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Modulation by prednisolone of calcium handling in skeletal muscle cells

*,[1]Laurent Metzinger, Anne-Catherine Passaquin, Wim J. Leijendekker, *Philippe Poindron & [2]Urs T. Rüegg

Pharmacology Group, School of Pharmacy, University of Lausanne, 1015 Lausanne, Switzerland and *Département d'Immunologie, d'Immunopharmacologie et de Pathologie, Faculté de Pharmacie, BP24, Université Louis Pasteur, Strasbourg, 67401 Illkirch, France

1 Increased calcium (Ca^{2+}) influx has been incriminated as a potential pathological mechanism in the chronic skeletal muscle degeneration exhibited by Duchenne muscular dystrophy (DMD) patients. We have studied the influence of the glucocorticoid α-methylprednisolone (PDN), the only drug known to have a beneficial effect on the degenerative course of DMD, on Ca^{2+} handling in the C2 skeletal muscle cell line.

2 PDN, when added 3 days (when myoblasts start to fuse into myotubes) after cell seeding, led to a 2 to 4 fold decrease in cellular Ca^{2+} uptake. This decrease was independent of the extracellular Ca^{2+} concentration applied to cells. The effect took at least 24 h in order to become established (PDN of 10^{-5} M) and took longer for lower PDN concentrations (EC_{50} of *ca.* 10^{-6} M at day 5, $10^{-6.5}$ M at day 7 and $10^{-7.5}$ M at day 9 in culture).

3 Cellular calcium accumulation was also decreased in PDN-treated myotubes exposed to $^{45}Ca^{2+}$-containing medium for 1 to 6 days.

4 No effect of PDN was seen on $^{45}Ca^{2+}$ efflux; a decrease in the amount of $^{45}Ca^{2+}$ released was observed due to the reduction of cellular $^{45}Ca^{2+}$ loading.

5 PDN treatment led to an approximately 2 fold decrease in basal cytosolic Ca^{2+} concentration.

6 Three antioxidant drugs (lazaroids), previously shown to enhance *in vitro* skeletal muscle cell differentiation to the same extent as PDN, induced a similar decrease in Ca^{2+} influx.

7 Our results suggest that long-term incubation of C2 cells with PDN leads to a decrease of the size of the cellular Ca^{2+} pools and to reduced resting cytosolic Ca^{2+} levels. Part of the beneficial effect of PDN in DMD patients could be attributed to a reduction of Ca^{2+} influx and of the size of Ca^{2+} pools in dystrophic muscle fibres.

Keywords: Duchenne muscular dystrophy; calcium; prednisolone; skeletal muscle; C2 cell line

Introduction

Duchenne muscular dystrophy (DMD) is a common X-linked myopathy caused by a defect of a gene that encodes the spectrin-like protein, dystrophin (Hoffmann *et al.*, 1987; Koenig *et al.*, 1987). Dystrophin is present mainly in skeletal muscle fibres (Zubrzycka-Gaarn *et al.*, 1988) where it probably links the cytoskeleton, *via* a complex composed of several proteins and glycoproteins, to the extracellular matrix protein laminin (for review, see Love *et al.*, 1993; Matsumura & Campbell, 1994). Dystrophin is believed to stabilize the sarcolemmal membrane and dystrophin-deficient muscle fibres have indeed been shown to be susceptible to exercise-induced contraction (Petrof *et al.*, 1993). This would in turn lead to the chronic muscle degeneration characteristic of DMD (for review, see Iannacone, 1992). Several other functions have also been proposed for dystrophin, including a role in signal transduction (Millner *et al.*, 1993; Madhavan & Jarret, 1994) and a regulation of intracellular calcium. Hoffman & Schwartz (1991) postulated that an instability of dystrophin-deficient sarcolemmal membranes would cause an increased Ca^{2+} entry into the muscle fibre. An enhancement of total Ca^{2+} content has indeed been found in the muscles of the *mdx* mouse (Dunn & Radda, 1991), which is the most commonly studied animal model of DMD (Sicinski *et al.*, 1989). Furthermore, cultivated skeletal muscle cells derived from both DMD patients and *mdx* mice exhibit an increase in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$ (Fong *et al.*, 1990; Turner *et al.*, 1991). This increase is localized mainly in areas near the sarcolemma and Ca^{2+} influx mechanisms would thus be implicated (Turner *et al.*, 1991). An enhancement of the open probability of leak channels (Fong *et al.*, 1990), an activation of stretch-sensitive channels (Franco & Lansman, 1990) and/or a fragile and leaky membrane (Hoffman & Schwartz, 1991) would contribute to this phenomenon. Several authors have proposed a correlation between the increased $[Ca^{2+}]_i$ and the enhanced protein degradation found in *mdx* muscle (Turner *et al.*, 1988; McLennan *et al.*, 1991; Kämper & Rodemann, 1992). They postulated that the elevated Ca^{2+} levels would activate Ca^{2+}-dependent proteases which would in turn, at least partly, be responsible for muscle necrosis. Turner *et al.* (1993) have shown that protease inhibitors can abolish the altered Ca^{2+} regulation found in dystrophic muscle.

Among several drugs tested in the treatment of DMD, only glucocorticoids have been shown to have a beneficial effect (Mendell *et al.*, 1989; for review, see Kahn, 1993). It was clinically demonstrated that the glucocorticoid α-methylprednisolone (PDN) is able to improve or sustain muscular strength and function in DMD boys for at least three years (Fenichel *et al.*, 1991). We and others have already shown that PDN and other glucocorticoids are able to enhance *in vitro* differentiation of skeletal muscle cells derived from normal (Guerriero & Florini, 1980; Braun *et al.*, 1989; Kaplan *et al.*, 1990; Sklar & Brown, 1991; Vilquin *et al.*, 1992; Passaquin *et*

[1]Present address: Molecular Genetics Group, Institute of Molecular Medicine, John Radcliffe Hospital, Headington, Oxford OX3 9DU, U.K.
[2]Author for correspondence.

al., 1994) and dystrophin-deficient muscle (Metzinger *et al.*, 1993; Passaquin *et al.*, 1993). Therefore the beneficial effect of PDN in DMD can be attributed, at least partly, to the preservation of functional muscle.

In this study, we have investigated a possible action of PDN on the dysregulation of Ca^{2+} handling observed in dystrophin-deficient muscle. To test this hypothesis, we have investigated the action of this agent on Ca^{2+} handling in C2 cells, a mouse skeletal muscle cell line (Yaffe & Saxel, 1977) which provides a model that recapitulates *in vitro* myogenesis. We have also tested the action of three lazaroids, compounds which are structurally related to PDN but show antioxidant properties and that have been shown to exhibit the same action as PDN with respect to *in vitro* skeletal muscle cell differentiation (Metzinger *et al.*, 1994).

Methods

Cell culture

The mouse skeletal muscle cell line C2 was maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco, Basel, Switzerland) supplemented with 20% foetal calf serum (FCS, Biological Industries, Kibbutz Beth Haemek, Israel) and 10 mg l^{-1} ciproxine (Bayer, Zürich, Switzerland). Cells were plated in 6- ($[Ca^{2+}]_i$ experiments) or 24-well (influx and efflux measurements) plates (coated with 2% gelatine, Sigma, Buchs, Switzerland) at 10^4 cells per cm^2 in this serum-rich medium and incubated at 37°C in a water-saturated atmosphere of 95% air and 5% CO_2. After 72 h in culture (3 days *in vitro*; DIV3), confluence was reached and the serum-rich medium was changed to a serum-poor medium (DMEM supplemented with 2% FCS) in order to induce the fusion of myoblasts into myotubes.

Culture treatment

PDN was obtained from Upjohn (Kalamazoo, MI, U.S.A.). The lazaroids, U-74006F (tirilazad mesylate; 21-[4-(2, 6-di-1-pyrrolidinyl-4-pyrimidinyl)-1-piperazinyl]-16-methyl-(16α)-pregna-1, 4, 9 (11)-triene-3, 20-dione monomethanesulphonate), U-74389F (21-[4-(2, 6-di-1-pyrrolidinyl-4-pyrimidinyl)-1-piperazinyl]-pregna-1, 4, 9 (11)-triene-3, 20-dione monomethane-sulphonate) and U-83836E (2-[(4-(2, 6-di-1-pyrrolidinyl-4-pyrimidinyl)-1-piperazinyl)-methyl]-3, 4-dihydro-2, 5, 7, 8-tetramethyl-2H-1-benzopyrane-6-ol, dichlorhydrate) were kindly provided by Drs E.D. Hall and J.M. McCall (Upjohn). Freshly prepared stock solutions were added to the cultures to be treated. Vehicle was sterile distilled water for U-83836E and PDN, and ethanol for U-74006F and U-74389F. Compounds were added at DIV 4 (unless specified otherwise), when myoblasts started to fuse into myotubes. This represents the optimal time for PDN (Metzinger *et al.*, 1993; Passaquin *et al.*, 1993) and lazaroids (Metzinger *et al.*, 1994) to develop their effects on skeletal muscle cell differentiation.

$^{45}Ca^{2+}$ influx measurements

The confluent monolayers of cells (in 24-well plates) were washed and pre incubated at 37°C for 10 min in 0.25 ml of physiological salt solution (PSS; composition in mM: NaCl 145, KCl 5, $MgCl_2$ 1, HEPES 5 and glucose 10, pH 7.6) containing 0.12 mM $CaCl_2$. Cells were washed three times with PSS and $^{45}Ca^{2+}$-influx was initiated by incubation with 0.2 ml PSS containing 1.2 mM $CaCl_2$ (unless specified otherwise) and 2 μCi of $^{45}Ca^{2+}$ (Amersham-Rahn, Zürich, Switzerland) for 20 min (unless indicated otherwise) at 37°C. The cells were subsequently washed 4 times (one column at a time, every 20 s) with ice-cold PSS containing no $CaCl_2$ but 0.2 mM EGTA (to remove extracellularly bound $^{45}Ca^{2+}$). NaOH (1N, 0.25 ml per well) was used to disintegrate the cells. The radioactivity of the lysate was measured by scintillation counting (Packard 460C, Zürich, Switzerland).

$^{45}Ca^{2+}$ efflux measurements

$^{45}Ca^{2+}$ loading and subsequent efflux measurements were performed on cells that had been washed twice with 0.5 ml PSS (containing 0.12 mM $CaCl_2$) and incubated for 20 min in 0.2 ml PSS (containing 0.12 mM $CaCl_2$) and 2 μCi of $^{45}Ca^{2+}$ at 37°C. After loading, cells were rapidly washed four times with 0.5 ml of ice-cold PSS (containing 1.2 mM $CaCl_2$). Efflux was initiated by addition to each well of prewarmed PSS (0.5 ml, 37°C) containing 1.2 mM $CaCl_2$ (one column at a time, every 20 s). Efflux media were collected after 3, 6, 9 and 11 min and immediately replaced with fresh medium (one column at a time, every 20 s). $^{45}Ca^{2+}$ radioactivity collected from each supernatant and from lysed cells was measured by scintillation counting.

$[Ca^{2+}]_i$ measurements with the fluorescent dye, fura-2

Myotubes were grown on glass coverslips (20 mm diameter) coated with 2% gelatine in 6-well plates as described above. They were washed twice with PSS containing 0.12 mM $CaCl_2$, and loaded for 45 min at 37°C in PSS containing 0.12 mM $CaCl_2$ and 5 μM of the acetoxymethylester of fura-2 (Molecular Probes, U.S.A.). After changing to fresh PSS the coverslips were mounted onto the thermostated (37°C) stage of a fluorescent microscope (Nikon Diaphot) and $[Ca^{2+}]_i$ levels were monitored by exciting an area comprising about 10 cells alternatively at 340 and 380 nm (PhoCal, Life Science Resources Ltd., Cambridge, UK). The emission was collected at 510 nm and the ratio of the emitted light from the 2 wavelengths (R) was used as a measurement of $[Ca^{2+}]_i$ (Grynkiewicz *et al.*, 1985). The $[Ca^{2+}]_e$ was increased stepwise from 0.12 mM $CaCl_2$ to 1.2 mM $CaCl_2$ and to 12 mM $CaCl_2$, and the fluorescence ratio (R) was determined during 2 min at each $[Ca^{2+}]$. At the end of each experiment calibration was performed by use of the Ca^{2+} ionophore, ionomycin (20 μM) (Gailly *et al.*, 1993; Berlin *et al.*, 1993). R_{max}, the fluorescence ratio under saturating $[Ca^{2+}]_i$ was calculated to be 5.6 ± 1.1 (value is mean ± s.d. ($n=6$)). R_{min}, the same ratio under Ca^{2+} free conditions was calculated by superfusing cells with PSS buffer containing 5 mM EGTA (20 min). Finally, the cells were exposed to PSS buffer with 2 mM $MnCl_2$ to quench the fura-2 fluorescence and to determine the level of background fluorescence. The background fluorescence was subtracted from all previous measurements. $[Ca^{2+}]_i$ was calculated from the following equation (Grynkiewicz *et al.*, 1985):

$$[Ca^{2+}]_i = K_d \beta \, (R - R_{min})/(R_{max} - R)$$

Apparent K_d was taken to be 224 nM (Grynkiewicz *et al.*, 1985) and β, the ratio of the 380 nm fluorescence under minimum and maximum $[Ca^{2+}]_i$ levels was determined to be 7.4 ± 2.8 (mean ± s.d. ($n=6$)). R_{max}, R_{min} and β determined in C2 cells treated with PDN (10^{-5} M) were not significantly different from untreated C2 cells.

Expression of results and statistical analysis

Cultures were established and experiments were performed at least in quadruplicate. Sets of experiments were carried out at least twice, one representative experiment being illustrated in the figures. Statistical differences between control and treated cultures were evaluated by a one-tailed Student's *t* test using the Statview 512+ software (Apple, Cupertino, CA, U.S.A.).

Results

PDN decreases $^{45}Ca^{2+}$ influx

The effects of α-methylprednisolone (PDN) and related compounds on Ca^{2+} handling of myotubes of the C2 skeletal muscle cell line were studied after 4 to 10 days in culture.

$^{45}Ca^{2+}$ influx was stimulated by increasing the Ca^{2+} concentration of the incubation buffer from 0.12 mM to 1.2 mM (see Methods) and cellular $^{45}Ca^{2+}$ accumulation was determined after various times of exposure to Ca^{2+} containing buffer. In control cells, a first rapid phase of increase of influx occurred (lasting 1 to 2 min) which was followed by a less rapid increase and, after 20 min, an apparent steady-state was reached (Figure 1). When cells were treated from day 4 (time of myoblast fusion) to day 10 in culture with 10^{-5} M PDN, the early rapid phase was abolished and $^{45}Ca^{2+}$ incorporation levelled off at values 3 to 4 times lower than in non-treated cells. The results described below were obtained at steady state, i.e. after 20 min of incubation with $^{45}Ca^{2+}$ containing buffer.

In order to test the sensitivity of Ca^{2+} influx to high extracellular Ca^{2+} concentrations ($[Ca^{2+}]_e$), cells were first incubated in low Ca^{2+} buffer (0.12 mM) and $[Ca^{2+}]_e$ was increased 10 and 100 fold in two steps (in buffers of the same $^{40}Ca^{2+}/^{45}Ca^{2+}$ ratio). This led to a 4 to 6 fold enhancement of $^{45}Ca^{2+}$ influx in both PDN-treated (10^{-5} M) and non-treated cells (Figure 2). Under all three conditions, PDN pretreatment resulted in a constant 2 to 2.2 fold decrease of the cellular $^{45}Ca^{2+}$ content.

Using cells after 8 days in culture the time of PDN treatment necessary for decreasing $^{45}Ca^{2+}$ influx was then determined. No significant effect was seen when the myotubes were pretreated for 3, 6, or 24 h. $^{45}Ca^{2+}$ flux decreased 2 fold only when PDN was added 96 h before the experiment (DIV 4) (Figure 3).

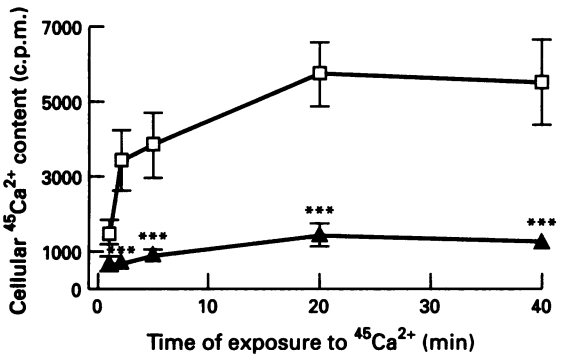

Figure 1 Effect of PDN on the time course of $^{45}Ca^{2+}$ influx. Myotubes were treated with PDN (10^{-5} M; ▲) from DIV 6 to 10 or were left untreated (□). $^{45}Ca^{2+}$ content was measured at DIV10 after various times of exposure to $^{45}Ca^{2+}$ containing buffer. Symbols indicate means ± s.d.; $^{***}P < 0.001$ ($n = 4$).

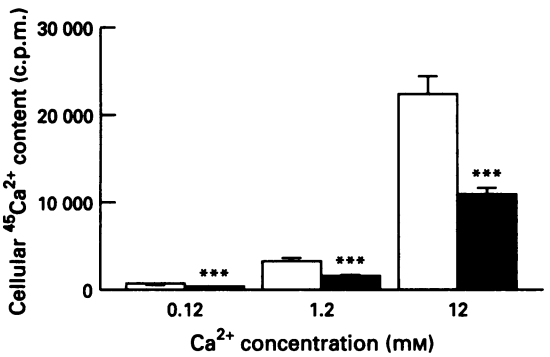

Figure 2 Effect of PDN on increased $[Ca^{2+}]_e$. Myotubes were preincubated for 10 min with a buffer containing 0.12 mM Ca^{2+}, and then for 20 min in a buffer containing various $[Ca^{2+}]_e$ (0.12, 1.2 or 12 mM) and $^{45}Ca^{2+}$. Open columns, control cells; solid columns, PDN-treated cells. Mean with s.d. are shown. $^{***}P$ controls/PDN < 0.001 ($n = 4$).

Sensitivity of the effects of PDN after various times in culture

$^{45}Ca^{2+}$ influx was measured at days 1, 3 and 5 in culture after pretreatment at DIV 4 with various concentrations of PDN. After 24 h (DIV 5), PDN had a slight but significant effect at concentrations of 10^{-4} M and 10^{-5} M (Figure 4), the effect becoming more pronounced at longer exposure times. For lower PDN concentrations the effect took longer to be established, i.e., at 10^{-6} M PDN after 3 days (DIV 7) and at 10^{-8} M after 5 days (DIV 9). EC_{50} values were *ca.* 10^{-6} M at DIV 5, $10^{-6.5}$ M at DIV 7 and $10^{-7.5}$ M at DIV 9 (Figure 4).

Long term effect of PDN on Ca^{2+} uptake

In order to assess the effect of PDN on chronic $^{45}Ca^{2+}$ accumulation in C2 cells, the tracer was added together with PDN at DIV 4 to the cultures. Myotubes were washed after 1 and 6 days and cell-associated $^{45}Ca^{2+}$ was measured. This chronic exposure to PDN led to a 50% decrease in $^{45}Ca^{2+}$ accumulation as compared to control (Figure 5). However, PDN had only a marginal effect on $^{45}Ca^{2+}$ chronic accumulation when cells were treated for only 24 h.

Effect of PDN on $^{45}Ca^{2+}$ efflux

The cellular pools were loaded to steady state with $^{45}Ca^{2+}$ (Figure 1) and efflux was initiated (see Methods). The amount of released $^{45}Ca^{2+}$ decreased with time, and the PDN-treated cells displayed a $^{45}Ca^{2+}$ release that was diminished by about 50% as compared to non-treated cells (not shown). The cell-associated $^{45}Ca^{2+}$ remaining after the efflux measurement was also 50% lower in PDN-treated cells. Therefore the kinetics of $^{45}Ca^{2+}$ release seem not to be affected by PDN, but the approximately 2 fold decrease in efflux in PDN treated cells reflects a reduced cellular $^{45}Ca^{2+}$ loading.

PDN treatment decreases basal $[Ca^{2+}]_i$

We next investigated the effect of PDN on $[Ca^{2+}]_i$ in myotubes using the fluorescent probe fura-2. Resting $[Ca^{2+}]_i$ in cells from DIV 6 to 9 was not statistically different (data not shown). $[Ca^{2+}]_i$ of cells treated for 4 days with PDN (10^{-5} M at DIV 4) was decreased by 58% during exposure with incubation buffer containing 0.12 mM Ca^{2+} (Table 1). A stepwise increase in $[Ca^{2+}]_i$ was triggered by enhancing the Ca^{2+} concentration of the incubation buffer from 0.12 mM to 1.2 mM and then to 12 mM. In control cells, this resulted in an increase in $[Ca^{2+}]_i$ of 138% and 192% respectively. In PDN-treated cells, the increase in $[Ca^{2+}]_i$ was of the same extent (162% and 214% respectively) but $[Ca^{2+}]_i$ values remained at least 50% lower than in controls.

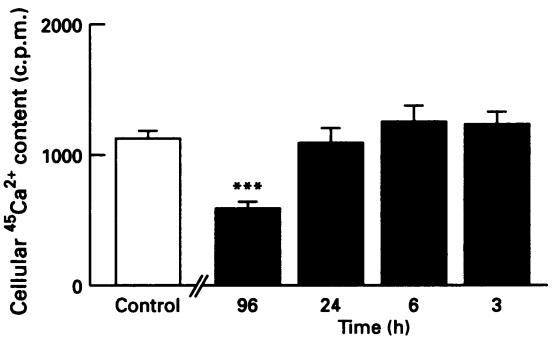

Figure 3 Kinetics of PDN action. PDN (solid columns) was added either as under standard conditions (96 h before the experiment), or 24 h, 6 h and 3 h before determining $^{45}Ca^{2+}$ influx. Measurements were done at DIV8; control cells, (open column). Results are expressed as cellular $^{45}Ca^{2+}$ content ± s.d.; $^{***}P < 0.001$ ($n = 4$).

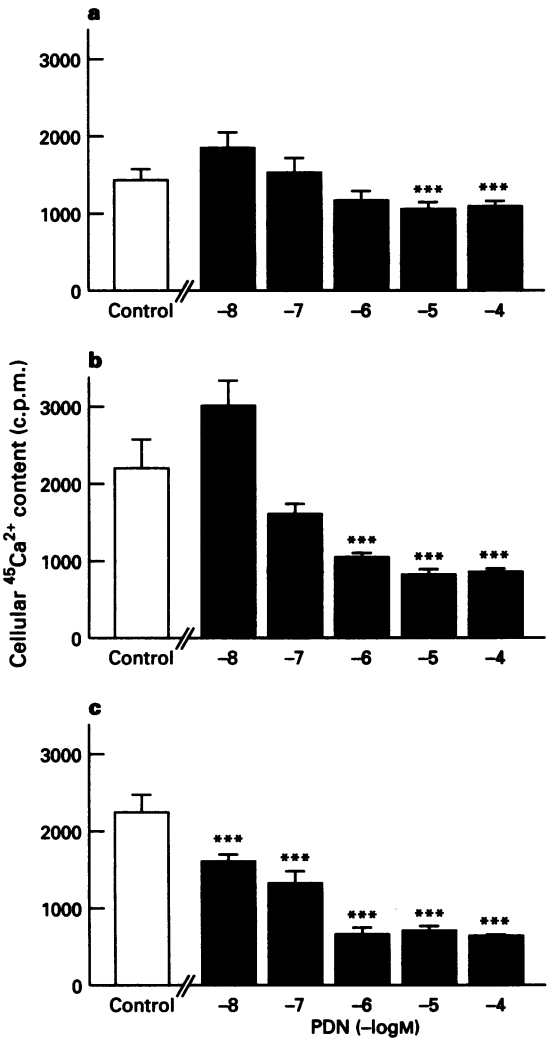

Figure 4 Concentration-response curves after various times of exposure to PDN in culture. PDN (solid columns) was added at day 4 at various concentrations. $^{45}Ca^{2+}$ influx was determined at (a) DIV 5, (b) 7 and (c) 9. Con = control cells (open column). Results are expressed as cellular $^{45}Ca^{2+}$ content ± s.d.; ***$P < 0.001$ ($n = 4$).

Antioxidant lazaroids decrease $^{45}Ca^{2+}$ influx

C2 cells were treated with PDN, with the vitamin-E derived lazaroid, U-83836E, or with the two glucocorticoid-derived lazaroids, U-74006F and U-74389F. All compounds were added at DIV 4. Lazaroids were added at a concentration of 10^{-6} M as this had previously yielded optimal results with respect to skeletal muscle cell differentiation (Metzinger *et al.*, 1994). The two glucocorticoid-derived lazaroids, U-74006F and U-74389F induced a decrease in $^{45}Ca^{2+}$ influx similar to the one of PDN, whereas treatment with the vitamin-E derived lazaroid, U-83836E, showed an approximately 25% decrease in $^{45}Ca^{2+}$ influx (Figure 6).

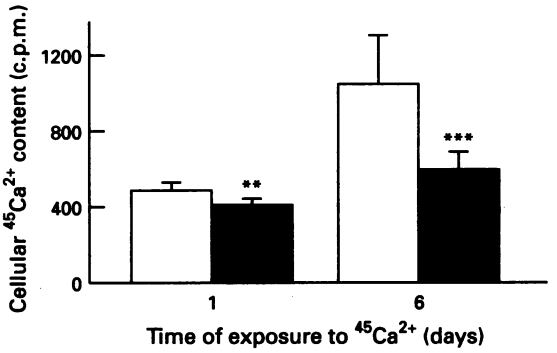

Figure 5 Effect of PDN on steady state $^{45}Ca^{2+}$ content. $^{45}Ca^{2+}$ and PDN were added together at DIV4 to the culture medium. Cellular $^{45}Ca^{2+}$ content was determined after 1 day and 6 days. Solid columns, PDN-treated cells and open columns, control cells. Results are expressed as cellular $^{45}Ca^{2+}$ content ± s.d.; ***$P < 0.001$ ($n = 8$).

Discussion

Clinical studies have shown beneficial effects of PDN for DMD patients (Fenichel *et al.*, 1991; Kahn, 1993). In a long-term effort to elucidate the basis for the action of PDN, we have previously shown that it promotes skeletal muscle cell differentiation (Braun *et al.*, 1989; Vilquin *et al.*, 1992; Metzinger *et al.*, 1993; Passaquin *et al.*, 1993; 1994). We have now investigated a possible link between the action of PDN and the known dysregulation of calcium homeostasis (Fong *et al.*, 1990; Dunn & Radda, 1991; Turner *et al.*, 1991; 1993). Here we report, for the first time, a major effect of PDN on Ca^{2+} handling in skeletal muscle cells.

First, PDN decreased $^{45}Ca^{2+}$ influx in differentiated skeletal muscle cultures, i.e. when added at DIV 4, the time when myoblast fusion into myotubes had occurred. PDN had no effect when added for periods of time shorter than 24 h, a lag time already noted for the promoting effect of PDN on skeletal muscle cell differentiation in several *in vitro* models (Braun *et al.*, 1989; Metzinger *et al.*, 1993; Passaquin *et al.*, 1993). The response of the cells to PDN increased between 1 and 5 days of PDN treatment, a concentration of PDN as low as 10^{-8} M resulting in a significant decrease after a 5 day treatment. The effect was independent of the $[Ca^{2+}]_e$ applied to the cells as PDN decreased $^{45}Ca^{2+}$ influx to the same extent when $[Ca^{2+}]_e$ was enhanced 10 and 100 fold. One could speculate that the PDN-triggered decrease in influx could simply be attributed to a decrease in the number of myogenic cells under the influence of this hormone. This is not the case, however, since PDN dramatically increases myotube numbers in C2 cell cultures (Passaquin *et al.*, 1994), a feature we constantly observed in our studies (data not shown). Second, PDN reduced chronic loading of $^{45}Ca^{2+}$ by approximately 50% in these skeletal muscle cells. This reduction of Ca^{2+} loading could be attributed to either a lowering of the loading capacity or a reduced size of the sarcoplasmic reticulum, the main Ca^{2+} storage system in skeletal muscle. Third, PDN treatment led to a reduced basal $[Ca^{2+}]_i$ (58% decrease versus untreated cells) even with elevated $[Ca^{2+}]_e$. Therefore, the main effect of PDN appears to be the lowering of cellular Ca^{2+} content which could

Table 1 Effect of prednisolone (PDN) on $[Ca^{2+}]_i$ in C2 cells exposed to various concentrations of $CaCl_2$

	0.12 mM $CaCl_2$		*1.2 mM $CaCl_2$*		*12 mM $CaCl_2$*	
	$R_{340/380}$	$[Ca^{2+}]_i$ (nM)	$R_{340/380}$	$[Ca^{2+}]_i$ (nM)	$R_{340/380}$	$[Ca^{2+}]_i$ (nM)
Control ($n = 6$)	0.48 ± 0.03	50 ± 9	0.54 ± 0.04	69 ± 14	0.62 ± 0.09	96 ± 32
PDN (10^{-5} M) ($n = 4$)	0.38 ± 0.01*	21 ± 10*	0.42 ± 0.02*	34 ± 11*	0.46 ± 0.01*	45 ± 10*

*Values significantly different from control ($P < 0.05$, Student's t test)

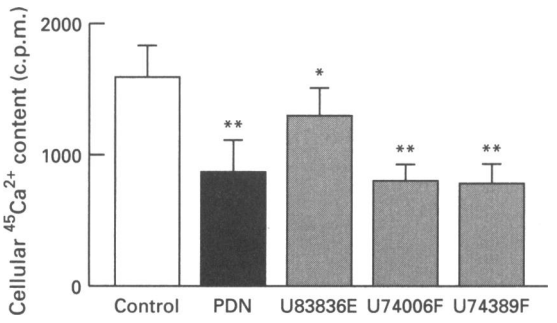

Figure 6 Effect of lazaroids on $^{45}Ca^{2+}$ influx. PDN (10^{-5}M; solid column) and lazaroids (10^{-6}M; stippled columns) were added at DIV4. $^{45}Ca^{2+}$ influx during 20 min was determined at DIV8. Control cells (open column). Results are expressed as $^{45}Ca^{2+}$ influx ± s.d.; $^{**}P<0.01$; $^{*}P<0.05$ ($n=4$).

explain the beneficial effect of this compound in DMD. It has already been shown that the Ca^{2+} entry blocker, diltiazem, alleviates the symptoms presented by a cardiomyopathic hamster model (Johnson & Bhattacharya, 1993). This compound also has a slight beneficial effect in DMD patients (Bertorini *et al.*, 1988) although no effect was found in another clinical study (Pernice *et al.*, 1988).

The mechanism involved in PDN-triggered reduction in $[Ca^{2+}]_i$ remains to be explained. It is interesting that the lazaroids tested, which are known antioxidants, mimic the stimulating effect of PDN on myogenesis (Metzinger *et al.*, 1994) and displayed the same effect as this steroid on Ca^{2+} influx. Since the vitamin E-derived U-83836E is devoid of glucocorticoid activity it is very unlikely that the mechanism responsible for the decline of Ca^{2+} influx involves the glucocorticoid receptor. Alternatively, these molecules could modulate Ca^{2+} handling via their antioxidant properties and/or by cell membrane stabilisation as does PDN (Massa *et al.*, 1975). Lazaroid and PDN actions could therefore be attributed to a decrease of Ca^{2+} exchange across the myotube membrane. Ca^{2+} entry through leak channels (Fong *et al.*, 1990; Franco & Lansman, 1990) appears to be increased in dystrophin-deficient muscle fibres (Fong *et al.*, 1990) and thereby leads to activation of Ca^{2+}-dependent proteases which in turn would be responsible for muscle necrosis. Therefore, the PDN-induced decrease in Ca^{2+} influx possibly compensates for an enhanced Ca^{2+} entry into dystrophin-deficient cells.

Several hypotheses, supported by many reports, have been put forward to explain the beneficial effects of PDN in DMD patients. (1) Reduction of muscle damage through immunosuppressive and anti-inflammatory properties of this glucocorticoid (Kissel *et al.*, 1991). In *mdx* mice, however, Weller *et al.* (1991) found that PDN did not change the *in vivo* prevalence of fibre necrosis and regeneration. (2) A direct effect on myogenesis has been noted (Guerriero & Florini, 1980; Askanas *et al.*, 1986; Misra & Entrikin, 1988; Braun *et al.*, 1989; Kaplan *et al.*, 1990; Sklar & Brown, 1991; Metzinger *et al.*, 1993; Passaquin *et al.*, 1993; 1994); an acceleration of the rate of fusion of quiescent myoblasts, so-called satellite cells (Bischoff, 1990), with DMD muscle fibres would therefore enhance muscle regeneration. (3) Protection of the newly formed myotubes by antioxidant action (Metzinger *et al.*, 1994) and/or inhibition of myotube apoptosis (Sklar & Brown, 1991). Our present work on the C2 skeletal muscle cells demonstrates that Ca^{2+} homeostasis is modulated by PDN and can account, at least in part, for the beneficial effect of PDN on muscular dystrophies.

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