Ca\textsuperscript{2+}-independent phospholipase A\textsubscript{2} enhances store-operated Ca\textsuperscript{2+} entry in dystrophic skeletal muscle fibers

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Summary
Duchenne muscular dystrophy is caused by deficiency of dystrophin and leads to progressive weakness. It has been proposed that the muscle degeneration occurring in this disease is caused by increased Ca\textsuperscript{2+} influx due to enhanced activity of cationic channels that are activated either by stretch of the plasma membrane (stretch-activated channels) or by Ca\textsuperscript{2+}-store depletion (store-operated channels). Using both cytosolic Ca\textsuperscript{2+} measurements with Fura-2 and the manganese quench method, we show here that store-operated Ca\textsuperscript{2+} entry is greatly enhanced in dystrophic skeletal flexor digitorum brevis fibers isolated from mdx\textsuperscript{5cv} mice, a mouse model of Duchenne muscular dystrophy. Moreover, we show for the first time that store-operated Ca\textsuperscript{2+} entry in these fibers is under the control of the Ca\textsuperscript{2+}-independent phospholipase A\textsubscript{2} and that the exaggerated Ca\textsuperscript{2+} influx can be completely attenuated by inhibitors of this enzyme. Enhanced store-operated Ca\textsuperscript{2+} entry in dystrophic fibers is likely to be due to a near twofold overexpression of Ca\textsuperscript{2+}-independent phospholipase A\textsubscript{2}. The Ca\textsuperscript{2+}-independent phospholipase A\textsubscript{2} pathway therefore appears as an attractive target to reduce excessive Ca\textsuperscript{2+} influx and subsequent degeneration occurring in dystrophic fibers.

Key words: Dystrophic skeletal muscle fibers, Store-operated channel, Ca\textsuperscript{2+}-independent phospholipase A\textsubscript{2}

Introduction
Duchenne muscular dystrophy (DMD) is an X-linked hereditary disease affecting 1 in 3500 male births. DMD is caused by a mutation in the p21 region of the X chromosome, which leads to a deficiency of dystrophin, a 427 kDa protein located in the inner face of the plasma membrane (Blake et al., 2002). In normal skeletal muscle fibers, dystrophin is linked to cytoskeletal actin and plasma membrane glycoproteins associated with the extracellular matrix. Dystrophin provides a link between the cytoskeleton and the extracellular matrix and its absence in dystrophic fibers leads to progressive weakness and muscle degeneration (Blake et al., 2002). Muscle degeneration occurring in DMD has been proposed to be due to increased Ca\textsuperscript{2+} influx and abnormal Ca\textsuperscript{2+} homeostasis, resulting in increased proteolysis and mitochondrial Ca\textsuperscript{2+} overload (Alderton and Steinhardt, 2000; Basset et al., 2004; Gailly, 2002; Robert et al., 2001; Ruegg et al., 2002; Vandebrouck et al., 2006). Mitochondrial Ca\textsuperscript{2+} overload occurring in dystrophic muscle may lead to cytochrome C release and apoptosis (Basset et al., 2006; Duchen, 2004). The increased Ca\textsuperscript{2+} permeability of dystrophic fibers sarcoclemma appears to be due to enhanced activity of non-selective cationic channels, which are activated either by Ca\textsuperscript{2+}-store depletion (store-operated channels: SOC) or by stretch of the plasma membrane (Allen et al., 2005; Vandebrouck et al., 2006; Vandebrouck et al., 2002; Yeung and Allen, 2004; Yeung et al., 2004). Furthermore, it has been recently proposed that channels responsible for the enhanced Ca\textsuperscript{2+} influx in dystrophic fibers belong to the TRP family (Allen et al., 2005; Iwata et al., 2003; Maroto et al., 2005; Vandebrouck et al., 2002).

Three models have been put forward to explain the link between the sarco-endoplasmic Ca\textsuperscript{2+} store and SOC, also called ‘capacitative Ca\textsuperscript{2+} entry’ (Parekh and Putney, Jr, 2005). The first model postulates that SOC located in the plasma membrane may be activated by a conformational coupling with the channels responsible for Ca\textsuperscript{2+} release, i.e. ryanodine and/or inositol 1,4,5-trisphosphate receptors (Kiselyov et al., 1998; Kiselyov et al., 2000). The second model proposes that capacitative Ca\textsuperscript{2+} entry is activated by docking or fusion of secretory vesicles containing SOC activators (or channels) with the plasma membrane (Parekh and Putney, Jr, 2005). Along these lines, STIM1, a recently discovered transmembrane protein, is a Ca\textsuperscript{2+} sensor activating SOC by migrating from the Ca\textsuperscript{2+} store to the plasma membrane (Zhang et al., 2005). Finally, the third model postulates that a diffusible messenger termed ‘calcium influx factor’ (CIF), most likely a phospholipid, is produced upon Ca\textsuperscript{2+}-store depletion and activates SOC (Randriamampita and Tsien, 1993; Rzigalinski et al., 1999). New evidence in favor of the idea of a diffusible messenger (such as CIF) produced by the sarcoplasmic reticulum (SR) upon Ca\textsuperscript{2+}-store depletion was recently obtained (Smani et al., 2004; Smani et al., 2003; Vandebrouck et al., 2004). It was further demonstrated that CIF can trigger SOC opening, but it
is not clear if CIF activates SOC directly or if it involves additional mechanisms (Bolotina and Csutora, 2005; Trepakova et al., 2000). Several reports suggest that CIF acts through the stimulation of a Ca^{2+}-independent PLA2 (iPLA2) (Smani et al., 2004; Smani et al., 2003; Vanden Abeele et al., 2004).

PLA2 are enzymes that catalyze the hydrolysis of fatty acid ester bonds at the second position of diacylglycerophospholipids (Chakraborti, 2003) leading to the release of a fatty acid (among which arachidonic acid) and lysophospholipids. Isoforms of PLA2 are distinguished according to their molecular mass, location and sensitivity to Ca^{2+}. Cytosolic PLA2 are Ca^{2+}-dependent but the iPLA2 family does not need Ca^{2+} for activation (Chakraborti, 2003). The involvement of PLA2 isoforms in capacitative Ca^{2+} entry has been demonstrated in several types of cells (Hichami et al., 2002; Martinez and Moreno, 2005; Rzigalinski et al., 1999; Smani et al., 2003; Vanden Abeele et al., 2004; Zablocki et al., 2000). CIF may act through the inhibition of calmodulin binding to iPLA2, resulting in iPLA2 stimulation (Smani et al., 2004; Vanden Abeele et al., 2004). The resulting PLA2 hydrolysis products would then be responsible for the stimulation of SOC (Smani et al., 2004). Indeed, it has been demonstrated that lysophospholipids or arachidonic acid and some metabolites of the latter can activate cationic channels such as SOC (Rzigalinski et al., 1999; Smani et al., 2004; So et al., 2005; Terasawa et al., 2002; Watanabe et al., 2003).

In dystrophic muscle, SOC have been proposed to be involved in enhanced Ca^{2+} influx (Vandebrouck et al., 2002). However, their regulation has not been studied so far. Here we have investigated the involvement of the PLA2 pathway in the regulation of Ca^{2+} entry in both normal and dystrophic fibers isolated from murine flexor digitorum brevis (FDB) muscles. Using both Ca^{2+} imaging and the manganese quench method, we show that store-operated Ca^{2+} entry is greatly enhanced in dystrophic fibers. We also demonstrate that this Ca^{2+} entry is controlled by iPLA2 and that exaggerated Ca^{2+} influx occurring in dystrophic fibers can be attenuated by iPLA2 inhibitors to a value close to the one of normal fibers. Finally, western blot analysis of muscle extracts revealed an increased expression of iPLA2 in dystrophic muscles, suggesting that enhanced store-operated Ca^{2+} entry occurring in dystrophic fibers may be due, at least in part, to overexpression of iPLA2. The iPLA2 pathway therefore appears to be an attractive target to reduce excessive Ca^{2+} influx and subsequent degeneration occurring in dystrophic fibers.

**Results**

In this study we have used Ca^{2+} imaging to investigate the regulation of store-operated Ca^{2+} entry in both normal (C57BL/6J) and dystrophic (mdx^{5cv}) isolated FDB fibers. In all experiments fibers were incubated with 30 µM BTS to inhibit contractions that may cause artifacts in Ca^{2+} measurements (Cheung et al., 2002). Because Ca^{2+} influx may occur through L-type voltage-gated Ca^{2+} channels during stimulation of fibers, experiments were performed in the presence of 1 µM nifedipine, a specific blocker of L-type voltage-gated Ca^{2+} channels in skeletal muscle (O’Connell and Dirksen, 2000).

**Ca^{2+} transients triggered by KCl depolarizations in normal and dystrophic fibers**

In skeletal muscle, depolarization of the plasma membrane triggers activation of L-type voltage-gated Ca^{2+} channels, allosterically activating Ca^{2+} release through the opening of ryanodine receptors (Berchtold et al., 2000).

Fig. 1A,B shows typical Ca^{2+} transients triggered by KCl depolarizations in C57BL/6J and mdx^{5cv} FDB fibers. Basal cytosolic Ca^{2+} concentrations and peak Ca^{2+} concentrations following KCl-induced Ca^{2+} transients in both C57BL/6J and mdx^{5cv} fibers, respectively. Top panels in A and B show time series of pseudocolor ratio images of isolated C57BL/6J and mdx^{5cv} fibers, respectively. Bottom panels represent global cytosolic Ca^{2+} transients triggered by high-KCl calculated from the whole fiber perimeter. Red circles on curves correspond to recording time of the 18 ratio images in both cases. (C) Plot of average [Ca^{2+}], at the base line (b) and at the peak of KCl-induced Ca^{2+} transients (p) for both C57BL/6J and mdx^{5cv} fibers. (D) Plot of average half time of decay of Ca^{2+} transients in both C57BL/6J and mdx^{5cv} fibers analyzed in C. Numbers of fibers tested (from six mice for C57BL/6J fibers and from 10 mice for mdx^{5cv} fibers) are indicated on bar graphs.

**Fig. 1.** KCl-induced cytosolic Ca^{2+} transients in isolated FDB fibers from C57BL/6J and mdx^{5cv} mice. (A,B) Calcium transients evoked by pressure ejection of a high KCl solution in isolated C57BL/6J and mdx^{5cv} fibers, respectively. Top panels in A and B show time series of pseudocolor ratio images of isolated C57BL/6J and mdx^{5cv} fibers, respectively. Bottom panels represent global cytosolic Ca^{2+} transients triggered by high-KCl calculated from the whole fiber perimeter. Red circles on curves correspond to recording time of the 18 ratio images in both cases. (C) Plot of average [Ca^{2+}], at the base line (b) and at the peak of KCl-induced Ca^{2+} transients (p) for both C57BL/6J and mdx^{5cv} fibers. (D) Plot of average half time of decay of KCl-induced Ca^{2+} transients in both C57BL/6J and mdx^{5cv} fibers analyzed in C. Numbers of fibers tested (from six mice for C57BL/6J fibers and from 10 mice for mdx^{5cv} fibers) are indicated on bar graphs.
receptor activation with acetylcholine or ryanodine receptor stimulation with caffeine (Fig. 7A).

However, kinetic properties of KCl-induced Ca2+ transients were altered in mdx5cv fibers compared with C57BL/6J fibers. Indeed, the decay phase of KCl-induced Ca2+ transients was slowed down in mdx5cv fibers, as measured by the increased half time of decay in dystrophic fibers as compared with normal fibers (3.03±0.25 seconds, n=90 and 2.22±0.09 seconds, n=87, respectively; Fig. 1D).

KCl-induced Ca2+ transients in the absence of extracellular Ca2+
Ca2+ transients triggered by KCl depolarizations were recorded in Ca2+-free solution in both types of fibers, indicating that Ca2+ influx is not needed to trigger Ca2+ release through ryanodine receptors, as previously reported for skeletal-type excitation-contraction coupling (Lamb, 2002; O’Brien et al., 2002).

Half times of decay of KCl-induced Ca2+ transients were significantly reduced for both types of fibers in Ca2+-free solution containing 1 mM EGTA (from 2.47±0.13 seconds to 1.34±0.12 seconds and from 3.14±0.74 seconds to 1.27±0.12 seconds for C57BL/6J and mdx5cv fibers, respectively; Fig. 2A,C), with no significant effect on peak Ca2+ transients in both types of fibers when exposed to 5 mM BTP2 (from 630.1±65 nM to 551.3±61 nM in the presence of BTP2; Fig. 3B). However, kinetic properties of L-type voltage-gated Ca2+ channels. We then tested the effect of the SOC blocker BTP2 on KCl-induced Ca2+ transients in both C57BL/6J and mdx5cv FDB fibers.

Preincubation of the cells with the PL2 inhibitor arachidonitrifluoromethyl ketone (AACOCF3, 50 μM) (Ackermann et al., 1995) reduced the half time of decay of KCl-induced Ca2+ transients in mdx5cv fibers about twofold (from 3.39±0.58 seconds to 1.71±0.15 seconds; Fig. 4A,C), suggesting that PL2 may be involved in the regulation of store-operated Ca2+ entry in dystrophic skeletal muscle fibers. Interestingly, preincubation of C57BL/6J fibers with

Effect of the SOC blocker BTP2 on KCl-induced Ca2+ transients
As all experiments were conducted in the continuous presence of nifedipine, it is unlikely that Ca2+ influx occurred through L-type voltage-gated Ca2+ channels. We then tested the hypothesis that store-operated Ca2+ influx could be responsible for the slow decay phase of KCl-induced Ca2+ responses. BTP2 has been described as a potent SOC blocker (He et al., 2005; Zitt et al., 2004). As shown in Fig. 3A,C, half times of decay of KCl-induced Ca2+ transients were significantly reduced in both types of fibers when exposed to 5 μM BTP2 (from 2.22±0.17 seconds to 1.43±0.11 seconds and from 2.91±0.37 seconds to 1.7±0.17 seconds for C57BL/6J and mdx5cv fibers, respectively). In mdx5cv fibers, KCl-induced Ca2+ peak values were not significantly affected by BTP2 (from 724.7±99 nM to 551.3±61.6 nM in the presence of BTP2; Fig. 3B). However, in C57BL/6J fibers, KCl-induced Ca2+ peaks were more strongly affected by BTP2 treatment (from 630.1±65 nM to 372±24.3 nM in the presence of BTP2; Fig. 3B). Altogether, these results suggest that Ca2+ influx through SOCC occurred during a single KCl-induced Ca2+ transient, and also that this store-operated Ca2+ entry is primarily responsible for the slow decay phase of KCl-induced Ca2+ transients in both types of fibers.

Effect of PL2 inhibitors on KCl-induced Ca2+ transients
Store-operated Ca2+ entry is regulated by PL2 in several cell types (Hichami et al., 2002; Martinez and Moreno, 2005; Rzigalinski et al., 1999; Smani et al., 2003; Vanden Abeele et al., 2004; Zablocki et al., 2000). We then investigated the effect of PL2 inhibitors on the kinetic properties of KCl-induced Ca2+ transients in both C57BL/6J and mdx5cv fibers. Preincubation of the cells with the PL2 inhibitor arachidonitrifluoromethyl ketone (AACOCF3, 50 μM) (Ackermann et al., 1995) reduced the half time of decay of KCl-induced Ca2+ transients in mdx5cv fibers about twofold (from 3.39±0.58 seconds to 1.71±0.15 seconds; Fig. 4A,C), suggesting that PL2 may be involved in the regulation of store-operated Ca2+ entry in dystrophic skeletal muscle fibers. Interestingly, preincubation of C57BL/6J fibers with
AACOCF$_3$ did not significantly reduce the half time of decay of KCl-induced Ca$^{2+}$ transients in these non-dystrophic fibers (from 2.03±0.14 seconds to 1.82±0.10 seconds; Fig. 4A,C). In both types of fibers, preincubation with AACOCF$_3$ did not significantly reduce peak Ca$^{2+}$ responses (Fig. 4B).

Since AACOCF$_3$ is known to inhibit Ca$^{2+}$-dependent PLA$_2$ (cPLA$_2$) as well as Ca$^{2+}$-independent PLA$_2$ (iPLA$_2$) (Ackermann et al., 1995), we next tested the effect of the specific iPLA$_2$ inhibitor bromoenol lactone (BEL) (Smani et al., 2003). Half time of decay of KCl-induced Ca$^{2+}$ transients recorded in mdx$^{cv}$ fibers was significantly reduced when cells had been treated with 5 µM BEL (from 3.05±0.37 seconds to 1.94±0.15 seconds for control and BEL treated fibers, respectively; Fig. 5A,C), suggesting that iPLA$_2$ may be involved in the regulation of store-operated Ca$^{2+}$ influx occurring during the late phase of KCl-induced Ca$^{2+}$ responses in dystrophic fibers. Consistent with the data obtained with AACOCF$_3$, pretreatment with BEL did not have a significant effect on the decay phase of KCl-induced Ca$^{2+}$ transient in normal fibers (half times of decay were 2.48±0.18 seconds and 2.04±0.22 seconds for control and BEL-treated fibers, respectively; Fig. 5C). With both types of fibers, pretreatment with BEL slightly decreased peak Ca$^{2+}$ transients (Fig. 5B). Similar experiments were conducted in mdx$^{cv}$ fibers stimulated with 100 µM acetylcholine (ACh). As shown in Fig. 5D, half times of decay of ACh-induced Ca$^{2+}$ transients were significantly reduced in BEL pretreated fibers (from 4.17±0.50 seconds to 2.77±0.35 seconds in control and BEL-treated fibers, respectively). These results suggest that iPLA$_2$ may be involved in the regulation of store-operated Ca$^{2+}$ influx occurring during a Ca$^{2+}$ transient in mdx$^{cv}$ fibers stimulated by either KCl depolarization or nicotinic receptor activation.

**Effect of BTP2 and BEL on Ca$^{2+}$ entry triggered by Ca$^{2+}$-store depletion**

To investigate more directly the possible involvement of iPLA$_2$ in store-operated Ca$^{2+}$ entry triggered by Ca$^{2+}$-store depletion and also to compare store-operated Ca$^{2+}$ entry in normal and dystrophin-deficient FDB fibers, we preincubated fibers in Ca$^{2+}$-free solution containing 1 µM thapsigargin, a potent inhibitor of the SR Ca$^{2+}$ ATPase (SERCA), to deplete SR Ca$^{2+}$ stores. Ca$^{2+}$ re-addition triggered an immediate Ca$^{2+}$ increase in both C57BL/6J and mdx$^{cv}$ fibers, as expected when SERCA is blocked (Fig. 6A). When both types of fibers were stimulated with 50 µM BTP2, the half time of decay in both C57BL/6J and mdx$^{cv}$ fibers was significantly reduced (Fig. 6B).}

![Fig. 3](image1.png)

**Fig. 3.** Effect of the store-operated channel blocker BTP2 on KCl-induced cytosolic Ca$^{2+}$ transients in isolated FDB fibers from C57BL/6J and mdx$^{cv}$ mice. (A) KCl-induced cytosolic Ca$^{2+}$transients in isolated FDB fibers from C57BL/6J and mdx$^{cv}$ mice in control conditions and after preincubation of fibers with 5 µM BTP2 for 10 minutes. (B) Plot of average [Ca$^{2+}$]$_i$, at the base line (b) and at the peak of KCl-induced Ca$^{2+}$ transients (p) for both C57BL/6J and mdx$^{cv}$ fibers in control conditions and in the presence of 5 µM BTP2. (C) Plot of average half time of decay of high KCl-induced Ca$^{2+}$ transients in both C57BL/6J and mdx$^{cv}$ fibers in control condition and in the presence of 5 µM BTP2. Data correspond to the fibers analyzed in B. Numbers of fibers tested (from two C57BL/6J mice and four mdx$^{cv}$ mice) are indicated on bar graphs.

![Fig. 4](image2.png)

**Fig. 4.** Effect of the PLA$_2$ inhibitor AACOCF$_3$ on KCl-induced cytosolic Ca$^{2+}$ transients in isolated FDB fibers from C57BL/6J and mdx$^{cv}$ mice. (A) KCl-induced cytosolic Ca$^{2+}$transients in isolated FDB fibers from C57BL/6J and mdx$^{cv}$ mice in control conditions and after preincubation of fibers with 50 µM AACOCF$_3$ for 10 minutes. (B) Plot of average [Ca$^{2+}$]$_i$, at the base line (b) and at the peak of KCl-induced Ca$^{2+}$ transients (p) for both C57BL/6J and mdx$^{cv}$ fibers in control condition and in the presence of 50 µM AACOCF$_3$. (C) Plot of average half time of decay of KCl-induced Ca$^{2+}$ transients in both types of fibers without and with 50 µM AACOCF$_3$. Data correspond to the fibers analyzed in B. Numbers of fibers tested (from two C57BL/6J mice and three mdx$^{cv}$ mice) are indicated on bar graphs.
preincubated with 5 µM BTP2 or 10 µM Gd3+, a well known SOC blocker (Allen et al., 2005), Ca2+ responses triggered by Ca2+ re-addition were nearly completely blocked, showing that they are due to Ca2+ influx through SOC (Fig. 6B,D). Ca2+ increases triggered by Ca2+ re-addition in SR-depleted fibers were 1100±161.6 nM and 402.6±51.9 nM in mdx5cv and C57BL/6J fibers, respectively (Fig. 6A,D), indicating that store-operated Ca2+ influx is enhanced about 2.5-fold in mdx5cv fibers compared with C57BL/6J fibers.

To investigate the possible involvement of iPLA2 in store-operated Ca2+ entry, fibers were treated with 5 µM BEL and then tested with a similar protocol. Results indicate that BEL significantly reduced store-operated Ca2+ entry in mdx5cv fibers (1100±161.6 nM to 488.4±119 nM for control and BEL treated cells, respectively; Fig. 6C,D). However, BEL had little effect in C57BL/6J fibers (402.6±51.9 nM to 267.7±69.9 nM for control and BEL-treated cells, respectively; Fig. 6C,D). Altogether these results indicate that iPLA2 is involved in the regulation of store-operated Ca2+ influx triggered by SR Ca2+-store depletion in mdx5cv fibers.

**Fig. 5.** Effect of the iPLA2 inhibitor BEL on KCl-induced cytosolic Ca2+ transients in isolated FDB fibers from C57BL/6J and mdx5cv mice. (A) KCl-induced cytosolic Ca2+ transients in isolated FDB fibers from C57BL/6J and mdx5cv mice in control conditions and after preincubation of fibers with 5 µM BEL for 20 minutes (B) Plot of average [Ca2+]i at the base line (b) and at the peak of KCl-induced Ca2+ transients (p) for both C57BL/6J and mdx5cv fibers in control condition and after preincubation of fibers with 5 µM BEL. (C) Plot of average half times of decay of KCl-induced Ca2+ transients in both C57BL/6J and mdx5cv fibers in control condition and after preincubation of fibers with 5 µM BEL. Data correspond to the fibers analyzed in B. (D) Acetylcholine (ACh)-induced cytosolic Ca2+ transients in isolated mdx5cv FDB fibers in control condition and in fiber pretreated with 5 µM BEL for 20 minutes. Right panel: average half time of decay of ACh-induced Ca2+ transients in control and BEL-treated fibers. Numbers of fibers tested (from two C57BL/6J mice and three mdx5cv mice) are indicated on bar graphs.

**Fig. 6.** Store-operated Ca2+ entry in C57BL/6J and mdx5cv fibers and effect of the iPLA2 inhibitor BEL. Both types of fibers were preincubated with thapsigargin (1 µM) for 10 minutes in Ca2+-free solution prior to calcium re-addition (2 mM), leading to store-operated Ca2+ entry. (A) Ca2+ increases recorded in C57BL/6J and mdx5cv fibers following preincubation with thapsigargin and Ca2+ re-addition. (B) Effect of the store-operated channel blocker BTP2 (5 µM, 10 minutes preincubation) on Ca2+ increases triggered by Ca2+ re-addition in both C57BL/6J and mdx5cv fibers treated with thapsigargin. (C) Effect of the iPLA2 inhibitor BEL (5 µM, 20 minutes preincubation) on Ca2+ increases triggered by Ca2+ re-addition in both C57BL/6J and mdx5cv fibers treated with thapsigargin. (D) Average values showing the effect of BTP2, Gd3+ and BEL on store-operated Ca2+ entry in both types of fibers. Numbers of fibers tested (from five C57BL/6J mice and four mdx5cv mice) are indicated on bar graphs.
Effect of BTP2 and BEL on caffeine-induced Mn²⁺ entry

Thapsigargin is known to trigger an almost complete Ca²⁺-store depletion. To investigate whether iPLA₂ is involved in SOC regulation for lower levels of Ca²⁺-store depletion, we investigated the effect of iPLA₂ inhibition on caffeine-induced Mn²⁺ entry. Caffeine is known to trigger Ca²⁺ release through ryanodine receptors (Frayssé et al., 2003). As shown in Fig. 7A, caffeine (50 mM) led to small Ca²⁺ transients, as previously reported for fast-twitch skeletal muscle fibers (Frayssé et al., 2003). Peak Ca²⁺ responses were not significantly different between C57BL/6J and mdx⁵cv fibers (220.3±20 nM and 196.6±30.8 nM in C57BL/6J and mdx⁵cv fibers; respectively, Fig. 7A). Although peak Ca²⁺ responses were identical in both types of fibers, caffeine-induced Mn²⁺ entry was strongly increased in mdx⁵cv fibers, in accordance with previous results (28.1±2.3% and 51.1±3.9% for C57BL/6J and mdx⁵cv fibers, respectively; Fig. 7B,C). When both types of fibers were incubated with BTP2 (5 µM), Mn²⁺ entry triggered by caffeine was nearly completely blocked (Fig. 7B,C). Similar results were obtained with the SOC blocker Gd³⁺ (not shown), indicating that caffeine triggered Mn²⁺ entry through SOC. Pretreatment of mdx⁵cv fibers with BEL (5 µM) strongly decreased caffeine-induced Mn²⁺ entry (51.1±3.9% to 14.3±1.9% for control and BEL treated mdx⁵cv fibers). In accordance with previous results, iPLA₂ inhibition had a much weaker effect in C57BL/6J fibers (28.1±2.3% to 18.9±1.3% for control and BEL treated mdx⁵cv fibers). These results indicate that whatever the extent of Ca²⁺-store depletion or the mechanism responsible for store discharge (SERCA inhibition by thapsigargin or Ca²⁺ release through ryanodine receptors), iPLA₂ is involved in the regulation of SOC in mdx⁵cv fibers.

Effect of melittin on Mn²⁺ entry in mdx⁵cv fibers

To further investigate the involvement of iPLA₂ in the regulation of store-operated Ca²⁺ entry in mdx⁵cv fibers, we tested the effect of melittin on Mn²⁺ entry assessed by the quench of Fura-2 fluorescence. Melittin, a toxin from bee venom, has been demonstrated to activate PLA₂ and subsequent release of arachidonic acid and lysophospholipids in various types of cells (Choi et al., 1992; Eintracht et al., 1998; Sharma, 1993). As illustrated in Fig. 8A, a 2 second application of melittin (5 µM) on mdx⁵cv fibers led to Mn²⁺ entry. Incubation of the cells with the SOC blocker BTP2 (5 µM) strongly reduced melittin-induced Mn²⁺ entry (from 39.2±3.3% to 10.6±2.1%; Fig. 8A,B), suggesting that melittin stimulates Mn²⁺ influx through SOC. Pretreatment of fibers with the iPLA₂ inhibitor BEL also reduced melittin-induced Mn²⁺ entry (from 39.2±3.3% to 15.6±4.8%; Fig. 8A,B). These results suggest that iPLA₂ is activated by melittin, in turn triggering Mn²⁺ entry through SOC.

Expression of iPLA₂ and SERCA1 in normal and dystrophic muscle

We investigated the expression of iPLA₂ and SERCA1 by western blot analysis of FDB and EDL (extensor digitorum longus) skeletal muscle extracts from both C57BL/6J and mdx⁵cv mice. Results in Fig. 8C show that the anti-iPLA₂ antibody recognized two specific bands of around 85 kDa for both muscles, corresponding to the range of molecular mass reported for iPLA₂ (Ackermann et al., 1995; Chakraborti, 2003). Quantitative analysis of the iPLA₂ bands revealed that the amount of iPLA₂ is increased in both FDB and EDL muscles from mdx⁵cv mice compared with C57BL/6J mice (Fig. 8C).

Fig. 8D shows that the anti-SERCA1 antibody recognized a specific band of around 110 kDa for C57BL/6J and mdx⁵cv FDB muscles, corresponding to the molecular mass of SERCA1, the major SERCA isoform in fast twitch muscle (Rossi and Dirksen, 2006). Quantitative analysis revealed that the expression of SERCA1 is essentially the same in FDB muscles from C57BL/6J and mdx⁵cv mice (Fig. 8D).
Fig. 8. Effect of BTP2 and BEL on Mn2+ entry triggered by the PLA2 activator melittin. (A) Melittin (5 μM) was applied for 2 seconds onto isolated mdx5cv fibers and Mn2+ entry was measured by the quench of Fura-2 fluorescence recorded at an excitation wavelength of 360 nm. Prior to melittin application, fibers were incubated with a solution containing 0.5 mM MnCl2 and 1.7 mM CaCl2. On the same graph are shown results from a control experiment and from experiments performed on fibers preincubated with either BTP2 (5 μM) for 10 minutes or BEL (10 μM) for 20 minutes (hatched trace). (B) Compiled data showing the percentage of fluorescence decrease of 360 nm. Prior to melittin application, fibers were incubated with a solution containing 0.5 mM MnCl2 and 1.7 mM CaCl2. On the same graph are shown results from a control experiment and from experiments performed on fibers preincubated with either BTP2 (5 μM) for 10 minutes or BEL (10 μM) for 20 minutes (hatched trace). (B) Compiled data showing the percentage of fluorescence decrease. Numbers of fibers tested (from 2 mdx5cv mice) are indicated on bar graphs. (C) Top: western blot of iPLA2 performed on FDB and EDL (extensor digitorum longus) C57BL/6J and mdx5cv muscles. Bottom: quantitative analysis of iPLA2 amounts in FDB and EDL muscles from C57BL/6J and mdx5cv mice. Numbers of mice used are indicated on bar graphs. (D) Left panel: western blot of SERCA1 performed on C57BL/6J and mdx5cv FDB muscles. Right panel: quantitative analysis of SERCA1 amounts in FDB muscles from C57BL/6J and mdx5cv mice. Numbers of mice used are indicated on bar graphs.

Discussion

We report here on our investigations regarding regulation of store-operated Ca2+ influx in dystrophic (mdx5cv) and normal (C57BL/6J) skeletal muscle fibers using cytosolic Ca2+ measurements and the manganese quench method.

In skeletal muscle, Ca2+ transients triggered by nictinic receptor activation or KCl-induced depolarization are due to Ca2+ release from the SR through ryanodine receptors, which are directly activated by L-type voltage-gated Ca2+ channels upon plasma membrane depolarization (Berchtold et al., 2000). Thus, L-type voltage-gated Ca2+ channels act as voltage-sensors and Ca2+ influx through these channels is not needed for Ca2+ release through ryanodine receptors (Lamb, 2002; O’Brien et al., 2002). Our results clearly show that the amplitude of Ca2+ transients triggered by KCl depolarization is not reduced in Ca2+-free solution in both normal and dystrophic fibers, indicating that FDB fibers used in this study have a characteristic skeletal-type excitation-contraction coupling mechanism.

Basal cytosolic Ca2+ concentrations were very similar in normal and dystrophic FDB fibers, in agreement with previous reports (Collet et al., 1999; De Backer et al., 2002; Tutdibi et al., 1999; Vandebrouck et al., 2002). Peak values of cytosolic Ca2+ transients triggered by KCl depolarization were also not significantly different between C57BL/6J and mdx5cv fibers in both normal and Ca2+-free solution, suggesting that mechanisms involved in Ca2+ release are not altered in dystrophic fibers, as previously reported for electrically-evoked Ca2+ transients (Collet et al., 1999; Gillis, 1996; Plant and Lynch, 2003; Tutdibi et al., 1999).

As opposed to the amplitudes of KCl-induced Ca2+ transients, the kinetic properties of these were altered in dystrophic fibers, which exhibited a slower decay phase than normal ones. Similar findings have been reported for electrically-evoked Ca2+ transients in mdx FDB fibers, ACh-evoked Ca2+ transients in dystrophic myotubes or relaxation speeds after electrical stimulation of dystrophic myotubes (Nicolas-Metral et al., 2001; Rivet-Bastide et al., 1993; Tutdibi et al., 1999; Woods et al., 2004).

A slower decay phase of Ca2+ transients in mdx5cv fibers may be due to lowered SERCA activity, to increased Ca2+ influx, to increased intracellular Ca2+ release or a combination of these effects. When experiments were performed in Ca2+-free solution, to prevent Ca2+ influx, half times of decay were significantly reduced in both types of fibers, indicating that Ca2+ entry is primarily responsible for the slow decay phase of these responses, although one can not exclude a minor involvement of intracellular Ca2+ release. Interestingly, in Ca2+-free solution, the half-time of decay of Ca2+ transients was similar for both types of fibers, suggesting that Ca2+ removal capabilities, mostly due to SERCA function, are not altered in mdx5cv fibers. In accordance with these findings, SERCA1, the major SERCA isoform in fast-twitch muscle (Rossi and Dirksen, 2006), was found to be similarly expressed in C57BL/6J and mdx5cv FDB muscles. Therefore Ca2+ influx appears to be mainly responsible for the slow decay of KCl-induced Ca2+ transients in both types of fibers, raising the question about the Ca2+ entry pathway.

It is unlikely that Ca2+ entry occurred through L-type voltage-gated Ca2+ channels, because nifedipine, known to block Ca2+ currents through L-type voltage gated Ca2+-
channels in skeletal muscle fibers, was used throughout our investigations (O’Connell and Dirksen, 2000). However, the SOC blocker BTP2 reduced the half-time of decay of Ca\(^{2+}\) transients in both types of fibers, suggesting that the slow decay could be due to opening of SOC. This is consistent with reports showing activation of store-operated Ca\(^{2+}\) entry when SR calcium stores were depleted in skeletal muscle cells (Kurebayashi and Ogawa, 2001; Ma and Pan, 2003). Slower decay phase in mdx\(^{cv}\) fibers could then be due to enhanced store-operated Ca\(^{2+}\) entry during Ca\(^{2+}\) responses.

This hypothesis was tested by experiments using thapsigargin and caffeine. Indeed, when SR Ca\(^{2+}\) stores were depleted with thapsigargin or caffeine, a greatly enhanced Ca\(^{2+}/\mathrm{Mn}^{2+}\) entry through SOC was found in dystrophic fibers. These results point to an increased activity of SOC following Ca\(^{2+}\)-store depletion in dystrophic FDB fibers, in accordance with other reports (Vandebrouck et al., 2006; Vandebrouck et al., 2002).

What could be the trigger for store-dependent Ca\(^{2+}\) entry and why is the decay of Ca\(^{2+}\) transients slower in dystrophic fibers? Our results suggest that iPLA\(_2\) is of central importance in regulating SOC in dystrophic fibers. We found that exposure of fibers with iPLA\(_2\) inhibitors normalized the decay of Ca\(^{2+}\) transients in mdx\(^{cv}\) fibers without affecting transients of C57BL/6J fibers. Experiments using thapsigargin or caffeine to deplete SR Ca\(^{2+}\) stores, showed that the iPLA\(_2\) inhibitor BEL greatly reduced store-operated Ca\(^{2+}/\mathrm{Mn}^{2+}\) entry in mdx\(^{cv}\) but had only a weak effect in C57BL/6J fibers. Altogether, these results indicate that iPLA\(_2\) activity may be the cause of enhanced store-operated Ca\(^{2+}\) entry in mdx\(^{cv}\) fibers, and therefore that the activity of SOC must be different between C57BL/6J and mdx\(^{cv}\) fibers.

The involvement of iPLA\(_2\) in the regulation of SOC in dystrophic fibers is further supported by the fact that melittin, a toxin from bee venom and potent PL\(_2\) activator (Choi et al., 1992; Eintracht et al., 1998; Sharma, 1993), triggered Mn\(^{2+}\) entry through SOC, which was blocked when iPLA\(_2\) was inhibited by BEL. The blockade of Mn\(^{2+}\) entry by both the SOC blocker BTP2 and the iPLA\(_2\) inhibitor BEL indicates that melittin triggers hyperactivation of iPLA\(_2\), resulting in SOC opening.

Various PL\(_2\) isoforms, including iPLA\(_2\), have been shown to regulate store-operated Ca\(^{2+}\) entry in other types of cells (Hichami et al., 2002; Martinez and Moreno, 2005; Rzigalinski et al., 1999; Smani et al., 2004; Smani et al., 2003; Zabolocki et al., 2000). Enhanced store-operated Ca\(^{2+}\) entry in mdx\(^{cv}\) fibers could be explained by higher expression levels of iPLA\(_2\) in dystrophic muscles compared with normal ones. Interestingly, a greatly increased total PL\(_2\) activity has been found in muscles from DMD patients (Lindahl et al., 1995). Since numerous PL\(_2\) products have been shown to be activators of cationic/SOC channels (Martinez and Moreno, 2005; Rzigalinski et al., 1999; Smani et al., 2004; So et al., 2005; Terasawa et al., 2002), enhanced production of the hydrolysis products of PL\(_2\) could be responsible for the enhanced Ca\(^{2+}\) entry observed in mdx\(^{cv}\) fibers, as proposed in Fig. 9.

To conclude, our results indicate that store-operated Ca\(^{2+}\) entry is greatly enhanced in dystrophic fibers compared with normal ones, and that enhanced store-operated Ca\(^{2+}\) entry is responsible for the altered kinetic properties of Ca\(^{2+}\) transients in mdx\(^{cv}\) fibers. Most interestingly, we show that the Ca\(^{2+}\)-independent isoform of PL\(_2\) (iPLA\(_2\)) is involved in the regulation of SOC channels in mdx\(^{cv}\) fibers, and that overexpression of this enzyme may be a key to the understanding of the increased store-operated Ca\(^{2+}\) entry occurring in dystrophic fibers. Recent evidence indicates that SOC or stretch-activated channels belonging to the TRP family are involved in the abnormal Ca\(^{2+}\) influx occurring in dystrophic fibers and that this could be responsible for fiber degeneration (Allen et al., 2005; Iwata et al., 2003; Vandebrouck et al., 2002; Yeung et al., 2004). Inhibition of iPLA\(_2\) leads to less store-operated Ca\(^{2+}\) entry in mdx\(^{cv}\) fibers, suggesting that the use of inhibitors of this enzyme could be of great interest for pharmacological treatment of Duchenne muscular dystrophy.

Materials and Methods

Cell preparation

Normal (C57BL/6J) or dystrophic (mdx\(^{cv}\)) mice (3-4 months old) were killed by cervical dislocation. Flexor digitorum brevis (FDB) muscles from both legs were removed quickly, and incubated in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 1 mg/ml collagenase type IA (Sigma) at 37°C for 60 minutes. Tissues were incubated for 30 minutes in PSS containing 1 M NaCl, 5.6 KCl, 1 MgCl\(_2\), 1.7 CaCl\(_2\), 11 glucose, and 10 Hepes, pH 7.4. Cells were kept in an incubator gassed with 95% air-5% CO\(_2\) at 37°C. Cells were used from 18-28 hours after isolation.

Calcium imaging

Intracellular Ca\(^{2+}\) concentration was monitored with the fluorescent Ca\(^{2+}\) indicator Fura-2AM (acetoxyxymethylester form of Fura-2, cell permeant). Before loading, cells were washed with physiological salt solution (PSS), consisting of (in mM): 130 NaCl, 5.6 KCl, 1 MgCl\(_2\), 1.7 CaCl\(_2\), 11 glucose, and 10 Hepes, pH 7.4. Cells were incubated for 30 minutes in PSS containing 1 μM Fura-2AM, washed to remove extracellular Fura-2, and allowed to de-esterify for 20 minutes. To minimize potential compartmentalization of the dye, Fura-2 loading was performed at room temperature.

Ca\(^{2+}\) measurements were performed using an inverted microscope (Zeiss...
Axiovert 200M with a ×20 objective, and images were acquired with an intensified CCD camera (Extended Isis, Photonic Science). The fiber culture chamber was placed on the stage of the microscope. Ultraviolet light, emitted from a 75 W xenon lamp (Visitron Systems), passed through a high-speed monochromator (Visitron Systems), which selected alternately excitation wavelengths of 340 and 380 nm. Fluorescence emitted by cells was band-passed filtered at around 510 nm and collected by the CCD camera. Metalfour software (Universal Imaging Corporation) was used to record and analyze fluorescence measurements. Fluorescence was processed by correcting each image for background fluorescence and calculating 340/380 nm fluorescence ratios on a pixel-by-pixel basis. \([\text{Ca}^{2+}]_i\) was calculated according to the following equation (Gryniewicz et al., 1985):

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\text{[Ca}^{2+}]_i = K_d (F_{\text{340nm}}/F_{\text{380nm}} - 1) / (F_{\text{340nm}} - R_{\text{min}}) - (4-[3,5-bis(trifluoromethyl)pyrazole derivative, BTP2. N-benzyl-p-toluene-sulfonamide (BTS) and thapsigargin were from Calbiochem.

Acetylcholine, caffeine, collagenase type IA and bromoelone lactone (BEL) were from Sigma. Fura-2 AM was from Molecular Probes. Ethylene glycol-bis(2-}

Statistics
Results are expressed as means ± s.e.m. Significance was tested by means of Student’s t-test and P values of <0.05 were considered as significant.

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