Creatine supplementation improves intracellular Ca^{2+} handling and survival in *mdx* skeletal muscle cells

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Received 18 September 1998; received in revised form 21 October 1998

Abstract Dystrophic skeletal muscle cells from Duchenne muscular dystrophy (DMD) patients and *mdx* mice exhibit elevated cytosolic Ca^{2+} concentrations ($[Ca^{2+}]_c$). Pretreatment of *mdx* myotubes for 6–12 days with creatine (20 mM) decreased the elevation in $[Ca^{2+}]_c$ induced by either high extracellular Ca^{2+} concentrations or hypo-osmotic stress to control levels. $^{45}Ca^{2+}$ influx measurements suggest that creatine lowered $[Ca^{2+}]_c$ by stimulating sarcoplasmic reticulum Ca^{2+}-ATPase. Creatine pretreatment increased levels of phosphocreatine but not ATP. Furthermore, myotube formation and survival were significantly enhanced by creatine pretreatment. Therefore, creatine supplementation may be useful for treatment of DMD.

Key words: Creatine; Calcium; Duchenne muscular dystrophy; α-Methylprednisolone; Skeletal muscle; *Mdx* mouse

1. Introduction

Duchenne muscular dystrophy (DMD) is a severe degenerative muscle disease caused by a mutation in the dystrophin gene, located on chromosome Xp21. Dystrophin is associated with a complex of transmembrane glycoproteins, the dystrophin-glycoprotein complex, and acts as a transsarcolemmal linker between the subsarcolemmal cytoskeleton and the extracellular matrix [1–4]. Loss of this complex results in mechanical instability of the sarcolemma [5], rendering dystrophic muscle more susceptible to stress [6,7] resulting in an increased activity of 'leak' [8,9], and/or stretch-inactivated Ca^{2+} channels [10–13]. The subsequent elevation of cytosolic calcium concentrations ($[Ca^{2+}]_c$) triggers Ca^{2+}-activated proteases such as calpain, further increasing the activity of calpain-sensitive Ca^{2+}-leak channels [14] and decreasing the function of calpain-sensitive plasma membrane Ca^{2+}-ATPases [15], which in turn leads to a cascade of Ca^{2+}-dependent catabolic events [16,17]. Denetclaw et al. have shown that transgenic introduction of dystrophin into *mdx* myotubes recovers $[Ca^{2+}]_c$ and open probabilities of Ca^{2+}-leak channels to normal levels [18], emphasizing the effect of dystrophin on cytosolic Ca^{2+} regulation.

Several enzyme systems, Ca^{2+}-specific pumps and ion channels are known to work together to maintain tight control of $[Ca^{2+}]_c$. One of these enzymes, creatine kinase (EC 2.7.3.2, CK), provides energy for the calcium-sequestering sarcoplasmic reticulum (SR) Ca^{2+}-ATPase and catalyzes the reversible transphosphorylation of the high energy *N*-phosphoryl group of phosphocreatine (PCr) to ADP, regenerating ATP by the following reaction [19]:

$$MgADP^- + PCr^{2-} + H^+ \leftarrow CK \rightarrow MgATP^{2-} + Cr$$

CK is expressed in a tissue-specific manner and is highly compartmentalized with an additional mitochondrial isoform (Mi-CK) [20]. The muscle-type MM-CK is localized in the vicinity of the SR Ca^{2+}-ATPase [21,22] which preferentially utilizes ATP generated by CK [23] and is therefore critically involved in controlling $[Ca^{2+}]_c$ homeostasis [19]. Inhibition of SR-bound CK has been shown to lead to a drastic decrease in SR Ca^{2+} uptake [21] and transgenic mice devoid of both muscle CK isoenzymes display serious problems with muscle relaxation due to elevated $[Ca^{2+}]_c$ [24].

Cellular creatine uptake is achieved by the specific creatine transporter protein (CreaT) in a sodium- and chloride-dependent process, which moves creatine against a concentration gradient [25]. CreaT is expressed in several tissues, including skeletal muscle [26].

Using dystrophic skeletal muscle cells from the *mdx* mouse model of DMD, we and others have demonstrated impaired $[Ca^{2+}]_c$ regulation in response to hypo-osmotic and high extracellular calcium ($[Ca^{2+}]_{extrac}$) stress [6,7]. With the aim of counteracting these stress-induced increases in $[Ca^{2+}]_c$, we have earlier examined the effects of prolonged administration of steroid hormones such as α-methylprednisolone (PDN) [7,26]. Our current results show that creatine increases cellular PCr levels and improves the regulation of $[Ca^{2+}]_c$ in *mdx* myotubes exposed to stress. In addition, increased myotube formation and cell survival was observed. To our knowledge, these data are the first to show that creatine supplementation acts to lower $[Ca^{2+}]_c$ and enhances survival of dystrophic skeletal muscle cells.

2. Materials and methods

2.1. Cell cultures

Primary cell cultures of mouse skeletal muscle cells were prepared from hind leg muscles of 1–4-day-old normal (C57BL/10) and *mdx* mice as described [27]. Cells were seeded at 5×10^4 cells/cm^2 in medium 199 (Gibco, Basel, Switzerland) containing 10% horse serum (Gibco) either on glass coverslips (9×18 mm) for $[Ca^{2+}]_c$ measurements or in 24-well polystyrene plates coated with 2% gelatin (Sigma, Buchs, Switzerland) for flux experiments. Fusion of myoblasts into myotubes occurred at day 3 in culture and was completed within the next 24 h.

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Abbreviations: $[Ca^{2+}]_c$, cytosolic Ca^{2+} concentration; $[Ca^{2+}]_{extrac}$, extracellular calcium concentration; CK, creatine kinase; Cr, creatine; DMD, Duchenne muscular dystrophy; EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; PCr, phosphocreatine; PDN, α-methylprednisolone; PSS, physiological salt solution; SL, sarcolemmal; SR, sarcoplasmic

PII: S0014-5793(98)01399-4

2.2. Culture treatment

α-Methylprednisolone (10^{-6} M, Upjohn Pharma, Brüttisellen, Switzerland) and creatine (20 mM, Sigma, Buchs, Switzerland) were added at the beginning of myoblast fusion (day 3 of culture) [7]. Fresh medium was added after 5 days in culture. Myotubes were used for the experiments between days 12 and 14 in culture.

2.3. Measurements of the cytosolic calcium concentration

$[Ca^{2+}]_c$ measurements were performed on single Fura-2-loaded myotubes, as described previously [7]. Cells cultured on a glass coverslip were washed twice with control PSS buffer (Table 1) and incubated for 30 min with Fura-2/AM (5 μM) (Molecular Probes, Eugene, OR, USA) in control PSS buffer. The coverslip was analyzed in a thermostatted chamber (37°C) on a Nikon Diaphot inverted epifluorescence microscope, with a PhoCal single cell fluorescence analyzer (Life Science Resources, Cambridge, UK). The myotubes were illuminated with alternating light of 340 and 380 nm from a rotating filter wheel (6.25 Hz). Emission was monitored at 510 nm. Results are given as calculated $[Ca^{2+}]_c$ as described [28].

2.4. Experimental protocols

In order to examine changes in $[Ca^{2+}]_c$ due to hypo-osmotic or high $[Ca^{2+}]_{extrac}$ stress, myotubes were exposed to two types of extracellular shock (100 mOsm, buffer 2, and 40 mM $[Ca^{2+}]_{extrac}$, buffer 1, see Table 1). The following protocol was used: control buffer (5 min) – 100 mOsm buffer (buffer 2) (10 min) – control buffer (5 min) – 40 mM $CaCl_2$ (buffer 1) (2 min) – control buffer (10 min) – calibration procedure [7]. The myotubes were also examined in the opposite sequence: control buffer (5 min) – buffer 1 (2 min) – control buffer (5 min) – buffer 2 (10 min) – control buffer (10 min) – calibration procedure. The osmolarity was determined by measuring the lowering of the freezing point (Osmometer, Infochroma, Zug, Switzerland).

2.5. $^{45}Ca^{2+}$ influx assay

$^{45}Ca^{2+}$ (from Amersham-Rahn, Zürich, Switzerland) influx measurements were performed as previously described [29]. Briefly, myotubes were preincubated for 10 min in control buffer (Table 1) containing 0.12 mM $CaCl_2$. Basal influx was determined by exposing the myotubes to 0.2 ml of control buffer containing 1.2 mM $CaCl_2$ and 0.4 μCi of $^{45}Ca^{2+}$. Hypo-osmotic and high $[Ca^{2+}]_{extrac}$ influx was determined by incubating myotubes for 15 min with 0.2 ml of the appropriate experimental buffer containing 0.4 μCi (buffer 2) or 1.6 μCi (for high $[Ca^{2+}]_{extrac}$ assay, buffer 3) of $^{45}Ca^{2+}$. Influx was stopped with EGTA (2 mM) and cells were lysed with trypsin (0.25%) and sodium dodecyl sulfate (1%). Radioactivity was determined by scintillation counting (Packard 460C, Zürich, Switzerland). Protein measurements were performed as described [7].

2.6. Determination of myotube numbers and survival

Myotubes were stained with May-Grünwald-Giemsa stain and counted as previously described [27]. Myotube survival was determined by trypan blue assay. Five randomly chosen fields were counted per dish and three dishes were examined for each condition.

2.7. ATP and phosphocreatine measurements

ATP and PCr levels were measured as previously described [30]. Luciferin and luciferase were purchased from Molecular Probes. Hypo-osmotic (buffer 2) and high $[Ca^{2+}]_{extrac}$ (buffer 1) shock were applied to 12-day-old myotubes grown in 24-well plates and treated with 20 mM creatine from day 3. Trichloroacetic acid (10%) at 0°C was used to extract ATP and PCr and was washed out with saturated ether. Luminescence was recorded on an ML 3000 plate reader (Dynatech, Embrach, Switzerland) for ATP and an LKB 410 luminometer (LKB-Pharmacia, Uppsala, Sweden) for PCr.

2.8. Statistics

All values are expressed as mean ± S.E.M., with the exception of Fig. 4 where S.D. was used. Student's *t*-test was used for statistical analysis.

3. Results

We have previously shown that exposure of myotubes to hypo-osmotic solution (100 mOsm) or buffer with increased extracellular Ca^{2+} concentrations results in a transient rise in $[Ca^{2+}]_c$, which is more pronounced in *mdx* myotubes than in controls [7]. Values for $[Ca^{2+}]_c$ for both *mdx* and C57BL/10 myotubes under these stress conditions are shown in Fig. 1.

3.1. Effect of creatine supplementation on hypo-osmotic stress-induced increases in $[Ca^{2+}]_c$

Following the onset of myotube formation (day 3 after seeding), the cultures were incubated for 9–11 days with creatine (20 mM). As a positive control, α-methylprednisolone (PDN, 10^{-6} M), which has been shown to inhibit stress-induced increases in $[Ca^{2+}]_c$ [7], was added to parallel cultures. Neither creatine nor PDN pretreatment had a significant effect on basal $[Ca^{2+}]_c$ in *mdx* or control C57BL/10 myotubes (Fig. 1). Exposure of *mdx* myotubes to hypo-osmotic shock resulted in an increased $[Ca^{2+}]_c$ within 2–10 min. Creatine (20 mM) pretreatment attenuated this increase and restored levels close to basal values (from 118 ± 12 nM to 62 ± 17 nM, $P < 0.001$) as did PDN (Fig. 1B).

3.2. Effect of creatine on high $[Ca^{2+}]_{extrac}$ stress-induced increases in $[Ca^{2+}]_c$

Superfusion of myotubes with buffer 1 containing 40 mM $[Ca^{2+}]_{extrac}$ more than doubled $[Ca^{2+}]_c$ in *mdx* myotubes, relative to untreated *mdx* and control C57BL/10 myotubes (Fig. 1). Pretreatment with 20 mM creatine inhibited this increase in $[Ca^{2+}]_c$ (from 112 ± 15 nM to 59 ± 17 nM, $P < 0.001$) to levels similar to control values, and to the same extent as PDN (Fig. 1B). In contrast to that elicited by hypo-osmotic shock, the high $[Ca^{2+}]_{extrac}$-induced increases in $[Ca^{2+}]_c$ in control C57BL/10 myotubes were slightly reduced by creatine pretreatment (Fig. 1A).

3.3. Effects of creatine supplementation on stress-induced $^{45}Ca^{2+}$ influx

Under both conditions of stress, Ca^{2+} influx more than doubled in *mdx* myotubes, while in control C57BL/10 myo-

Table 1
Composition of experimental buffers

	Control buffer (PSS)	Buffer 1 (high Ca^{2+})	Buffer 2 (low osm)	Buffer 3 (high Ca^{2+})
HEPES	5	5	5	5
KCl	5	5	5	5
$MgCl_2$	1	1	1	1
NaCl	145	85	6	135
$CaCl_2$	1.2	40	1.2	10
Glucose	10	10	1	10
Sucrose	–	–	50	–
Osmolarity (mOsm)	300	308	100	300

All buffers were at pH 7.4.

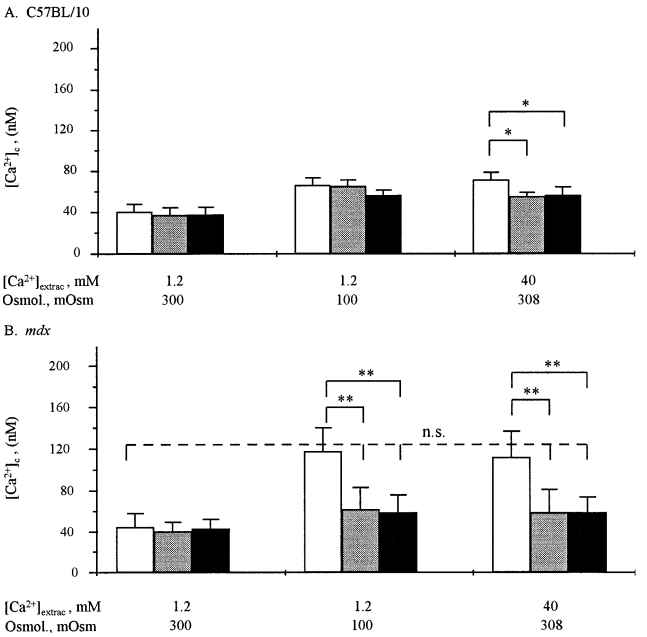

Fig. 1. Effects of creatine and PDN on $[Ca^{2+}]_c$ changes in control C57BL/10 (A) or mdx myotubes (B). Myotubes (day 12–14) were exposed to hypo-osmotic shock (buffer 2) or high $[Ca^{2+}]_{extrac}$ (buffer 1) as described in Section 2. Untreated myotubes (open columns), myotubes pretreated with creatine (20 mM, gray columns), myotubes pretreated with PDN (10^{-6} M, black columns). Five experiments from three separate primary cultures were performed in duplicate. Asterisks indicate significantly different values (*$P < 0.01$, **$P < 0.001$, Student's t-test). ns: not significant.

tubes a milder increase was noted (Fig. 2). In control myotubes, creatine and PDN produced a reduction of Ca^{2+} influx when myotubes were exposed to high $[Ca^{2+}]_{extrac}$ (Fig. 2A). In contrast, in *mdx* myotubes creatine pretreatment did not affect stress-induced Ca^{2+} influx while PDN was effective (Fig. 2B).

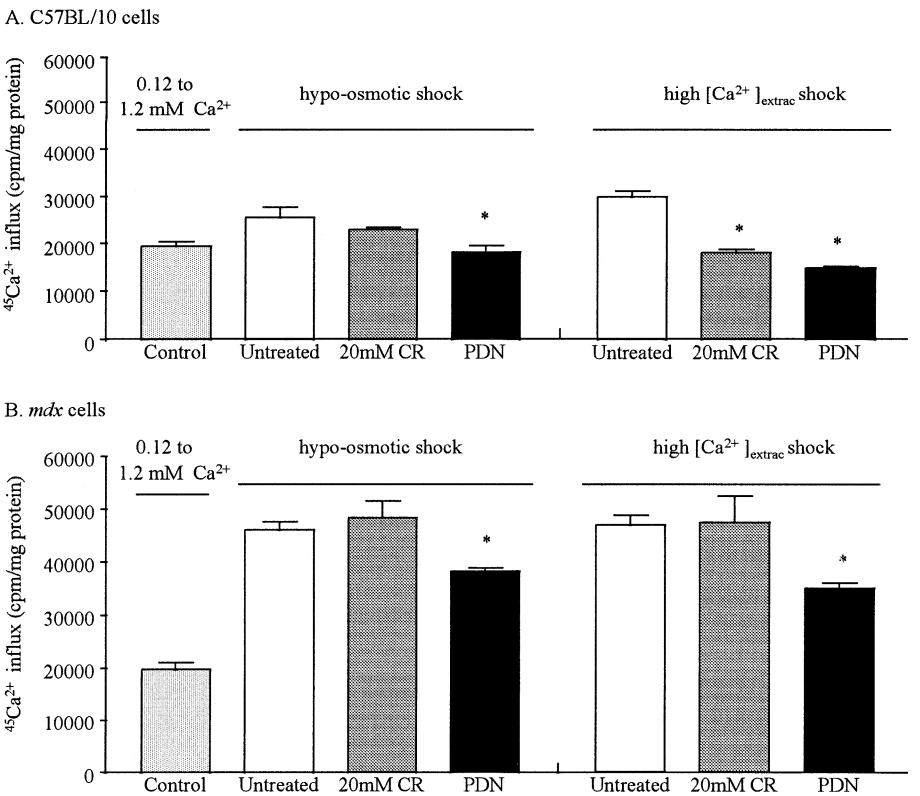

Fig. 2. Effects of creatine and PDN on $^{45}Ca^{2+}$ influx in C57BL/10 (A) or *mdx* (B) myotubes. Untreated myotubes (open columns), myotubes pretreated with creatine (20 mM, gray columns), myotubes pretreated with PDN (10^{-6} M, black columns). Both agents were added on day 3 of culture, and $^{45}Ca^{2+}$ influx was measured at day 12, as described in Section 2. Three experiments from separate primary cultures were each performed in quadruplicate. Asterisks indicate significantly different values (*$P < 0.01$, Student's t-test), compared to control.

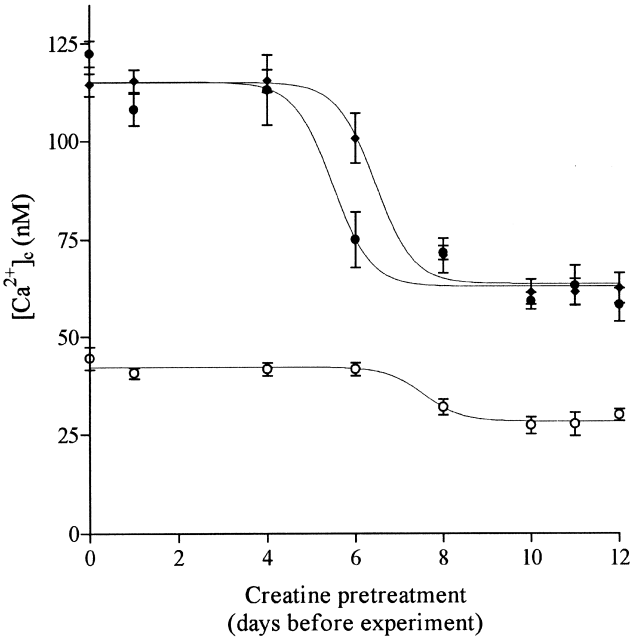

Fig. 3. Time course of creatine effect on $[Ca^{2+}]_c$ increases elicited by stress in *mdx* myotubes. Myotubes (day 13) were exposed to basal conditions (○, control buffer), hypo-osmotic shock (●, buffer 2) or high $[Ca^{2+}]_{extrac}$ (♦, buffer 1) as described in Section 2. Creatine (20 mM) was added to the cultures for the periods indicated before $[Ca^{2+}]_c$ measurements on day 13. Five experiments from three separate primary cultures were performed in duplicate. Asterisks indicate significantly different values (**$P < 0.001$, Student's *t*-test), compared to control.

3.4. Time course of creatine supplementation on stress-induced increases in $[Ca^{2+}]_c$

The cultures were incubated for 1–12 days after seeding with 20 mM creatine and the effect of hypo-osmotic or high $[Ca^{2+}]_{extrac}$ stress on $[Ca^{2+}]_c$ was then determined. Pretreatment for at least 6 days before the experiment inhibited the increase in $[Ca^{2+}]_c$ due to stress (Fig. 3). No effect was observed if creatine was added to myotubes for shorter periods before the experiment or acutely, 5 min to 1 h prior to measurement (data not shown).

3.5. Creatine enhances differentiation, myotube number and survival in mdx and C57BL/10 cultures

The numbers of multinucleated myotubes in skeletal muscle cell cultures from *mdx* and C57BL/10 cells (after 7- or 11-day pretreatment with either creatine or PDN) were determined as described in Section 2, at days 10 and 14 of culture. The results (Table 2) show that myotube numbers in *mdx* cultures were increased 4–6-fold after pretreatment with creatine or with PDN. Both agents had similar effects on C57BL/10 cultures, but to a smaller extent (2–4-fold increase, Table 2).

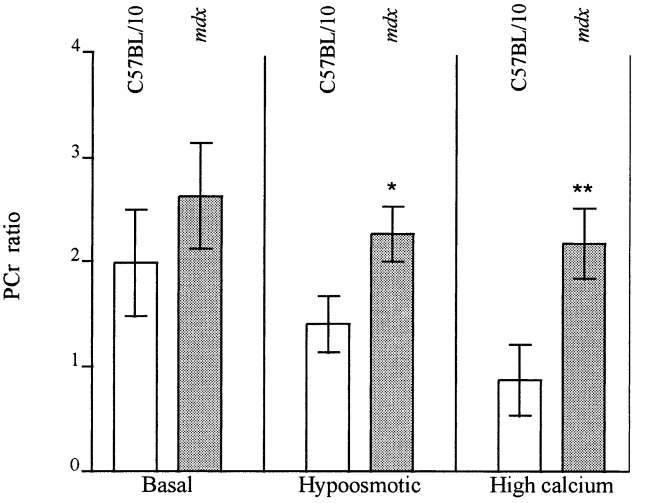

Fig. 4. Effects of creatine pretreatment on cellular PCr levels. Myotubes were prepared and PCr was measured as described in Section 2. Results are expressed as ratios of creatine-pretreated versus untreated C57BL/10 (open columns) or *mdx* (hatched columns) myotubes exposed to stress protocols. Asterisks indicate significantly different values (*$P < 0.01$, **$P < 0.001$, Student's *t*-test).

Creatine-treated *mdx* cultures exhibited more myotube branching and an increase in size relative to untreated *mdx* or control cultures. Increased myotube survival was apparent up to 17 days after creatine pretreatment and percentages of trypan blue-positive cells were markedly decreased in creatine-treated versus untreated controls (data not shown).

3.6. ATP and phosphocreatine measurements in mdx and C57BL/10 myotubes exposed to stress

ATP levels in *mdx* or C57BL/10 myotubes were not significantly different from each other and were not affected by either stress treatment resulting in ratios close to 1 (Table 3). In contrast, PCr levels in *mdx* myotubes were about half of those in controls, and high $[Ca^{2+}]_{extrac}$, but not hypo-osmotic shock, decreased these further (Table 3). Supplementation with 20 mM creatine increased PCr levels in both control and *mdx* cells (Table 3, Fig. 4), this effect being even more pronounced in *mdx* cells (Fig. 4).

4. Discussion

Calcium homeostasis in dystrophin deficient skeletal muscle cells derived from DMD patients and *mdx* mice has been shown to be dysfunctional, leading to a chronic Ca^{2+} overload [9,11,12,14,31–34]. We have exposed *mdx* and C57BL/10 control myotubes to reduced osmolarity or high $[Ca^{2+}]_{extrac}$, and have found increases in $[Ca^{2+}]_c$ (Fig. 1) as well as in

Table 2
Creatine increases myotube numbers in C57BL/10 and *mdx*-derived skeletal muscle cultures

Mouse strain	Day	Myotubes/field		
		Control	20 mM CR	1 μM PDN
C57BL/10	10	10.3 ± 1.99	24.3 ± 1.27	20.6 ± 2.99
	14	6.3 ± 1.11	23.2 ± 1,39	193. ± 1.56
mdx	10	8.6 ± 1.13	38.6 ± 1.26	35.1 ± 2.85
	14	6.6 ± 1.14	39.2 ± 1.66	34.3 ± 1.89

Analyses were performed as described in Section 2.

Table 3
Effect of creatine on ATP and PCr ratio (*mdx*/C57BL/10) under basal or stress conditions

	mdx/C57BL/10 ration (±S.D.)			
	untreated		Creatine (20 mM)	
	ATP	PCr	ATP	PCr
Basal conditions	0.98 ± 0.02	0.46 ± 0.02	0.92 ± 0.05	0.61 ± 0.08
Osmotic shock	0.91 ± 0.02	0.42 ± 0.05	0.93 ± 0.06	0.81 ± 0.01
High $[Ca^{2+}]_{extrac}$	0.90 ± 0.25	0.33 ± 0.01	0.91 ± 0.12	0.65 ± 0.11

$^{45}Ca^{2+}$ influx (Fig. 2) in *mdx* but not in control myotubes. This is consistent with an increase in stretch or 'leak' Ca^{2+} channel activity [6,9]. Chronically elevated $[Ca^{2+}]_c$ is known to cause activation of Ca^{2+}-dependent proteases (e.g. the calpains), which in turn can increase Ca^{2+} leak channel activity [14], reduce mitochondrial function (and therefore ATP synthesis) and eventually lead to muscle cell apoptosis or necrosis [35].

The hypo-osmotic or hypercalcemic stress-induced increases in $[Ca^{2+}]_c$ in *mdx* myotubes were significantly inhibited by several days of pretreatment with creatine (Fig. 1). The extent of inhibition was comparable to that which we have previously reported for the glucocorticoid PDN or other steroids [7,27]. This effect of creatine could be due to a decrease in sarcolemmal (SL) leak channel-mediated Ca^{2+} influx, or to improved sarcoplasmic (SR) uptake of cytosolic calcium. Creatine pretreatment of *mdx* myotubes did, however, not affect the stress-induced increase in $^{45}Ca^{2+}$ influx (Fig. 2), thus ruling out that creatine affected cellular Ca^{2+} entry. An effect on the SL Ca^{2+} ATPase or on the SR Ca^{2+}-ATPase is likely. Stimulation of the SL Ca^{2+} ATPase would lead to increased cellular Ca^{2+} extrusion and of the SR Ca^{2+} ATPase to increased Ca^{2+} sequestration. As $^{45}Ca^{2+}$ efflux was not altered by creatine treatment (our own unpublished observations), an effect on the SL Ca^{2+} ATPase can be ruled out and the SR Ca^{2+} pump is the most likely target for creatine action. Such an effect would lead to a more efficient SR Ca^{2+} uptake which would not alter total cellular $^{45}Ca^{2+}$ influx, which is what was observed (Fig. 2B).

The direct coupling of creatine kinase (CK) to the SR Ca^{2+}-ATPase, the energetically most highly demanding Ca^{2+} pump in muscle [21], has been shown to convey a functional and kinetic advantage for Ca^{2+} sequestration [21–23]. The SR Ca^{2+} pump derives its ATP preferentially from PCr via the SR-bound CK which leads locally to a very high ATP/ADP ratio [36]. The crucial involvement of SR-bound CK in Ca^{2+} homeostasis of skeletal muscle has been corroborated by the phenotype of CK null-mutant transgenic mice which have severe problems in calcium handling and relaxation of their skeletal muscles and which overproduce as a compensatory measure a grossly enlarged tubular SR membrane network [24].

Mdx cells showed a reduced PCr content and long-term creatine exposure led to increased PCr levels (Table 3). These results are consistent with those previously reported which demonstrate similar ATP levels but decreased PCr levels in human and murine dystrophic muscles relative to control [37].

One way that elevated [PCr] could lead to long term metabolic changes is by preventing the activation of the AMP-dependent protein kinase. This enzyme, which has recently been shown not only to be regulated by the ATP/AMP ratio, but also by the PCr/Cr ratio [38] can specifically phosphorylate CK downregulating its activity [38]. Therefore, increasing the cellular PCr levels by creatine supplementation, should increase the local ATP concentrations, and this is bound to lower ADP and even more so AMP concentrations [19]. These combined events, higher PCr and lower AMP levels, would keep the AMP-dependent protein kinase inactive [38], thus preventing the downregulation of CK activity [35]. Taken together, creatine supplementation should increase the availability of PCr for an active form of the SR-bound CK, which increases the local supply of the SR-Ca^{2+} pump with ATP and consequently the efficiency of Ca^{2+} sequestration [35].

In addition to this energy-donating effect of creatine, it has recently been shown that binding of creatine to the mitochondrial isoform of CK directly inhibits the formation of the mitochondrial permeability transition pore, thus inhibiting apoptosis [35].

Production of new myotubes is dependent on the rapid production and fusion of myoblasts and/or satellite cells, which is an energy and calcium requiring process. Creatine-treated *mdx* and C57BL/10 cultures were found to exhibit a greater number of myotubes, an elevated degree of branching, and an increase in size relative to untreated control myotube cultures (Table 2). Creatine also increased *mdx* and C57BL/10 myotube viability, extending the life of primary cells in culture to 20 days. We hypothesize that adding creatine at the time of fusion of the myoblasts provides energy for dystrophic cells and prevents an accumulation of Ca^{2+} in the cytoplasm. Due to an increased influx of Ca^{2+} into dystrophic myotubes, the Ca^{2+}-ATPases may be functioning at a higher rate during differentiation, relative to control cells. The energy provided by the increased PCr could be used continuously to regulate cytosolic Ca^{2+} levels during fusion and differentiation and may reduce the activity of Ca^{2+}-activated proteases and thus reduce membrane instability and further Ca^{2+} leakage.

Oral supplementation with creatine has been shown, in normal subjects, to lead to elevated levels of both total creatine and phosphocreatine in muscles, thereby improving the cellular energy status and muscle performance and recovery following strenuous exercise [39]. Based on our results, showing a marked improvement in *mdx* muscle cell survival and Ca^{2+} homeostasis, especially after stress, and on preliminary findings with patients with neuromuscular diseases [40,41] we propose that creatine is a substance with potential therapeutic properties which should be investigated as a possible adjuvant to established therapies [42].

Acknowledgements: We thank Drs. M. Markert, Y. Dunant and T. Buetler for use of equipment and helpful discussion, and Philippe Lhote for excellent technical assistance. This work was supported by the Swiss Foundation for Research on Muscle Diseases, the Association Française contre les Myopathies, the Roche Research Foundation, and Swiss National Science Foundation Grants 3100.47294.96 (to U.T.R.) and 3100.50824.97 (to T.W.).

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