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Essential role of TRPV2 ion channel in the sensitivity of dystrophic muscle to eccentric contractions

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ABSTRACT

Duchenne myopathy is a lethal disease due to the absence of dystrophin, a cytoskeletal protein. Muscles from dystrophin-deficient mice (*mdx*) typically present an exaggerated susceptibility to eccentric work characterized by an important force drop and an increased membrane permeability consecutive to repeated lengthening contractions. The present study shows that mdx muscles are largely protected from eccentric work-induced damage by overexpressing a dominant negative mutant of TRPV2 ion channel. This observation points out the role of TRPV2 channel in the physiopathology of Duchenne muscular dystrophy.

1. Introduction

Duchenne muscular dystrophy (DMD) is a progressive neuromuscular disease affecting 1/3500 male birth (reviewed in [1,2]). It is characterized by an important muscle degeneration (foci of necrotic fibers) followed, at early stages, by an intense regeneration process. As the regenerative capacity diminishes, muscle tissue is progressively replaced by adipose and fibrous connective tissue, leading to an important muscle weakness. This pathology results from a mutation in the Xp21 locus, which leads to the lack of expression of dystrophin, a 427 kDa protein located at the cytoplasmic face of the sarcolemma [3,4]. In normal skeletal muscle cells, dystrophin is associated, at its carboxy-terminal domain, with a complex of transmembrane proteins constituted of β-dystroglycan and α-, β-, γ- and δ-sarcoglycans and called the dystrophin–glycoprotein complex (DGC [5,6]). The complex also binds to merosin (laminin-2), a component of the extracellular matrix. At its amino-terminal domain, dystrophin binds to cytoskeletal F-actin filaments (i.e. non-sarcomeric actin). Dystrophin thus constitutes a link between the cytoskeleton and the extracellular matrix [7]. The alteration of dystrophin expression has two main consequences: (i) it affects DGC targeting to the membrane and therefore causes a disruption of the link between the cytoskeleton and the extracellular matrix that could induce membrane fragility; (ii) it induces a disorganization of the cytoskeleton and leads to a disregulation of several ion channel types such as the acetylcholine receptor [8] and stretch-activated and stretch-inactivated channels [9–11]. The mechanism by which the absence of dystrophin leads to muscle fiber death remains partially unknown, but an abnormal entry of Ca^{2+} through membrane tears caused by the absence of dystrophin or through abnormally regulated channels seems to play a major role (reviewed in [12,13]). Indeed, in muscles from *mdx* mice, a genetic model of Duchenne muscular dystrophy, this abnormal entry of Ca^{2+} has been proved to activate several signaling cascades involved in cell damage such as Ca^{2+}-activated proteases (calpains) [14–16], phospholipase A_2 [17,18], accumulation of Ca^{2+} in the mitochondria [19] and production of ROS [20,21].

Recently, the involvement of a specific ion channel called TRPV2 in the pathophysiology of DMD was proposed [22]. TRPV2 protein belongs to the large family of TRP cationic channels involved in various processes of sensory signaling (sensing heat and cold, pH, osmolarity, etc. for review, see [23]). TRPV2 is activated by membrane stretch [24]. It normally localizes in the intracellular membrane compartments but translocates to the plasma membrane in dystrophic muscle fibers [25]. Its specific inhibition was shown to ameliorate muscular dystrophy. Indeed, *mdx* mice expressing a dominant negative mutant TRPV2 channel presented a reduced entry of Ca^{2+} in muscle fibers; as a consequence, they exhibited reduced muscle degeneration (necrosis and apoptosis) and regeneration (central nuclei, etc.). These observations suggest that TRPV2 channel constitutes the principal Ca^{2+} entry pathway in dystrophic muscle fibers.

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The most specific characteristic of dystrophic muscles is their particular sensitivity to eccentric (lengthening) contractions [26]. Indeed, repeated eccentric work of dystrophic muscles has been shown to induce a large and progressive loss of force as well as an important increase of membrane permeability which can be detected by the release of creatine kinase or by the uptake of membrane-impermeable dyes such as procion orange and Evans Blue dye. It is generally accepted that this is due to an abnormal fragility of the membrane, given the lack of connection between the cytoskeleton and the extracellular matrix. We tested an alternative hypothesis according to which muscle sensitivity to stretch would be primarily due to the abnormal presence at the plasma membrane or to the abnormal regulation of TRPV2 channels. We therefore studied eccentric contractions of EDL muscles from *mdx* mice expressing or not the dominant negative mutant of TRPV2. The progressive loss of force developed by muscles of these mice was very much reduced in comparison to *mdx* control mice, suggesting the involvement of TRPV2 channel in the increased sensitivity of dystrophic muscle to eccentric contractions. Interestingly, the uptake of procion orange dye was also significantly reduced, suggesting that the entry of Ca^{2+} through TRPV2 precedes the large increase of non-specific membrane permeability.

2. Materials and methods

2.1. Animals

Dystrophin-deficient C57BL/10ScSn-*DMD*^{mdx} (*mdx*) mice and their control C57 were from Jackson Laboratories. Generation of TRPV2 dominant negative (TRPV2-DN) transgenic mice (expressing the hemagglutinin (HA)-tagged E604 K mutant TRPV2 channel under the control of the α-skeletal actin promoter in skeletal muscle) has been described previously [22]. Male homozygous TRPV2-DN mice were mated with female *mdx* mice and the resulting male *mdx*/TRPV2-DN were analyzed.

All mice analyzed were 12–16 weeks of age. The experiments were approved by the Animal Ethics Committee of the Catholic University of Louvain, Brussels.

2.2. Muscle mechanical protocol

Mice were deeply anesthetized by intraperitoneal injection of a solution containing ketamine (10 mg ml^{-1}) and xylazine (1 mg ml^{-1}) in order to preserve muscle perfusion during dissection of extensor digitorum longus (EDL) and tibialis anterior muscles. Depth of anesthesia was assessed by the abolition of eyelid and pedal reflexes. After dissection, the animals were killed by cervical dislocation.

EDL muscles were bathed in a 1 ml horizontal chamber continuously superfused with HEPES buffered Krebs solution (100% O_2) containing (in mM): NaCl 135.5, KCl 5.9, $MgCl_2$ 1.2, $CaCl_2$ 2.5, HEPES sodium 11.6 and glucose 11.5, and maintained at a temperature of 20 ± 0.1 °C. One end of the muscle was tied to an isometric force transducer, and the other to an electromagnetic motor and length transducer. Stimulation (125 Hz) was delivered through platinum electrodes running in parallel to the muscles. Optimum muscle length (L_0) was carefully adjusted for maximal isometric force using 300 ms maximally fused tetani. Force was digitalized at a sampling rate of 1 kHz, using a PCI 6023E i/o card (National Instruments). Normalized stress was expressed relative to cross-sectional area, obtained by multiplying absolute force by the quotient "muscle fiber length (mm)/muscle blotted weight (mg)" and considering the fiber length equal to 0.5 × L_0 for EDL [27].

The muscles were subjected to a series of seven eccentric contractions consisting in 500 ms tetani during which a stretch of 1 mm (L_0 to L_0 + 8%) at a speed of 1 L_0 s^{-1} was applied 160 ms after the start of stimulation and maintained up to 250 ms after the start of stimulation (10 s interval between two successive tetani). Isometric force was measured for each tetanus just before the onset of the stretch and the percentage force drop was calculated.

2.3. Assessment of sarcolemmal damage

The stimulated muscle and its non-stimulated contralateral control were both bathed for 1 h in the oxygenated Krebs solution containing 1% procion orange. This dye is known to be excluded from intact cells but to penetrates damaged membranes [26,28]. The muscles were then rinsed twice in normal Krebs solution and were finally rapidly frozen in isopentane precooled with liquid N_2. Transverse 10 μm thick sections were cut from muscle midbelly region, mounted with vectashield solution and viewed in epifluorescence (Zeiss S100 inverted microscope, FITC filters). The area of procion orange positive fibers was measured as a percentage of the entire muscle cross-sectional area using ImageJ program (NCBI).

2.4. Reagents

The GsMTx4 toxin, isolated from *Grammostola spatulata* spider [29], was obtained from PeptaNova (Sandhausen, Germany); SFK-96365 (1-[B-[3-(4-methoxyphenyl)propoxy]-4-methoxypheneth-yl]-1H-imidazole) from Alexis Corporation (Lausen, Switzerland)

2.5. Statistical analysis

Data are presented as means ± S.E.M. Unpaired *t*-tests were used to compare two groups and ANOVA to compare many groups. Significance level was fixed at $P < 0.05$.

3. Results

3.1. Dominant-negative inhibition of endogenous TRPV2 reduces the sensitivity of dystrophic muscle (mdx) to eccentric contractions

Isolated EDL muscles from *mdx* mice were submitted to successive eccentric contractions. We observed that dystrophin-deficient muscles presented a specific and progressive force drop (Fig. 1A and B). After six lengthening contractions, *mdx* muscles were able to develop less than 10% of the initial force; in the same conditions, their controls (C57) still developed 80% of the initial force. We and others previously reported that the channels abnormally activated in *mdx* fibers were sensitive to store depletion and membrane stretch [11,30,32]. In particular, we showed that they were sensitive to SKF-96365, a non-specific inhibitor of TRP channels [33] and to GsMTx4 toxin, the most specific inhibitor of mechanosensitive channels known today [29]. We therefore investigated the effect of these compounds on the sensitivity of *mdx* muscles to eccentric contractions. We observed that muscles incubated in the presence of 30 μM SKF-96365 (10 min preincubation) or in the presence of 10 μM GsMTx4 (2 h preincubation) developed significantly more force after a series of lengthening contractions than in the absence of the inhibitors (data not shown). These results confirm data reported by other groups using different eccentric contractions protocols [34] and suggest the involvement of these channels in the sensitivity to eccentric contractions. Since the best candidate of stretch-activated channel in skeletal muscle is TRPV2 [25], we studied the process on EDL muscles from normal C57 and dystrophic *mdx* mice expressing or not a dominant-negative mutant of TRPV2 (TRPV2-DN) [22]. We first observed that EDL muscles from *mdx* mice were bigger but developed less isometric

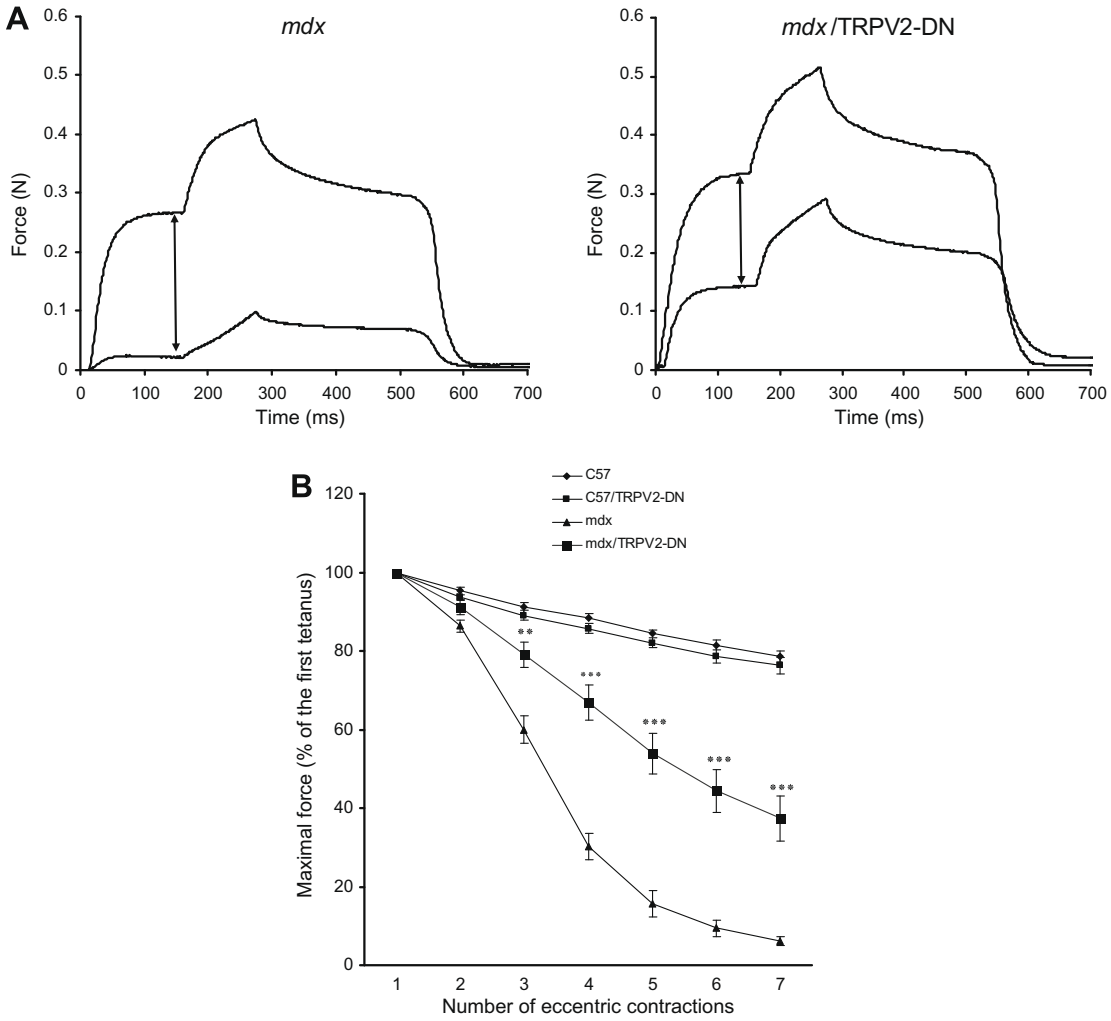

Fig. 1. Sensitivity to eccentric contractions. (A) Force traces of EDL muscle from *mdx* (left panel) and *mdx*/TRPV2-DN (right panel) mice. First and seventh tetanus presented. (B) Quantification of the force produced during repetitive eccentric contractions of EDL muscles from C57, C57/TRPV2, *mdx* and *mdx*/TRPV2. $^{**}P < 0.01$; $^{***}P < 0.001$ (one way repeated measures ANOVA, $n = 5–8$).

Table 1
Mechanicals properties of EDL muscles.

	C57	C57/TRPV2-DN	*mdx*	*mdx*/TRPV2-DN
Muscle weight (mg)	10.11 ± 0.22	9.84 ± 0.17	13.99 ± 0.49[a]	12.48 ± 0.31[a,b]
L_0 (mm)	11.75 ± 0.17	11.96 ± 0.26	11.82 ± 0.09	11.95 ± 0.09
F_0 (mN)	327.50 ± 32.88	358.46 ± 22.01	345.71 ± 21.87	330.73 ± 15.28
S_0 (mN/mm^2)	188.88 ± 16.93	219.23 ± 16.53	147.14 ± 9.85[a]	157.95 ± 3.98
n	5	8	8	6

[a] $P < 0.05$ vs C57.
[b] $P < 0.05$ vs *mdx*.

tension than controls (Table 1; Two way ANOVA, $P < 0.05$). This muscle pseudohypertrophy has been previously described in *mdx* mice [35]. Interestingly, in *mdx*/TRPV2-DN mice, EDL pseudohypertrophy was reduced (muscle weight significantly lower in *mdx*/TRPV2-DN than in *mdx*, and isometric tension slightly but not significantly higher in *mdx*/TRPV2-DN than in *mdx*, Table 1). When submitted to a series of eccentric contractions, EDL muscles from *mdx*/TRPV2-DN presented a much lower force drop than EDL from *mdx* mice (Fig. 1, one way repeated measures ANOVA, $P < 0.01–0.001$). The expression of TRPV2-DN had no effect on the force developed by C57 muscles during lengthening contractions.

These results strongly suggest a major role of TRPV2 channel in the stretch-induced force drop observed in *mdx* muscles.

3.3. Effect of dominant-negative inhibition of TRPV2 on stretch-induced membrane permeability in isolated muscles

To test whether the force drop in *mdx* muscle was correlated with the membrane permeability, isolated muscles were incubated for 1 h after a series of eccentric contractions in oxygenated Krebs solution containing 1% procion orange (a fluorescent dye substance which is excluded from intact cells but penetrate cells with membrane damage). Muscle cross-sections were analyzed for the percentage of procion orange positive fibers area related to the global area of the muscle section. As illustrated in Fig. 2, *mdx* muscles expressing the dominant negative mutant of TRPV2 presented a large reduction of the procion orange positive area compared to

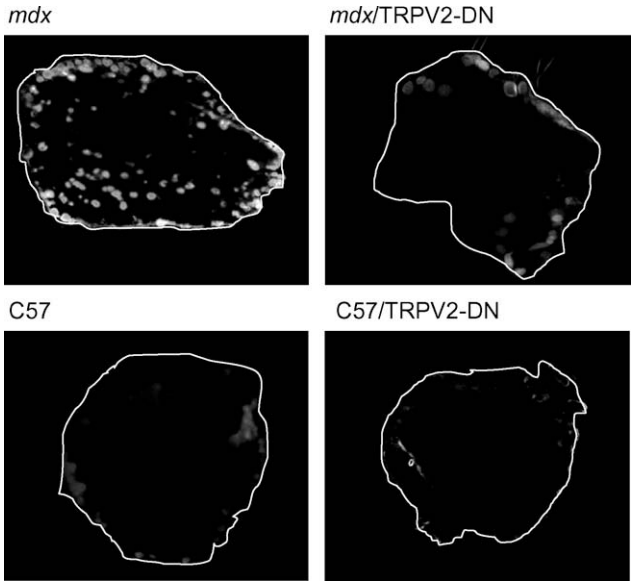

Fig. 2. Permeability to procion orange dye. Transverse sections of EDL muscles submitted to a series of seven lengthening contractions and then immersed during 1 h in procion orange containing Krebs solution. Sections examined under FITC fluorescence microscopy.

their *mdx* controls (11.46 ± 1.01% in *mdx*/TRPV2-DN vs 29.00 ± 3.72% in *mdx*, $n = 3$, $P < 0.05$). We checked that C57 and C57/TRPV2-DN muscles submitted to a series of eccentric contractions had a procion orange positive area that was always very low (below 5% of the total area) and limited to the periphery of muscles. Similarly, unstretched muscles (from C57, C57/TRPV2-DN, *mdx* and *mdx*/TRPV2-DN mouse strains) also present a negligible basal level of procion orange positive area.

4. Discussion

Since a long time, the importance of an abnormal influx of Ca^{2+} has been studied in skeletal myotubes from DMD patients [36] and in myotubes and adult fibers from *mdx* mice [14,37–40]. This influx has been shown to implicate various spontaneously active, store-dependent and/or mechanosensitive cationic channels [36,41] whereas the voltage-dependent Ca channels did not seem to be involved [41]. Accordingly, the channels involved were inhibited by lanthanides (Gd^{3+}, La^{3+}) and by pharmacological agents such as SKF-96365 and GsMTx4 toxin. We and others also showed that their occurrence at the membrane increased after myocyte stretch and after stimulation with IGF-1 [25,31,42], suggesting that they could be constituted of TRPV2 proteins [25,43,44]. This was clearly demonstrated recently. Indeed, transgenic or adenoviral expression of a dominant-negative TRPV2 in *mdx* muscles reduced several indices of the severity of the disease such as elevated serum creatine kinase level, number of apoptotic and necrotic fibers, intensity of the regeneration (central nuclei) and the consecutive fibrosis [22].

In the present study, we investigated the possible involvement of TRPV2 ion channel in the sensitivity of dystrophic muscle of *mdx* mice to eccentric contraction. This sensitivity constitutes the characteristic feature of the disease: forced lengthenings produce an important loss of force in dystrophic muscles [26,45]. This property is clearly related to membrane permeability and does not arise from factors within myofibrils [46]. Muscle fiber lesions resulting from eccentric contractions can be visualized by penetration of membrane-impermeant dyes like orange procion, suggesting that stretch of dystrophin-deficient membrane induces membrane tears, at least transiently [28]. Interestingly a linear relationship was observed between the percentage of orange procion positive fibers and the peak of tension observed at the end of eccentric contraction [45] and the force drop [47], suggesting that the high strain imposed on the fiber during forced lengthening produces localized disruptions of the plasma membrane that impairs mechanical response to electrical stimulation. However, the percentage of orange procion positive fibers was always smaller than the force drop (slope of relationship < 1). This was attributed to the fact that membrane lesions are localized; a single localized lesion completely prevents fiber contraction but as diffusion of the dyes is restricted, histochemical detection of these lesions could be under-estimated (few sections analyzed in a complete muscle) [2]. In this view, the increased fragility of the membrane due to the absence of dystrophin would explain the sensitivity of *mdx* muscles to eccentric contraction. However, this explanation was challenged by the observation that in muscles strained up to the point of membrane rupture, the absence of dystrophin had no detectable effect on the stress, the strain, or the energy absorbed, regardless of the state of muscle activation [48]. An alternative explanation was proposed by the group of Allen, who showed that short-term damage of *mdx* muscle fibers caused by stretch was partially prevented by blocking mechanosensitive channels [34,49]. This was confirmed here on isolated EDL muscles with a much milder protocol (8% stretch at $1\ L_0\ s^{-1}$ in our experiments vs 40% stretch at $4\ L_0\ s^{-1}$ in [49]). Interestingly, eccentric contractions also allowed an entry of Na^+ in *mdx* fibers through the same stretch-activated channels [50]. The prolonged increase of intracellular Na^+ concentration consecutive to eccentric contractions suggests that the loss of tension might be due, at least partially, not only to membrane damage but also to a prolonged membrane depolarization. This would explain the discrepancy between the percentage of damaged fibers (permeabilized to orange procion) and the amplitude of the force drop (see above). Here we compared the sensitivity to eccentric contractions in muscles from dystrophic mice expressing or not a dominant-negative TRPV2 channel. We show that muscles expressing the mutated channel are partially protected against eccentric contractions. Not only the force drop was very reduced, but the permeability to procion orange dye was significantly decreased, suggesting that the entry of ions through these channels precedes and is implicated in membrane lesions. This is in agreement with a recent study showing that reducing extracellular Ca^{2+} concentration or inhibiting calpain activity with leupeptin during lengthening contractions not only improved the force maintained but minimized membrane disruption [51]. This does not rule out an involvement of membrane fragility. Indeed, a significant force drop consecutive to repeated eccentric contractions is nevertheless still observed in dystrophic muscles expressing the dominant-negative TRPV2 mutant. Alternatively, this might be due to the fact that the entry of Ca^{2+} through native TRPV2 channels is not completely abolished in these muscles [22].

In conclusion, this study identifies TRPV2 as the mechanosensitive channel involved in eccentric work-induced force drop observed in dystrophic muscles. It suggests that the entry of cations, among which Ca^{2+}, through this channel is, at least partially, responsible for the observed membrane disruptions. Therefore, it constitutes a potentially interesting pharmacological target for the treatment of Duchenne muscular dystrophy.

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