British Journal of Pharmacology (2001) 132, 1047–1054 © 2001 Nature Publishing Group All rights reserved 0007–1188/01 $15.00

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Alteration of excitation-contraction coupling mechanism in extensor digitorum longus muscle fibres of dystrophic mdx mouse and potential efficacy of taurine

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1 No clear data is available about functional alterations in the calcium-dependent excitation-contraction (e-c) coupling mechanism of dystrophin-deficient muscle of mdx mice.

2 By means of the intracellular microelectrode 'point' voltage clamp method, we measured the voltage threshold for contraction (mechanical threshold; MT) in intact extensor digitorum longus (EDL) muscle fibres of dystrophic mdx mouse of two different ages: 8–12 weeks, during the active regeneration of hind limb muscles, and 6–8 months, when regeneration is complete.

3 The EDL muscle fibres of 8–12-week-old wildtype animals had a more negative rheobase voltage (potential of equilibrium for contraction- and relaxation-related calcium movements) with respect to control mice of 6–8 months. However, at both ages, the EDL muscle fibres of mdx mice contracted at more negative potentials with respect to age-matched controls and had markedly slower time constants to reach the rheobase.

4 The *in vitro* application of 60 mM taurine, whose normally high intracellular muscle levels play a role in e-c coupling, was without effect on 6–8-month-old wildtype EDL muscle, while it significantly ameliorated the MT of mdx mouse.

5 HPLC determination of taurine content at 6–8 months showed a significant 140% rise of plasma taurine levels and a clear trend toward a decrease in amino acid levels in hind limb muscles, brain and heart, suggesting a tissue difficulty in retaining appropriate levels of the amino acid.

6 The data is consistent with a permanent alteration of e-c coupling in mdx EDL muscle fibres. The alteration could be related to the proposed increase in intracellular calcium, and can be ameliorated by taurine, suggesting a potential therapeutic role of the amino acid.

British Journal of Pharmacology (2001) **132,** 1047–1054

Keywords: Muscular dystrophy; mdx mouse; excitation-contraction coupling; taurine; calcium handling mechanisms

Abbreviations: e-c coupling, excitation-contraction coupling; EDL, extensor digitorum longus; HPLC, high pressure liquid cromatography; MT, mechanical threshold; NMR, nuclear magnetic resonance; R, rheobase voltage; SR, sarcoplasmic reticulum; TA, tibialis anterior

Introduction

Dystrophin is a subsarcolemmal cytoskeletal component that is missing in Duchenne muscular dystrophy (DMD) as well as in animal models of the disease, such as the mdx mouse (Matsumura & Campbell, 1994). Although the exact function of dystrophin is still unclear, it has been claimed that it confers mechanical reinforcement to the muscle membrane during the stress of contraction (Petrof *et al*., 1993; McArdle *et al*., 1995). As a consequence of its absence, the sarcolemma could undergo focal damage during eccentric contraction or osmotic shock, that may in turn account for a calcium overload by enhancing the activity of calcium-selective mechanosensitive and/or leak channels (Franco & Lansman, 1990; Turner *et al*., 1991; Gillis, 1996; Tutbiti *et al*., 1999; Berchtold *et al*., 2000). The increase in cytosolic calcium can trigger fibre necrosis and degeneration probably through the activation of calcium-dependent proteases (Turner *et al*., 1993; Spencer *et al*., 1995; Alderton & Steinhardt, 2000). In many experimental situations using calcium-sensitive fluorescent dyes, no clear rise of basal cytosolic calcium has been found, the differences between controls and mdx muscle fibres being evident under extreme conditions, such as high extracellular calcium and/or hypo-osmotic shock (Leijendekker *et al*., 1996; Pulido *et al*., 1998; Collet *et al*., 1999). However, the high activity of calcium-activated potassium channels, as established by cell-attached patch clamp recordings on freshly dissociated muscle fibres, is clear functional evidence in favour of a basal subsarcolemmal increase of calcium ions (Mallouk *et al*., 2000). Similarly, some controversies have been found when the time course of calcium transients has been evaluated, since some authors reported no change while others showed a longer decay time of the transient (Khammari *et al*., 1998; Tutdibi *et al*., 1999; Collet *et al*., 1999). This latter event can result from a decreased activity of calcium ATPase pump of sarcoplasmic reticulum (SR) for the

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impairment of energy products, due to calcium overload in mitochondria (Pulido *et al*., 1998; Kargacin & Kargacin, 1996; Rüegg & Gillis, 1999). Changes in calcium homeostasis or in related handling mechanisms should lead to alteration of contractile properties already detectable in intact muscle. Controversial results have been obtained in this regard; for instance some authors reported no changes in twitch and tetanic tension of extensor digitorum longus (EDL) muscle of 7-week-old mdx animals but a significant decrease at 16 weeks, whereas others found no change in adult and a decrease in aged (about 20-month-old) mdx mice (Mechalchuck & Bressler, 1992; Hayes & Williams, 1998). Moreover, the contractile properties of diaphragm, a muscle that in the mdx phenotype undergoes a progressive degeneration, are significantly impaired in both young and aged dystrophic mice (Dupont-Versteegden & McCarter, 1992; Petrof *et al*., 1993). An attempt to reconcile the available findings is that the mdx mouse develops a mild form of dystrophy, since the limb muscles, such as EDL, can functionally overcome the genetic lack of dystrophin due to a high regeneration potential (Anderson *et al*., 1988; De Luca *et al*., 1999). However, the identification in the murine phenotype of muscle functional parameters that are closely related to the genetic defect is an important task, especially when using this animal for testing pharmacological trials. In the light of the above findings various questions arise: (1) Can the effect of the alteration in calcium homeostasis on contractile properties of mdx limb muscles be minimized by regeneration events? (2) Does a significant alteration in calcium homeostasis occur in intact dystrophin-deficient muscle, since all the available data comes from isolated fibres or cell cultures? (Rüegg & Gillis, 1999); (3) Are there any other calcium-sensitive functional indexes of intact muscle of mdx mouse more sensitive to the changes in calcium homeostasis? We attempted to answer some of these questions by measuring the voltage threshold for mechanical activation as a measure of excitation-contraction (e-c) coupling, i.e. the mechanism linking the membrane depolarization to calcium release and contraction. The mechanical threshold has been measured in single fibres of intact EDL muscle in mdx mouse at two ages: 8–10 weeks when the active regeneration is still ongoing and 6–8 months when regeneration cycle has been completed (Anderson *et al*., 1988; De Luca *et al*., 1997; 1999). Furthermore, we tested on both wildtype and mdx EDL muscles the effect of *in vitro* application of taurine, an amino acid that is abundantly present in skeletal muscle and is able to control e-c coupling mechanism of rat striated fibres through a stimulation of the Ca^{2+}-ATPase pump of SR (Huxtable, 1992; De Luca *et al*., 1996). The hypothesis that taurine can have a therapeutic potential in this condition derives from recent results obtained with H^1-NMR spectroscopy showing that its levels in mdx muscles fluctuate in relation to damage and repair events (McIntosh *et al*., 1998), suggesting that a taurine deficit can contribute to the alteration of calcium homeostasis in relation to the pathology progression.

Methods

Animals

The experiments were carried out on 8–12-week-old and 6–8-month-old male mdx and related wildtype (C57/BL10) mice. The electrophysiological experiments were made *in vitro* on extensor digitorum longus (EDL) muscles. The muscles were removed under urethane anaesthesia (1.2 g kg^{-1} i.p.). Soon after the removal of the muscle, the other tissues were collected for determination of taurine content by HPLC. In particular the tibialis anterior (TA) muscle, a fast twitch muscle similar to EDL, was removed from both legs, during anaesthesia. Then the animals were killed by decapitation and trunk blood was collected for preparation of plasma. Then heart and brain were also collected. TA, heart and brain were immediately frozen for subsequent taurine determination (see below). The EDL muscle was stretched to about 1.5 times its resting length on a 3 mm plastic rod in a temperature-controlled muscle chamber at 30°C and perfused with a physiological solution in the absence and in the presence of the test compounds.

Measurement of mechanical threshold

The mechanical threshold of the fibres was determined using a two microelectrode 'point' voltage clamp method as previously described (Dulhunty, 1988; De Luca *et al*., 1996; Pierno *et al*., 1998). In brief, a voltage-sensing electrode (3 M KCl) and a current-passing electrode (2 M potassium citrate) were inserted within 5 μm of each other into the central region of a randomly selected superficial fibre which was continuously viewed using a stereomicroscope (100× magnification). The holding potential was set at −90 mV and depolarizing command pulses of variable duration were given at a rate of about 0.3 Hz. Below this rate the fibres could not be clamped for an appropriate length of time to perform the required measurements. Tetrodotoxin (3 μM) was continuously present during recordings to prevent action potential generation (Dulhunty, 1988; De Luca *et al*., 1996; Pierno *et al*., 1998). As a standard protocol the command-pulse duration was usually set sequentially to each of the following values: 500, 50, 5, 200, 20, 100 and 10 ms. At each duration, the command voltage was increased using an analogue control until contraction was just visible, and then backed down until the contraction just disappeared. A digital sample-and-hold millivoltmeter stored the value of the threshold membrane potential at this point. We estimated the uncertainty of any single measurement for a given fibre to be 1–2 mV (Dulhunty, 1988; De Luca *et al*., 1996; Pierno *et al*., 1998). Particular care was taken to perform the measurements in any experimental condition in an identical fashion, with about the same length of time involved in each determination so as to exclude any effect on the mechanical threshold of intracellular citrate ions from the electrodes (Dulhunty, 1988). The experiments with each muscle lasted less than 2 h. The threshold membrane potential V (mV) for each fibre was averaged at each pulse duration t (ms) and then mean values plotted against duration giving us a 'strength-duration' relationship. A fit estimate of the rheobase voltage (R) and of the rate constant ($1/\tau$) to reach the rheobase was obtained by non-linear least square algorithm using the following equation:

$$V = [H - R \exp(t/\tau)]/[1 - \exp(t/\tau)] \quad (1)$$

where H is the holding potential (mV), R, is the rheobase (mV) and τ is the time constant. In the fitting algorithm, each point was weighed by the reciprocal of the variance of that

mean V and the best fit estimates of the parameters R and l/τ were made (Miledi *et al*., 1983). We used this procedure in order to be able to incorporate all of our determination points and their associated errors into our estimate of R under each condition. The mechanical threshold values are expressed as the fitted rheobase (R) and rate constant (l/τ) parameters ± standard error (s.e.mean) which were determined from the variance-covariance matrix in the non-linear least square fitting algorithm. The statistical significance from each other was estimated by a Student's *t* distribution using these standard errors and a number of degrees of freedom equal to the total number of threshold values determining the curves minus the number of means minus two for the free parameters (De Luca *et al*., 1996; Pierno *et al*., 1998).

HPLC analysis

Trunk blood was collected in centrifuged tubes rinsed with 10 μl of ethylendiaminotetraacetic acid (150 mM) and centrifuged at $600 \times g$ for 10 min. The plasma was separated and stored at -20°C until used for taurine determination. TA muscles, heart and brain were weighed and homogenized with 10 ml of $HClO_4$ (0.4 N) per g of tissue. The homogenized muscles were buffered with 80 μl K_2CO_3 (5.5 g 10 ml^{-1}) for each ml of $HClO_4$ used. The homogenates were centrifuged at $600 \times g$ for 10 min at 4°C. The supernatants were stored at -80°C until assay. Derivatization with ophthalaldehyde was performed as previously described and samples were processed for HPLC taurine determination (De Luca *et al*., 1996; Pierno *et al*., 1998).

Solutions and drugs

The normal physiological solution had the following composition (in mM): NaCl 148; KCl 4.5; $CaCl_2$ 2.0; $MgCl_2$ 1.0; $NaHCO_3$ 12.0; NaH_2PO_4 0.44 and glucose 5.55. The solution was continuously gassed with 95% O_2 and 5% CO_2. For *in vitro* application of taurine on EDL muscle of mdx and wildtype mice, the drug was dissolved in the physiological solution in the mM concentration needed. Equimolar concentrations of sucrose were used to establish any possible effects due to the osmotic change of the solutions. Particular care was taken to maintain the physiological pH at 7.2 when taurine was applied *in vitro* since its actions have been described to be strongly dependent upon external pH.

Results

Mechanical threshold of EDL muscle fibres of mdx and wildtype mice

The threshold potential for contraction (mechanical threshold, MT) of EDL muscle fibres of both mdx and control mice showed the typical dependence on command pulse duration; i.e. it was the more negative the longer the duration of the pulse. Under the experimental conditions used, a constant rheobase value was almost fully reached at the longest pulses used, a behaviour commonly seen with mammalian muscle fibres (De Luca *et al*., 1996; Pierno *et al*., 1998).

In wildtype mice, a difference in mechanical threshold has been found with age. In fact at 8–12 weeks, the MT was significantly more negative with respect to that recorded at 6–8 months of age, while no remarkable difference was found in the rate at which the rheobase is reached (l/τ) (Table 1). The post-natal development changes occurring in membrane electrical properties responsible for excitation pattern, i.e. the ionic conductances, are fully completed during the first 2 months of age in wildtype mice (De Luca *et al*., 1997). In the light of this finding and taking into account that recordings are made in voltage clamp conditions, the present result suggests that the structures strictly involved in the e-c coupling mechanism can undergo age-related changes (independently from post-natal development) likely as adaptive modifications of the contractile apparatus to functional demand. The EDL muscle fibres of mdx mice of 8–12 weeks of age showed a mechanical threshold significantly different with respect to that of age-matched controls. In fact, at each pulse duration, the voltage threshold for contraction was significantly more negative with respect to that of controls (Figure 1, upper panel). The strength-duration curve was significantly shifted by almost 6 mV in the negative potential range with respect to that of age-matched controls, as also estimated by the rheobase value (Figure 1, Table 1). Also l/τ, indicating the speed for reaching the rheobase, was significantly slower in the mdx than in controls. Similarly to that observed in wildtype animals, in the 6–8-month-old mdx EDL the mechanical threshold showed a tendency, albeit not significant, to occur at more positive voltages with respect to 8–12-week-old mice of the same strain; however the contraction occurred always at potentials that were significantly more negative with respect to age-matched wildtype animals. Actually, the shift of the strength-duration curve was even more evident than that observed at 8–12 weeks, the difference being about 8 mV (Figure 1, Table 1). Again, l/τ was slower in mdx with respect to controls. Thus, mdx muscle fibres require less depolarization to contract and this feature is independent from age and progression of the pathology.

Effect of in vitro *taurine application on mechanical threshold of mdx and wildtype mice*

The effect of *in vitro* application of taurine was evaluated on fully regenerated EDL muscles of 6–8-month-old mdx animals and related age-matched wildtype animals. Milli-

Table 1 Mechanical threshold parameters of extensor digitorum longus muscle fibres of mdx mice

Experimental condition	*Rheobase* (mV)	$1/\tau$ (s^{-1})
8–12 weeks		
Wildtype	−66.6 ± 0.4	0.142 ± 0.005
mdx	−72.2 ± 0.8*	0.114 ± 0.01*
6–8 months		
Wildtype	−62.0 ± 0.9	0.151 ± 0.01
mdx	−70.1 ± 1.3*	0.124 ± 0.02

The values ± s.e.mean of rheobase voltage and rate constant ($1/\tau$) have been obtained from the fit of the experimental data points describing the strength-duration curve with the equation described in the Methods section. *Significantly different with respect to the age-matched wildtype value ($P < 0.005$ and less). The rheobase value of wildtype muscles at 8–12 weeks of age is also significantly different with respect to the value at 6–8 months.

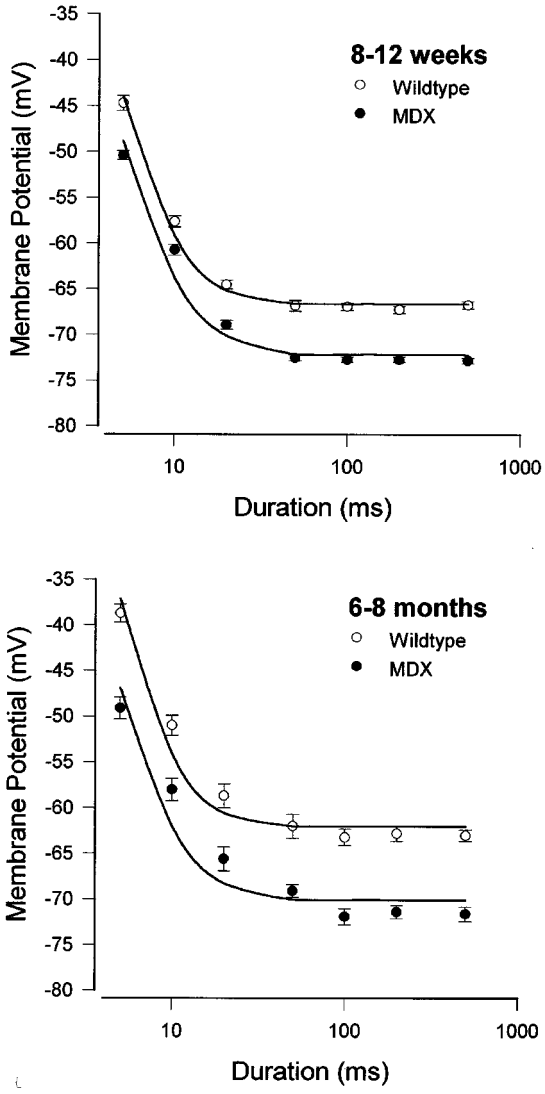

Figure 1 Strength–duration curves of the threshold potentials for mechanical activation of extensor digitorum longus muscle fibres of 8–12-week-old (upper panel) and 6–8-month-old (lower panel) mdx and wildtype mice. Each point is the mean±s.e.mean of the threshold potentials (in mV) recorded at each pulse duration from 30–40 fibres sampled from 6–7 preparations (8–12 weeks) and from 15–25 fibres from 3–5 preparations (6–8 months). The curve fitting the experimental points has been obtained using the equation described in the Methods section. The fitting procedure allowed also to obtain the calculated values±s.e. of the rheobase voltage (R) and of the rate constant of the process ($1/\tau$) in each experimental condition, shown in Table 2.

molar concentrations of the amino acid have been chosen based on previous findings on mechanical threshold of taurine-depleted muscle (De Luca *et al*., 1996) and also taking into account that high taurine concentrations are often required *in vitro* in order to saturate both low- and high-affinity sites on the sarcolemma, also in consideration of the high intracellular concentration of the amino acid (Pierno *et al*., 1994; Huxtable, 1992). The application of taurine up to 60 mM did not produce any change in the mechanical threshold of control mice. In fact, as it can be seen in Table 2, no effect was observed at any threshold potential value after application of 60 mM taurine (Table 2), the rheobase voltage being -61.6 ± 1.4 mV, a value almost overlapping that observed before the application of the amino acid (see value in Table 1; Table 2). Also no effect of taurine was observed in the $1/\tau$ value that was 0.155 ± 0.018 s^{-1}. On the contrary the *in vitro* application of taurine on mdx EDL muscle produced an amelioration of the mechanical threshold by shifting the voltage for contraction toward more positive potentials. The effect started to be detectable after application of 20 mM taurine that shifted the rheobase of about 2 mV toward the control value (data not shown), however it became statistically significant after application of 60 mM, a concentration at which taurine was able to markedly shift the voltage for contraction toward more positive potentials at any pulse duration (by 4–5 mV toward the value of control; Table 2). The fit of the experimental points determining the strength-duration curve in the presence of 60 mM taurine led to a rheobase voltage of -65.0 ± 1.3 mV, significantly less negative with respect to that recorded in the absence of the compound (-70.1 ± 1.3 mV; $P<0.01$). Interestingly, the application of taurine also speeded up the rate for reaching the rheobase that was almost overlapping that observed in control muscle fibres, being 0.156 ± 0.02 s^{-1} from 0.124 ± 0.02 s^{-1}.

To see whether the effect of taurine was homogeneous within the whole mdx EDL muscle, we evaluated the distribution of single fibres as a function of threshold potential in wildtype and mdx mice before and after the application of 60 mM taurine, using the pulse duration of 100, 200 and 500 ms (when the contraction occurs at constant potentials) and a sampling value of 2 mV (Figure 2). As it can be seen in both wildtype and mdx mice, the fibres showed a clear unimodal distribution, with mean values of -63.0 ± 0.4 mV ($n=57$) and -71.6 ± 0.4 mV ($n=74$), respectively, values significantly different between each other with a $P<0.001$. The application of taurine did not change the unimodal distribution and uniformly shifted the Gaussian curve toward a mean value that was in between those of wildtype and mdx ones, being -67.1 ± 0.3 mV ($n=88$), significantly different with respect to both mean values. This data suggests that the change in the threshold voltage observed in mdx EDL muscle is due to a homogeneous modification of an e-c coupling step in all muscle fibres, and that taurine is able to modulate this step in a positive manner. Higher concentrations were not tested for avoiding large change in osmolarity of the bathing solution. At this regard, since mdx muscle fibres are known to be susceptible to change in osmotic pressure (Leijendekker *et al*., 1996; Pulido *et al*., 1998), we tested the possibility that taurine could have produced an osmotic-sensitive non-specific effect. This was ruled out by the observation that the addition of 60 mM sucrose did not produce any change of the mechanical threshold, while the following application of 60 mM taurine on the same preparations produced the already described shift of the strength-duration curve toward more positive voltages (Figure 3).

Plasma and tissue content of taurine in mdx and C57/BL10 mice

The plasma and tissue contents of taurine in mdx and control mice are shown in Table 3. As it can be seen, no significant differences were found in the taurine tissue content, although

Table 2 Effect of *in vitro* application of 60 mM taurine on the mechanical threshold of EDL muscle fibres of 6–8 months mdx mice

Experimental condition	*Duration* (ms) 5	10	20	50	100	200	500
Wildtype ($n=3$)	-38.7 ± 1.0 (17)	-51.0 ± 1.1 (15)	-58.7 ± 1.3 (16)	-62.0 ± 1.3 (18)	-63.2 ± 0.9 (21)	-62.8 ± 0.8 (16)	-63.0 ± 0.6 (20)
Wildtype + taurine	-39.8 ± 1.2 (12)	-49.0 ± 2.2 (11)	-58.1 ± 1.3 (10)	-61.0 ± 1.4 (13)	-62.0 ± 0.4 (15)	-63.9 ± 1.1 (16)	-63.8 ± 0.8 (15)
mdx ($n=5$)	-49.1 ± 0.9* (19)	-58.0 ± 1.1* (20)	-65.5 ± 1.2* (21)	-69.1 ± 0.8* (20)	-71.9 ± 0.7* (25)	-71.4 ± 0.6* (24)	-71.6 ± 0.6* (25)
mdx + taurine	-45.7 ± 0.9*§ (27)	-54.9 ± 1.1* (22)	-60.1 ± 1.0§ (26)	-63.8 ± 0.7§ (32)	-66.3 ± 0.7*§ (26)	-67.0 ± 0.4*§ (29)	-67.4 ± 0.5*§ (33)

The columns from left to right are as follows: Experimental conditions: the fibres sampled are from extensor digitorum longus muscles from three wildtype and five mdx mice of 6–8 months of age before and after the *in vitro* application of 60 mM taurine. For each experimental condition are shown the threshold membrane potential values obtained with depolarizing command pulse of duration ranging from 5 up to 500 ms. The values are expressed as mean ± s.e.mean from the number of fibres shown in parantheses below each value. Significantly different * with respect to wildtype and § with respect to related values in the absence of taurine ($P<0.05$ and less).

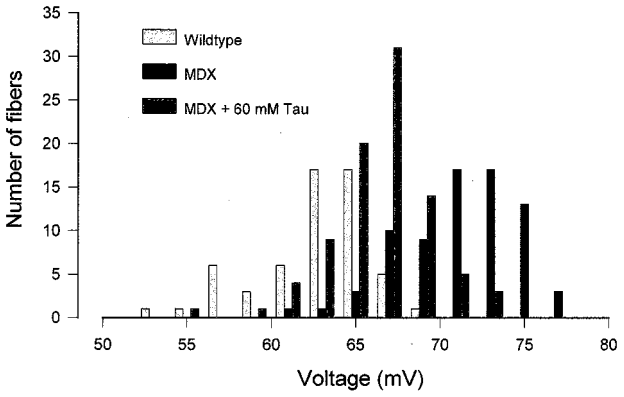

Figure 2 Distribution of single fibres as a function of threshold potential in 6–8-month-old wildtype and in age-matched mdx mice before and after the application of taurine. The distribution has been evaluated at the pulse duration of 100, 200 and 500 ms (pulse duration at which the threshold potential remains constant) using a sampling value of 2 mV. In all experimental conditions, the fibres showed a clear unimodal distribution, with mean values of -63.0 ± 0.4 mV ($n=57$) for wildtype, -71.6 ± 0.4 mV ($n=74$) for mdx and -67.1 ± 0.3 mV ($n=88$) in mdx after the application of taurine (mdx + 60 mM Tau). The three values are significantly different between each other with $P<0.001$.

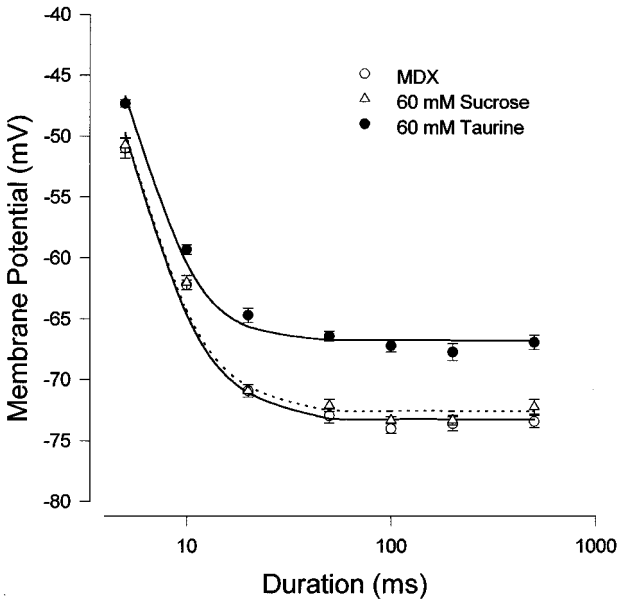

Figure 3 Lack of osmolarity-induced effect on the strength-duration curves of extensor digitorum longus muscle fibres of 6–8-month-old mdx mice. As it can be seen, the addition of 60 mM sucrose failed to produce any shift of the curve (dashed line), while the following application of 60 mM taurine produced the shift of the curve toward more positive potentials, already described in Table 1. Each point is the mean ± s.e.mean of the threshold potentials (in mV) recorded at each pulse duration from 12–13 fibres sampled from two preparations of different mice. The curve fitting the experimental points has been obtained using the equation described in the Methods section and the fitted parameters obtained in each of the experimental conditions are the following: No drug (mdx): $R=-73.2\pm0.6$ mV; $1/\tau=0.11\pm0.007$ s^{-1}, 60 mM sucrose $R=-72.6\pm0.6$ mV; $1/\tau=0.113\pm0.007$ s^{-1}, 60 mM Taurine: $R=-66.7\pm0.4$ mV; $1/\tau=0.154\pm0.006$ s^{-1}.

a clear trend toward a decrease was observed. In fact the taurine content in TA muscle was 14% lower in mdx with respect to control mice. Similarly in the other excitable tissues tested of mdx mice (heart and brain) the levels of taurine tended to be lower with respect to the same tissues of wildtype animals. However, it has to be underlined that the decrease observed in heart and brain was very slight, ranging about 5%, thus being much less than that observed in skeletal muscle. Interestingly, the plasma level of the amino acid was instead markedly increased in mdx vs controls, the increase being about 140%, suggesting that, in spite of the apparent compensation, the tissues, and especially the skeletal muscle might have an impaired ability to retain the appropriate amount of taurine.

Discussion

The mdx mouse is largely used as a model for studying Duchenne muscular dystrophy and the efficiency of gene-therapy, since a deletion mutation leads to a similar lack of dystrophin (Matsumura & Campbell, 1994; Gillis, 1996). However, an important task in this field is the rapid identification of drug therapies able to alleviate symptoms and/or prolong life expectation of patients. In this respect, the validity of the murine phenotype for testing pharmacological trials is hindered by the mild form of pathology that the animals develops, since an effective muscle regeneration allows the mice to survive in spite of the genetic defect (Anderson *et al*., 1988; Dupont-Versteegden *et al*., 1992). The identification of those functional parameters that, in spite of the regeneration-induced functional recovery, stay perma-

Table 3 Taurine content in different tissues of 6–8-month-old wildtype and mdx mice

Tissue	*Control*	*mdx*
Plasma (μmol ml^{-1})	0.729±0.062 (5)	1.734±0.267* (6)
Skeletal muscle (μmol g^{-1})	28.0±2.07 (7)	24.09±2.67 (6)
Brain (μmol g^{-1})	9.01±0.29 (7)	8.61±0.29 (5)
Heart (μmol g^{-1})	39.2±3.13 (6)	37.97±2.63 (6)

Taurine content has been evaluated by HPLC analysis from samples collected from 6–8-month-old wildtype and mdx mice. Each value is the mean±s.e.mean from the number of animals shown in brackets. *Significantly different with respect to related wildtype value.

nently altered in mdx muscle fibres can therefore have an important application for both *in vitro* and *in vivo* pharmacological studies. There is a general consensus about the raise of cytosolic calcium ions as a direct consequence of the lack of dystrophin, but no clear data is available about the functional effect of this alteration on contractile properties of intact mdx muscle (Mechalchuck & Bressler, 1992; Gillis, 1996; Hayes & Williams, 1998; Rüegg & Gillis, 1999). Our electrophysiological investigation showed an alteration of the excitation-contraction (e-c) coupling mechanism, since we observed that the mdx muscle fibres needed significantly less depolarization to contract with respect to age-matched wildtype animals, resulting in a rheobase voltage significantly shifted toward more negative potentials. The mechanical threshold is an integrate function of the structures contributing to e-c coupling and specifically matches the muscle functional requirement (Dulhunty & Gage, 1983; Larsson & Salviati, 1989; Berchtold *et al*., 2000). The e-c coupling mechanism starts with the depolarization of the sarcolemma activating the dihydropyridine (DHP) voltage sensor in the transverse tubules that in turn is able to mechanically open the ryanodine-sensitive calcium release channel in the sarcoplasmic reticulum, finally leading to contraction (Rios *et al*., 1992). The contraction is then stopped when the calcium is buffered by the SR Ca^{2+}-ATPase pump and by low affinity calcium binding protein, such as parvalbumin. The rheobase is the voltage at which an equilibrium is reached between the speed at which calcium is released and buffered. Any intervention that would increase the release of calcium or reduce the uptake would produce a shift of the rheobase toward more negative potentials (Walsh *et al*., 1988; Dulhunty, 1988). In fact, previous observations have shown that various pathophysiological conditions related to an impairment of calcium homeostasis, such as aging, are paralleled by a shift of the rheobase voltage of fast-twitch muscle towards more negative potentials (Larsson & Salviati, 1989; De Luca & Conte Camerino, 1992; Pierno *et al*., 1998). A similar alteration has been presently observed in mdx muscle fibres at both 8–12 weeks and 6–8 months of age, suggesting that it is strictly connected to the proposed increase of cytosolic calcium and that it is unrelated to degeneration-regeneration events occurring in the mdx phenotype. In fact, at 8–12 weeks of age, the successful regeneration characterizing the hind limb muscle in the mdx phenotype is still ongoing and at this age it is possible to observe temporary biochemical and electrophysiological changes that are likely important to adapt the functional demands of the regenerating muscle (Anderson *et al*., 1988; McIntosh *et al*., 1998; De Luca *et al*., 1997; 1999). As opposite, the regeneration process does not appear to exert any compensatory change of the e-c coupling, suggesting that this latter may be a permanent functional alteration, more strictly related the genetic defect.

The available findings may help to identify the structures contributing to e-c coupling mechanism possibly targeted by the dystrophic condition. No significant modification of dihydropyridine receptor expression has been found in mdx TA muscle, while the parvalbumin expression is even increased in fast-twitch mdx limb muscles, suggesting that this latter can actually work to compensate the calcium buffering capacity of dystrophic fibres (Pereon *et al*., 1997; Gillis, 1996). Thus, the alteration of the e-c coupling mechanism observed may be finally related to a chronic change in calcium homeostasis that may in turn affect the function of the SR structures responsible for calcium release and reuptake (Takagi *et al*., 1992; Kargacin & Kargacin, 1996). For instance a higher basal level of calcium can add to the calcium transients and contribute to saturate the Ca^{2+}-ATPase pump, especially if the function of this structure is already affected. An impairment of Ca^{2+}-ATPase pump has been indeed described and may be due to two concomitant events: (1) the loss of creatine kinase which works to produce ATP nearby SR, and (2) the calcium overload in mitochondria that can similarly reduce the furnishing of energy products. In fact, creatine supplementation of mdx myotubes is able to counteract the increase in cytosolic calcium (induced by hypo-osmotic shock or by rise of extracellular calcium) by stimulating the activity of Ca^{2+}-ATPase pump, whereas both influx and efflux are rather unaffected (Pulido *et al*., 1998). This hypothesis is strongly supported by our present finding that the *in vitro* application of taurine significantly ameliorates the e-c coupling mechanism of dystrophic fibres. Experiments performed on isolated vesicles of rat muscle SR showed that taurine, physiologically present in muscle cells at high levels, is able to directly stimulate the calcium reuptake by the Ca^{2+}-ATPase pump (Huxtable & Bressler, 1973). Through this mechanism taurine works physiologically by modulating e-c coupling mechanism of striated fibres. In fact, a shift of the mechanical threshold toward more negative potentials is commonly observed in conditions of taurine depletion either naturally occurring (as in aged muscle) or induced pharmacologically (De Luca *et al*., 1996; Pierno *et al*., 1999). In both conditions taurine supplementation, either *in vitro* or *in vivo*, has beneficial effects on mechanical threshold. In the present experimental conditions only a slight and not significant decrease of taurine level has been found in mdx muscle, although the large increase in the plasma is in favour of a tissue suffereance in retaining adequate level of the amino acid. Recent results by H^1-NMR spectroscopy have revealed that the fluctuation of the levels of creatine and taurine can be taken as markers of dystrophic progression, the level of both being strongly decreased in mdx muscles at the very young age (3–6 weeks) when degeneration occurs, while they recovered at older ages, likely in relation to muscle regeneration (McIntosh *et al*., 1998). Thus, in the light of the available information, we can propose that a positive

effect of taurine on e-c coupling mechanism of striated fibres can be detectable not only when the cellular levels of the amino acid are clearly reduced, but also when Ca^{2+}-ATPase pump activity is affected, as in the present case.

Finally, our results indicate that the e-c coupling mechanism of dystrophin-deficient muscle fibres of mdx mouse is permanently affected and is therefore a useful functional index for investigating the effectiveness of drug treatment in muscular dystrophy. The alteration of the e-c coupling mechanism can be a direct consequence of the biochemical and metabolic effects triggered in muscle fibres by the increased cytosolic calcium. This condition can be ameliorated by supplementation of the intracellular component taurine, able to modulate calcium handling mechanisms. Our results, along with the facts that the progression of the dystrophic condition can trigger more dramatic fluctuation of tissue taurine content and that the amino acid is almost free of side effects upon *in vivo* administration, lead us to propose that taurine can be one possible effective drugs for dystrophic patients.

The contribution of Telethon-Italy to the project No 1150 is gratefully acknowledged. The authors wish to thank Prof Saverio Cellamare for helpful advice during the experiments.

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(Received September 25, 2000
Revised December 11, 2000
Accepted December 18, 2000)