise observed in cultured skeletal muscle cells exposed to a high potassium solution (27, 40). According to their kinetic properties, these two phases were identified as fast and slow calcium signals. In these works, the slow calcium signal was eliminated in the presence of IP3 inhibitors, whereas the fast signal was abolished by ryanodine, showing that the calcium increase observed in these experimental conditions was mainly originating from sarcoplasmic release.

Similar results were observed in our previous works with the demonstration of the enhancement of the IP3-dependent release phase and of spontaneous calcium release events in dystrophin-deficient cells (4, 5). Furthermore, forced expression of mini-dystrophin in dystrophin-deficient cells contributed, both during stimulation and in resting condition, to recover a more controlled calcium homeostasis.

Here we report the modulation effect of cyclosporin A (CsA) on calcium signaling in dystrophin-deficient cells [SolC1(−)] and in mini-dystrophin-transfected cells [SolD(+)], and that this effect was mediated through the IP3 signaling pathway as well as through the gene expression of IP3 receptor. Furthermore, the use of a reliable and demonstrative cell viability assay adapted for dystrophin-deficient muscle cells allowed us to show that pharmacological or small interfering RNA (siRNA) manipulations of these two types of regulation pathway could have actual physiological consequences on the level of natural calcium-dependent cell death. A clear protective effect of CsA and other pharmacological IP3 modulators on the cell survival was demonstrated.

**MATERIALS AND METHODS**

**Cell Lines**

Sol8 myogenic cell line (a gift from I. Martelly, University of Paris XII, Creteil, France) derived from the Sol8 cell line originally was obtained from primary culture of normal C3H mouse soleus muscle (38). The method for obtaining cell lines [SolC1(−) and SolD(+)] was described elsewhere (33). In brief, after several steps of cloning, a dystrophin-deficient cell line was obtained, named SolC1(−). The SolD(+) cell line was obtained by transfection of the SolC1(−) cell line with a retrovirus encoding for mini-dystrophin (22 kDa), SolC1(−) and SolD(+) cell lines (“Sol” cell lines in the following text) maintain a high ability to fuse and form myotubes. Cells were seeded on gelatin-coated glass coverslips in plastic dishes. Myoblasts were grown to around 80% of confluence in Ham F12/DMEM (1:1) medium supplemented with 10% fetal calf serum, 1% l-glutamine, and 1% antibiotics. To induce differentiation, the growth medium was changed to a fusion medium [DMEM supplemented with 2% heat-inactivated horse serum, insulin (10 μg/ml, Sigma-Aldrich), 1% l-glutamine, and 1% antibiotics] to promote fusion of myoblasts into myotubes. The cells were stored at 37°C in water-saturated atmosphere of 95% air and 5% CO\(_2\).

**RT-PCR**

To observe the effect of CsA on IP3R-1 expression, myotubes were incubated with CsA (100 and 300 nM) 3 days after promoting fusion (D3) for 24 h, and RNA was extracted for RT-PCR (IP3R-1) at D4.

Reverse transcription. Total cellular RNA was extracted using RNeBlot kit (Eurobio, Courtaboeuf, France). Twenty microliters of RNAs were reverse transcribed in a total volume of 44 μl consisting of first 10 μl of strand buffer (Tris·HCl 250 mM, pH 8.3; KCl 375 mM; MgCl\(_2\) 15 mM), 4.8 μg of pd(N)6 random hexamer (Amer sham), 22.6 mM of DTT (GIBCO), 2.2 mM of each dNTP (Invitrogen), and water for a final volume of 44 μl. This mix was incubated at 65°C for 2 min. Then 80 U of RNAsine (Promega) and 800 U of M-murine leukaemia virus reverse transcriptase (Promega) were added and incubated for 1 h at 37°C, in a final volume of 100 μl.

Real-time PCR. The 15 μl reaction mixture contained 7.5 μl Taqman Universal Master Mix (Applied Biosystems, Foster City, CA), 5 μl of RT products, and appropriate primers and probes (900 and 200 nM, respectively). All probes contained 6-carboxy-tetramethylrhodamine (3′-TAMRA) quencher dye and were labeled at the 5′ end with 6-carboxyfluorescein (FAM) reporter fluorescent dye (4). Amplification was carried out 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 carried out in 2× MicroAmp optical 96-well reaction plates (Applied Biosystems) using an ABI PRISM 7700 sequence detection system (Applied Biosystems). All measurements were normalized to the mitochondrial ribosomal protein S6 (MṛpS6, an endogenous control) to account for the variability in the initial concentration and quality of the total RNA.

**Cell Survival Assay: MTT Test**

To determine natural cell death and effects of agents on cell viability in myotubes, colorimetric 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich) assay was performed (37). Cell survival was evaluated by measuring the mitochondrial-dependent reduction of MTT. Myoblasts were incubated and differentiated to myotubes in 96-well plates. One day after fusion, cells were incubated during 4 days in the presence of 2-aminoethoxydiphenyle borate (2-APB) or CsA at 37°C, and the MTT test was performed the day after (5 days after promotion of fusion). At each point of treatment, cells were washed with PBS (in mM: 130 NaCl, 2 KCl, 1.5 Na\(_2\)HPO\(_4\), and 8 K\(_2\)HPO\(_4\), pH 7.4). The medium was removed and 100 μl of MTT solution (5 mg/ml in PBS) were added to each well. After 4 h of incubation at 37°C, the supernatant was removed and the purple formazan crystals were dissolved by addition of 100 μl dimethylsulfoxide (DMSO, Sigma-Aldrich). The plates were agitated, and the optical density was read at the wavelength of 570 nm and a reference wavelength at 630 nm in a microplate reader (SpectraCount microplate photometer, Packard). Report values were the mean of three replicates and are expressed as percentage of the control values.

**Intracellular Calcium Activities**

Calcium activities were recorded by confocal laser scanning microscopy using a Bio-Rad MRC 1024 equipped with a 15 μW Ar-Kr gas laser. The confocal unit was attached to an inverted microscope (Olympus IX70). Fluorescence signal collection was performed through the control software Lasersharp 3.2 (Bio-Rad). Myoblasts were loaded with 3 μM fluo-4 acetoxyxymethyl (AM) ester (Molecular Probes) for 20 min at room temperature (20°C) in a resting solution (in mM: 130 NaCl, 5.4 KCl, 2.5 CaCl\(_2\), 0.8 MgCl\(_2\), 10 HEPES, and 5.6 glucose, pH 7.4). Experiments were performed at D4 after fusion for SolC1(−) and SolD(+) myotubes.

**K\(^+\)-Evoked Calcium Increase**

Confocal calcium measurements. The fluorescence images were visualized through the ×60 oil immersion objective and collected every 30 ms (256 × 256 pixels). Regions of interest (ROIs) have been selected in myotubes, and fluorescence transients from these ROIs were analyzed. Myotubes exposed to high K\(^+\) solution (KCl 47
mM) were depolarized by a fast change of solution by perfusion inducing global calcium variations. 2-APB was incubated at 50 μM for 30 min to inhibit IP3R. CsA was also tested for the inhibition of IP3R at 10 and 100 μM for 30 min and 300 mM for 16 h.

**Calcium transient processing.** Fluorescence transients from ROIs in fluo-4-loaded myotubes were analyzed with a computer program developed in our laboratory under IDL 5.3 structured language on a PC computer. Three parameters were extracted from analyzed curves for the characterization of calcium-release amplitude and kinetics. These parameters were the area under the curve (AUC: in arbitrary units·second (AU·s)) of the normalized curve, the increase slope (IS: in AU·s), and the half-decay time (HD: in s). The use of these kinetic parameters allowed us previously to demonstrate considerable differences in kinetics between Sol cells, supporting the presence of two processes of excitation-calcium release coupling in these cells (4). These parameters were represented in histograms as relative values to control conditions in SolD (+) set at 100%.

**Localized Calcium Release Events: Release Site Density at Rest**

Confocal calcium measurements. Density of release sites (RSD) was obtained by recording sequences of 30–50 images (256 × 256 pixels) with an ×20 objective in fast scanning mode. The time resolution was 300 ms for each pixel position. 2-APB was incubated at 50 μM for 30 min to inhibit IP3R. CsA was also tested for the inhibition of IP3R at 100 nM, 1, 10, and 100 μM for 30 min.

**Image processing.** Calcium images were analyzed with a computer program developed in our laboratory under IDL 5.3 structured language. Sequences of images in fast mode were analyzed with a program allowing us to calculate the standard deviation of the recorded fluorescence in each pixel as a function of time. During the acquisition sequence, when several releases were observed in the same location, the calculated standard deviation of pixels in this location was higher than in areas without calcium increase. The result obtained was an image with discrete spots of elevated standard deviation on which it was easy to compute the number of release sites in the sequence. From that process a parameter can be derived that describes the RSDs in myotubes: the number of events/μm² (in μm−2).

**Downregulation of IP3R1 by siRNA**

siRNA transfection was performed using Amaxa Cell Line Nucleofactor Kit V (Amaza). Myoblasts were grown until the required number of cells for siRNA transfection was attained. They were replaced in a suspension by trypsinization and were transfected with 3 μg of siRNA targeting IP3R-1 (5'-ACCAAGGUAGGUGCUGA-3') or control siRNA, according to the manufacturer’s protocol. After transfection was completed, myoblasts were incubated in 96-well plates or 6-well plates and differentiated to myotubes 1 day after transfection. At D3 it was possible to obtain well-differentiated myotubes in the temporal window of effective transitory extinction effect of siRNA. MTT assay, calcium measurements, or Western blot analysis were further performed on these transfected myotubes.

**Propagation of Cell Membrane Stains by siRNA**

Preparation of cell lysates and Western blot analysis were performed as previously described (4).

**Propidium Iodide Staining**

Propidium iodide (PI, Sigma-Aldrich), a nucleic acid stain, was used to identify dead cells. PI was not cell permeant and was only penetrated into dead cells. Cells were incubated with 50 μg/ml of PI during 5 min at room temperature under agitation. Cells were washed twice with control solution before experiments. The PI was excited with the 568-nm yellow line of a confocal microscope (Bio-Rad MRC 1024), and the acquisition of PI labeling images was performed at the wavelength higher than 600 nm via a photomultiplier through a band-pass filter centered at 605 nm.

**Drugs**

2-APB was from Calbiochem, and CsA and cypermethrin were from Sigma-Aldrich. CsA solution was prepared in ethanol at 60 μM. 2-APB solution was prepared in DMSO (Sigma-Aldrich) at 50 mM concentration, and cypermethrin solution was prepared at 20 mM in methanol.

**Statistical Analysis**

All results are expressed as means ± SE of n observations. Sets of data were compared using two-way ANOVA and Tukey post hoc test. Main comparative analyses were performed by using two-factor repeated-measures analysis of variance to test the effect of time (within subject factor) and cell types (between-subject factor) and the time-cell types interaction. All statistical tests were performed using GraphPad Prism version 5.0 for Windows (GraphPad Software).

**RESULTS**

**Protective Effect of CsA Against Natural Cell Death of SolC1(−)**

To investigate the death of muscular cells lacking dystrophin, PI staining was previously used in our group (34). This fluorescent probe enters cells only through damaged plasma membranes and stains nuclei by its intercalating property. Counting of dead myotubes was performed with the help of fluorescent-stained myonuclei (Fig. 1A, inset), and cell viability was expressed (Fig. 1A) as a percentage relative to the number of SolD (+) viable myotubes 4 days after promoting fusion (D4, 100% living cells). Starting from the same amount of cells when promoting fusion (D0), the cell viability was higher at D4 in SolD (+) myotubes than in SolC1(−) (Fig. 1A). Cell survival of SolD (+) myotubes slightly decreased during the time course of fusion from D4 to D7, whereas a stronger decrease of cell viability was observed in SolC1(−) myotubes. As it has been previously demonstrated (34), dystrophin-deficient cells from the SolC1(−) line were progressively dying after promoting differentiation, whereas SolD (+) remained living at a more extent. To perform a large-scale analysis, we have adapted the MTT assay to our cell culture protocols (proliferation of myoblasts and differentiation into myotubes). MTT assays were performed during the time course of culture, starting from D4 to D7 in SolC1(−) and SolD (+) myotubes (Fig. 1B). Results were expressed as the percentage relative to the number of SolD (+) viable myotubes at D4 (100% living cells). In the same way of results obtained with PI staining (Fig. 1A), cell viability at these differentiation stages was lower in SolC1(−) myotubes (open bars) than in SolD (+) myotubes (closed bars). Statistical analyses revealed, first, a significant effect of time on cell survival parameter in both cell types. Hence, cell viability was significantly decreasing, from D4 to D7, in both SolD (+) and SolC1(−) myotubes. Second, the decrease of cell viability significantly differed between the two cell types (P < 0.001). The decrease observed in SolC1(−) was stronger (47%) than that in SolD (+) (33%) between D4 and D7.

We further used the MTT cell viability assay to quantify the protective effect of various agents against the natural cell death observed in these cell lines. At D5, there was clear evidence of natural cell death in SolC1(−) myotubes, whereas the decrease
Effects of CsA on K⁺ exposure on calcium release properties in our cellular models. Consequently, we investigated the effect of CsA/H₁₁₀₀₂ deregulation could be involved in the natural cell death of phin-deficient cells, and we have hypothesized that such a and characterized a deregulation of calcium release in dystro-

squares). These data suggest that exposure to these two com-

A similar effect (Fig. 1 D) was observed with CsA treatment [SolC₁(−) control vs. each conditions of SolC₁(−)] (two-way ANOVA analysis followed by Tukey post test).

of cell viability in SolD(+) myotubes remained rather weak. We then decided to apply the MTT assay at this stage of differentiation (red boxes), allowing us to reveal a potential protective effect of the several agents used in this study. Protective effect of 2-APB (Fig. 1 C) and CsA (Fig. 1 D) was investigated in our preparations. With each agent, incubation was performed from D1 to D5 and MTT test was applied at D5. On one hand, regarding the cell survival, exposure of SolD(+) myotubes to 2-APB had no effect (Fig. 1 C, closed squares) with a cell viability of around 85%. On the other hand, when SolC₁(−) was incubated with 2-APB (from 12.5 to 50 μM), cell viability was found to increase with the concentration with a maximal effect at 25 μM (Fig. 1 C, open squares), evidencing a protective effect of this agent against SolC₁(−) natural cell death. A similar effect (Fig. 1 D) was observed with CsA exposure (75 to 600 nM) with a large protective effect on SolC₁(−) (Fig. 1 D, open squares) and a very weak toxicity of this compound on SolD(+) myotubes (Fig. 1 D, closed squares). These data suggest that exposure to these two compounds leads to the protection of dystrophin-deficient cells against death. In our previous works (4, 5), we have described and characterized a deregulation of calcium release in dystro-

Effects of CsA on K⁺-Evoked Calcium Increase

To explore the effects of 2-APB and CsA on global calcium release in SolD(+) and SolC₁(−), large-scale analysis has been performed on K⁺-evoked calcium transients recordings. Previous studies (27, 40) provided evidence for at least two phases in the calcium rise observed in cultured skeletal muscle cells exposed to a high potassium solution. According to their kinetic properties, these two phases were identified as slow, through IP₃Rs, and fast, through RyRs, calcium releases. Figure 2A shows representative examples of K⁺-evoked calcium increases in our preparations in control conditions and with incubation of CsA for SolC₁(−) myotubes (Fig. 2A, black lines). As previously described (4) an intracellular calcium increase was observed in both cell types upon superfusion with a depolarizing solution (47 mM KCl), and it was demonstrated that an IP₃-dependent slow release was more increased in SolC₁(−) compared with SolD(+) (Fig. 2A, black lines). As previously described (4) an intracellular calcium increase was observed in both cell types upon superfusion with a depolarizing solution (47 mM KCl), and it was demonstrated that an IP₃-dependent slow release was more increased in SolC₁(−) compared with SolD(+) (Fig. 2A, black line).

To quantify the effects of CsA in calcium release after depolarization, three parameters have been measured: the AUC (Fig. 2B), the HD time (Fig. 2C), and the IS (Fig. 2D). In control conditions, the AUC (Fig. 2B) was significantly higher in SolC₁(−) myotubes than in SolD(+) ones (160% higher). Incubation with 2-APB (50 μM, 30 min) led to a strong and significant decrease for this parameter in SolC₁(−) myotubes (81% decrease) and in SolD(+) (53% decrease). Whatever its concentration and its incubation time, CsA had also a significant decreasing effect on this parameter but only in SolC₁(−) cells. Short-time incubation (30 min) with high concentrations (10 and 100 μM) induced a decrease of AUC until 30%, but long-time incubation (16 h) with low concen-
tration (300 nM) led to the strongest decrease (36%). Figure 2, C and D, illustrates the effects of 2-APB or CsA on the HD and IS parameters. The 2-APB exposure leads to faster calcium transients. With CsA incubation, a significant decrease of HD parameter was observed with a marked effect for the long-time incubation (300 nM for 16 h). In control conditions the IS (Fig. 2D) was significantly higher (50%) in SolC1(+/H11002) myotubes than in SolD(+/H11001) ones. Like the AUC parameter, 2-APB exposure significantly decreased IS in both myotubes types [35% and 33% in SolC1(−) and SolD(+), respectively]. CsA had a similar effect to that observed with 2-APB, the extent of which was depending on its concentration and its incubation time. Effects on kinetics parameters (HD and IS) can be interpreted as a reduction of the slow calcium release. Moreover, it can be noticed that CsA exposure in SolC1(+/H11002) led to pattern of calcium release similar, at a lesser extent, to the one in SolD(+/H11001) myotubes in control conditions. Table 1 summarizes parameters obtained in Sol myotubes in the presence of CsA and 2-APB and with other inhibitors. We observed, in reducing IP3 production with the use of a PLC inhibitor (U73122), a significant decrease of K+/H11001-evoked calcium release in Sol myotubes. U73122 significantly reduced the AUC in SolC1(+/H11002) (73%) and to a lesser extent in SolD(+/H11001) myotubes (15%). U73122 also decreased the HD in SolC1(+/H11002) (30%) and not in SolD(+). Xestospongin C, an inhibitor of IP3Rs, also decreased Ca2+ release signal with a significant reduction of...
AUC but not HD, this later observation being probably explained by the secondary inhibition effect of this compound on calcium pumps. By comparison, in previous results (4) ryano- dine, known to inhibit RyRs at high concentrations (24), has been shown to induce a weak and slow (increased HD) K⁺-evoked calcium increase in both SolC1(−) and SolD(+) myotubes with a slope of calcium rise significantly reduced [92% and 88% reduction, in SolC1(−) and SolD(+), respectively]. Taken together the results showed that ryanodine exposure led to both an inhibitory effect higher than during IP3R blockade (2-APB and U73122) and a similar decrease in the IS parameter in SolC1(−) and SolD(+) (35% and 33%, respectively). Thus, although SolC1(−) and SolD(+) myotubes showed a fast phase of calcium release, whereas the blockade of IP3Rs showed a fast phase. The previous data (4) also showed that, when the two types of inhibitor (Rya + 2-APB) are applied together, no significant inhibition of calcium increase could be obtained, suggesting that K⁺-evoked calcium response mainly depends on sarcoplasmic reticulum releases through both RyRs and IP3Rs and that the ryanodine-dependent release may be a prerequisite for the IP3-dependent one (4, 5). It must be noticed that IP3 pathway inhibitors (2-APB, U73122, and xestospongin C) exerted a stronger effect in SolC1(−) myotubes compared with SolD(+). These results are in agreement with our previous works (4, 5) indicating an overactivation of the IP3 pathway in dystrophin-deficient cells. The present results also show that incubation with CsA led to a significant reduction of both HD and IS, meaning that CsA could have a modulatory (inhibitory) effect on this overactivated IP3-dependent calcium increase. To test the involvement of calcineurin in K⁺-evoked calcium release, we tested the effect of cypermethrin, known to directly inhibit calcineurin (18, 31). Cypermethrin, at a concentration of 170 nM, significantly reduced the AUC (Table 1) of K⁺-evoked calcium release (67%) in SolC1(−) and to a lesser extent (13%) in SolD(+) myotubes. Cypermethrin slightly decreased the HD in SolC1(−) (32%). Also, cypermethrin showed a similar decrease in the IS as shown with 2-APB or CsA. Thus exposure with either CsA or cypermethrin led to the same pattern of calcium release kinetics. These results favor the involvement of calcineurin in the IP3-mediated K⁺-evoked calcium release. Furthermore, despite the differences between SolC1(−) and SolD(+) in K⁺-evoked calcium rise, application of cyper- methrin led to similar patterns of release in both myotube types. Furthermore, with application of the same concentration of cypermethrin (170 nM), cell viability was found higher than in control conditions, in SolC1(−) (92.4 ± 0.1% higher, n = 9), and not in SolD(+) (63.4 ± 0.1%, n = 15), suggesting that calcineurin inhibition leads to the protection of dystrophin-deficient cells.

Effect of CsA on Localized Calcium Release Events at Rest

In a previous work (5), the number of sites discharging calcium was found greater in SolC1(−) than in SolD(+) myotubes, and it was quantified with the use of a parameter describing the density of calcium-release sites in myotubes (RSD in the following text). Time sequences of fast confocal images have been recorded in fluo-4-loaded myotubes. In each sequence, 30 to 50 fluorescence images with 300 ms time resolution were recorded on the same microscope field in myotubes preparations. Figure 3 A–C, shows examples of three different fast-recorded images extracted from the same sequence. Each spot (white arrows, α and β in top B and C) of high fluorescence corresponded to a localized calcium release event in one myotube. Images without release event and reflecting intracellular calcium levels at rest were found inside the sequence (Fig. 3A), as well as localized calcium discharges at several sites (top of Fig. 3, B and C, site α and β, respectively). It was observed that several release events could originate from the same location in myotubes during the acquisition sequence. An analysis of the images sequence allowed us to compute the standard deviation of the recorded fluorescence of each pixel as a function of time. Hence, during the acquisition sequence, when several fluorescence increases were observed at the same location, the computed standard deviation of the corresponding pixel was more elevated compared with areas without calcium increase. Consequently, the result obtained (Fig. 3D) was an image with discrete spots of elevated standard deviation, corresponding to release sites, on which it was easy to visually number them (α, β, and four other smaller sites). This number of sites on the cell surface provides the RSD parameter (in μm⁻¹). As displayed in Fig. 3E, in control conditions, RSD was found 49% higher in SolC1(−) myotubes (open bars) than in SolD(+) myotubes (closed bars).

Table 1. Area under the curve and kinetics parameters of K⁺-evoked Ca²⁺ release in SolC1(−) and SolD(+) myotubes in the presence of several inhibitors

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<th>SolC1(−)</th>
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<th>SolD(+)</th>
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<tr>
<td></td>
<td>AUC</td>
<td>HD</td>
<td>IS</td>
</tr>
<tr>
<td>Control</td>
<td>259±15(176)</td>
<td>110±5(176)</td>
<td>152±8(176)</td>
</tr>
<tr>
<td>CsA (300 nM–16 h)</td>
<td>172±12(79)</td>
<td>78±3(79)</td>
<td>87±6(79)</td>
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<tr>
<td>2-APB</td>
<td>49±6(84)</td>
<td>49±6(84)</td>
<td>98±6(84)</td>
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<td>U73122</td>
<td>70±5(218)</td>
<td>78±3(217)</td>
<td>96±4(218)</td>
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<tr>
<td>XeC</td>
<td>70±8(21)</td>
<td>117±7(19)</td>
<td>51±4(22)</td>
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<tr>
<td>Rya</td>
<td>5±2(120)</td>
<td>177±5(120)</td>
<td>11±1(120)</td>
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<tr>
<td>2-APB + Rya</td>
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<td>210±8(5)</td>
<td>15±1(5)</td>
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<td>Cypermethrin (16 h)</td>
<td>192±9(10)</td>
<td>93±10(10)</td>
<td>84±9(10)</td>
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</table>

Values are means ± SE (n, number of observation in parentheses) in percent. SolC1(−) dystrophin-deficient cells; SolD(+), mini-dystrophin-transfected cells. Myotubes were incubated with 2-aminoethoxydiphenyle borate (2-APB, 50 μM, 30 min), cyclosperine A (CsA, 300 nM, 16 h), ryanodine (Rya, 100 μM, 10 min), U73122 (10 μM, 20 min), xestospongin C (XeC, 5 μM, 20 min), or 2-APB + Rya, cypermethrin (170 nM, 16 h). Values in italic are for comparison, a reminder of data from our group (4).
Incubation with 50 μM 2-APB strongly reduced the RSD in both cell types, leading it to the same low level. Interestingly, short-time incubation (30 min) CsA also reduced the RSD value in SolC1/H11002 myotubes in a clear significant way for 10 and 100 μM concentration of the compound (44% decrease and 52% decrease, respectively). In these cases the RSD was driven back to the level observed in SolD/H11001 myotubes in control conditions. By contrast, CsA did not affect the RSD parameter in SolD/H11001 myotubes. These results are in line with our previous data about the involvement of IP3Rs in calcium release activity in dystrophin-deficient cells (5) and with the K+-evoked calcium increase experiments described above. Taken together, these data suggest that CsA could damp down the enhanced IP3-dependent release processes in dystrophin-deficient cell lines.

**Effect of CsA on IP3R-1 mRNA Expression**

It has been previously demonstrated that a long-time incubation with CsA could reduce both IP3R-1 mRNA levels and IP3R-1 protein levels in primary neuronal cultures (23). To explain the decrease of calcium signals described above, with long-time exposure of CsA in our preparations, IP3R-1 mRNA expression was assessed by quantitative RT-PCR. As shown previously (4), in control conditions, IP3R-1 mRNA levels were found more elevated in SolC1/H11002 myotubes than in SolD/H11001 myotubes (160% higher). Surprisingly, CsA exposure at low concentrations during 48 h had a strong effect on IP3R-1 mRNA expression in SolC1/H11002 myotubes (Fig. 4). In SolC1/H11002, incubation of 100 and 300 nM CsA reduced mRNA levels of IP3R-1 of 57% and 81%, respectively (Fig. 4, open bars). By contrast, no significant reduction of IP3R-1 mRNA expression was observed in SolD/H11001 myotubes (Fig. 4, closed bars). Importantly, incubation of SolC1/H11002 with CsA led to IP3R-1 mRNA levels close to those in SolD/H11001 in control conditions. This reduction was found significant after 24 h of CsA exposure (data not shown) and was also observed at the protein level with Western blots (Fig. 4, B and C). Whole cell extracts of control and CsA-
treated cell lines were electrophoresed, blotted, and incubated with IP3R-1 antibody (Fig. 4B). Immunodetection of α-tubulin was used to control for protein loading. The IP3R-1 bands were quantified by densitometry and plotted in Fig. 4C. Exposure with CsA (300 nM, 48 h) produced a significant reduction of IP3R-1 mRNA levels in SolC1(−) and not in SolD(+) (Table 2). All these data suggest that a long-time incubation of CsA can affect the IP3R-1 expression leading to a decrease of resting and stimulated IP3-dependent calcium releases.

**Knockdown of IP3R-1 Reduced Both Calcium Releases and Natural Cell Death in Dystrophin-Deficient Cells**

To confirm that type 1 of IP3R is involved in the IP3 pathway overactivation in dystrophin-deficient cells, experiments have been performed using siRNA to knockdown IP3R-1 expression. Myoblasts of Sol cell lines were transfected with control or IP3R-1 targeting siRNA 1 day before promoting fusion (D1), and experiments were performed on myotubes at D3 (3 days after promoting fusion). At this day of fusion it was possible to obtain well-differentiated myotubes in the temporal window of effective transitory extinction effect of siRNA. Knockdown of IP3R-1 was confirmed by quantitative RT-PCR (Fig. 5A), leading to a reduction of IP3R-1 mRNA levels in both SolC1(−) and SolD(+) myotubes, driving to a final IP3R-1 mRNA level in SolC1(−) similar to the SolD(+) control one. This reduction was also observed at the protein level with Western blots (Fig. 5, B and C). Whole cell extracts of control and siRNA-transfected Sol cell lines were electrophoresed, blotted, and incubated with IP3R-1 antibody (Fig. 5B). The IP3R-1 bands were quantified by densitometry and plotted in Fig. 5C. In control conditions, SolC1(−) contained ~53% more IP3R-1 protein than SolD(+). IP3R-1 extinction by siRNA produced a 62% reduction in SolC1(−) and 55% in SolD(+). Because transfection with siRNA significantly reduced the level of IP3R-1 calcium channels, recordings of both K+-evoked and spontaneous calcium releases were performed in transfected myotubes, and the data were summarized in Table 2. In SolC1(−), IP3R-1 extinction led to a 58% decrease of AUC and 38% decrease of HD, reflecting the partial loss of the slow phase of calcium release. This reduction effect was observed at a lower extent in SolD(+) (43% reduction for AUC and 40% reduction for HD). According to the AUC parameter, similar values were observed in dystrophin-deficient myotubes knocked down for IP3R-1 and in myotubes expressing mini-dystrophin (109% and 100%, respectively, Table 2). With regard to spontaneous calcium releases at rest, extinction of IP3R-1 significantly reduced the number of sites discharging calcium (RSD parameter) in SolC1(−) leading to an equivalent value of RSD in SolD(+). Hence, both at rest and during K+ stimulation, calcium release activities were highly depending on IP3R-1 calcium channels, in correlation with the amount of these proteins in the sarcoplasmic reticulum.

Furthermore, when cells were knocked for IP3R-1, cell viability was found higher than in control conditions, particularly in SolC1(−) (Fig. 6), suggesting that IP3R-1 extinction leads to the protection of dystrophin-deficient cells. Taken together, these data demonstrate that IP3R-1 calcium channels are key elements in both calcium deregulation and natural cell death observed in dystrophin-deficient cells.

**DISCUSSION**

With the aim to investigate the involvement of Ca2+ release in the calcium deregulation and the cell death of dystrophin-deficient muscle cells, we performed experiments on two types of myotubes originating from the same Sol8 cell line: 1) dystrophin-deficient myotubes SolC1(−), and 2) myotubes transfected to express the BMD mini-dystrophin SolD(+). This mini-dystrophin-forced expression allows readressing of members of the DAP complex and recovery of intracellular Ca2+ levels similar to those observed in myotubes from mouse primary cell culture (33). Furthermore, this model allows the physiological exploration of two cellular systems (4, 5, 47) with the only difference being the transfection-induced presence (or not) of mini-dystrophin and has also been used to demonstrate the restoration of sarcolemmal store-dependent channels with forced expression of mini-dystrophin (46). We previously provided evidence that K+-evoked calcium rise was...
Cells were transiently transfected with control or IP3R-1-targeting siRNA and the expression of IP3R-1 mRNA expression was normalized to SolD](H11001) IP3R-1/H11001 control; § vs. SolC1](H11002) control for normalization. §§: abundance of IP3R-1 was quantified by using siRNA.

**Fig. 5. Effect of small interfering RNA (siRNA) against IP3R-1 expression.**

All measurements of IP3R-1 mRNA expression were normalized to SolD](H11002) control; * vs. SolC1](H11021) control vs. SolD](H11021) control vs. SolC1](H11002) control vs. SolC1](H11002) control vs. SolD](H11001) control for normalization. **§**: abundance of IP3R-1 was quantified by using α-tubulin as a loading control for normalization. §§§: IP3R-1 MODULATION AND DYSTROPHIN-DEFICIENT CELL DEATH

**A**

**Effect of SiRNA on IP3R-1 expression**

- Control
- SiRNA

**B**

**Control**

- SolD](H11001)
- SolC1](H11001)

**IP3R-1 SiRNA**

- SolD](H11001)
- SolC1](H11001)

**C**

**Effect of SiRNA on IP3R-1 expression**

- Control
- SiRNA

**Fig. 5.** Effect of small interfering RNA (siRNA) against IP3R-1 expression. Cells were transiently transfected with control or IP3R-1-targeting siRNA and quantitative RT-PCR or Western blot analysis was performed at D3. A: IP3R-1 mRNA expression performed by quantitative RT-PCR (40 cycles). S6 ribosomal mRNA was used as an internal standard, and mRNA expression level of IP3R-1 was normalized with S6 ribosomal mRNA expression. All measurements of IP3R-1 mRNA expression were normalized to SolD](H11001) control. §§ P < 0.05 for SolC1](H11010) control vs. SolD](H11001) control vs. SolC1](H11010) IP3R-1 siRNA expression level in control conditions (set at 1.0). § P < 0.05 for SolD](H11001) IP3R-1 mRNA level in control conditions (set at 1.0). §§§ P < 0.05 for SolD](H11001) IP3R-1 mRNA level in control conditions (set at 1.0). **§**: abundance of IP3R-1 was quantified by using α-tubulin as a loading control for normalization. §§§ P < 0.01 for SolD](H11001) control vs. SolC1](H11010) control vs. SolD](H11001) control vs. SolC1](H11010) IP3R-1 siRNA (two-way ANOVA analysis followed by Tukey post test to compare the control with each of the conditions).

very different in these two cell types and were almost abolished with addition of IP3 pathway inhibitors (4). We have also previously demonstrated that signaling of resting release events was different in dystrophin-deficient SolC1](H11001) and in SolD](H11001) myotubes. The number of sites discharging calcium was more elevated in SolC1](H11001) than the number in SolD](H11001) myotubes (5). Blockade of IP3 pathway significantly reduced these spontaneous activities in both cell types but at a larger extent in dystrophin-deficient SolC1](H11001) myotubes (5). Here, we demonstrate that CsA exposure displayed a dual modulator effect on calcium signaling in SolC1](H11001). First, short-time incubation induced a decrease of IP3-dependent calcium release, leading to patterns of release similar to that observed in SolD](H11001). Second, long-term incubation had an additional effect on calcium signaling by reducing the expression of IP3R-1 mRNA levels. Moreover, IP3 blockade, through 2-APB or CsA induced improved survival of dystrophin-deficient myotubes, showing an actual cellular effect and demonstrating the dependence of cell death on the IP3-dependent calcium signaling. Moreover, results obtained with cypermethrin favor the involvement of calcineurin in both cell death and IP3-dependent calcium signaling. Here we also demonstrated with IP3R-1 knockdown that this isotype strongly contributes to the over-activation of IP3-dependent pathway in dystrophin-deficient cells. We propose a hypothetical model of the effect of mini-dystrophin on IP3 production, depicting the interaction with calcineurin pathway and the possible effect of CsA on this mechanism (Fig. 7).

Many studies already reported the histological damages of dystrophin-deficient muscles and fibers with specific staining as Evans blue as well as the properties of regenerated muscle (central nuclei, presence of macrophages) (42). Nevertheless, there are only few data about the death and survival of muscle cells during development. Here, the use of MTT test allowed us to quantify both the death of dystrophin-deficient myotubes and the protective effect of various agents, through cell survival measurements. This MTT assay, usually used for investigating cytotoxic effects of compounds or the cells proliferation (19), was found in this study to be a powerful tool to identify experimental conditions for the survival of muscle cells in development in culture.

In our preparations, IP3R-1 knockdown allowed a higher cell survival, suggesting that direct IP3R-1 expression inhibition leads to the protection of dystrophin-deficient cells against death. Furthermore, cell exposure to IP3 inhibitors during potassium depolarization significantly decreased Ca2+ release, particularly in dystrophin-deficient myotubes. This blockade allowed dystrophin-deficient myotubes to recover Ca2+ transient kinetics similar to that observed in control conditions in myotubes expressing mini-dystrophin (4). In the case of dystrophic cells, basal levels of IP3 mass were found three- to sixfold higher than in their normal counterparts (29). All these data lead us to think that IP3 production is significantly enhanced by membrane depolarization in dystrophin-deficient myotubes and that, conversely, the presence of mini-dystrophin under the membrane leads to reduced IP3 production.

One of our other studies (5) indicates a possible role of local Ca2+ release from the SR in Ca2+ mishandling in dystrophin-deficient cells at rest. In the present study, IP3R-1 knockdown lead to a strong reduction of sparks activity in SolC1](H11001) myotubes corroborating the results obtained in a previous study.
with application of 2-APB (4). The IP3R-related component of release activity could be enhanced when dystrophin is absent [SolC1(−)] and reduced when mini-dystrophin is experimentally expressed [SolD(+)]. When this component is inhibited, both cell types displayed a similar release of events activity. These data enlighten first the importance of the IP3-signaling pathway in resting calcium signaling and homeostasis, particularly in dystrophin-deficient skeletal muscle cells, and second that an overactivation of this pathway, via the overexpression of IP3R-1, could lead to a global calcium deregulation in muscle cells that can be cytotoxic for the myotubes.

Our previous results (4) obtained with pertussis toxin on depolarization-evoked calcium release showed a strong reduction in calcium release that was more pronounced in SolC1(−) than in SolD(+). These data suggest an inhibitory effect of mini-dystrophin on IP3 production through the modulation of a Gi protein. It could be hypothesized that a possible way of mini-dystrophin-induced modulation of this protein could occur through the demonstrated binding of heterotrimeric G proteins to syntrophin, a cytoskeletal protein known to interact with dystrophin (47, 51).

During the last two decades, CsA has been one of the most important immunosuppressive drugs used for the clinical prevention of transplant rejection and the therapy of various diseases (10, 48). CsA is also known to block the mitochondrial permeability transition (MPT) and to prevent necrotic cell death from oxidative stress, drug toxicity, and other models of cell injury (28). But CsA also acts by inhibiting calcineurin (30), a calcium-activated serine/threonine phosphatase, leading to modulation of the signaling cascade. In the skeletal muscle context, it is well established that calcium activates calcineurin via calmodulin, which in turn affects muscle characteristics by stimulating the expression of a slower and more oxidative phenotype (17). Calcineurin dephosphorylates nuclear factor of activated T cells (NFAT) and thereby unmask its nuclear localization signal, allowing NFAT to translocate from cytosol to nucleus (3). Furthermore, a physical interaction between calcineurin and IP3Rs, via the anchoring with the immunophilin FKBP12, has been previously shown (12) and has functional consequence in regulating the receptor’s phosphorylation status induced by Ca2+−activated kinases and phosphatases. Inhibition of calcineurin by CsA is a consequence of the phosphorylation status alteration of IP3R, affecting Ca2+ permeation and reducing release from calcium stores (36). The effect of calcineurin seems not to be restricted to the regulation of the phosphorylation state of the IP3R since, in neurons, a long time incubation (4 days) with CsA induced IP3R mRNA changes in parallel with protein levels (23). Thus it was proposed that calcineurin plays a dual role in IP3R-mediated Ca2+ signaling in, first, regulating IP3R function by dephosphorylation at the short-term time scale (see Fig. 7 and Ref. 1), and second, IP3R expression over more extended periods through transcription factors (Fig. 7, and Ref. 2). The same dual effect is observed in our preparations, generalizing the modulator concept of the calcineurin pathway regulating/modulating the IP3Rs expression.

In addition, calcium-dependent signaling pathways such as those linked to calcineurin and protein kinase C were found to contribute to nuclear factor-κB (NF-κB) activation by depolarization (45). This activation depends on the duration of elevated intracellular calcium. Long-lasting calcium increases, mediated by ryanodine and IP3 receptors, obtained by prolonged electrical stimulation protocols of various frequencies induce maximal NF-κB activation. It has also been hypothesized that NF-κB contributes to the perpetuation of the dystrophic damage, and its blockade produces beneficial effects on

Table 2. Parameters of spontaneous and K+-evoked Ca2+ releases in IP3R-1 siRNA transfected myotubes

<table>
<thead>
<tr>
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<th>SolC1(−)</th>
<th>SolD(+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium Release Parameters</td>
<td>K+-Evoked Calcium Increase Parameters, %</td>
<td>Release Site Density, μm−2</td>
</tr>
<tr>
<td>Control</td>
<td>AUC 259±15 (176)</td>
<td>110±5 (176)</td>
</tr>
<tr>
<td>siRNA IP3R1</td>
<td>109±8 (61)</td>
<td>68±4 (61)</td>
</tr>
</tbody>
</table>

Values are means ± SE (n, number of observations in parentheses) for K+-evoked calcium increase parameters. Values are means (× 10−4) ± SE (× 10−4) (n, number of observations in parentheses) for release site density. Myotubes were transitory transfected with control or inositol 1,4,5-trisphosphate receptor 1 (IP3R-1) small interfering RNA (siRNA), and calcium releases were performed at D3.
functional, biochemical, and morphological parameters in mdx mice (35). Our results in dystrophin-deficient cells showed a sustained and elevated calcium release, via spontaneous release events observations, due to the overactivation of the IP3 pathway as well as a cell death inhibition with CsA exposure. In this case, a continuous activation of calcineurin pathway could be involved in the cascade of events that leads to natural cell death of dystrophin-deficient cells.

Overactivation of the IP3 pathway may have many consequences on the general calcium homeostasis in dystrophin-deficient cells. It is well known that release from IP3Rs strongly depends on intracellular calcium concentrations, leading to a calcium-induced calcium release (CICR) phenomenon with high levels of Ca$^{2+}$ (9). It is possible that calcium ions initially released from RyRs may activate IP3Rs and then amplify the release mediated by the IP3 pathway. Previous studies hypothesized a possible interaction between RyRs and IP3Rs in smooth muscle cells and that Ca$^{2+}$ release events could originate from a “cross-talk” between these channels (24, 50). Hence, conditions where IP3 pathway could be overactivated may lead to an overactivation of the RyRs-dependent CICR phenomenon due to this potential cross-talk. Finally, the overactivation of one calcium release pathway may increase all calcium-sensitive properties (including CICR properties) both at rest and in stimulation conditions, participating in the overall calcium dysregulation. Furthermore, our studies (4) lead us to propose that IP3 production after membrane depolarization is significantly elevated in dystrophin-deficient myotubes and that the presence of mini-dystrophin under the membrane leads to reduced IP3 production. If IP3 production is reduced, a reduced Ca$^{2+}$ release will follow, and the consequences of the negative interaction could be propagated inward through the reduction of the diffusible CICR mechanism. Conversely, the absence of dystrophin may have the opposite effect in enhancing the CICR mechanism, even in thicker structures, such as adult muscle fibers. Furthermore, by producing two second messengers, namely IP3 and DAG, the regulation of PLC activity by dystrophin and DAPs could be a common pathway linking IP3R-dependent calcium release from SR and capacitative calcium entries through the sarcolemma. Indeed, various data suggest that heterotetrameric TRPCs, which are known to form store-operated channels (SOCs), can be modulated either indirectly or directly by DAG and PKC (39). In dystrophin-deficient muscle cells, DAG- or PKC-dependent overactivation of TRPC channel may be involved in parallel to the elevated production of IP3 by PLC during muscle activity and the calcium influx mediated by TRPC1-dependent SOCs.

Finally, inhibiting the IP3 pathway that is overactivated in dystrophin-deficient cells by reducing the levels of IP3 receptors protein could be a very interesting approach for limiting the cell death of dystrophin-deficient cells. It is now questionable whether modulation of the IP3 pathway would have any therapeutic value on dystrophic fibers. Nevertheless, a complete extinction of these calcium channels would be not wanted, according to their crucial roles in cellular signaling. The interest in the use of CsA could be the partial inhibition of IP3R expression and consequently a modulatory effect (reduction) of the IP3 pathway. Taking into account all these data, one can think that the clinical effect of CsA observed in DMD patients could be due for a part to this inhibitory mechanism, and the approach of calcineurin pathway modulation could find interest in new therapies for myopathies.

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