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Bcl-2 overexpression prevents calcium overload and subsequent apoptosis in dystrophic myotubes

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Duchenne muscular dystrophy (DMD) is a lethal disease caused by the lack of the cytoskeletal protein dystrophin. Altered calcium homeostasis and increased calcium concentrations in dystrophic fibres may be responsible for the degeneration of muscle occurring in DMD. In the present study, we used subsarcolemmal and mitochondrial-targeted aequorin to study the effect of the anti-apoptotic Bcl-2 protein overexpression on carbachol-induced near-plasma membrane and mitochondrial calcium responses in myotubes derived from control C57 and dystrophic (*mdx*) mice. We show that Bcl-2 overexpression decreases subsarcolemmal and mitochondrial calcium overload that occurs during activation

of nicotinic acetylcholine receptors in dystrophic myotubes. Moreover, our results suggest that overexpressed Bcl-2 protein may prevent near-plasma membrane and mitochondrial calcium overload by inhibiting IP₃Rs (inositol 1,4,5-trisphosphate receptors), which we have shown previously to be involved in abnormal calcium homeostasis in dystrophic myotubes. Most likely as a consequence, the inhibition of IP₃R function by Bcl-2 also inhibits calcium-dependent apoptosis in these cells.

Key words: aequorin, apoptosis, Bcl-2, Duchenne muscular dystrophy, inositol 1,4,5-trisphosphate receptor, staurosporine.

INTRODUCTION

Duchenne muscular dystrophy (DMD) is an X-linked neuromuscular disease that affects approx. 1 in 3500 males and which results in progressive muscle degeneration [1]. DMD ultimately leads to premature death by respiratory or cardiac failure during the third decade and is caused by the absence of dystrophin, a 427 kDa protein localized under the plasma membrane [2]. Numerous studies have shown that the absence of dystrophin in DMD impairs the stability of the plasma membrane, resulting in a greater fragility towards mechanical stress and increased permeability to calcium (Ca²⁺) [3]. Indeed, it has been proposed that an alteration of Ca²⁺ homeostasis might be responsible for the muscle degeneration that occurs in muscle fibres from DMD patients or in those of the mouse model of DMD, the *mdx* mouse [4]. Moreover, elevations of basal Ca²⁺ concentration in myotubes and skeletal muscle fibres from DMD patients or *mdx* mice have been reported [5].

Long-term elevation of cytosolic Ca²⁺ is linked to apoptosis [6] and mitochondrial Ca²⁺ overload activates the PTP (permeability transition pore) leading to cell death [7]. Because Ca²⁺ overload occurs in dystrophic myotubes and fibres, the involvement of apoptosis in these cells has been studied but is still a matter of debate. Indeed, it remains unclear if apoptosis causes or is secondary to muscle degradation [8,9]. Thus some groups have suggested that necrosis only occurs in dystrophin-lacking cells [10]. Other groups have shown that apoptosis occurs in dystrophic cells [11] and finally that both apoptosis and necrosis occur in DMD [12]. Therefore apoptosis, linked to necrosis or not, could be responsible for the muscle degeneration that occurs in *mdx* cells and DMD patients [13].

Apoptosis is a highly regulated process and numerous studies have shown that the Bcl-2 protein, which belongs to the Bcl-2 family, could regulate apoptosis by modulating the release of

mitochondrial apoptogenic factors like cytochrome *c* or apoptosis-inducing factor that activate proteases such as caspases [14]. Even if the exact role of the Bcl-2 protein is still unclear and debated [15], Bcl-2 could act by decreasing Ca²⁺ concentration in the SR (sarcoplasmic reticulum) [16] and other cellular compartments such as mitochondria [17]. Recent studies have postulated that Bcl-2 could interact directly with IP₃R (inositol 1,4,5-trisphosphate receptor) [18,19]. This receptor is known to be overexpressed in dystrophic myotubes [20]. We have shown recently that IP₃R is involved in increased CCh (carbachol)-induced near-plasma membrane Ca²⁺ responses in dystrophic myotubes [21]. However, at present, the effect of Bcl-2 overexpression on both near-plasma membrane and mitochondrial Ca²⁺ transients has not been studied in dystrophic myotubes.

In the present paper, we have studied the effect of overexpression of the anti-apoptotic protein Bcl-2 on CCh-induced Ca²⁺ responses in subcellular compartments. We have also investigated the effect of Bcl-2 overexpression on cell survival and apoptosis of myotubes derived from control C57 and dystrophic mice. We show that Bcl-2 overexpression decreases near-plasma membrane and mitochondrial CCh-induced Ca²⁺ transients in dystrophic myotubes. We also show that Bcl-2 overexpression prevents Ca²⁺-dependent apoptosis in dystrophic myotubes and that the beneficial effect of Bcl-2 overexpression may be mediated by a direct Bcl-2-dependent IP₃R inhibition.

EXPERIMENTAL

Cell culture

Cultures of purified myoblasts were prepared in Petri dishes (Falcon, Becton Dickinson) and maintained at 37 °C in a water-saturated atmosphere of 95 % air/5 % CO₂. They were obtained as described previously [21].

Abbreviations used: 2-APB, 2-aminoethoxydiphenyl borate; CCh, carbachol; DMD, Duchenne muscular dystrophy; IP₃, D-myo-inositol 1,4,5-trisphosphate; IP₃R, inositol 1,4,5-trisphosphate receptor; m[Ca²⁺], mitochondrial Ca²⁺ concentration; nAChR, nicotinic acetylcholine receptor; pm[Ca²⁺], subsarcolemmal Ca²⁺ concentration; PSS, physiological salt solution; PTP, permeability transition pore; SNAP-25, 25 kDa synaptosome-associated protein; SOCC, store-operated calcium channel; SR, sarcoplasmic reticulum; STS, staurosporine.

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Cell permeabilization

To permeabilize myotubes, a Ca^{2+} -free PSS (physiological salt solution; 145 mM NaCl, 5 mM KCl, 1 mM MgCl_2 , 5 mM Hepes and 10 mM glucose, pH 7.6) containing 50 $\mu\text{g/ml}$ saponin (Sigma) was used. Cells were incubated in the presence of saponin for 60 s. Directly after permeabilization, myotubes were perfused with a PSS containing 1.2 mM Ca^{2+} and either inositol 1,4,5-trisphosphate [50 μM ; IP_3 (D-*myo*-inositol 1,4,5-trisphosphate); Calbiochem] or its L-enantiomer (50 μM ; L-*myo*-inositol 1,4,5-trisphosphate; Calbiochem) as a negative control.

Transfection

Control C57 and *mdx* myoblasts were plated at 15 000 cells per cm^2 on 13 mm Thermanox coverslips (Nalge Nunc International) in 4-well plates. When 80–90% confluent, growth medium was removed and replaced with a serum-free medium, Optimem 1 (Gibco). Cells were transfected overnight using LipofectamineTM 2000 (Invitrogen, Life Technologies) at a ratio of 1 μg of DNA per 2 μl of transfection reagent. The DNA–LipofectamineTM 2000 complex was prepared in Optimem 1 medium. After overnight incubation, this medium was replaced by differentiation medium. Myotubes were used after 3 or 4 days of differentiation.

Plasmids

The aequorin plasmids were gifts from Professor T. Pozzan (University of Padova, Padova, Italy). Cells were transfected with a pcDNAI expression vector containing a cDNA encoding aequorin for Ca^{2+} measurement, fused with the SNAP-25 (25 kDa synaptosome-associated protein) sequence to measure $\text{pm}[\text{Ca}^{2+}]$ (subsarcolemmal Ca^{2+} concentration) [22] or mitochondrial cytochrome *c* oxidase subunit VIII to measure $\text{m}[\text{Ca}^{2+}]$ (mitochondrial Ca^{2+} concentration) [23]. The Bcl-2 plasmid [24] was a gift from Professor Karl Heinz Krause (University of Geneva). The IP_3 sponge plasmid was a gift from Dr H. L. Roderick and Dr M. D. Bootman (Calcium Group, Babraham Institute Laboratory of Molecular Signalling, University of Cambridge, U.K.). Cells were transfected with a pdc515 expression vector (Microbix Biosystems) containing a cDNA encoding enhanced green fluorescent protein and the high-affinity IP_3 sponge [25].

Immunocytochemistry

After 3–4 days of differentiation, myotubes were washed with PSS. Then cells were fixed with methanol/acetic acid (95:5, v/v). After saturation with PSS containing 3% (w/v) BSA, antibody raised against the Bcl-2 protein (1:500 dilution; sc-509; Santa Cruz Biotechnology) was added for 1 h at 37 °C and revealed by an Alexa Fluor 488-conjugated anti-mouse antibody (1:1000 dilution; Molecular Probes). Cells were washed four times with PSS after each incubation.

Confocal microscopy

Mdx and control C57 myotubes were grown in plastic culture dishes and transfected with pcDNA3 expression vector containing cDNA encoding Bcl-2. The immunolabelled samples were examined by confocal laser scanning microscopy using a Bio-Rad MRC 1024 ES (Bio-Rad, Hemel Hempstead, U.K.) equipped with an argon–krypton gas laser, 3–4 days after differentiation. The Alexa Fluor fluorochrome was excited with the 488 nm green line and the emission was collected at 520 nm. Mitotracker was excited with the 550 nm red line and the emission was collected at 580 nm. Data were acquired using an inverted microscope (Olympus IX70) and processed with the LaserSharp software (version 3.0; Bio-Rad).

Western blot

Bcl-2 protein content of control C57 and *mdx* myotubes was determined using Western blotting. Equal amounts of protein extracted were loaded on to SDS/12% PAGE. The relative content of muscle protein in the samples was adjusted according to the actin (antibody dilution 1:500; A2172; Sigma) band. Proteins were transferred to nitrocellulose membranes (Bio-Rad). The Bcl-2 protein was detected using the Bcl-2-specific antibody at a dilution of 1:2500. Subsequently, alkaline phosphatase-conjugated goat anti-mouse (170-6520; Bio-Rad) was used at a dilution of 1:1000. Specific signals were detected with the ECF (enhanced fluorescence) Western blotting detection reagent (Amersham Biosciences).

Intracellular calcium measurements

After 3–4 days of differentiation, Ca^{2+} concentration was determined in a population of myotubes as described previously [21]. Briefly, aequorins were reconstituted in a PSS containing 5 μM coelenterazine (Calbiochem) for 1 h before the experiment. Particular conditions are needed for the subsarcolemmal-targeted aequorin because the removal of intracellular Ca^{2+} is required for the complete aequorin reconstitution [26]. Thus SNAP-25–aequorin was reconstituted in a Ca^{2+} -free PSS containing 0.1 mM EGTA in order to decrease the $[\text{Ca}^{2+}]$ within the cell. Mitochondrial-targeted aequorin was reconstituted in 1.2 mM Ca^{2+} . Cells were perfused at a rate of 1 ml/min in a custom made 0.5 ml chamber thermostatically maintained at 37 °C (MecaTest, Geneva, Switzerland). Emitted luminescence was detected at 466 nm with a photomultiplier apparatus (EMI 9789A; Electron Tubes, U.K.) and recorded every 1 s using a computer photon-counting board (EMI C660) as described previously [27]. The relationship between recorded emitted light and $[\text{Ca}^{2+}]$ was calculated using a previously described equation [28]. Total light output was obtained by exposing cells to 10 mM CaCl_2 after permeabilization with 100 μM digitonin to consume all the aequorin.

Survival assay and apoptosis

The enzymatic colorimetric measurement of the phosphatase acid activity was used to determine the effect of Bcl-2 overexpression on the survival of the myotubes. Myotubes were cultured in 24-well plates coated with collagen. Briefly, after 3–4 days of differentiation, cells were washed twice for 5 min with 500 μl of PBS (0.2 g/l KCl, 0.2 g/l KH_2PO_4 , 8 g/l NaCl and 1.15 g/l NaH_2PO_4 , pH 7.2). Then 250 μl of substrate solution containing citrate buffer aqueous solution (0.1 M, pH 5.5), 0.1% Triton X-100 and 1 mg/ml *p*-nitrophenyl phosphate substrate was added to each well for 90 min. The reaction was stopped by adding 25 μl of 1 M NaOH. Absorbance was measured at 405 nm.

The APO percentage apoptosis assay (Biocolor, Newtownabbey, U.K.) was used to assess the effect of Bcl-2 overexpression on apoptosis. Cellular dye uptake was analysed according to the manufacturer's method modified for our myotubes that were cultured in 24-well plates coated with collagen. Briefly, after a 90 min incubation at 37 °C with APO dye reagent (dilution 1:20), cells were washed with PSS to remove extracellular dye. Subsequently, APO dye release reagent was applied for 10 min and absorbance of the released dye was measured at 550 nm with a plate reader (Fluostar Galaxy; BMG Labtechnologies).

Data analysis

Data analysis was performed using the software GraphPad Prism 4 (GraphPad Software, San Diego, CA, U.S.A.) and Matlab 7 SP1 (The MathWorks, Natick, MA, U.S.A.). Results are expressed

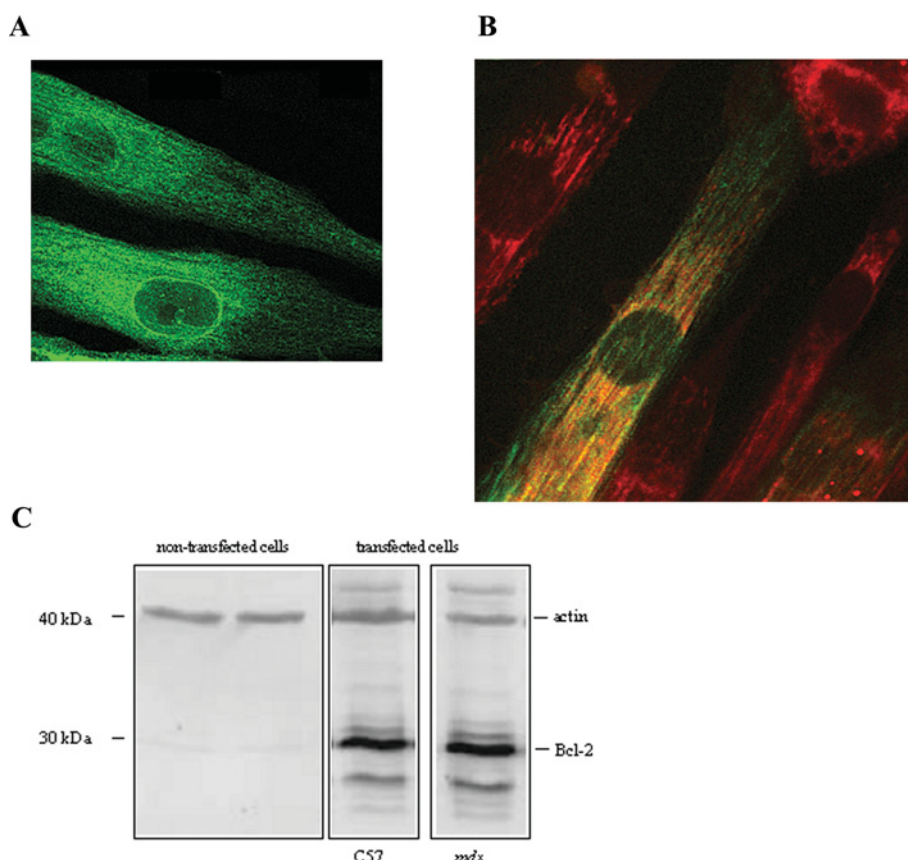


Figure 1 Bcl-2 localization and overexpression

(A) Confocal section of *mdx* myotubes labelled with anti-Bcl-2 antibody for protein localization (green fluorescence). (B) Mitotracker (red fluorescence) and co-localization of mitochondria and Bcl-2 (yellow fluorescence). (C) Western-blot analysis of cell lysates from non-transfected and transfected control C57 and *mdx* myotubes. The blot was probed with anti-Bcl-2 antibody. Actin was used as control.

as means \pm S.E.M. Statistical significance of differences between the values was assessed with the unpaired Student's *t* test and one-way ANOVA followed by Dunnett post-hoc test when necessary. *P* values ≤ 0.05 were considered significant.

RESULTS

Bcl-2 localization and overexpression

In order to investigate the localization of the Bcl-2 protein, myoblasts were transfected with cDNA encoding Bcl-2. Localization of Bcl-2 was assessed using immunocytochemistry and confocal imaging. Figure 1 shows representative confocal sections of dystrophic myotubes after 3 days of differentiation. The green fluorescence alone corresponds to overexpressed Bcl-2 protein (Figure 1A), as no fluorescence was detected in non-transfected myotubes (results not shown). Overexpressed Bcl-2 protein appears to be located on membranes which could be SR membranes and also in the perinuclear area. Double staining of Bcl-2 protein and mitochondria (with mitotracker, red fluorescence) indicates that overexpressed Bcl-2 proteins were located in part in mitochondria (yellow fluorescence, Figure 1B). Similar results were obtained with control C57 myotubes (results not shown). Bcl-2 overexpression was confirmed by Western-blot analysis of both control C57 and *mdx* myotubes (Figure 1C). Indeed, Bcl-2 protein was only detected in transfected myotubes. Moreover, results shown in Figure 1(C) indicate that Bcl-2 was similarly expressed in both cell types.

Effect of Bcl-2 overexpression on pm[Ca²⁺]

In order to study the effect of Bcl-2 overexpression on Ca²⁺ homeostasis in both control C57 and *mdx* myotubes, we investigated increases in pm[Ca²⁺] and m[Ca²⁺] triggered by nAChR (nicotinic acetylcholine receptor) stimulation using CCh. CCh-induced pm[Ca²⁺] increases were measured with plasma membrane-targeted aequorin as previously described [21]. Due to very high [Ca²⁺] in this compartment, aequorin was reconstituted in Ca²⁺-free PSS containing 0.1 mM EGTA for 1 h before the experiment in order to decrease the [Ca²⁺] within the cell. This allows complete reconstitution of aequorin [26]. Stimulation of myotubes with CCh triggered near-plasma membrane Ca²⁺ increases with average amplitudes of 1.10 ± 0.11 and 5.04 ± 0.81 μ M for control C57 and dystrophic myotubes respectively. Bcl-2 overexpression did not affect significantly the CCh-induced near-plasma membrane Ca²⁺ increases in control C57 myotubes but decreased this response in dystrophic myotubes approx. 2-fold (to 2.35 ± 0.35 μ M; Figure 2). These results demonstrate that the CCh-induced Ca²⁺ increase is five times higher in dystrophic myotubes as compared with control C57 cells. These results also show that Bcl-2 overexpression decreases CCh-induced near-plasma membrane Ca²⁺ increases only in dystrophic myotubes. Thus Bcl-2 overexpression appears to correct exaggerated near-plasma membrane Ca²⁺ transients that occurred during stimulation of nAChRs in dystrophic myotubes. We have previously shown that the increased CCh-induced Ca²⁺ response in dystrophic myotubes involves Ca²⁺ release through IP₃Rs [21], which

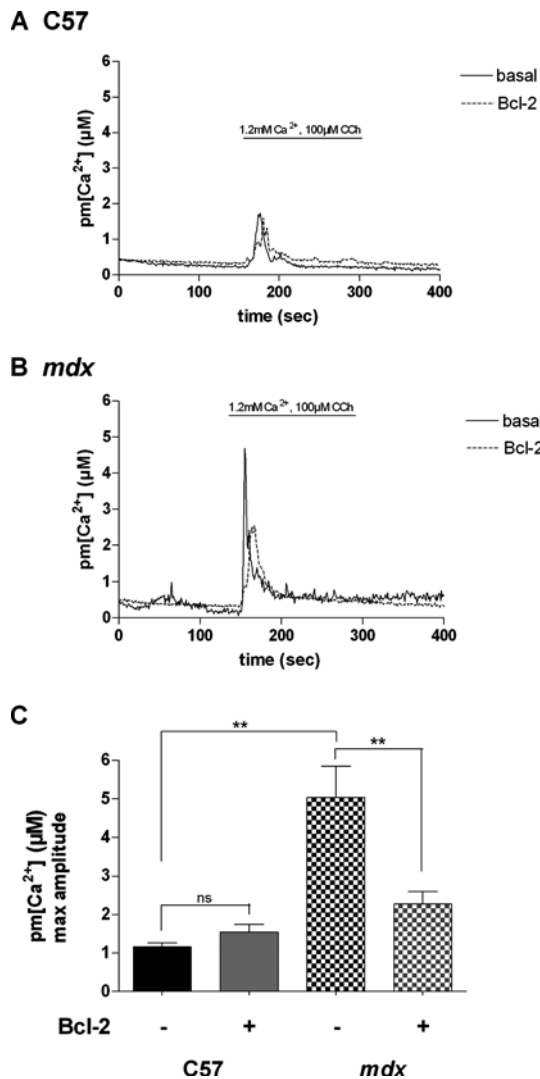


Figure 2 Effect of Bcl-2 overexpression on pm[Ca²⁺]. Representative traces of pm[Ca²⁺] increases in a population of (A) control C57 and (B) *mdx* myotubes showing the effect of nAChR stimulation and Bcl-2 overexpression on the pm[Ca²⁺]. (C) Summarizing histogram of CCh-induced subsarcolemmal Ca²⁺ responses measured in control C57 (*n* ≥ 4) and *mdx* (*n* ≥ 14) myotubes. The bar graphs correspond to mean pm[Ca²⁺] values ± S.E.M. ns, not significant (*P* > 0.05); ** corresponds to a significant inhibition (0.01 < *P* < 0.001) using unpaired Student's *t* test.

have been shown to be overexpressed in dystrophic myotubes [20].

Effect of IP₃R inhibitors on the near-plasma membrane Ca²⁺ response in dystrophic myotubes that overexpress Bcl-2

As Bcl-2 protein has been shown to interact directly with IP₃R and to inhibit its opening [18], we next investigated the effect of potent IP₃R inhibitors on CCh-induced near-plasma membrane Ca²⁺ increases in dystrophic myotubes. As shown in Figure 3, incubation of dystrophic myotubes that overexpress Bcl-2 with either 2-APB (2-aminoethoxydiphenyl borate; 75 μM) [29] or xestospongine D (0.1 μM) [30] for 10 min did not affect the CCh-induced Ca²⁺ response when compared with non-treated cells (2.32 ± 0.30 and 1.63 ± 0.21 μM respectively for 2-APB; 2.32 ± 0.30 and 2.20 ± 0.15 μM respectively for xestospongine D) (Figure 3C). In contrast, in dystrophic myotubes that do not

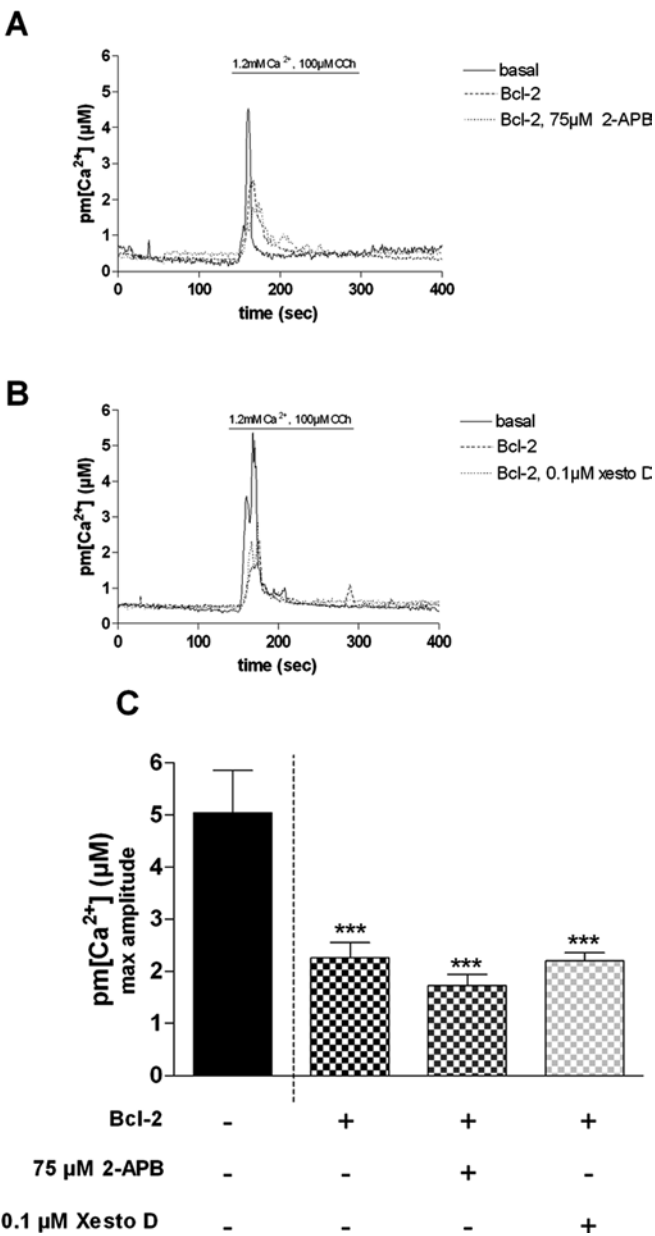


Figure 3 Effect of IP₃R inhibitors on Bcl-2-transfected dystrophic myotubes. Representative traces of Ca²⁺ increases in a population of *mdx* myotubes showing the effect of Bcl-2 overexpression alone and Bcl-2 overexpression together with (A) 75 μM 2-APB or (B) xestospongine D (xesto D; 0.1 μM) on the CCh-induced Ca²⁺ response. (C) Summarizing histogram. The bar graphs correspond to mean pm[Ca²⁺] values ± S.E.M.; *n* ≥ 8; *** corresponds to a significant inhibition (*P* < 0.001) using one-way ANOVA followed by a Dunnett post-hoc test when compared with the control bar, e.g. 1.2 mM Ca²⁺, 100 μM CCh, no Bcl-2 overexpression.

overexpress Bcl-2, the two IP₃R inhibitors have been shown to strongly reduce CCh-induced Ca²⁺ responses [21]. Altogether, these results suggest that Bcl-2, when overexpressed in dystrophic myotubes, may reduce CCh-induced Ca²⁺ responses by inhibiting the IP₃R, as shown in other cell types [18]. To confirm that Bcl-2 could act by inhibiting IP₃R, we used saponin-permeabilized dystrophic myotubes perfused with 50 μM IP₃. As shown in Figure 4, perfusion with this second messenger induced a subsarcolemmal Ca²⁺ increase with a maximal amplitude of 2.10 ± 0.14 μM, whereas Ca²⁺ readdition alone triggered a maximal Ca²⁺ transient of 0.92 ± 0.13 μM. In myotubes

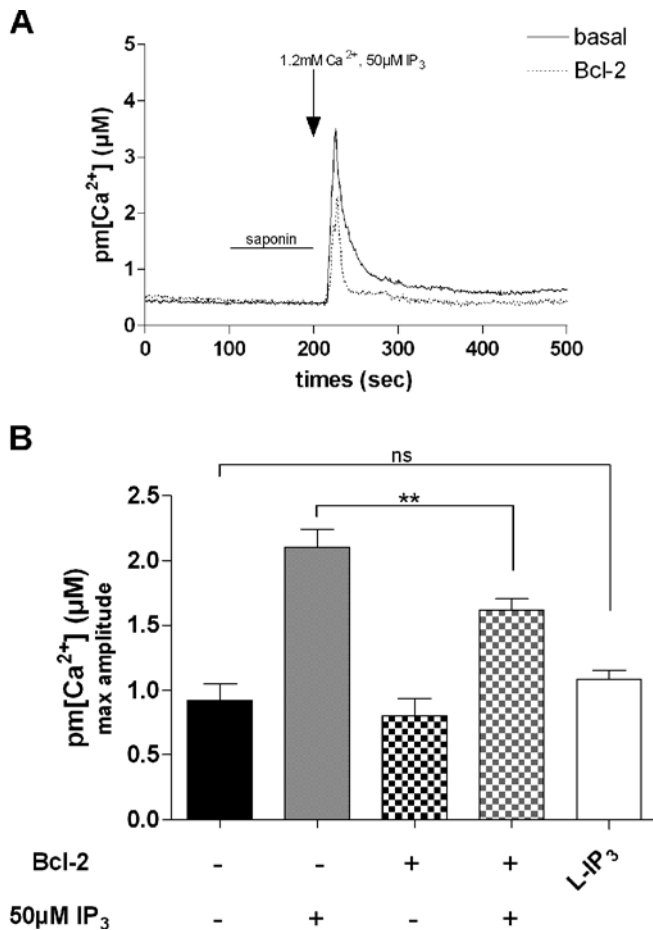


Figure 4 IP₃-induced Ca²⁺ response is modulated by Bcl-2 overexpression

(A) Representative trace of pm[Ca²⁺] increases in a population of saponin-permeabilized *mdx* myotubes showing the effect of Bcl-2 overexpression on the IP₃ (50 μM)-induced pm[Ca²⁺] response. (B) Summarizing histogram. The L-enantiomer of IP₃ (L-IP₃; 50 μM) is used as a negative control. The bar graphs correspond to mean pm[Ca²⁺] values ± S.E.M.; *n* ≥ 6; ns, not significant (*P* > 0.05); ** corresponds to a significant inhibition (0.01 < *P* < 0.001) using unpaired Student's *t* test.

overexpressing Bcl-2, the IP₃-induced Ca²⁺ response was significantly decreased when compared with myotubes not overexpressing the anti-apoptotic protein (from 2.10 ± 0.14 to 1.62 ± 0.09 μM). Perfusion with the L-enantiomer of IP₃ (50 μM), known to have very little affinity for IP₃R, did not significantly change the subsarcolemmal Ca²⁺ response when compared with Ca²⁺ readdition (1.08 ± 0.07 and 0.92 ± 0.13 μM respectively). Thus Ca²⁺ transients induced by both CCh, which triggers an IP₃-dependent Ca²⁺ response [21], and IP₃, which acts directly on IP₃R, are inhibited by Bcl-2 overexpression. However, Bcl-2 overexpression has also been shown to affect SR Ca²⁺ content in some cases [16]. To test this hypothesis, we investigated the effect of caffeine (20 mM), an activator of the ryanodine receptor [31], on pm[Ca²⁺]. Caffeine-induced near-plasma membrane Ca²⁺ increases were not significantly affected in dystrophic myotubes overexpressing Bcl-2 when compared with myotubes not overexpressing the anti-apoptotic protein (results not shown). This suggests that Bcl-2 overexpression does not correct the CCh-induced Ca²⁺ response by reducing the Ca²⁺ content of SR Ca²⁺ stores. Altogether, these results therefore suggest that Bcl-2 overexpression is likely to inhibit IP₃R-dependent Ca²⁺ release

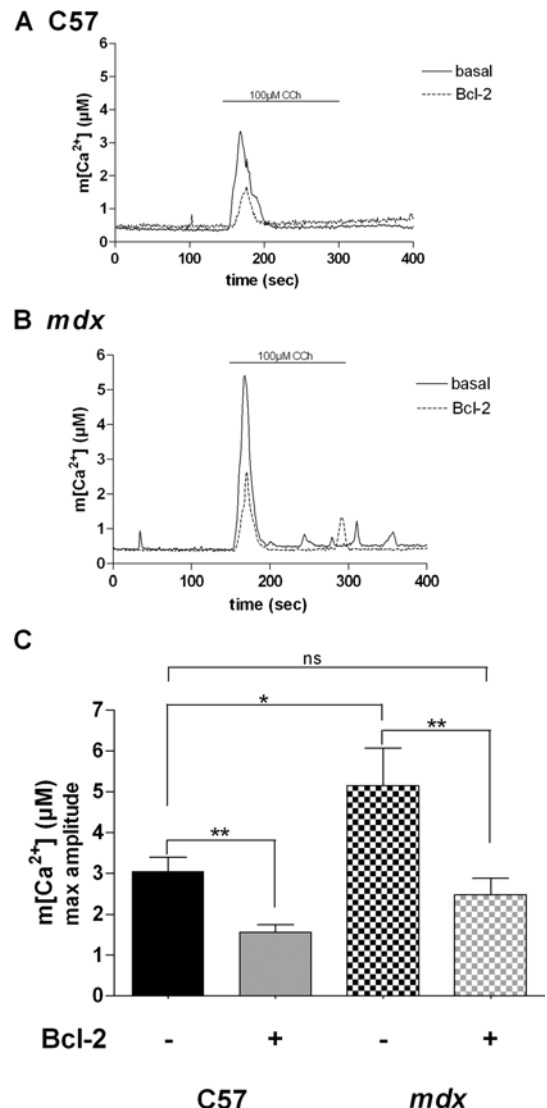


Figure 5 Effect of the Bcl-2 overexpression on m[Ca²⁺]

Representative traces of m[Ca²⁺] increases in a population of (A) control C57 and (B) *mdx* myotubes showing the effect of nAChR stimulation and Bcl-2 overexpression on m[Ca²⁺]. (C) Summarizing histogram of CCh-induced m[Ca²⁺] responses measured in control C57 (*n* = 5) and *mdx* myotubes (*n* = 14). The bar graphs correspond to mean m[Ca²⁺] values ± S.E.M. ns, not significant (*P* > 0.05); * and ** correspond to a significant inhibition (*P* < 0.05 and 0.01 < *P* < 0.001 respectively) using unpaired Student's *t* test.

in dystrophic myotubes, thus leading to a reduced near-plasma membrane Ca²⁺ transient during activation with CCh.

Effect of Bcl-2 overexpression on m[Ca²⁺]

It has been shown that a tight coupling between SR Ca²⁺ release channels (IP₃Rs and ryanodine receptors) and mitochondrial Ca²⁺ uptake sites operates in various types of cells [32]. CCh-induced m[Ca²⁺] increases were measured with aequorin targeted to mitochondria. As shown in Figure 5, the amplitudes of CCh-induced Ca²⁺ response were 3.05 ± 0.34 and 5.15 ± 0.92 μM in control C57 and dystrophic myotubes respectively. In myotubes overexpressing Bcl-2, CCh-induced mitochondrial Ca²⁺ increases were significantly decreased in both control C57 and *mdx* myotubes (1.56 ± 0.18 and 2.47 ± 0.4 μM respectively). These results show that CCh-induced mitochondrial Ca²⁺ transients are

approx. 1.5-fold higher in dystrophic myotubes than in control C57 cells. This demonstrates that elevated mitochondrial Ca^{2+} responses occur in dystrophic myotubes during nicotinic activation and also that Bcl-2 overexpression decreases these transients to a value close to control C57 levels. Altogether these results suggest that Bcl-2 overexpression decreases exaggerated subsarcolemmal and mitochondrial Ca^{2+} responses during physiological activation of dystrophic myotubes.

Effect of Bcl-2 overexpression on near-plasma membrane STS (staurosporine)-dependent Ca^{2+} responses

As Bcl-2 is known to have anti-apoptotic effects mediated by modifications of Ca^{2+} homeostasis in various cell types [33], we investigated the effect of Bcl-2 overexpression on Ca^{2+} responses triggered by the pro-apoptotic agent STS. STS is known to trigger Ca^{2+} -dependent apoptosis [34]. To investigate the effect of STS on the Ca^{2+} homeostasis in our dystrophic myotubes, we measured $\text{pm}[\text{Ca}^{2+}]$ during 75 min under basal conditions (1.2 mM Ca^{2+}) and in the presence of 0.1 μM STS. This limited recording time was chosen to prevent elevated aequorin consumption that may disturb calibration at the end of the experiment. Dystrophic myotubes were transfected with the plasma membrane-targeted aequorin for control experiments and with both Bcl-2- and plasma membrane-targeted aequorin to study the effect of the anti-apoptotic protein. When perfused with a PSS containing 1.2 mM Ca^{2+} , STS induced a 3.5-fold increase in near-plasma membrane Ca^{2+} spike frequency as compared with the frequency occurring in these myotubes when perfused with 1.2 mM Ca^{2+} alone (Figures 6A and 6C). Moreover, when the IP_3 R inhibitor 2-APB (75 μM) was perfused together with STS, the Ca^{2+} spike frequency decreased approx. 1.5-fold (Figures 6A and 6C). This suggests that the increased Ca^{2+} spike frequency is due to Ca^{2+} release through IP_3 Rs. In Bcl-2-overexpressing myotubes, near-plasma membrane Ca^{2+} spike frequency was only increased 2.5-fold in the presence of STS (Figures 6B and 6C), which is consistent with Bcl-2 overexpression inhibiting Ca^{2+} spikes triggered by STS. The addition of the IP_3 R inhibitor xestospongin D did not further increase the inhibition already triggered by Bcl-2 overexpression. Moreover, Ca^{2+} spike frequency in Bcl-2-overexpressing dystrophic myotubes was similar to that recorded in dystrophic myotubes perfused with 2-APB but which do not overexpress Bcl-2. Altogether, these results show that STS increases near-plasma membrane Ca^{2+} spike frequency in dystrophic myotubes and that overexpression of Bcl-2 counteracts the effect of STS. Moreover, near-plasma membrane Ca^{2+} spike frequencies are reduced in myotubes by Bcl-2 or by 2-APB, suggesting that the decrease in Ca^{2+} spike frequency may be dependent on reduction of IP_3 R-dependent Ca^{2+} release.

Effect of Bcl-2 overexpression on cell survival and apoptosis

We next investigated the effect of Bcl-2 overexpression on cell survival. In addition to STS, we used H_2O_2 , which induces reactive oxygen species formation in similar cultures [35]. Control C57 and dystrophic myotubes were exposed for 18 h to H_2O_2 (1 mM) or STS (0.1 μM) followed by cell survival assay using acid phosphatase activity. As shown in Figure 7, both compounds decreased cell survival of control C57 and *mdx* myotubes. Indeed, in the presence of STS, cell survival was $74.64 \pm 3.07\%$ for control C57 cells and $40.26 \pm 4.12\%$ for *mdx* myotubes. In the presence of H_2O_2 , cell survival was $57.85 \pm 7.20\%$ for control C57 cells and $13.73 \pm 4.80\%$ for *mdx* myotubes. These results show that dystrophic myotubes are more sensitive to H_2O_2 and STS compared with control C57 myotubes. However, when myotubes

were treated with STS, cell survival of Bcl-2-overexpressing myotubes was significantly increased (from 40.26 ± 4.12 to $57.50 \pm 6.17\%$) only in dystrophic myotubes (Figure 7B). In contrast, cell survival of control C57 myotubes was not affected by Bcl-2 overexpression (Figure 7A). When myotubes were treated with H_2O_2 , Bcl-2 overexpression had no effect on cell survival in both cell types (Figures 7A and 7B). It is therefore likely that Bcl-2 overexpression protects only dystrophic myotubes against cell death triggered by STS.

As acid phosphatase measurement does not discriminate between necrosis and apoptosis, we used a specific apoptosis assay. When myotubes were treated with STS, $28.35 \pm 3.30\%$ of the control C57 cells and $59.91 \pm 2.56\%$ of the dystrophic cells were apoptotic (Figure 8A), confirming the fact that dystrophic myotubes are more sensitive to STS than control C57 cells. When Bcl-2 was overexpressed in these myotubes, the number of apoptotic cells decreased in dystrophic cells only (from 59.91 ± 2.56 to $45.55 \pm 3.35\%$), showing that Bcl-2 overexpression does not protect control C57 myotubes. When cells were treated with H_2O_2 , Bcl-2 overexpression did not change the apoptotic rate significantly in both control C57 and dystrophic myotubes (Figure 8B). These results show that Bcl-2 overexpression counteracts the STS-induced apoptosis in dystrophic myotubes only and that H_2O_2 -induced apoptosis remains unchanged in Bcl-2-overexpressing myotubes.

IP_3 sponge protects dystrophic myotubes from STS-induced apoptosis

Dystrophic myotubes were transfected with a plasmid containing either Bcl-2 or the IP_3 sponge. IP_3 sponge has been shown to behave as a very specific IP_3 chelator and to inhibit IP_3 -mediated Ca^{2+} responses [25]. After 3–4 days of differentiation, cells were treated with STS (0.1 μM) for 18 h. As shown above (Figure 8A), Bcl-2 decreased STS-induced apoptosis in dystrophic myotubes. More interestingly, apoptosis was also decreased from 59.91 ± 2.56 to $39.09 \pm 1.32\%$ in myotubes transfected with the IP_3 sponge (Figure 9). The decrease observed in Bcl-2-overexpressing myotubes was not statistically different from the value obtained with the IP_3 sponge. This result strongly suggests that the IP_3 pathway is involved in STS-induced apoptosis in dystrophic myotubes.

DISCUSSION

In these experiments we have investigated the effect of Bcl-2 overexpression on Ca^{2+} homeostasis and apoptosis in both control C57 and dystrophic myotubes. Plasma membrane- and mitochondrial-targeted aequorin fusion proteins have been used to study the effect of Bcl-2 overexpression on $\text{pm}[\text{Ca}^{2+}]$ and $\text{m}[\text{Ca}^{2+}]$ following nAChR stimulation of both types of myotubes.

First, we found that CCh-induced near-plasma membrane Ca^{2+} responses are 4.5 times higher in dystrophic myotubes compared with control C57 cells, indicating that exaggerated near-plasma membrane Ca^{2+} responses occur in dystrophic myotubes during physiological activation. These results are in agreement with previous reports on dystrophic myotubes and fibres showing that a subsarcolemmal Ca^{2+} overload occurs in these cells [23,36]. Secondly, we show here for the first time that Bcl-2 overexpression counteracts the elevated Ca^{2+} transients upon physiological activation. On the other hand, Bcl-2 overexpression had no effect in control C57 myotubes, indicating that overexpressed Bcl-2 protein reduces CCh-induced near-plasma membrane Ca^{2+} responses in dystrophic myotubes by a mechanism that is present or visible only in these cells. We have previously shown that

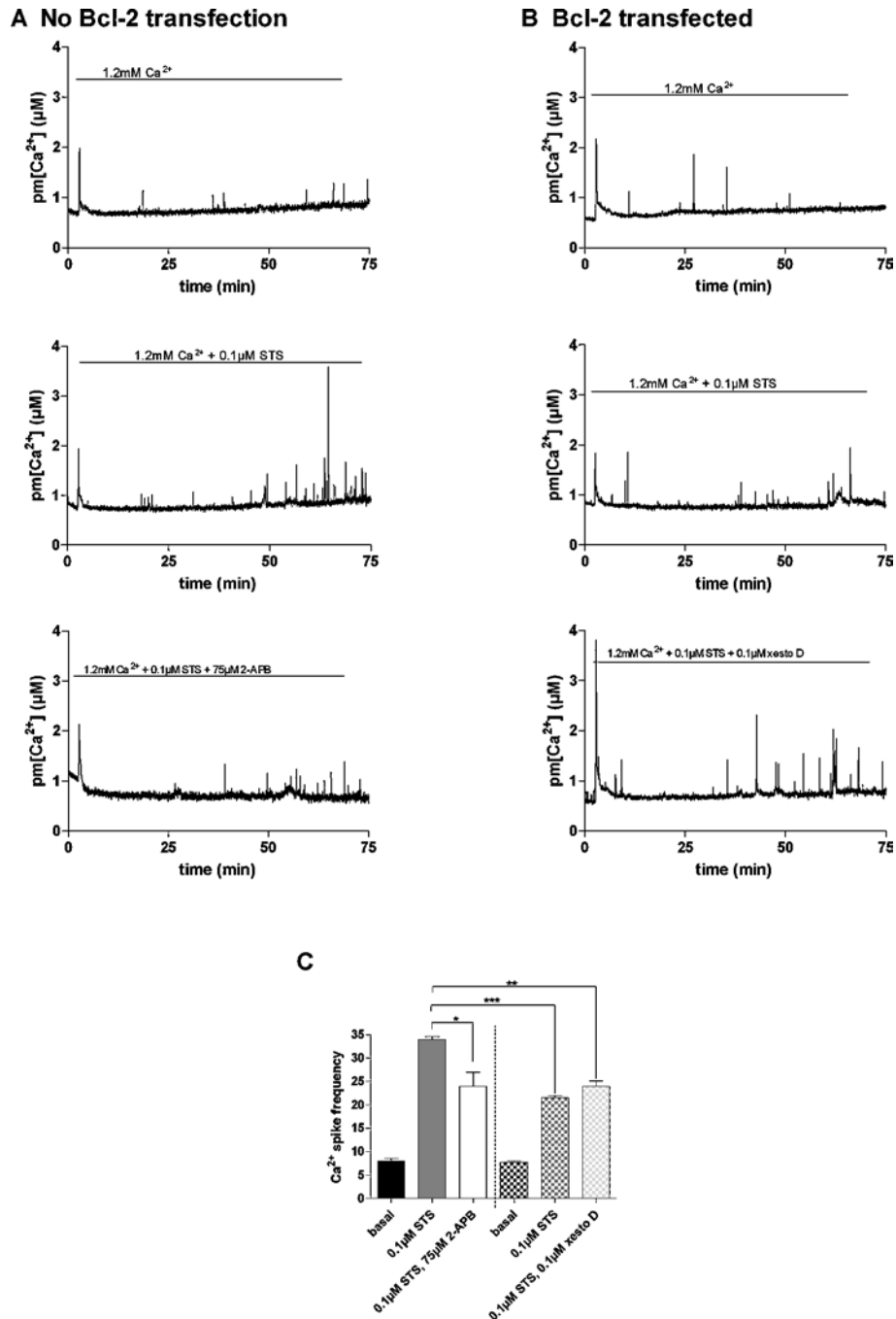


Figure 6 Effects of STS on plasma membrane Ca^{2+} spikes in dystrophic myotubes

Representative trace of the effect of STS (0.1 μM) in the presence or absence of IP_3R inhibitors on the $\text{pm}[\text{Ca}^{2+}]$ spikes in (A) control dystrophic myotubes and (B) in dystrophic myotubes that overexpress Bcl-2. (C) Summarizing histogram of the mean plasma membrane Ca^{2+} spike frequency ($n \geq 3$). Frequency was calculated manually and represents spikes that point out from the basal $\text{pm}[\text{Ca}^{2+}]$. Plain columns represent dystrophic myotubes that were not transfected with Bcl-2. Hatched columns represent Bcl-2-overexpressing dystrophic myotubes. *, ** and *** correspond to a significant decrease ($P < 0.05$, $0.01 < P < 0.001$ and $P < 0.001$ respectively) using Student's *t* test. xesto D, xestospongine D.

Ca^{2+} release mechanisms are different between control C57 and dystrophic myotubes [21]. Indeed, CCh-induced near-plasma membrane Ca^{2+} responses in dystrophic myotubes appear to selectively involve Ca^{2+} release through IP_3Rs as demonstrated with blockers of this receptor (2-APB, xestospongine D or by chelating IP_3 with an IP_3 sponge), or by reducing the SR Ca^{2+} store content with the SERCA (sarcoplasmic/endoplasmic-reticulum Ca^{2+} -ATPase) inhibitor thapsigargin [21]. These findings suggest

that IP_3Rs are likely to be very close to the plasma membrane but also that SOCCs (store-operated calcium channels) which could be controlled by IP_3R [37] may contribute to CCh-induced near-plasma membrane Ca^{2+} increases. In the present paper, we show that in dystrophic myotubes overexpressing Bcl-2, IP_3R blockers did not affect CCh-induced near-plasma membrane Ca^{2+} responses, suggesting that Bcl-2 may reduce CCh-induced Ca^{2+} responses by inhibiting IP_3R -dependent Ca^{2+} release,

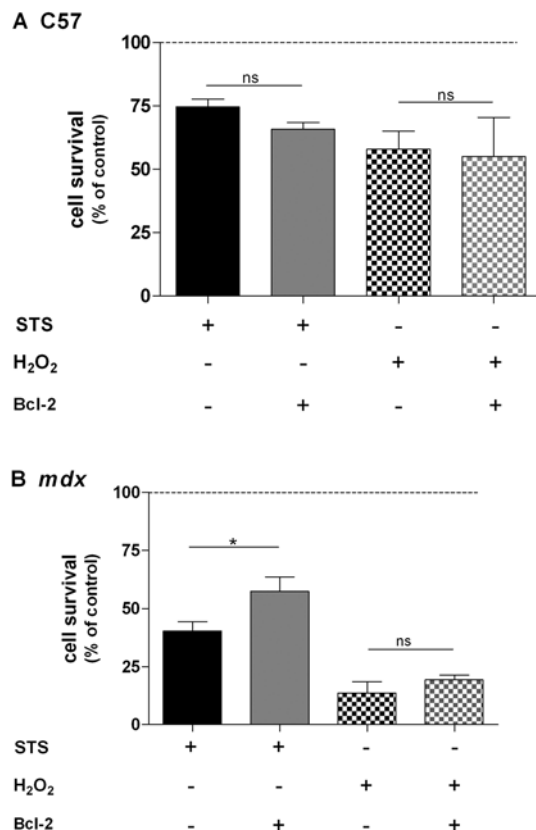


Figure 7 Effects of STS and H₂O₂ on cell survival of control C57 and *mdx* myotubes

Summarizing histogram of the sensitivity of control C57 (**A**) and *mdx* (**B**) myotubes to STS (0.1 μ M; plain bar) and H₂O₂ (1 mM; hatched bar) combined with the effect of Bcl-2 overexpression. STS and H₂O₂ were applied for 18 h. The bar graphs correspond to mean cell survival \pm S.E.M.; $n \geq 6$; ns, not significant ($P > 0.05$); * corresponds to a significant increase ($P < 0.05$) using unpaired Student's *t* test.

as proposed in other cell types [18]. Indeed, it has been shown that Bcl-2 protein interacts allosterically with the C-terminal part of the channel and in this way decreases its open probability [18]. Our results indicate that such an inhibition may operate in our dystrophic myotubes as IP₃-induced near-plasma membrane Ca²⁺ responses were reduced by Bcl-2 overexpression. However, Bcl-2 overexpression has also been shown to reduce SR Ca²⁺ content in some cell types [16]. In our experiments, such an effect of Bcl-2 overexpression on SR Ca²⁺ content is unlikely, as the amount of releasable Ca²⁺ from the SR (estimated using caffeine-induced near-plasma membrane Ca²⁺ responses) was not significantly different in dystrophic myotubes overexpressing Bcl-2 or not. Our results therefore suggest that the Bcl-2 protein, when overexpressed in dystrophic myotubes, is likely to inhibit IP₃R-dependent Ca²⁺ release and possibly subsequent IP₃R-dependent SOCC-dependent influx after CCh stimulation.

We also found that CCh induced mitochondrial Ca²⁺ increases in both control C57 and dystrophic myotubes, suggesting that mitochondrial Ca²⁺ uptake sites are closely linked to SR in our myotubes, as demonstrated in other cell types [32]. Moreover, we show that CCh-induced mitochondrial Ca²⁺ responses are 1.5 times higher in dystrophic myotubes as compared with control C57 cells, indicating that transient mitochondrial Ca²⁺ overload occurred during nicotinic activation of dystrophic myotubes, similar to what was observed by Robert et al. [23] during high-potassium stimulations. In both control C57 and dystrophic

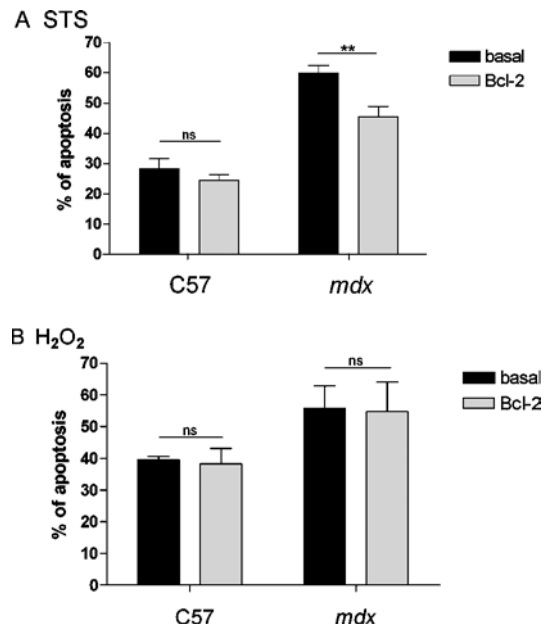


Figure 8 Apoptosis of control C57 and *mdx* myotubes overexpressing Bcl-2

(**A**) Summarizing histogram of the sensitivity of control C57 ($n \geq 6$) and *mdx* ($n \geq 15$) myotubes to STS (0.1 μ M). (**B**) Summarizing histogram of the sensitivity of control C57 ($n \geq 3$) and *mdx* ($n \geq 5$) myotubes to H₂O₂ (1 mM). The bar graphs correspond to mean apoptosis \pm S.E.M. ns, not significant ($P > 0.05$); ** corresponds to a significant decrease ($0.01 < P < 0.001$) using Student's *t* test.

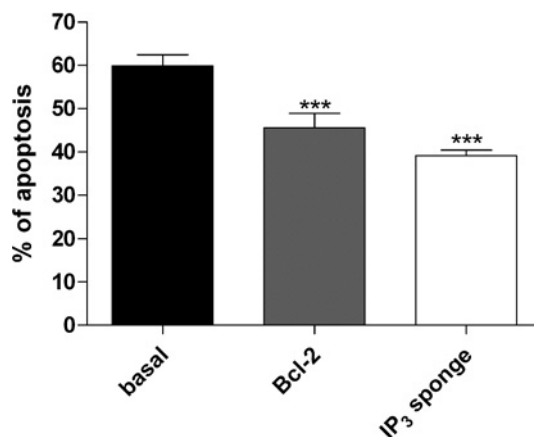


Figure 9 IP₃ sponge protects cells against STS-induced apoptosis

Histogram showing the effects of Bcl-2 overexpression and IP₃ sponge on apoptosis induced by STS in dystrophic myotubes. The bar graphs correspond to mean apoptosis rate \pm S.E.M.; $n \geq 15$; *** corresponds to a significant decrease ($P < 0.001$) using one-way ANOVA followed by a Dunnett post-hoc test when compared with the control bar. The Bcl-2 bar and IP₃ sponge bar were not significantly different (using Student's *t* test).

myotubes, Bcl-2 overexpression decreased CCh-induced mitochondrial Ca²⁺ responses. In dystrophic myotubes overexpressing Bcl-2, reduced mitochondrial Ca²⁺ responses may be linked to reduced SR Ca²⁺ release through IP₃R, as CCh-induced near-plasma membrane Ca²⁺ responses were decreased. Links between IP₃R or SOCC and mitochondria have been demonstrated [38,39]. In our dystrophic myotubes overexpressing Bcl-2, reduced mitochondrial Ca²⁺ uptake during CCh stimulation may be explained by an inhibitory effect of Bcl-2 on IP₃R-dependent Ca²⁺

release and subsequent SOCC-dependent influx. However, in control C57 myotubes overexpressing Bcl-2, reduced mitochondrial Ca^{2+} responses cannot be explained by reduced SR Ca^{2+} release, as CCh-induced near-plasma membrane Ca^{2+} increases were not affected by Bcl-2 overexpression. Bcl-2 proteins, when overexpressed in these control C57 myotubes, are likely to act on mitochondria by a direct mechanism such as inhibition of cytochrome *c* release [40] or by decreasing Ca^{2+} influx into mitochondria through the Ca^{2+} uniporter [41], which is consistent with the co-localization of Bcl-2 and mitochondria observed in our myotubes.

Ca^{2+} overload is known to play a key role in apoptosis by activating a cascade of proteolytic enzymes [42]. In particular, mitochondrial Ca^{2+} overload can induce PTP opening, resulting in the release of pro-apoptotic factors such as cytochrome *c* [40]. Moreover, cytochrome *c* has been shown to bind to IP_3R and to induce Ca^{2+} release from the endoplasmic reticulum, amplifying Ca^{2+} -dependent apoptosis [43]. As Bcl-2 overexpression counteracted transient near-plasma membrane and mitochondrial Ca^{2+} overload, we have investigated the effect of Bcl-2 overexpression on cell survival and apoptosis in our myotubes. When myotubes were exposed to two types of stress, both control C57 and dystrophic myotubes displayed increased apoptosis and decreased cell survival. However, dystrophic myotubes were more sensitive to H_2O_2 - and STS-induced stresses. These results are in agreement with previous studies indicating that dystrophin-deficient cells are more susceptible to free radicals [35]. However, Bcl-2 overexpression was unable to protect both control C57 and dystrophic myotubes against reactive oxygen species produced by H_2O_2 . On the other hand, Bcl-2 overexpression decreased STS-induced apoptosis selectively in dystrophic myotubes. As STS increased the frequency of near-plasma membrane Ca^{2+} spikes in dystrophic myotubes, our results suggest that STS is likely to trigger Ca^{2+} -dependent apoptosis, as shown previously [34]. Recently, it was proposed that once apoptosis is induced by STS in HeLa cells, caspase 3 can remove the IP_3R -binding site and most of the regulatory domain of type 1 IP_3R , thus triggering formation of a 'channel only' domain that remains constitutively open and which leads to Ca^{2+} leak in the cytosol [44]. Our results show that both an IP_3R inhibitor (2-APB) and Bcl-2 overexpression reduced the STS effect on Ca^{2+} spike frequency, which suggests that leaky IP_3R may be involved in Ca^{2+} spikes and subsequent Ca^{2+} overload triggered by STS.

Overall, our results suggest that the anti-apoptotic effect of Bcl-2 may be explained by its inhibitory effect on STS-triggered Ca^{2+} overload, possibly by a direct inhibition of IP_3R [18]. Moreover, the fact that the transiently transfected IP_3 sponge inhibited STS-dependent apoptosis indicates that the IP_3 pathway is involved in this form of cell death. Overexpressed Bcl-2 proteins may therefore exert their anti-apoptotic effect by inhibiting IP_3Rs , which are overexpressed in dystrophic myotubes [20] and could lead to abnormal Ca^{2+} homeostasis near the plasma membrane (Ca^{2+} leak) and in mitochondria.

To conclude, our results indicate that Bcl-2 overexpression reduces both near-plasma membrane and mitochondrial Ca^{2+} responses occurring during nicotinic activation of dystrophic myotubes, most probably by inhibiting IP_3R . Our results also indicate that Bcl-2 overexpression can prevent STS-induced apoptosis selectively in dystrophic myotubes, presumably by reducing near-plasma membrane Ca^{2+} overload. As the IP_3 pathway is involved in CCh-induced Ca^{2+} transients and in STS-induced apoptosis, the beneficial effect of Bcl-2 overexpression in dystrophic myotubes may be explained by a direct inhibition of IP_3Rs , which are indeed involved in abnormal Ca^{2+} homeostasis in these cells. Thus overexpression of Bcl-2 or other anti-apoptotic proteins

could represent an interesting approach to prevent muscle damage occurring in DMD.

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