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

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INTRODUCTION

Duchenne muscular dystrophy (DMD) is a lethal disease caused by the lack of the cytoskeletal protein dystrophin. Altered calcium homoeostasis 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 homoeostasis 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.
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 µg/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-trisphosphat); 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 Thermaxano 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 Lipofectamine\(^\text{TM}\) 2000 (Invitrogen, Life Technologies) at a ratio of 1 µg of DNA per 2 µl of transfection reagent. The DNA–Lipofectamine\(^\text{TM}\) 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 pcDNA expression vector containing a cDNA encoding aequorin for Ca\(^{2+}\) measurement, fused with the SNAP-25 (25 kDa synaptosome-associated-protein) sequence to measure pm\([\text{Ca}^{2+}]\) (subsarcolemmal Ca\(^{2+}\) concentration) [22] or mitochondrial cytochrome c oxidase subunit VIII to measure 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 Biostems) containing a cDNA encoding enhanced green fluorescent protein and the high-affinity IP\(_3\) sponge [25].

Imunochemistry

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\(_2\)PO\(_4\), 8 g/l NaCl and 1.15 g/l NaH\(_2\)PO\(_4\), 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 APODye 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
Bcl-2 overexpression in dystrophic myotubes

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 ± 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 immunochemistry 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[Ca^{2+}]

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

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

**Effect of IP\(_3\)R inhibitors on the near-plasma membrane Ca\(^{2+}\) response in dystrophic myotubes that overexpress Bcl-2**

As Bcl-2 protein has been shown to interact directly with IP\(_3\)R and to inhibit its opening [18], we next investigated the effect of potent IP\(_3\)R inhibitors on CCh-induced near-plasma membrane Ca\(^{2+}\) 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 \(\mu\)M) [29] or xestospongin D (xesto D; 0.1 \(\mu\)M) on the CCh-induced Ca\(^{2+}\) response. (C) Summarizing histogram. The bar graphs correspond to mean pm(Ca\(^{2+}\)) values ± S.E.M.; n \(\geq 8\); *** 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 Ca\(^{2+}\), 100 \(\mu\)M CCh, no Bcl-2 overexpression.

To confirm that Bcl-2 could act by inhibiting IP\(_3\)R, we used saponin-permeabilized dystrophic myotubes perfused with 50 \(\mu\)M IP\(_3\). As shown in Figure 4, perfusion with this second messenger induced a subsarcolemmal Ca\(^{2+}\) increase with a maximal amplitude of 2.10 ± 0.14 \(\mu\)M, whereas Ca\(^{2+}\) readdition alone triggered a maximal Ca\(^{2+}\) transient of 0.92 ± 0.13 \(\mu\)M. In myotubes
overexpressing Bcl-2, the IP$_3$-induced Ca$^{2+}$ 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 IP$_3$ (L-IP$_3$;50 µM), known to have very little affinity for IP$_3$R, did not significantly change the subsarcolemmal Ca$^{2+}$ response when compared with Ca$^{2+}$ readdition (1.08 ± 0.07 and 0.92 ± 0.13 µM respectively). Thus Ca$^{2+}$ transients induced by both CCh, which triggers an IP$_3$-dependent Ca$^{2+}$ response [21], and IP$_3$, which acts directly on IP$_3$R, are inhibited by Bcl-2 overexpression. However, Bcl-2 overexpression has also been shown to affect SR Ca$^{2+}$ 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[Ca$^{2+}$]. Caffeine-induced near-plasma membrane Ca$^{2+}$ 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 Ca$^{2+}$ response by reducing the Ca$^{2+}$ content of SR Ca$^{2+}$ stores. Altogether, these results therefore suggest that Bcl-2 overexpression is likely to inhibit IP$_3$R-dependent Ca$^{2+}$ release in dystrophic myotubes, thus leading to a reduced near-plasma membrane Ca$^{2+}$ transient during activation with CCh.

**Effect of Bcl-2 overexpression on m[Ca$^{2+}$]**

It has been shown that a tight coupling between SR Ca$^{2+}$ release channels (IP$_3$Rs and ryanodine receptors) and mitochondrial Ca$^{2+}$ uptake sites operates in various types of cells [32]. CCh-induced m[Ca$^{2+}$] responses measured in control C57 ($n=5$) and mdx myotubes ($n=14$). The bar graphs correspond to mean m[Ca$^{2+}$] 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.

Representative traces of m[Ca$^{2+}$] increases in a population of (A) control C57 and (B) mdx myotubes showing the effect of Bcl-2 overexpression on m[Ca$^{2+}$]. (C) Summarizing histogram of CCh-induced m[Ca$^{2+}$] responses measured in control C57 ($n=5$) and mdx myotubes ($n=14$). The bar graphs correspond to mean m[Ca$^{2+}$] 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.
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 pm[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\(_2\)O\(_2\), which induces reactive oxygen species formation in similar cultures [35]. Control C57 and dystrophic myotubes were exposed for 18 h to H\(_2\)O\(_2\) (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 ± 3.07% for control C57 cells and 40.26 ± 4.12% for mdx myotubes. In the presence of H\(_2\)O\(_2\), cell survival was 57.85 ± 7.20% for control C57 cells and 13.73 ± 4.80% for mdx myotubes. These results show that dystrophic myotubes are more sensitive to H\(_2\)O\(_2\) 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 ± 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\(_2\)O\(_2\), 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 ± 3.30% of the control C57 cells and 59.91 ± 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 ± 3.35%), showing that Bcl-2 overexpression does not protect control C57 myotubes. When cells were treated with H\(_2\)O\(_2\), 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\(_2\)O\(_2\)-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 ± 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 pm[Ca\(^{2+}\)] and m[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...
Ca\textsuperscript{2+} release mechanisms are different between control C57 and dystrophic myotubes [21]. Indeed, CCh-induced near-plasma membrane Ca\textsuperscript{2+} responses in dystrophic myotubes appear to selectively involve Ca\textsuperscript{2+} release through IP\textsubscript{3}Rs as demonstrated with blockers of this receptor (2-APB, xestospongin D or by chelating IP\textsubscript{3} with an IP\textsubscript{3} sponge), or by reducing the SR Ca\textsuperscript{2+} store content with the SERCA (sarcoplasmic/endoplasmic-reticulum Ca\textsuperscript{2+}-ATPase) inhibitor thapsigargin [21]. These findings suggest that IP\textsubscript{3}Rs are likely to be very close to the plasma membrane but also that SOCCs (store-operated calcium channels) which could be controlled by IP\textsubscript{3}R [37] may contribute to CCh-induced near-plasma membrane Ca\textsuperscript{2+} increases. In the present paper, we show that in dystrophic myotubes overexpressing Bcl-2, IP\textsubscript{3}R blockers did not affect CCh-induced near-plasma membrane Ca\textsuperscript{2+} responses, suggesting that Bcl-2 may reduce CCh-induced Ca\textsuperscript{2+} responses by inhibiting IP\textsubscript{3}R-dependent Ca\textsuperscript{2+} release.
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 IP3, R-dependent Ca2+ release and possibly subsequent IP3, R-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+ overloading 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 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 IP3, R, as CCh-induced near-plasma membrane Ca2+ responses were decreased. Links between IP3, R 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 IP3, R-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\(_3\)R, 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 free radicals [35]. However, Bcl-2 overexpression was unable to protect both control C57 and dystrophic myotubes against reactive oxygen species produced by H\(_2\)O\(_2\). 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\(_3\)-binding site and most of the regulatory domain of type 1 IP\(_3\)R, 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\(_3\)R inhibitor (2-APB) and Bcl-2 overexpression reduced the STS effect on Ca\(^{2+}\) spike frequency, which suggests that leaky IP\(_3\)R 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\(_3\)R [18]. Moreover, the fact that the transiently transfected IP\(_3\)R, 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\(_3\)R, which are overexpressed in dystrophic myotubes [20] and could lead to abnormal Ca\(^{2+}\) homoeostasis 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\(_3\)R. 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\(_3\)R, which are indeed involved in abnormal Ca\(^{2+}\) homoeostasis 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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