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New insights in the regulation of calcium transfers by muscle dystrophin-based cytoskeleton: implications in DMD

Bruno Constantin · Stéphane Sebille ·
Christian Cognard

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Abstract Calcium mishandling in Duchenne muscular dystrophy (DMD) suggested that dystrophin, a membrane-associated cytoskeleton protein, may regulate calcium-signalling cascades such as calcium entries. Calcium overload in human DMD myotubes is dependent on their contractile activity suggesting the involvement of channels being activated during contraction and/or calcium release. Forced expression of mini-dystrophin in dystrophin-deficient myotubes, reactivates appropriate sarcolemmal expression of dystrophin-associated proteins and restores normal calcium handling in the cytosol. Furthermore, the recombinant mini-dystrophin reduced the store-operated calcium influx across the sarcolemma, and the mitochondrial calcium uptake during this influx. A slow component of calcium release dependent on IP3R, as well as the production of IP3, were also reduced to normal levels by expression of mini-dystrophin. Our studies provide a new model for the convergent regulation of transmembrane calcium influx and IP3-dependent calcium release by the dystrophin-based cytoskeleton (DBC). We also suggest molecular association of such channels with DBC which may provide the scaffold for assembling a multiprotein-signalling complex that modulates the channel activity. This suggests that the loss of this molecular association could participate in the alteration of calcium homeostasis observed in DMD muscle cells.

Keywords Skeletal muscle · Duchenne muscular dystrophy · Dystrophine · Calcium signalling · Store-operated calcium entries · Inositol [1,3,5] triphosphate receptor · Mitochondrial calcium uptake

Calcium overload and muscle injury generation

A tight control of intracellular Ca^{2+} is essential for the survival and normal function of skeletal muscle. The skeletal muscle cells use a transient increase in cytosolic free calcium concentration ($[\text{Ca}^{2+}]_i$) for intracellular signalling, and maintain resting calcium activity at a low level (around 100 nM) in order to keep a large dynamic range for the calcium signal. Membrane ionic channels and transporters, cytosolic calcium buffers and calcium buffering organelles regulate calcium influx, storage and extrusion to maintain $[\text{Ca}^{2+}]_i$ below the activation thresholds and extraphysiological values. Several observations suggest that a sustained increase in the intracellular calcium concentration exceeding the cell's buffering capacity could be involved in the aetiology of muscle fibre injury, especially in some muscular dystrophies, e.g. in Duchenne muscular dystrophy (DMD). Indeed, strong perturbations of calcium handling were observed in DMD muscle fibres (Bodensteiner and Engel 1978; Bertorini et al. 1982; Jackson et al. 1985). Moreover, studies performed on dystrophic muscle cells from *mdx* mice, the animal model for DMD (Bulfield et al. 1984), have suggested that a persistent intake of Ca^{2+} activates Ca-sensitive proteolytic and phospholipolytic activities (Turner et al. 1991; Alderton and Steinhardt 2000) resulting in the degradation of dystrophic muscle fibres. However,

B. Constantin (✉) · S. Sebille · C. Cognard
Institut de Physiologie et Biologie Cellulaires, CNRS,
UMR-6187, University of Poitiers, 86022 Poitiers, France
e-mail: Bruno.Constantin@univ-poitiers.fr

the question of global elevation in the resting $[Ca^{2+}]_i$; values remains controversial in isolated muscle fibres from *mdx* mice and in cultured *mdx* or DMD myotubes (see Gillis 1999 for extended review) as well. While some observations reported global elevation in resting $[Ca^{2+}]_i$ of *mdx* myotubes and myofibres (Turner et al. 1988, 1991; Bakker et al. 1993; Denetclaw Jr et al. 1994), other groups did not confirm this elevation in steady-state cytosolic calcium levels (Gailly et al. 1993; Head 1993; Pressmar et al. 1994; Collet et al. 1999). Different observations obtained in our group suggested that the global elevation of the resting $[Ca^{2+}]_i$ could be dependent on differentiation and activity of muscle cells. While human DMD myotubes in primary culture did not develop a significant increase in $[Ca^{2+}]_i$ (Rivet-Bastide et al. 1993; Pressmar et al. 1994), well-differentiated and contracting human DMD myotubes co-cultured with neural explants (Imbert et al. 1995) displayed elevated steady-state calcium levels dependent on muscle spontaneous activity. This latter study has suggested that calcium perturbations are linked to activation of specific channels or to membrane alterations occurring during contraction. Moreover, a recent work has suggested that increases in $[Ca^{2+}]_i$ were related to the degree of mechanical stress and that calcium elevations and muscle damage were prevented by stretch-activated channel (SAC) blockers (Yeung et al. 2005).

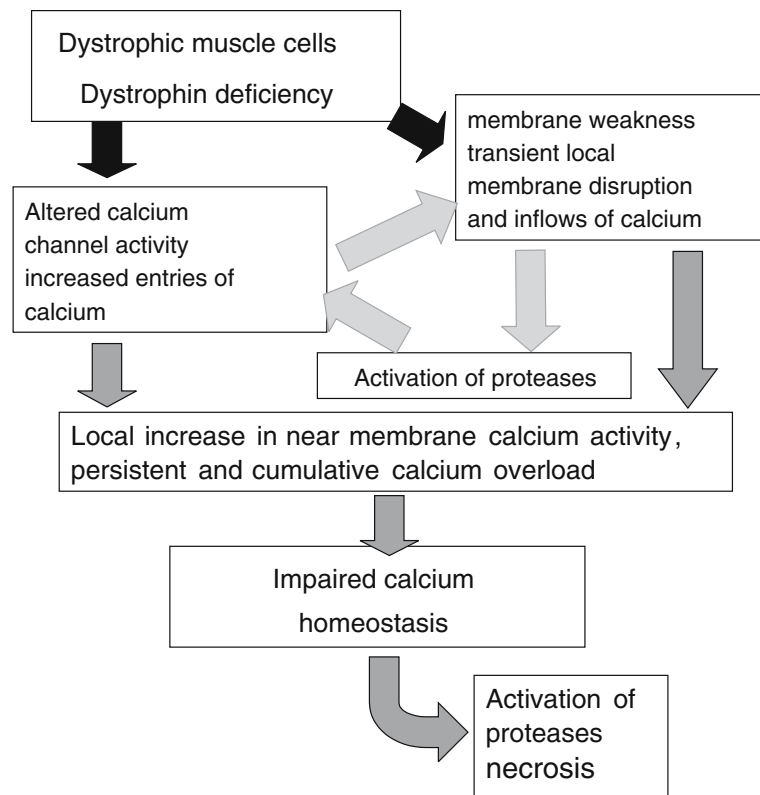
Global sarcolemmal membrane permeability increase or more specific alterations?

Duchenne muscular dystrophy results from the lack of dystrophin, a cytoskeletal protein associated with the inner surface membrane in skeletal muscle and other tissues. Important progress in the understanding of muscular dystrophies in human and animal models have been gained by the classical studies showing that the linkage of the dystrophin-related cytoskeleton to the extracellular matrix (ECM), mediated by the dystrophin-associated-proteins (DAP) complex, is involved in membrane stability, force transduction and membrane protein functions (Ervasti and Campbell 1993; Blake et al. 2002). This led to the assertion that the loss of cytoskeleton–ECM linkage due to a defect in one of this component could be the primer of cellular necrosis, and that dystrophin and associated proteins play a structural role in ensuring that membrane stresses associated with periodic contractions do not lead to membrane disruptions and leakage (Fig. 1). This hypothesis is based on the observation of (i) “delta lesions” in dystrophic muscle, (ii) the require-

ment of contraction for the appearance of lesions and of a dystrophic phenotype (Mokri and Engel 1975; Morizumi et al. 1984; Peterson et al. 1986) and (iii) the susceptibility of dystrophin-deficient muscle cells to hypotonic shocks (Menke and Jockusch 1995) and to membrane tension during contraction (Petrof et al. 1993). This idea was reinforced by the observations that forced expression in dystrophic *mdx* mouse muscle of a minigene encoding for dystrophin domains increases sarcolemmal stability and protects fibres against mechanical injury (Phelps et al. 1995; Wells et al. 1995; Deconinck et al. 1996; Decrouy et al. 1997). Several studies have also proposed that dystrophin could interact with another membrane-signalling system, namely the neural isoform of the nitric oxide synthase (nNOS), through α 1-syntrophin (Brenman et al. 1995, 1996; Grozdanovic et al. 1996; Decrouy et al. 1998).

Alterations of the sodium permeability were not observed in DMD or *mdx* muscle cells (Turner et al. 1991), ruling out a general (non-specific) increase of ionic permeability through membrane wounds. In contrast, several experiments have supported the idea that a small and persistent inward calcium leak in dystrophin-deficient muscle cells could be the consequence of the alteration of the control normally supported by dystrophin on transmembrane channels (Fig. 1). Because dystrophin is a plasma-membrane-associated protein, it was first thought that it could influence the behaviour of sarcolemmal channels involved in calcium entries. Furthermore, in muscle cells from *mdx* mice, the lack of dystrophin is associated with an increase in the calcium concentration underneath the plasma membrane (Mallouk et al. 2000). This observation was in accordance with the hypothesis that calcium mishandling in DMD cells could result from an exaggerated entry of calcium via channels from the sarcolemma (Fig. 1). Indeed, experimental data had shown that *mdx* myotubes and fibres exhibit an increase permeability to divalent cations that enter the cell through channel-blocker-sensitive pathways (Hopf et al. 1996; Tutdibi et al. 1999), and uncontrolled stretch-activated currents (Franco Jr and Lansman 1990). The studies conducted by our group on co-cultured human myotubes (Vandebrouck et al. 2001) also confirmed that SACs were more active in the plasma membrane of DMD myotubes. As stated above, the recent work of Yeung et al. (2005) also suggested the involvement of SACs in calcium alteration and muscle damage in the *mdx* mouse. Turner et al. (1991) have also proposed that leak calcium channels could also be activated by proteolysis near transient membrane microruptures. The weaker dystrophic

Fig. 1 Current hypothesis for physiopathological pathways leading to impaired calcium homeostasis and necrosis in dystrophin-deficient muscle cells



sarcolemma may thus increase the occurrence of membrane ruptures leading to localized calcium entries and activation of calcium-dependent proteolysis which could in turn activate cationic channels (Fig. 1). This reinforced the idea that alternative pathways of calcium entry mediated by background cationic channels are specifically altered in dystrophic muscles.

Other studies have also suggested that other pathways of calcium entry could be altered in dystrophic muscles. The studies performed by our group on DMD myotubes have shown that the main voltage-gated calcium entry through L-type calcium channels displayed altered properties in DMD myotubes although it did not lead to a global increase in calcium influx (Imbert et al. 2001). A work performed on transgenic *mdx* mice also suggested that mini-dystrophin restored normal properties of L-type calcium currents (Friedrich et al. 2004), supporting the idea that dystrophin influences L-type Ca^{2+} channels via a direct or indirect linkage. These views were followed by a recent study conducted on *mdx* muscle cells (Johnson et al. 2005). This work suggested that dystrophin and actin cytoskeleton may modulate the role of PKA in voltage-dependent potentiation of I_{Ca} , L, presumably via anchoring to an AKAP complex. Finally a study performed in our laboratory with DMD muscle co-culture suggested an indirect involvement of

the Na/Ca exchanger in calcium-handling alterations (Deval et al. 2002). We observed that the difference between calcium responses induced by alteration of the outside sodium concentration in normal and DMD myotubes were more likely due to differences in intracellular calcium-release systems of the two types of cells than to sarcolemmal mechanisms.

Using recombinant dystrophins in muscle cell lines

The extensive comparison of dystrophic cells from human or *mdx* mice with normal muscles has not allowed researchers to definitively determine if the alterations of calcium handling could be directly the result of the lack of dystrophin or DAP. Indeed, calcium mishandling could alternatively occur indirectly through other proteins or even only during the degeneration process of dystrophic cells and dystrophin might not exert primary control on calcium-signalling proteins.

One could address this question more directly by introducing a recombinant protein into dystrophin-deficient muscle cells displaying major calcium abnormalities and by observing relatively rapid changes in calcium-handling and -signalling properties. For this purpose we selected and developed a subline of muscle

cells deficient in dystrophin and derived from Sol8 cells (Guo et al. 1996). We took advantage of some characteristics of these muscle cells, namely the lack of dystrophin, and a clear increase in $[Ca^{2+}]_i$ at the later stages of the development associated with calpain-dependent cell death (Marchand et al. 2001). We demonstrated with this cellular model that forced expression of full-length dystrophin (by plasmid microinjection) prevented the development of the calcium-handling abnormalities by maintaining the resting $[Ca^{2+}]_i$ at low levels and decreasing global calcium-release amplitudes (Marchand et al. 2004). These observations are in agreement with results previously reported for myotubes from transgenic *mdx* mice (Denetclaw Jr et al. 1994) or for *mdx* transfected myotubes (McCarter et al. 1997). This effect occurred rapidly within 2 to 4 days after transfection, which is compatible with the idea of a direct control of calcium handling.

Since the BMD mini-dystrophin was reported to mimic some of the functions of the 427 kDa dystrophin, we also explored the abilities of the truncated protein to regulate the resting $[Ca^{2+}]_i$ and the calcium-release process. The inclusion of the cDNA in a

retroviral vector allowed us to obtain stable clones of myoblast constitutively expressing the BMD mini-dystrophin (Fig. 2). Stable transfection of mini-dystrophin from Becker Muscular Dystrophy also resulted in a lower resting $[Ca^{2+}]_i$ in differentiated (4 days after fusion) myotubes (Marchand et al. 2004). This restoration of a lower steady-state cytosolic calcium level was also accompanied by a decrease in the amplitude of KCl-induced calcium release and changes in spontaneous calcium-release properties at the subcellular level. These results demonstrated a specific effect of mini- and full-length dystrophin expression on calcium-handling abilities and calcium-release properties of cultured skeletal muscle cells. Furthermore, mini-dystrophin was also shown to restore normal properties of L-type calcium current in fibres from transgenic *mdx* mice (Friedrich et al. 2004), also suggesting that dystrophin may influence excitation- Ca^{2+} -release coupling from the sarcoplasmic reticulum (SR). Our observations obtained with Sol8 myotubes developing in culture were also in accordance with calcium-handling properties described on developing *mdx* myotubes (Robert et al. 2001). Direct measurements with recombinant aequorin of $[Ca^{2+}]_i$ in

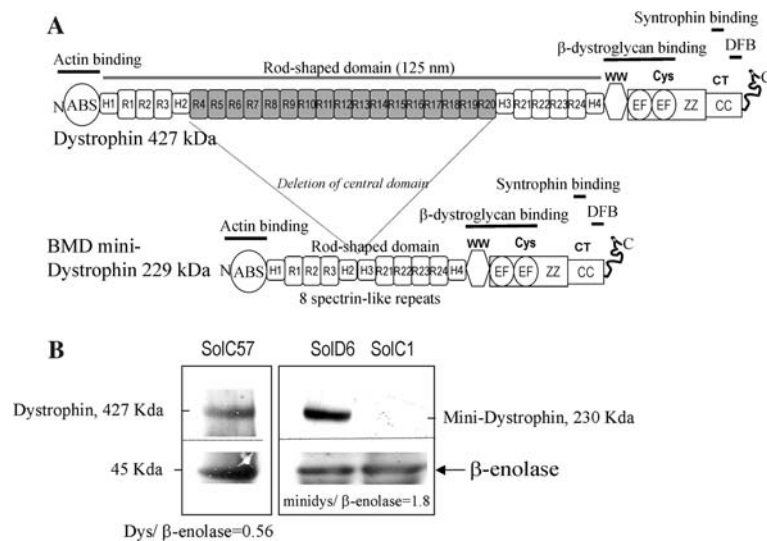


Fig. 2 **A** Schematic showing the organization of “full-length” dystrophin and of BMD mini-dystrophin used for forced expression. At the N-terminal region actin-binding sequences are shown (ABS) followed by the rod-shaped domain where are identified the four proline-rich hinge regions (HI–H4) and the spectrin-like repeats (R1–R24). At the cystein-rich domain (Cys) and C-terminal region (CT) are shown the WW domain, the EF hands, the ZZ domain and the paired coiled-coil (CC). Binding sites for β -dystroglycan, syntrophin and the dystrophin family binding site are represented by black bars. The spectrin-like repeats from R4 to R20 lost in mini-dystrophin are represented in grey. **B** Forced expression of recombinant mini-dystrophin in

muscle cell lines. Total cell lysates were applied at the same amount of proteins and migrated in parallel for comparing by Western blotting the expression levels of the β -enolase and of full-length dystrophin or of mini-dystrophin. A monoclonal antibody against the N-terminal part of the dystrophin (NCL-DYS1, Novocastra) was used—for each band, levels were expressed as ratio of densities from bands of dystrophin over densities from bands of β -enolase, taken as an internal control. SolC57 normal mouse myotubes, SolD6 myotubes expressing recombinant mini-dystrophin, SolC1 myotubes deficient in dystrophin

cytosol, mitochondrial matrix and under the plasma membrane at day 11 of culture showed a larger increase after KCl-induced depolarization in *mdx* myotubes compared with control myotubes. Measurements of $[Ca^{2+}]_i$ in the SR also showed the intraluminal steady-state level is significantly higher in *mdx* myotubes. In contrast, depolarization-induced SR calcium release in *mdx* fibres from postnecrotic mice (8–18 weeks old) were reduced compared to normal fibres (Woods et al. 2005). This could highlight adapting secondary changes at postnecrotic stages in calcium-release properties and in SR calcium loading, since calsequestrin-like Ca^{2+} binding protein (Culligan et al. 2002) was shown to be depressed in *mdx* fibres from 8-week-old mice.

Store-operated channels contribution

Although dystrophin-deficient Sol8 cell lines cannot be considered as a pathological model reproducing all the aspects of DMD, it provides the ability to isolate specific changes linked to the expression of mini-dystrophin and occurring rapidly after the sorting of mini-dystrophin to the sarcolemma. It especially allowed us to analyse specific changes occurring at early stages in culture, before the appearance of necrotic myotubes. Further studies were thus conducted to determine whether mini-dystrophin was able to control calcium influx through specific plasma-membrane channels that were shown to be altered in dystrophic muscles. The work of Kurebayashi and Ogawa (2001) previously showed that store-operated calcium entries, also named capacitative calcium entries (CCEs), can be activated in muscle fibres even by a partial depletion of the stores. More recently, the study on muscle cells lacking mg29 (Pan et al. 2002) has shown that the slow cumulative calcium entry through store-operated channels (SOC) is crucial for long-term calcium homeostasis, and reduced SOC activity exaggerates muscle fatigue (Pan et al. 2002). These different studies have revealed that CCEs that were well described in other non-excitabile cells also support cumulative calcium entries in skeletal muscle fibres in physiological conditions, and are necessary for long-term activity of muscles. These properties suggest that CCEs could be a good candidate for mediating sustained and cumulative calcium entries during activity of dystrophic muscles. Indeed, the early work of Hopf et al. (1996), suggested first that the “leak channels” that are overactivated in *mdx* and DMD muscle cells could be store-operated cationic channels and generate elevated

CCEs. Another important study (Vandebrouck et al. 2002) conducted in *mdx* and C57bl10 muscle fibres showed that the activity of cationic channels in adult skeletal muscle fibres was indeed activated by the depletion of the stores of calcium with thapsigargin or caffeine. More importantly, the activity of SOCs was increased in fibres from dystrophic mice. By analogy with the situation observed in non-excitabile cells, it was therefore hypothesized that these SOCs could belong to the transient receptor potential channel (TRPC) family. The expression of TRPC isoforms in normal and *mdx* adult skeletal muscles fibres was also explored and among the seven known isoforms, five were detected (TRPC1, 2, 3, 4 and 6) by RT-PCR and western blot analysis. The immunocytochemistry of normal and *mdx* muscle fibres demonstrated the localization of TRPC1, 4 and 6 proteins at the plasma membrane. Therefore, an antisense strategy was used to repress these TRPC isoforms. In parallel with the repression of the TRPC1 and TRPC4, the authors observed that the occurrence of calcium leak channels was decreased to one tenth of its control value (patch-clamp technique), showing the involvement of these TRPC isoforms in the abnormal calcium influx observed in dystrophic fibres.

These observations prompted us to explore if BMD mini-dystrophin (deletion of the central rod-shaped domain) was able to restore normal calcium handling in Sol8 myotubes through a better control of CCEs. The analysis of CCEs properties has been conducted by means of fluorescence quenching of fura-2 by the entry of manganese ions into the cells (Vandebrouck et al. 2005). We gained insight concerning the level of CCEs in Sol8 myotubes deficient in dystrophin (SolC1) or in myotubes expressing the truncated BMD dystrophin (Sold7, Sold6). These experiments clearly showed that after depleting calcium stores from the SR, store-dependent calcium influx can be activated in both myotube types, and that myotubes expressing mini-dystrophin display reduced CCEs. Measurements of store-dependent calcium influxes have also shown that the properties of the responses recorded in myotubes expressing mini-dystrophin are similar to the ones in normal myotubes expressing native dystrophin (SolC57 myotubes). This clearly shows that mini-dystrophin expression restores normal level of CCEs in muscle cells. The analysis with the cytosolic fluorescent calcium probe indo-1 also showed that depletion of calcium stores and reintroduction of calcium in the bath solution resulted in less sustained calcium increases in the myoplasm of myotubes expressing mini-dystrophin.

The dystrophin-based cytoskeleton hypothesis

Taken together these data led us to propose that the dystrophin-based cytoskeleton (DBC), provides the scaffold anchoring signalling molecules that can negatively regulate the amount of store-dependent calcium influx during muscle activation (Vandebrouck et al. 2005). This interaction with DBC could involve a mechanism of channels accumulation and immobilization at specific membrane domains (accumulation machine) and/or the building of complexes including signalling molecules modulating channel activity. Mini-dystrophin, which lacks 17 repeats of the central rod domain (Fig. 2), retains the ability to negatively regulate store-dependent calcium influx and to restore a global normal calcium homeostasis (Marchand et al. 2004). Altogether with previous studies based on expression of mini-dystrophin in *mdx* mice, our observations provide additional support for the use of mini-dystrophin for functional recovery of dystrophin-deficient muscles. The domains involved in the negative control of SOCs may be situated in the C-terminal domain where binding sites for dystrobrevins and syntrophins can be found (Fig. 2). These views are in accordance with the current idea that the DBC provides the scaffold for anchoring and regulating calcium transporters and adhesion-mediated-signalling molecules (Zhou et al. 2004; Johnson et al. 2005; Batchelor and Winder 2006).

The mitochondrial contribution

The contribution of transmembrane calcium influx in global or subcellular calcium handling is highly dependent on cell buffering capacities. New insights concerning calcium homeostasis regulation have highlighted the dynamic interplay between plasma membrane and subcellular compartments such as the SR and mitochondria. These latter organelles seem to be of particular importance for the regulation of CCEs in different cellular systems. Various studies suggest that close contacts between mitochondria and SR exist permitting Ca^{2+} exchanges during depolarization (Challet et al. 2001; Arnaudeau et al. 2001). Moreover, other studies (Hoth et al. 1997; Gilabert and Parekh 2000) have shown that mitochondria are able to rapidly capture Ca^{2+} ions that enter into the cells via store-operated calcium influx and regulate the pattern and rate of these CCEs. During cell stimulation, continuous Ca^{2+} fluxes (uptake and release) through mitochondria are necessary for generating subplasmalemmal microdomains of low Ca^{2+} , which facilitates CCEs (Malli

et al. 2003). Moreover, these continuous fluxes through mitochondria relay Ca^{2+} from the plasma membrane to the endoplasmic reticulum. These various data suggest that calcium uptake by mitochondria during CCEs or calcium release from SR stores may be crucial for maintaining a normal calcium homeostasis in skeletal muscle cells. Indeed, the use of a targeted calcium probe (m-aequorin) into the mitochondrion (Robert et al. 2001) has demonstrated an increase in the calcium uptake by mitochondria of *mdx* myotubes during depolarization. In our hands (dystrophin-deficient muscle cell lines), the inhibition of mitochondrial Ca^{2+} uptake by mitochondrial depolarization (FCCP treatment) revealed that the prolonged duration of store-dependent calcium increase in dystrophin-deficient myotubes was shortened. These long-lasting CCEs were thus dependent on mitochondrial Ca^{2+} buffering (Vandebrouck et al. 2005). This could be explained by the fact that a high and rapid mitochondrial uptake generates a subplasmalemmal microdomain of low Ca^{2+} , which prevents calcium inactivation of SOCEs. This suggested that, in dystrophin-deficient myotubes, mitochondria might uptake cytosolic calcium more actively, presumably for compensating the increase in store-dependent calcium influx. This idea was explored by measurements, after CCEs stimulation, of calcium changes in mitochondria by means of a recombinant m-aequorin. These experiments have shown that the amount of calcium entering mitochondria after store depletion was higher in dystrophin-deficient myotubes (Vandebrouck et al. 2005). This is in complete accordance with the previous observation that, in activated *mdx* dystrophic myotubes, mitochondrial calcium uptake was higher than in non-dystrophic cultures (Robert et al. 2001). The higher uptake of cytosolic calcium by mitochondria in response to an increased store-dependent influx may potentiate prolonged activation of CCEs, which in turn increases the amount of calcium buffered by mitochondria. This loop is likely a critical point that controls the calcium overload of mitochondria and may lead to alterations of mitochondrial metabolism that will accelerate the process of necrosis. Interestingly, the restoration of normal calcium handling in myotubes expressing mini-dystrophin was concomitant with an increased cell-survival during the time-course of culture (Fig. 3). Moreover, it has been previously shown that apoptosis can occur, preceding necrosis, in *mdx* fibres (Tidball et al. 1995) and that expression of the anti-apoptotic Bcl-2 protein can prevent apoptosis in muscle (Behrens et al. 1997). We recently collaborated to a study showing that the overexpression of Bcl-2 decreases subsarcolemmal and mitochondrial

calcium overload that occurs in *mdx* muscle cells during activation of nicotinic acetylcholine receptors (Basset et al. 2006). It was also shown that *bcl2* overexpression prevents Ca^{2+} -dependent apoptosis. Altogether, this data strongly suggests that mitochondrial calcium overload during activation of dystrophic muscles is also involved in calcium mishandling and triggering of muscle death. Although inflammatory mediators contribute to the DMD phenotype and can provide efficient targets to counteract muscle necrosis (Porter et al. 2002; Grounds and Torrisi 2004; Messina et al. 2006), regulation of Ca^{2+} -dependent apoptosis and mitochondrial Ca^{2+} handling could open new therapeutic approaches.

SR and IP₃ pathway contributions

In order to understand how the DBC is involved in calcium homeostasis, we need to consider, as noticed above, not only the transfers through plasma membrane but also the dynamic interplay between the different Ca^{2+} pathways through the different connected compartments. Previous works of our group (Deval et al. 2002) have suggested that increased calcium release from SR can be involved in calcium dysregulation in DMD human myotubes. This study suggested that the influx of Ca^{2+} transported through the inverted mode of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger was unchanged in DMD myotubes, but that the resulting calcium-induced calcium released (CICR) was increased. These increases in the CICR component revealed a higher sensitivity of the calcium-release apparatus in dystrophic muscle cells, which could also explain the higher spontaneous calcium-release activity observed in contracting DMD myotubes (Imbert et al. 1996). Such a release was classically attributed to ryanodine receptors (RyRs), but could either involve the inositol 1,4,5-trisphosphate receptors (IP₃R). Skeletal muscle fibres possess the basic molecular machinery for a functioning IP₃ messenger system (Carrasco and Figueroa 1995), and *in vitro* studies in developing muscle cells have suggested that the IP₃-induced Ca^{2+} release plays a role in gene expression regulation (Powell et al. 2001; Araya et al. 2003). Moreover, a previous study has shown that basal levels of IP₃ were increased two- to threefold in dystrophic human and murine cell lines as compared to normal cell lines (Liberona et al. 1998). After potassium depolarization, IP₃ mass rose in normal human cells and in cells of the myogenic mouse cell line. However, in the human dystrophic cell line, IP₃ levels remained elevated a longer time (Liberona et al. 1998). This

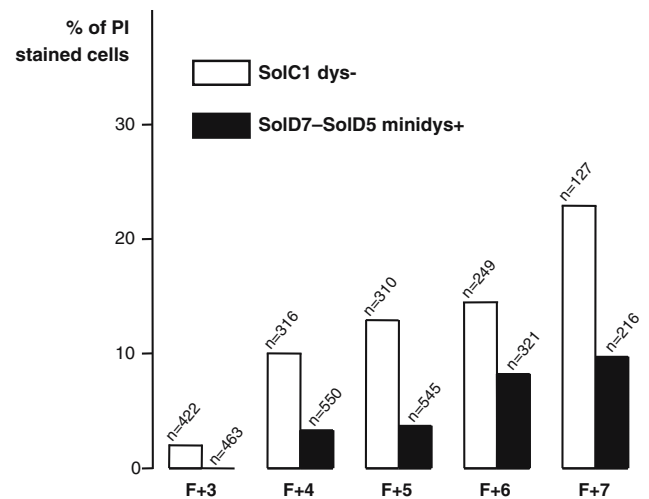


Fig. 3 Quantification of dying myotubes stained with propidium iodide (PI) in dystrophin-deficient SolC1 culture (*white bars*) and in SolD7 and SolD5 myotubes expressing the recombinant mini-dystrophin (*black bars*), all issued from the same passage number. *Bar graphs* correspond to the percentage of PI-stained myonuclei. Age of cells is indicated under the corresponding *bar graph* ($F+n = n$ days after promotion of myoblast fusion). n corresponds to the number of cells observed

prompted us to explore the involvement of IP₃-dependent calcium release in developing myotubes and to determine whether forced expression of mini-dystrophin could control this Ca^{2+} pathway. First, both RT-PCR and Western blot experiments demonstrated the presence of the three isoforms of IP₃ receptors in normal primary cultures as well as in dystrophin-deficient cell lines or in myotubes expressing mini-dystrophin. Immunocytochemistry allowed us to localize IP₃R-1 and IP₃R-2 in the three types of myotubes (Balghi et al. 2006), the first one being mainly distributed in a perinuclear area, near the sarcolemma, and in striated SR. The IP₃R-2 was distributed in a striated pattern and in subplasmalemmal areas. Using laser scanning confocal microscopy, measurements of calcium response during perfusion of high KCl solution (47 mM) have been performed in myotubes loaded with the Ca^{2+} probe Fluo-4. The use of ryanodine at very high concentration abolished Ca^{2+} release in almost every control myotubes in primary culture expressing dystrophin or in myotubes expressing mini-dystrophin. In contrast a significant Ca^{2+} release still remained at a high ryanodine concentration in myotubes that do not express dystrophin or BMD mini-dystrophin (SolC1). These results suggested that SolC1 myotubes displayed a calcium-release component that is insensitive to ryanodine treatment. By contrast, cells exposure to IP₃ inhibitors during potassium depolarization significantly decreased Ca^{2+} release particularly in dystrophin-deficient myotubes.

2-APB and U73122 displayed indeed a strong effect on dystrophin-deficient SolC1 myotubes while myotubes expressing mini-dystrophin were less or not affected by these treatments. This blockade allowed dystrophin-deficient myotubes to recover Ca^{2+} transient kinetics similar to that observed in control conditions in myotubes expressing mini-dystrophin (Balghi et al. 2006). These data led us to measure kinetics parameters and perform statistics to address such differences. Depolarization of dystrophin-deficient cell line (SolC1) induced a large increase with slower kinetics as compared to the other cells, expressing dystrophin (primary culture) or mini-dystrophin. Forced expression of mini-dystrophin allowed recovery of fast calcium release very similar to that observed in primary myotubes. These results are in favour of the

hypothesis that dystrophin could play a regulator role on the release during cell excitation (Balghi et al. 2006), by building the cellular environment in favour of a fast calcium release dependent on RYR. The lack of dystrophin in dystrophic muscle could exaggerate the slow calcium-release component dependent on IP3R, likely by the removal of a negative control on this pathway (Fig. 4).

Furthermore, the observation of resting fluo4-loaded cells revealed a higher occurrence of spontaneous release events in dystrophin-deficient myotubes as compared to that observed in mini-dystrophin or dystrophin expressing cells. Since these spontaneous events are known to contribute to the resting level of calcium (Berridge 1997), they could contribute with other mechanisms to calcium mishandling. It is known

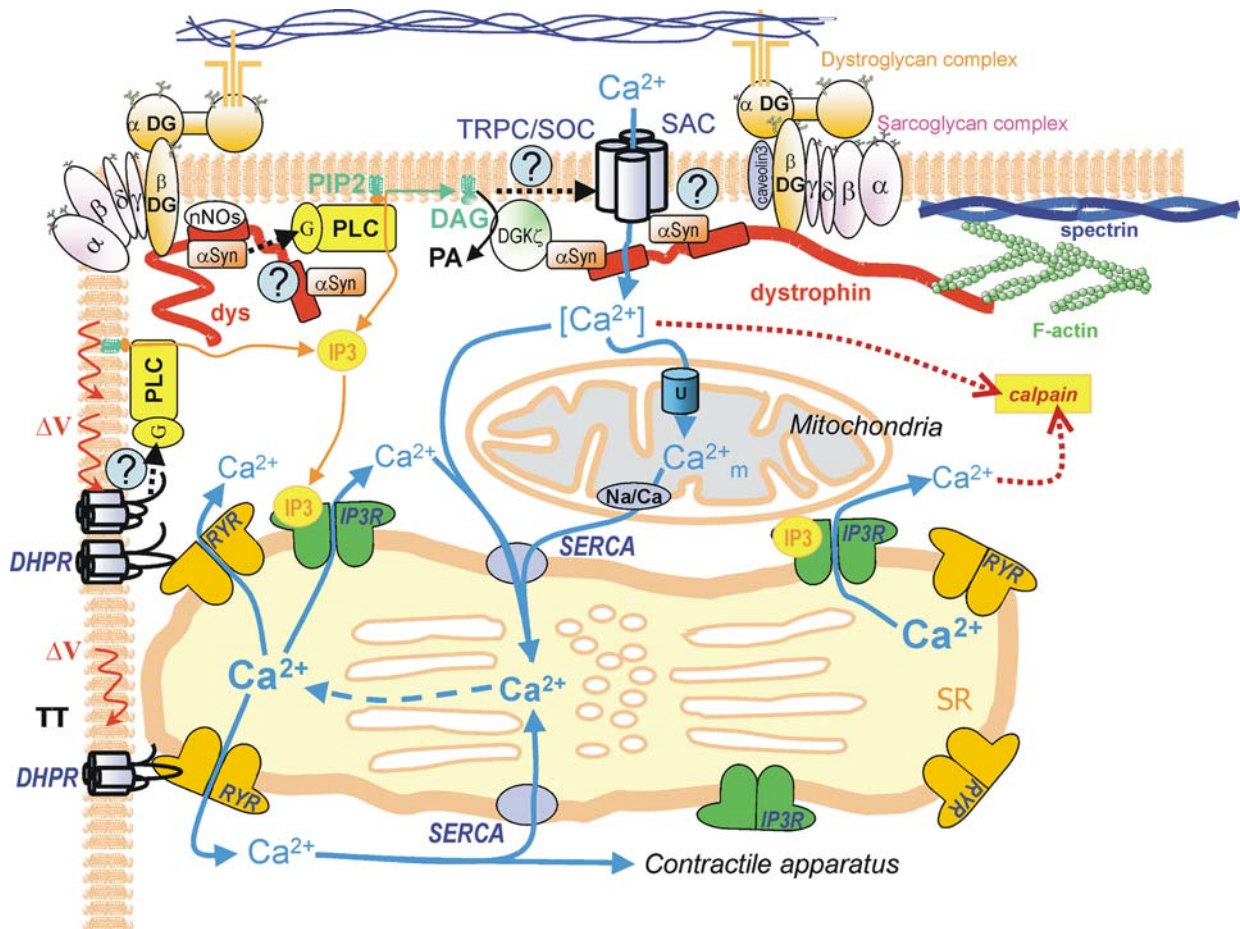


Fig. 4 A working model for regulation of calcium signalling by dystrophin-based cytoskeleton in skeletal muscle cells. The DAP/dystrophin complex may provide a scaffold for signalling molecules, which regulates the activity of channels. *SOC* store-operated channel, *SAC* stretch-activated channel, *DG* dystroglycan, α *SYN* α -syntrophin, *RYR* ryanodine receptor, *DHPR* dihydropyridine receptor, *IP3R* inositol 1,3,5-trisphosphate

receptor, *SERCA* sarco-endoplasmic reticulum calcium ATPase, *Na/Ca* $\text{Na}^+/\text{Ca}^{2+}$ exchanger, *U* uniporter, *G* heterotrimeric G protein, *PLC* phospholipase C, *IP3* inositol 1,3,5-trisphosphate, *DAG* diacylglycerol, *DGK* ζ diacylglycerol kinase ζ , *nNOS* neuronal nitric oxide synthase. For commodity, dystrophin (*dys*) was sketched in two different forms, but representing the same molecule

that RYRs and IP3Rs can be involved in these spontaneous release events. For that reason, the release initiation mechanisms as well as the implicated pathways have to be investigated for the understanding of resting calcium homeostasis in dystrophin-deficient cells.

Interplay between signalling pathways anchored to the DBC scaffold

What can be the link between the scaffold of DBC and the calcium channels of the sarcolemma and of the SR?

One could think that a molecular link exist between the DBC and the sarcolemmal channels, maintaining the calcium influx low and thus moderating the CICR that could overactivate IP3Rs and the slow component of calcium release. For instance, (α 1-syntrophin, a cytosolic member of DAPs, was proposed to bind a heterotrimeric Gs protein, which in turn is proposed to moderate the calcium entry through nifedipine-sensitive calcium channels (Zhou et al. 2004). It is thus possible that DAPs and mitochondria, by controlling the level of calcium influx through sarcolemmal SOC may contribute to the generation of subplasmalemmal microdomains of low Ca^{2+} (Fig. 4), which in turn moderate the activation of near-plasma membrane IP3Rs by CICR.

Additional results however are also in favour of a higher activation of IP3Rs in dystrophic myotubes. The IP3 production was quantified in muscle cell lines and an over-production of IP3 was clearly observed in dystrophin-deficient myotubes at rest and during stimulation. This suggests that phospholipase C (PLC), the enzyme that catalyses IP3 production, could be over-activated in dystrophin-deficient myotubes. Moreover, this IP3 production could be activated during depolarization through a heterotrimeric G pathway, blocked by Pertussis Toxin (Balghi et al. 2006). It is thus possible that like the heterotrimeric Gs protein (Zhou et al. 2004) the heterotrimeric G protein activating PLC could bind the DBC through α -syntrophin (Fig. 4). This association to dystrophin and DAPs could regulate the proposed activation of the heterotrimeric G protein and PLC by the DHPR-voltage sensor during depolarization (Araya et al. 2003; Balghi et al. 2006).

By producing secondary messengers, namely IP3 and DAG, the regulation of PLC activity by dystrophin and DAPs could be a common pathway linking IP3R-dependent calcium release from SR and CCEs through the sarcolemma (Fig. 4). Various data suggest that heterotetrameric TRPCs, which are known to form

SOCs, can be modulated either indirectly or directly by DAG and PKC (see review of Pedersen et al. 2005). In dystrophin-deficient muscle cells, DAG- or PKC-dependent over-activation of SOC may be involved in parallel to the elevated production of IP3 by PLC during depolarization, which in turn exaggerates a IP3R-dependent component of slow calcium release. Moreover, the over-activation of SOC by DAG production could be favoured in dystrophic muscle cells by a decrease of DAG metabolization by the enzyme DAG kinase ζ (DGK ζ). Recent studies conducted on skeletal muscle cells have shown that (α 1-syntrophin links also DGK ζ , which means that the DGK ζ is part of the multimeric complex associated to dystrophin and DAPs (Abramovici et al. 2003). The reduction of (α 1-syntrophin in dystrophin-deficient *mdx* mice is accompanied by a reduction of the amount of this enzyme at the sarcolemma (Abramovici et al. 2003), which may have functional consequences due to the reduction of DAG metabolization.

The role of the scaffolding proteins like syntrophins may be more extensive in regulating calcium signalling. For instance, the anchoring of SACs to the DAPs complex may determine the mechanosensitivity of these cationic channels, and thus modulate calcium influx during contraction. A regulation of channels by syntrophin has been proposed by Ou et al. (2003) who showed that the γ 2-syntrophin, in intestinal smooth muscle, regulates the gating of SNC5A mechanosensitive channels by a PDZ domain-mediated interaction.

Conclusion: a scaffold of proteins as a signalling complex

It was already proposed that the assembly of multi-protein-signalling complexes at sites of membrane specialization might be a widespread function of DAP complexes. The mechanical susceptibility of dystrophic fibres has also shown that DAPs plays a crucial role in the protection against membrane ruptures. Here we propose that the scaffold of DAPs, through the assembly of multiprotein-signalling complexes regulating calcium channels (SOCs, SACs and IP3Rs) has important consequences in maintaining normal calcium homeostasis in the skeletal muscle cell. The lack of dystrophin and the consequent disorganization of membrane-associated cytoskeleton could induce a dysfunction of calcium influxes and calcium releases, which have cumulative consequences on mitochondrial, subplasmalemmal and myoplasmic Ca^{2+} homeostasis. This may be a crucial determinant in triggering the calcium-dependent dysregulation of mitochondria

metabolism as well as the activation of calcium-dependent proteases such as calpains, and ultimately in promoting cell necrosis.

Although dystrophin can have a role in mechano-transduction and membrane protection, results from our group and others clearly show that dystrophin is implicated in a cell-signalling network. Further studies exploring the molecular links between DBC and proteins involved in calcium signalling will help to understand this interplay between membrane-associated cytoskeleton and calcium homeostasis.

Finally, demonstrating that subsarcolemmal cytoskeleton, namely dystrophin and the horde of interacting proteins/adaptator molecules, can modulate calcium transfer pathways, raises the hope of the possibility to pharmacologically or genetically modulates each one of the steps of involved pathways. The identification of the pathways involved in the dystrophic calcium mishandling provides therapeutic targets for counteracting the calcium overload in myoplasm and mitochondria. Such a way has to be thought as a various and rich therapeutic approach in the way to correct the diseases, or at least to bring relief to DMD patients.

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