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ORIGINAL ARTICLE

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Sarcoplasmic reticulum function in slow- and fast-twitch skeletal muscles from mdx mice

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Abstract The aim of the present study was to establish whether alterations in sarcoplasmic reticulum function are involved in the abnormal Ca^{2+} homeostasis of skeletal muscle in mice with muscular dystrophy (*mdx*). The properties of the sarcoplasmic reticulum and contractile proteins of fast- and slow-twitch muscles were therefore investigated in chemically skinned fibres isolated from the extensor digitorum longus (EDL) and soleus muscles of normal (C57BL/10) and *mdx* mice at 4 and 11 weeks of development. Sarcoplasmic reticulum Ca^{2+} uptake, estimated by the Ca^{2+} release following exposure to caffeine, was significantly slower in *mdx* mice, while the maximal Ca^{2+} quantity did not differ in either type of skeletal muscle at either stage of development. In 4-week-old mice spontaneous sarcoplasmic reticulum Ca^{2+} leakage was observed in EDL and soleus fibres and this was more pronounced in *mdx* mice. In addition, the maximal Ca^{2+}-activated tension was smaller in *mdx* than in normal fibres, while the Ca^{2+} sensitivity of the contractile apparatus was not significantly different. These results indicate that *mdx* hindlimb muscles are affected differently by the disease process and suggest that a reduced ability of the Ca^{2+}-ATPase to load Ca^{2+} and a leaky sarcoplasmic reticulum membrane may be involved in the altered intracellular Ca^{2+} homeostasis.

Keywords · Sarcoplasmic reticulum · Skeletal muscle · *Mdx* mouse · Skinned fibres · Caffeine · EDL · Soleus

Introduction

Duchenne's muscular dystrophy (DMD), an hereditary disease affecting man and characterised by muscle fibre necrosis and progressive muscle wasting and weakness, is one of the best-known and most common muscular dystrophies, affecting around 1 in 3,500 male births [17]. Although the primary defect in DMD was recognised more than 10 years ago [25], the relationship between the lack of dystrophin and the pathogenic degenerative processes of the dystrophy still remains unclear. DMD, today classified as a dystrophinopathy, is related to the failure of the expression of dystrophin, a subsarcolemmal cytoskeletal protein normally present in muscle [9, 22, 46, 50]. The dystrophin protein ensures a continuous mechanical link between the cytoskeleton and extracellular matrix, its primary function is thus to stabilise the sarcolemma [32, 39]. The *mdx* mouse, used widely as an animal model of dystrophin deficiency, has a genotype homologous to that responsible for human DMD and a milder clinical phenotype [3, 49]. For the limb muscles of *mdx* mice a short period of intense muscle degeneration occurring in the first 3–4 weeks of life is followed by a phase of very active regeneration at 10–12 weeks [6, 7, 42].

It is suggested that, in the absence of dystrophin, the sarcolemma could undergo focal damage during eccentric contraction or osmotic shock, and that this may in turn cause Ca^{2+} overload related to abnormally increased Ca^{2+} entry in the dystrophic muscle fibres [2, 16]. The elevated cytosolic Ca^{2+} may then trigger necrosis and degeneration in DMD and *mdx* fibres, probably through the activation of Ca^{2+}-dependent proteases [1, 38]. The recent description of reduced contraction-relaxation dynamics in *mdx* myotubes, characterised by longer times of contraction and relaxation, supports the concept of impaired Ca^{2+} removal mechanisms in dystrophin-deficient cells leading to alteration of the contractile properties, including muscle relaxation [33].

The muscle fibre, the functional unit of skeletal muscle, shows an enormous variability in its morphological, biochemical and physiological properties. This variability also applies to the sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA), the SERCA1 and -2 isoforms of which are expressed in fast- and slow-twitch skeletal

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muscles, respectively. Due to these functional differences and the variable expression of the main proteins isoforms involved in the Ca^{2+} cycle, the dystrophic process might affect the Ca^{2+} cycle in slow- and fast-twitch muscles differently.

Despite numerous studies of the Ca^{2+} homeostasis of the limb muscles of *mdx* mice, the fundamental role of the sarcoplasmic reticulum in the contraction/relaxation cycle in fast-and slow-twitch skeletal muscle fibres in the different phases of the degeneration-regeneration process remains unknown. The present study was designed to analyse the function of the sarcoplasmic reticulum in limb muscles affected by the dystrophy. Experiments were conducted on the extensor digitorum longus (EDL), a fast-twitch muscle, and the soleus, a slow-twitch muscle, from 4- and 11-week-old *mdx* mice and the results compared with those from age-matched wild-type (control) mice. Ca^{2+} handling by the sarcoplasmic reticulum and the properties of contractile proteins were investigated using saponin- or Triton-skinned fibre techniques, respectively.

Sarcoplasmic reticulum Ca^{2+} uptake was slowed in fast- and slow-twitch fibres from *mdx* mice while the maximal Ca^{2+} content was not altered. Furthermore, spontaneous leakage of Ca^{2+} from the sarcoplasmic reticulum was significantly increased in *mdx* fibres. In good agreement with previous reports, measured tension was lower, and the Ca^{2+} sensitivity of the contractile proteins not significantly different in *mdx* mice at the two stages of development tested compared with age-matched control mice. Furthermore, for the 11-week-old *mdx* soleus, tension was increased, sarcoplasmic reticulum Ca^{2+} uptake was faster and Ca^{2+} leakage reduced compared with the 4-week-old *mdx* soleus.

Materials and methods

All procedures were performed in accordance with the stipulations of the Helsinki Declarations for the care and use of laboratory animals. Male wild-type (C57BL/10) and *mdx* (C57BL/10*mdx*) mice at 4 (12.4±0.7 g; 8.8±0.4 g respectively, n=14) or 11 (25.8±0.6 g; 26.3±0.5 g respectively, n=14) weeks of age were killed by cervical dislocation. These ages correspond to the peak of degeneration in *mdx* muscle and to complete regeneration respectively [6, 7]. The soleus and EDL muscles were excised and placed in oxygenated HEPES-buffered physiological solution in a dissecting dish at room temperature. Small bundles of two to five fibres were isolated manually from tendon to tendon from both freshly isolated muscles and, with the aid of a microscope, short portions (100–250 μm in diameter; 2–3 mm in length) excised. Chemical skinning was carried out immediately after dissection. All experiments were conducted with fibres that had been skinned using either Triton X-100 or saponin.

Experimental apparatus

After the skinning procedure, fibres were transferred to a chamber and mounted between two stainless-steel tubes [21]. One end of the fibre was snared in a loop of fine hair pulled into a tube glued to a fixed rod. The other end of the preparation was similarly snared to a tube glued to a flexible rod that supported a metal target facing the sensor of a transducer (model 0.5 SU, Displacement Measuring System KD 2300, Kaman Instrumentation, Colorado Springs, Colo., USA). The output voltage of the system is proportional to the distance between the face of the sensor and the target. The diameter and length of the skinned muscle fibres were measured with the aid of a binocular microscope. At the beginning of each experiment, the fibre was adjusted to slack length and then stretched progressively until the tension developed at pCa 4.5 became maximal. Maximal tension was generally reached at resting length plus 20%. All experiments were performed at 22 °C.

Tension/pCa relationships

The maximal Ca^{2+}-activated tension (T_{max}) and the Ca^{2+} sensitivity of the contractile apparatus were investigated in Triton-skinned fibres. Preparations were incubated for 1 h in relaxing solution (pCa 9.0, see composition below) containing 1% Triton X-100 (v/v) and subsequently washed several times in relaxing solution without detergent. This treatment solubilises the sarcolemma and the sarcoplasmic reticulum without affecting the biochemical and mechanical properties of the myofibrils. Following skinning, fibres were stored at –20 °C in relaxing solution containing 50% glycerol (v/v).

Tension/pCa relationships were obtained by exposing Triton-skinned fibres sequentially to solutions of decreasing pCa until maximal tension was reached (at pCa 4.5), whereafter fibres were returned to a low $[Ca^{2+}]$ (pCa 9.0). At the beginning of each experiment, a full set of solutions containing different $[Ca^{2+}]$ was prepared and each solution then divided into appropriate aliquots, one serving as the control and the other containing caffeine (10 mM). Isometric tension was recorded continuously using a chart recorder (model 1200, Linear, Reno, Nev., USA), and baseline tension was established at the steady-state measured in relaxing solution. To obtain the Ca^{2+} sensitivity curve, data for the relative tension (T/T_{max}) were fitted by using a modified Hill equation [21]:

$$T/T_{max} = \frac{[Ca^{2+}]^{n_H}}{[K]^{n_H} + [Ca^{2+}]^{n_H}}$$

where K is the $[Ca^{2+}]$ at which activation is half maximal. pCa_{50}, the negative decadian logarithm of K, is a measure of the apparent Ca^{2+} sensitivity of contractile proteins and the Hill coefficient (n_H) is an estimate of the degree of cooperativity. These two parameters were calculated for each experiment using linear regression analysis. n_H was calculated for each fibre as the slope of the fitted straight lines. The tension obtained at each $[Ca^{2+}]$ was normalised to fibre cross-sectional area.

Ca^{2+} uptake and release in sarcoplasmic reticulum

The saponin-skinned fibre technique was used largely to assess the properties of the sarcoplasmic reticulum. Saponin skinning was performed by incubating the preparation for 20 min in relaxing solution at pCa 9.0 containing 50 μg ml^{-1} saponin. The skinned fibres were then washed several times in relaxing solution without detergent. This treatment permeabilises the sarcolemmal and T-tubule membranes, leaving the sarcoplasmic reticulum intact and functional [10]. With this preparation, it is possible to load the sarcoplasmic reticulum with different levels of Ca^{2+} and then to release the latter with pharmacological tools such as caffeine, an activator of the ryanodine receptors [20]. For experiments on saponin-skinned fibres, the preparation was immersed sequentially in five different solutions (Table 1 and below) to load the sarcoplasmic reticulum with Ca^{2+}, and the Ca^{2+} then released with caffeine (10 mM), which generates a transient contracture.

The ionic composition of these solutions was the same as that of the relaxing and activating solutions (pCa 9.0 and pCa 4.5, respectively). However, the concentrations of EGTA, Mg^{2+} and Ca^{2+} were varied as described below. Solution 1 (pCa 9.0, 10 mM

Table 1 Composition of solutions used for saponin and Triton X-100 experiments (pCa=–$\log_{10}[Ca^{2+}]$)

Solution and function	Parameters			
	pCa	[EGTA] (mM)	[Mg^{2+}] (mM)	[Caffeine] (mM)
Basic solutions				
Relaxing	9.0	10	1	0
Maximal activating	4.5	10	1	0
Saponin experiment solutions				
1. Depleting	9.0	10	1	25
2. Washing	9.0	10	1	0
3. Loading	7.0	10	1	0
4. Washing	7.5	0.4	0.1	0
5. Releasing	7.5	0.4	0.1	10

EGTA, 1 mM Mg^{2+}, 25 mM caffeine) was used to deplete the sarcoplasmic reticulum of Ca^{2+}. Solution 2 (pCa 9.0, 10 mM EGTA, 1 mM Mg^{2+}) was the caffeine-free wash solution. Solution 3 (pCa7.0, 10 mM EGTA, 1 mM Mg^{2+}) was the sarcoplasmic reticulum Ca^{2+}-loading solution. Solution 4 (pCa 7.5, 0.4 mM EGTA, 0.1 mM Mg^{2+}) was used to wash out solution 3 and to prepare for Ca^{2+} release. Solution 5 (pCa 7.5, 0.4 mM EGTA, 0.1 mM Mg^{2+}, caffeine) was similar to solution 4, but contained different caffeine concentrations (0.5, 1.0, 2.5, 5.0 or 10 mM) to release Ca^{2+} from the sarcoplasmic reticulum. During a control Ca^{2+} load-release cycle, the skinned fibre was incubated for 1 min in solutions 1 and 2 and for 2 min in solutions 3 and 4. In solution 5, the incubation time was based on contracture duration. The caffeine induced a transient contracture, for which the amplitude and half-relaxation time were measured.

For these experiments, Ca^{2+} uptake by the sarcoplasmic reticulum was realised in a solution containing 1 mM Mg^{2+}, a physiological concentration. In contrast, caffeine-induced Ca^{2+} release proceeded in a solution containing a low [Mg^{2+}] (0.1 mM) that favours the release of Ca^{2+} by caffeine [13, 23]. At the beginning of each experiment, two or three 10 mM caffeine contractures were generated to ensure the integrity of the sarcoplasmic reticulum following the saponin skinning treatment and control caffeine contractures were recorded regularly. The amplitude of the control caffeine contractures did not change significantly, suggesting that the sarcoplasmic reticulum remained in a suitably functional state. Fibres that developed a contractile response in which the amplitude decreased by more than 5% were discarded.

The concentration/response relationship for caffeine-induced contractures was assessed at caffeine concentrations of 0.5, 1.0, 2.5, 5.0 or 10 mM in solution 5. The caffeine sensitivity of the Ca^{2+} release mechanism of the sarcoplasmic reticulum was indicated by the EC_{50} (the caffeine concentration producing half-maximal contracture).

To modify the level of Ca^{2+} in the sarcoplasmic reticulum and to evaluate the kinetics of Ca^{2+} loading, fibres were loaded for various periods (15 s, 30 s, 1 min, 2 min or 10 min in solution 3) and Ca^{2+} released with 10 mM caffeine.

Spontaneous Ca^{2+} leakage from the sarcoplasmic reticulum was assayed by increasing the duration of incubation in solution 4 (the post-loading, pre-release washing solution) between the Ca^{2+} loading and Ca^{2+} release solutions. After the sarcoplasmic reticulum had been loaded with Ca^{2+} in solution 3 (2 min), the fibre was incubated in solution 4 for 2 min under control conditions and for 4 or 10 min and contracture subsequently induced by 10 mM caffeine. The rate of Ca^{2+} leakage was evaluated from the decrease in the caffeine-induced contracture amplitude with increasing duration of incubation in solution 4.

Estimation of Ca^{2+} uptake by the sarcoplasmic reticulum

The accumulation of Ca^{2+} by the sarcoplasmic reticulum during the loading period was estimated by calculating the [Ca^{2+}] "seen" by the myofilaments during caffeine application. That 10 mM caffeine is sufficient to release all the Ca^{2+} from the sarcoplasmic reticulum was confirmed by the fact that additional application of 25 mM caffeine failed to induce any further contracture. The contractile response following exposure to 10 mM caffeine is thus an indirect estimate of the amount of Ca^{2+} stored in the sarcoplasmic reticulum. Conventionally, the Ca^{2+} released by caffeine in skinned-fibre preparations is estimated from the isometric force transient resulting from the Ca^{2+}-evoked activation of the troponin/tropomyosin complex. The sarcoplasmic reticulum Ca^{2+} loading capacity can be estimated by using the contractile proteins as an internal sensor. It is well known that the sensitivity of the contractile proteins to Ca^{2+} is different between slow- and fast-twitch fibres. The same amount of Ca^{2+} released from the sarcoplasmic reticulum should therefore cause different force transients [47]. Furthermore, caffeine used to release Ca^{2+} from the sarcoplasmic reticulum also affects the properties of the contractile proteins and these effects were different in fast- and slow-twitch muscle [12]. Consequently, to compare all the results presently obtained on both types of muscle and to allow any effects of the dystrophic process on contractile proteins to be discounted, the amplitude of each caffeine contracture was converted into a Ca^{2+} transient by reference to the individual pCa/force relationship established for each saponin-skinned fibre in the presence of 10 mM caffeine [30, 45].

Solutions and chemicals

The control physiological solution (Ringer) contained (in mM): NaCl 140, KCl 6, $CaCl_2$ 3, $MgCl_2$ 2, glucose 5 and HEPES 5. The pH was adjusted to 7.4 with TRIS base. The composition of skinned-fibre solutions was calculated according to [18]. The relaxing (pCa 9.0) and activating (pCa 4.5) solutions consisted of (mM): EGTA 10, imidazole 30, Na^+ 30.6, Mg^{2+} 1, MgATP 3.16, phosphocreatine 12, with pH adjusted to 7.1. The ionic strength of these solutions was 160 mM. In saponin-skinned fibre experiments, solutions also contained phosphocreatine kinase (17.5 IU ml^{-1}) and sodium azide (1 mM). For both Triton X-100- and saponin-skinned fibre experiments solutions with intermediate [Ca^{2+}] were obtained by mixing the pCa 9.0 and pCa 4.5 solutions in appropriate proportions. EGTA and phosphocreatine were obtained from Sigma (St. Louis, Mo., USA).

Statistical analysis

Values are expressed as means±SEM. To compare the different parameters, data were analysed using Student's t-test, for paired or unpaired samples as appropriate. $P<0.05$ was considered significant.

Results

In saponin-skinned fibres from 4-week-old *mdx* mice, following a loading step (pCa 7.0, 2 min) the release of Ca^{2+} by application of 10 mM caffeine induced a transient contracture, the amplitude of which was decreased

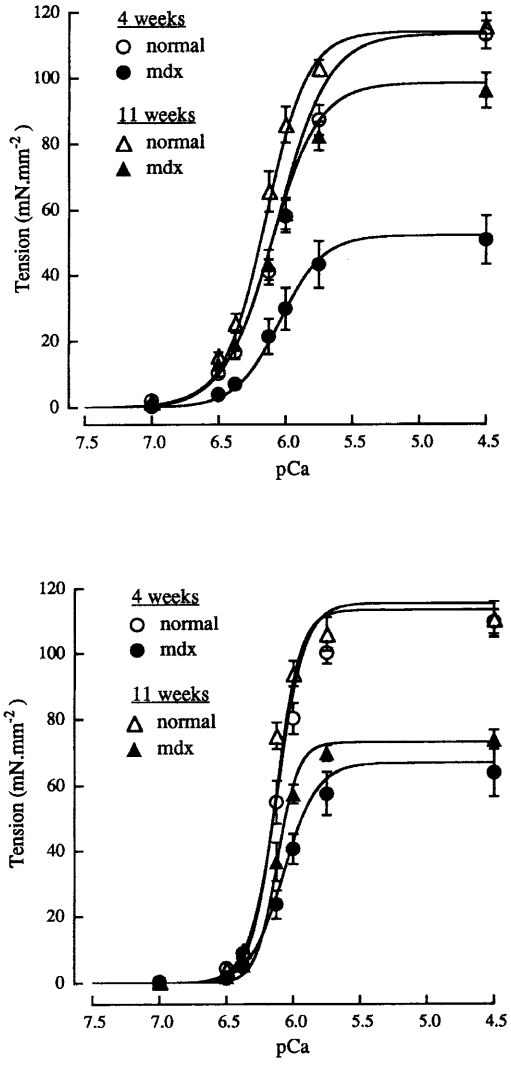

Fig. 1 Tension/pCa relationships of slow- (soleus, *upper panel*) and fast-twitch [extensor digitorum longus (EDL), *lower panel*]), Triton-skinned muscle fibres 4- and 11-week-old normal or dystrophic (*mdx*) mice. Curves were fitted using the modified Hill equation (see Materials and methods and Table 2 for values). Experiments were carried out at 22 °C

compared with that obtained in control mice. The mean values for control and *mdx* soleus were 61.2±2.4 and 26.7±3.5 mN mm^{-2} respectively (n=10 per group, P<0.001). Furthermore, the half-relaxation time for *mdx* soleus was increased significantly (17.1±2.8 s, n=10, vs. control 9.7±0.9 s, n=10, P<0.05). These results could be explained by a reduced tension developed by the contractile proteins and/or to a reduced Ca^{2+} release and/or to reduced Ca^{2+} uptake by the sarcoplasmic reticulum.

Ca^{2+}-activated tension

T_{max} and the apparent Ca^{2+} sensitivity in the absence and presence of caffeine were estimated first in Triton-skinned EDL and soleus fibres from 4- and 11-week-old normal and *mdx* mice. Figure 1 illustrates the tension/pCa curves in skinned soleus and EDL fibres. The isometric tension developed at each [Ca^{2+}] in both types of skeletal muscle from *mdx* mice was lower than in normal muscles at either stage of development. The dystrophic process induced a decrease in the force developed by contractile proteins in both fast- and slow-twitch fibres from young and old *mdx* mice.

Maximal Ca^{2+}-activated tension (T_{max})

In muscle fibres from 4- and 11-week-old *mdx* and normal mice, T_{max} was reached at pCa 5.5. The tension generated by the contractile proteins of EDL and soleus fibres when maximally activated by Ca^{2+} was not significantly different in 4- and 11-week-old normal mice (Table 2). In both EDL and soleus muscle fibres of 4-week-old *mdx* mice, T_{max} was decreased compared with age-matched normal mice, on average by 40% and 55%, respectively. In EDL *mdx* fibres at 11 weeks of development, T_{max} was reduced significantly compared with that in normal mice, but was not significantly different from that at 4-week-old *mdx* EDL fibres. In contrast, comparison of the values for the soleus from 4- and 11-week-old *mdx* mice showed that the reduction in T_{max} in old *mdx* mice was significantly less (14% vs. age-matched controls) than in young *mdx* mice (55%).

Ca^{2+} sensitivity

In muscle fibres of both *mdx* and normal mice of both age groups, the [Ca^{2+}] required for activation of contraction was not modified (pCa 7.0 and pCa 6.5 for soleus and EDL respectively). In both muscles, the apparent Ca^{2+} sensitivity of contractile proteins was not affected significantly by the dystrophic process (Table 2). Therefore, in EDL and soleus muscle of 4- and 11-week-olds *mdx* mice the pCa_{50} was unchanged compared with age-matched normal mice. Thus, the amount of Ca^{2+} required to develop 50% of T_{max} was not different in *mdx* compared with wild-type muscles. In fibres of 4-week-old *mdx* mice, the Hill coefficient n_H was enhanced in soleus muscle fibres. In contrast, n_H did not differ significantly in soleus fibres from 11-week-old normal and *mdx* mice.

Effects of caffeine on contractile proteins

The differences in the caffeine contracture amplitudes between normal and *mdx* mice could be explained by the fact that caffeine, used to release Ca^{2+} from the sarcoplasmic reticulum, affects the properties of the contractile apparatus. Caffeine also has various other side effects, in particular an increase in the Ca^{2+} sensitivity of the myofilaments [47]. Accordingly, the effects of caffeine on T_{max} and on the apparent Ca^{2+} sensitivity of the contractile proteins were tested in Triton X-100-skinned normal and *mdx* fibres.

Table 2 Maximal Ca^{2+}-activated tension (T_{max}, obtained in pCa 4.5), the pCa required for half-maximal activation (pCa_{50}) and the Hill coefficient (n_H) in Triton-skinned fibres from soleus and extensor digitorum longus (*EDL*) muscles from 4- and 11-week-old normal and dystrophic (*mdx*) mice. Values were obtained in either the absence (*control*) or in the presence of 10 mM caffeine. Means±SEM. The data for pCa_{50} were obtained by fitting the curves to the Hill equation. The data for n_H were obtained from the Hill plot curves; *n* is the number of fibres

	4 Weeks				11 Weeks			
	Normal		*mdx*		Normal		*mdx*	
	Control	Caffeine	Control	Caffeine	Control	Caffeine	Control	Caffeine
Soleus								
T_{max} (mN mm^{-2})	113.3±4.6	95.7±6.0§	52.2±7.8*	43.4±6.4§	114.0±4.2	97.2±3.6§	98.4±4.6*	82.1±5.0§
pCa_{50}	6.06±0.03	6.15±0.04§	6.04±0.03	6.10±0.03§	6.15±0.03	6.19±0.02§	6.11±0.05	6.20±0.05§
n_H	2.28±0.10	1.96±0.10§	2.69±0.12*	2.21±0.11§	2.76±0.12	2.32±0.08§	2.44±0.19	2.12±0.16
n	10	10	9	9	11	11	9	9
EDL								
T_{max} (mN mm^{-2})	115.5±4.0	90.9±4.4§	66.8±8.0*	54.5±6.8§	113.4±3.3	89.6±3.7§	73.0±2.8*	57.9±3.2§
pCa_{50}	6.12±0.02	6.14±0.02	6.07±0.03	6.09±0.02	6.13±0.03	6.16±0.03§	6.12±0.03	6.19±0.03§
n_H	4.20±0.38	3.55±0.31§	3.51±0.18	2.89±0.09§	4.78±0.38	4.15±0.31§	5.62±0.90	4.48±0.61§
n	10	9	8	8	11	11	9	9

*$P<0.05$ vs. normal age-matched mice; $^{\S}P<0.05$ vs. control

T_{max} in the presence of caffeine

In the presence of caffeine (10 mM), the tension developed by the contractile apparatus when maximally activated by Ca^{2+} was reduced in both normal and *mdx* mice, with values equalling 80% of control values without caffeine (Table 2).

Ca^{2+} sensitivity in the presence of caffeine

Exposure to10 mM caffeine induced a similar shift to the left in the force/pCa relationships for EDL and soleus muscles from normal and *mdx* muscles. This means that caffeine increased the apparent Ca^{2+} sensitivity of contractile proteins and thereby decreased the amount of Ca^{2+} required to elicit 50% of T_{max}. In both normal and *mdx* muscles, soleus fibres showed a significant increase in pCa_{50} above control values and this effect was more pronounced than in fast muscles (Table 2). In EDL fibres a significant increase was observed only in normal and *mdx* mice at 11 weeks of development. The Hill coefficient was reduced in the presence of caffeine in both normal and *mdx* muscles in young and old mice The decrease in the amplitude of caffeine-induced contracture and the increase in half-relaxation time observed for *mdx* muscles thus did not involve a change in the Ca^{2+} sensitivity of the contractile protein either in the absence or presence of caffeine.

Concentration/response relationship for caffeine contractures

In saponin-skinned fibres in which a functional sarcoplasmic reticulum is preserved, caffeine induces a transient contracture reflecting a specific amount of Ca^{2+} released by the sarcoplasmic reticulum. In our experiments, five concentrations of caffeine (0.5, 1.0, 2.5, 5.0 and 10 mM) were chosen to compare the caffeine sensitivity of the Ca^{2+} release mechanism of the sarcoplasmic reticulum in normal and *mdx* mice. Caffeine induced a concentration-dependent transient contracture and, for each concentration of caffeine tested, the contracture amplitude was lower in *mdx* than in normal fibres. However, the EC_{50} for the caffeine-induced contracture did not differ significantly between normal and *mdx* muscles in either 4- or 11-week-old mice, except for the 11-week-old *mdx* EDL fibres, in which the EC_{50} was increased (Table 3).

Table 3 The caffeine concentration eliciting 50% of the maximal caffeine contracture (EC_{50}) in saponin-skinned soleus and EDL fibres from 4- and 11-week-old normal and *mdx* mice. Means±SEM. The EC_{50} data were obtained by fitting the curves to the Hill equation. n is the number of fibres

	4 Weeks		11 Weeks	
	Normal	*mdx*	Normal	*mdx*
Soleus				
EC_{50} (mM)	2.67±0.29	2.47±0.29	1.27±0.21	1.54±0.24
n	8	8	6	8
EDL				
EC_{50} (mM)	0.89±0.01	0.73±0.07	0.49±0.09	1.35±0.19*
n	7	7	8	5

*$P<0.05$ vs. age-matched normal mice

Taken together, the results show that the dystrophic process does not modify the caffeine sensitivity in slow- or fast-twitch muscles, although the caffeine sensitivity is decreased in 11-week-old *mdx* EDL fibres. Thus, properties of the sarcoplasmic reticulum need to be studied further in dystrophic muscles. The functions of the sarcoplasmic reticulum in respect of Ca^{2+} uptake and leakage were assessed in saponin-skinned fibres.

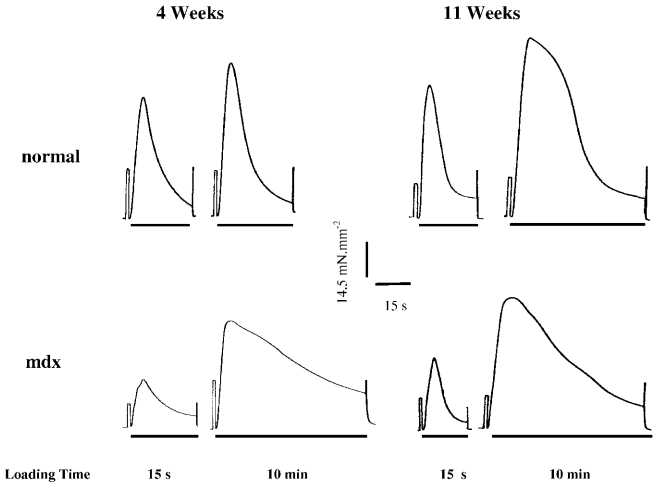

Fig. 2 Effects of loading period on Ca^{2+} release from the sarcoplasmic reticulum in saponin-skinned soleus fibres from control or *mdx* mice. Records were obtained after 15 s or 10 min loading in solution 3. The fibre was then bathed for 2 min in solution 4 and the Ca^{2+} released by exposure to caffeine 10 mM (see Materials and methods and Table 1)

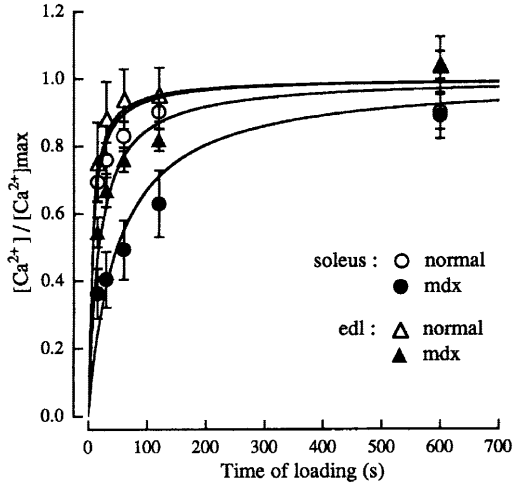

Fig. 3 Relation between the duration of Ca^{2+} loading and the Ca^{2+} released by exposure to 10 mM caffeine in saponin-skinned soleus and EDL fibres from 4-week-old normal or *mdx* mice. The free $[Ca^{2+}]$ estimated at the peak of the contracture was normalised to the calculated maximal $[Ca^{2+}]$ (Ca^{2+}/Ca^{2+}_{max}, see Materials and methods). The solid lines show the fits of the data using a hyperbolic equation

Effects of loading period on sarcoplasmic reticulum Ca^{2+} uptake

Experiments were performed by incubating saponin-skinned soleus and EDL fibres in a loading solution (pCa 7.0, solution 3) for increasing periods (15 and 30 s, 1, 2 and 10 min). After a given loading period, fibres were bathed for 2 min in a solution of pCa 7.5 (solution 4) and then exposed to caffeine (10 mM) to release Ca^{2+} from the sarcoplasmic reticulum (see Materials and methods). The amplitude of the caffeine contracture was dependent on the time of loading and could be fitted to a hyperbolic equation (Fig. 2). As shown in Table 4 for EDL and soleus muscles, the maximal amplitude of the 10 mM caffeine contracture (A_{max}) did not differ significantly between 4- and 11-week-old normal mice. However, in EDL and soleus fibres from 4-week-old *mdx* mice, A_{max} was significantly lower (65% and 70% of control values, respectively). For the older *mdx* mice, the decrease in A_{max} was less for soleus fibres (20%), than for EDL (50%) compared with age-matched normal mice.

To compare the results obtained on both types of skeletal muscle and to discard the effects of the dystrophic process on the contractile proteins, caffeine-induced contractures were converted into Ca^{2+} transients using the tension/pCa relationship established in the presence of 10 mM caffeine (see Materials and methods). The amount of Ca^{2+} loaded was dependent on the duration of Ca^{2+} loading and could be fitted to a hyperbolic equation (Fig. 3). The amount of Ca^{2+} corresponding to maximal Ca^{2+} loading (Ca^{2+}_{max}) and the loading time corresponding to the time to load 50% of the maximal Ca^{2+} (T_{50}) were then calculated for each fibre. Ca^{2+}_{max} did not differ significantly between age-matched normal and *mdx* mice. Thus, the dystrophic process does not alter the maximal sarcoplasmic reticulum Ca^{2+} loading capacity. However, for each loading duration the amount of Ca^{2+} loaded in the sarcoplasmic reticulum was reduced in *mdx* fibres. The results indicate that in soleus and EDL fibres from both young and old *mdx* mice the sarcoplasmic reticulum Ca^{2+} loading was significantly slower than in normal

Table 4 Parameters of the caffeine-induced contracture (A_{max} maximal amplitude, Ca^{2+}_{max} maximal $[Ca^{2+}]$, T_{50} loading time required to elicit 50% of the response to Ca^{2+}_{max}) in saponin-skinned soleus and EDL fibres from 4- and 11-week-old normal and *mdx* mice. Means±SEM. n is the number of fibres

	4 Weeks		11 Weeks	
	Normal	*mdx*	Normal	*mdx*
Soleus				
A_{max} (mN mm^{-2})	64.7±3.1	44.6±2.6*	71.3±3.6	57.3±4.1*
Ca^{2+}_{max} (nM)	1065±94	1111±100	1074±111	1027±108
T_{50} (s)	7±2	49±10*	7±3	25±5*
n	7	9	7	8
EDL				
A_{max} (mN mm^{-2})	67.6±4.2	44.2±3.8*	76.4±2.8	38.2±4.9*
Ca^{2+}_{max} (nM)	942±66	1021±50	989±79	775±67
T_{50} (s)	6±2	18±4*	5±1	16±2*
n	8	10	6	6

*$P<0.05$ vs. normal age-matched mice

Table 5 Rate of Ca^{2+} leakage from the sarcoplasmic reticulum of saponin-skinned slow- (soleus) and fast- (EDL) twitch fibres from 4- and 11-week-old normal and *mdx* mice, as reflected by the decrease in the amplitude of the caffeine contracture with increasing duration of leakage. Means±SEM. n is the number of fibres

Leakage period	Contracture amplitude (% decrease)			
	4 Weeks		11 Weeks	
	Normal	*mdx*	Normal	*mdx*
Soleus				
4 min	6.6±1.4	13.6±2.4*	2.4±0.6	6.4±1.1*
n	8	8	6	8
10 min	12.8±2.3	35.9±4.7*	3.8±1.2	7.7±1.9
n	8	8	6	8
EDL				
4 min	5.4±0.4	12.8±1.5*	5.7±0.7	7.5±1.1
n	8	9	9	9
10 min	10.9±1.7	29.2±2.5*	11.8±1.4	12.7±2.2
n	8	9	9	8

*$P<0.05$ vs. normal age-matched mice

mice. T_{50} was thus strikingly increased in *mdx* muscles, and this effect was more pronounced at 4 weeks. For example, T_{50} was increased by a factor of 7 in *mdx* soleus, vs. 3 for *mdx* EDL fibres (Table 4). In 11-week-old *mdx* soleus fibres, T_{50} remained higher than control values, but was nevertheless significantly lower (50%) than in 4-week-old *mdx* mice. In contrast, T_{50} values for EDL fibres were not different between 4- and 11-week-old *mdx* mice.

These results show that in both types of skeletal muscle, the mechanisms involved in the Ca^{2+} uptake were altered by the dystrophic process. The kinetics of loading were significantly slower in *mdx* muscles, while the sarcoplasmic reticulum maintained its maximal Ca^{2+} loading capacity, suggesting a dysfunction of the Ca^{2+}-ATPase and/or abnormal Ca^{2+} leakage.

Ca^{2+} leakage from the sarcoplasmic reticulum

To evaluate Ca^{2+} leakage from the sarcoplasmic reticulum, experiments were performed by increasing the period of incubation of saponin-skinned fibres in solution 4 (washing solution; after Ca^{2+} loading solution and before Ca^{2+} release, see Materials and methods). The progressive decrease in the amplitude of the caffeine-induced contracture with the increasing duration of incubation in solution 4 ("leakage period") reflected the decreasing amount of Ca^{2+} remaining in the sarcoplasmic reticulum and hence the rate of Ca^{2+} leakage. As shown in Table 5 a small spontaneous Ca^{2+} leakage occurred in 4-week-old normal EDL and soleus fibres. The spontaneous Ca^{2+} leakage from Ca^{2+}-loaded fibres was significantly larger in 4-week-old *mdx* mice than in age-matched normal mice and was similar in fast- and slow-twitch fibres (Table 5). Furthermore, at 11 weeks of development in both types of *mdx* muscle, the Ca^{2+} leakage was significantly reduced compared with 4-week-old *mdx* muscles and similar to that in normal muscles.

Discussion

The present functional findings demonstrate that in fast- and slow-twitch skeletal muscle from *mdx* mice, the rate of Ca^{2+} loading was slowed and associated with a Ca^{2+} leakage while the sarcoplasmic reticulum maintained its maximal Ca^{2+} loading capacity.

In DMD, skeletal muscle failure is the most prominent manifestation. In accordance with previous observations, our results with Triton X-100 skinned fibres indicate that in both types of skeletal muscle from *mdx* mice at 4 and 11 weeks of development, T_{max} and the tension developed for each submaximal [Ca^{2+}] were decreased. This decline in force development is in agreement with previous results obtained in other *mdx* muscles, such as the tibialis anterior and the diaphragm [34, 36] and in muscles from DMD patients [11]. The loss of force-generating capacity may be due to necrotic muscle fibre replacement by fibrous tissue [34] and also to modification of the muscle fibre architecture with disorganisation of the sarcomere and, hence, of the contractile apparatus [6]. However, the decrease in force developed by the contractile proteins of *mdx* mice is not related to differences in sensitivity of the myofilaments to Ca^{2+}. Similar contractile and activation properties have also been reported for muscle fibres from normal and *mdx* animals [48]. These observations support the suggestion that the absence of the subsarcolemmal protein dystrophin has little influence on the expression of contractile and regulatory protein in *mdx* muscle. In slow (soleus) *mdx* fibres at 11 weeks of development average T_{max} was higher than in the 4-week-old *mdx* soleus, possibly reflecting spontaneous regeneration in this muscle [7].

To analyse the uptake of Ca^{2+}, experiments were performed with saponin, a chemical detergent that permeabilises the sarcolemma and maintains a functional sarcoplasmic reticulum. In these experiments, the trimethylxanthine caffeine was used to release Ca^{2+} from the sarcoplasmic reticulum and the Ca^{2+} content estimated. Caffeine is an important tool for exploring the functional properties of the structurally intact sarcoplasmic reticulum from skinned muscle fibres including Ca^{2+} uptake, Ca^{2+} content and Ca^{2+} release [20].

It is commonly acknowledged that caffeine activates the ryanodine receptors and that fast-twitch fibres are less sensitive than slow-twitch fibres [12]. Under our experimental conditions, EDL fibres were more sensitive to caffeine than soleus fibres in both control and *mdx* mice. Although this result is quite different from other observations on intact or skinned fibres, similar results have been obtained in normal rat skeletal muscle [4]. The ability of caffeine to induce Ca^{2+} release is reduced markedly by lowering the level of sarcoplasmic reticulum loading or by raising the free [Mg^{2+}] [13, 23]. The caffeine sensitivity depends not only on the sarcoplasmic

reticulum Ca^{2+} content but also of the cytosolic [Ca^{2+}]. At low [Ca^{2+}], as in the present experiments (pCa 7.5), caffeine triggered Ca^{2+} release in soleus fibres at much higher concentrations than in EDL fibres. However, the caffeine sensitivity of saponin-skinned fibres was similar in control and *mdx* mice. Previous studies have reported that the amplitude of Ca^{2+} transients elicited by electrical stimulation are not affected by the dystrophic process, suggesting that Ca^{2+} release is not altered in *mdx* muscles [5, 19, 43, 44]. Furthermore, at 11 weeks of development, the EC_{50} for caffeine was increased in *mdx* EDL fibres. This could be explained by different Ca^{2+} release properties in these fast fibres where the dystrophic process was maintained. On the other hand, caffeine also exerts also non-specific effects, particularly at the level of the contractile proteins by increasing the Ca^{2+} sensitivity of the myofilaments [47]. Thus, caffeine may affect the contractile apparatus properties of normal and *mdx* mice differently. These possibilities were tested in Triton-skinned fibres, in which 10 mM caffeine had similar effects on the contractile proteins, decreasing T_{max} and increasing the apparent Ca^{2+} sensitivity of myofilaments in both normal and dystrophic muscles.

In this context, the Ca^{2+} uptake properties of the sarcoplasmic reticulum were investigated by examining the time course of Ca^{2+} loading into the sarcoplasmic reticulum of saponin-skinned fibres. Our principal finding was that the mechanisms involved in sarcoplasmic reticulum Ca^{2+} uptake were altered strikingly by the dystrophic process. Indeed, the loading time T_{50} was increased significantly in both *mdx* muscles, whereas the maximal amount of Ca^{2+} loaded was not modified. These results suggest a dysfunction of the Ca^{2+} handling mechanism and are consistent with previous results obtained in sarcoplasmic reticulum vesicles from *mdx* muscles [24]. Another possible explanation for the reduced sarcoplasmic reticulum Ca^{2+} content is Ca^{2+} leakage and our results show that spontaneous leakage of Ca^{2+} from Ca^{2+}-loaded fibres was indeed larger in *mdx* muscles. Thus Ca^{2+} uptake is reduced and Ca^{2+} leakage enhanced in dystrophic muscle. The reduction in net Ca^{2+} uptake may therefore have been simply the consequence of increased Ca^{2+} leakage occurring simultaneously with loading the sarcoplasmic reticulum. However, the differences in sarcoplasmic reticulum loading and leakage between normal and dystrophic muscle could be explained by the deleterious effects of saponin [27, 28]. The skeletal muscle sarcoplasmic reticulum of different species responds differently to saponin treatment. In anuran skeletal muscle fibres the sarcoplasmic reticulum is not affected by saponin concentrations below 100 μg/ml, while the Ca^{2+} loading ability of rat fibres is reduced. Although we can not exclude the possibility that the saponin treatment itself induced the observed difference in sarcoplasmic reticulum function, our results are in good agreement with those obtained in *mdx* tibialis anterior [40]. A clear rise of basal cytosolic Ca^{2+} is still controversial and the differences between controls and dystrophin-deficient muscle fibres being more evident under extreme conditions of membrane stress [5, 14, 29, 35]. The increase in sarcoplasmic reticulum Ca^{2+} leakage alone would not lead to a significant elevation of intracellular Ca^{2+}, but may nevertheless maintain an elevated [Ca^{2+}] in the vicinity of the sarcoplasmic reticulum in *mdx* muscles. Furthermore, the higher activity of Ca^{2+}-activated potassium channels in *mdx* than control mice is clear functional evidence in favour of a basal subsarcolemmal increase of Ca^{2+} [31]. Some authors have suggested that exposure of fibres to high [Ca^{2+}] reduces the ability of the sarcoplasmic reticulum to load and retain Ca^{2+} [26]. This sarcoplasmic reticulum Ca^{2+} efflux mechanism is not well understood and could occur by reversal of the Ca^{2+}-ATPase, via a distinct channel or through the ryanodine receptor/Ca^{2+}-release channels [15, 41].

Our results in 11-week-old *mdx* mice suggest that the regenerative process is not similar in slow- and fast-twitch *mdx* hindlimb muscles. Soleus fibres showed an increasing T_{max}, a decline in Ca^{2+} leakage from the sarcoplasmic reticulum and, above all, a rise in the rate of Ca^{2+} uptake. In contrast, EDL muscle demonstrated only a decrease in Ca^{2+} leakage. At this stage, necrotic fibres in the *mdx* muscle are partially replaced by regeneration from dormant satellite cells, which are potential myogenic cells that participate in repair and growth of muscle fibres. Our finding is consistent with other studies indicating that, in general, slow muscles have much higher density of satellite cells and a greater regenerative capacity than fast muscles [8, 37].

Finally, the results of this study suggest that *mdx* hindlimb muscles are affected differently by the disease process and indicate a perturbation in the mechanisms of sarcoplasmic reticulum Ca^{2+} sequestration in both types of dystrophic skeletal muscle. The reduced ability of the Ca^{2+}-ATPase to load Ca^{2+} and the leaky sarcoplasmic reticulum membrane could disturb intracellular Ca^{2+} homeostasis and signalling. The presence of Ca^{2+} in the cell for a more prolonged time could participate in the process of necrosis observed during pathology and would accelerate further muscle degeneration by activating Ca^{2+}-dependent proteolysis. Sarcoplasmic reticulum and Ca^{2+} have many important and essential functions for skeletal muscle performance. Therefore, any defect in Ca^{2+} handling could impair the efficiency of Ca^{2+} homeostasis and the regulation of muscle contraction. Further investigation will be required to identify the mechanisms involved in such alteration of sarcoplasmic reticulum Ca^{2+} sequestration.

In conclusion, the sarcoplasmic reticulum in situ is leakier in dystrophic muscle fibres than in normal muscles. The abnormality in intracellular [Ca^{2+}] in dystrophic muscle is not due solely to the abnormal influx of extracellular Ca^{2+}. The question is whether the abnormal sarcoplasmic reticulum function is a consequence of increased influx of extracellular Ca^{2+} or is related more directly to the absence of dystrophin. It is not clear whether sarcoplasmic reticulum dysfunction is an early or a late step in the dystrophic process. Since

dystrophin is linked to the sarcolemma, but not associated with the triads [9, 22, 46, 50], it is unlikely that the sarcoplasmic reticulum functions are altered as a primary manifestation of the disease. Increased influx of Ca^{2+} into the subsarcolemmal space can lead to the overload and dysfunction of Ca^{2+} cycle and storage systems.

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