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Calcium influx inhibition by steroids and analogs in C2C12 skeletal muscle cells

[1]Anne-Catherine Passaquin, [1]Philippe Lhote & [1,2]Urs T. Rüegg

[1]Pharmacology Group, School of Pharmacy, University of Lausanne, 1015 Lausanne, Switzerland

1 Glucocorticoids, namely α-methylprednisolone (PDN) and deflazacort, are the only drugs reported to have a beneficial effect on the degenerative course of Duchenne muscular dystrophy (DMD). Increased cytosolic calcium concentrations ($[Ca^{2+}]_c$) have been implicated as one of the pathological events responsible for the degeneration of dystrophic skeletal muscles. In previous studies, we have demonstrated that PDN treatment of both normal and dystrophic murine skeletal muscle cells was able to normalize elevated $[Ca^{2+}]_c$ and improved myogenesis. Here we have investigated the mechanism underlying the effects of glucocorticoids on cellular Ca^{2+} influx into C2C12 skeletal muscle cells.

2 Long-term incubation of C2C12 myocytes with PDN was necessary to observe a reduction of $^{45}Ca^{2+}$ influx. PDN was most effective in inhibiting $^{45}Ca^{2+}$ uptake when added for 4 days (at the time of fusion of myoblasts into myotubes) and to a lesser extent, when added after fusion. It was ineffective when added to C2C12 cells at the myoblast stage. Short PDN incubation times, at the time of fusion were insufficient to elicit a response.

3 Several steroids were tested for their ability to inhibit $^{45}Ca^{2+}$ influx in C2C12 myocytes. All four glucocorticoids examined were able to reduce Ca^{2+} influx, dexamethasone being the most potent (IC_{50} $3.14 \pm 0.34 \times 10^{-8}$ M). Mineralocorticoids (aldosterone and 11-deoxycorticosterone) were also able to reduce Ca^{2+} influx.

4 The vitamin E-derived lazaroid U-83836E and the glucocorticoid-derived lazaroid U-74389G also elicited a decrease in Ca^{2+} influx, but higher concentrations were necessary. Because both glucocorticoids and lazaroids display antioxidant properties, but U-83836E is devoid of glucocorticoid activity, the reduction in Ca^{2+} influx was suspected to be triggered *via* an antioxidant mechanism.

5 To test this hypothesis, we assessed the action of several antioxidants, such as vitamin E, vitamin C, 2-*tert*.-butyl-4-methoxyphenol (BHA), 2,6-di-*tert*.-butyl-4-methyl-phenol (BHT) and nordihydroguaiaretic acid (NDGA), on $^{45}Ca^{2+}$ influx. None of these agents had an effect on $^{45}Ca^{2+}$ influx. In addition, several oxidants were tested (either acutely or chronically) for their ability to elicit $^{45}Ca^{2+}$ influx in C2C12 myocytes and were found to be inactive.

6 The involvement of the glucocorticoid receptor on the modulation of Ca^{2+} influx was investigated. The glucocorticoid receptor antagonist mifepristone (code name RU38486, 10^{-6} M) caused a shift of two orders of magnitude of the PDN response. However, neither actinomycin D nor cycloheximide affected the response to PDN.

7 Results with the phospholipase A_2 inhibitor, manoalide, suggest that glucocorticoid-induced protein synthesis (e.g. enhanced stimulation of lipocortin) does not play a role in the reduction of calcium influx.

8 Our results suggest that steroids elicit a decrease in calcium influx in C2C12 skeletal muscle cells. This decrease is not due to an antioxidant mechanism or to a mechanism which requires gene expression. Since mineralocorticoids and U-83836E also had similar effects, the mechanism could belong to the non-genomic effects of corticoids (e.g. membrane stabilization). The beneficial effect of glucocorticoids in DMD could be attributed to a reduction of the pathological increase in Ca^{2+} influx *via* an effect on the sarcolemma.

Keywords: Antioxidant; C2C12 cells; Duchenne muscular dystrophy; calcium; glucocorticoid; lazaroid; manoalide; mifepristone; oxidant; skeletal muscle

Introduction

Glucocorticoids represent the only pharmacological treatment providing a beneficial effect for Duchenne muscular dystrophy (DMD) patients (Mendell *et al*., 1989; for review, see Kahn, 1993; Dubowitz, 1996). The glucocorticoids α-methylprednisolone (PDN) and deflazacort were shown to prolong ambulation and sustain muscular strength and function in DMD patients for at least three years (Fenichel *et al*., 1991; Angelini *et al*., 1994).

DMD is a common X-linked myopathy caused by a defect of the gene that encodes the spectrin-like protein dystrophin (Koenig *et al*., 1987). Dystrophin is present mainly in skeletal muscle fibres (Zubrzycka-Gaarn *et al*., 1988) where it links the cytoskeleton, *via* a complex composed of several proteins and glycoproteins, to the extracellular matrix protein laminin (for review, see Sunada & Campbell, 1995). Recently it was proposed that utrophin, a homologous protein, encoded by a gene located on human chromosome 6, could compensate for the lack of dystrophin (Tinsley *et al*., 1996).

Dystrophin is believed to stabilize the sarcolemmal membrane and dystrophin-deficient muscle fibres have been shown to be susceptible to exercise-induced contraction (Petrof *et al*., 1993) but it could also play a role in signal transduction (Madhavan & Jarret, 1994). Hoffman and Schwartz (1991)

[2] Author for correspondence.

postulate that an instability of dystrophin-deficient sarcolemmal membranes would cause an increased Ca^{2+} entry into muscle fibres. An enhancement of total Ca^{2+} content has been reported in the muscles of the *mdx* mouse (Turner *et al*., 1988; Dunn & Radda, 1991), the most commonly studied animal model of DMD (Sicinski *et al*., 1989). This increase is localized mainly in areas near the sarcolemma and Ca^{2+} influx mechanisms would thus be implicated (Turner *et al*., 1993). 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 fragilized and leaky membrane (Hoffman & Schwartz, 1991) have been characterized in dystrophic muscle.

We have recently shown that α-methylprednisolone (PDN) was able to reduce both elevated calcium influx and cytosolic calcium concentration ($[Ca^{2+}]_c$) in skeletal muscle cells from the C2C12 cell line (Metzinger *et al*., 1995) or from *mdx* mice (Leijendekker *et al*., 1996). PDN was also able to enhance utrophin expression (Passaquin *et al*., 1993; Pasquini *et al*., 1995) and to enhance *in vitro* differentiation (Braun *et al*., 1989; Kaplan *et al*., 1990; Metzinger *et al*., 1993; Passaquin *et al*., 1993). Lazaroids, a class of antioxidant compounds structurally related to PDN, exhibit the same action as PDN on *in vitro* skeletal muscle cell differentiation (Metzinger *et al*., 1994).

In this study, we have investigated the mechanism of action of glucocorticoids and lazaroids on Ca^{2+} influx in C2C12 skeletal muscle cells (Yaffe & Saxel, 1977) which provide a useful model for the study of *in vitro* myogenesis. In particular, we examined the underlying mechanisms in terms of antioxidant properties and genomic actions of glucocorticoids.

Methods

Cell culture

C2C12 skeletal muscle cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco, Basel, Switzerland) supplemented with 20% foetal calf serum (FCS, Gibco, Basel, Switzerland) and 10 mg/l ciproxine (Bayer, Zürich, Switzerland) as previously described (Metzinger *et al*., 1995). Cells were seeded in 24-well plates at 10^4 cells per cm^2 and incubated at 37°C in a water-saturated atmosphere of 95% air and 5% CO_2. After 72 h in culture, confluence was reached and the medium was switched to DMEM supplemented with 2% FCS in order to induce the fusion of myoblasts into myotubes.

Chemicals

Prednisolone was obtained from Upjohn (Kalamazoo, MI, U.S.A.); dexamethasone, prednisone, hydrocortisone, and 11-deoxycorticosterone were from Sigma (Buchs, Switzerland). The lazaroids U-74389G (21-[4-(2, 6-di-1-pyrrolidinyl-4-pyrimidinyl)-1-piperazinyl]-pregna-1,4,9 (11)-triene-3,20-dione monomethanesulphonate) 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, Kalamazoo, MI, U.S.A.) and were also purchased from Biomol (Wangen, Switzerland). Vehicle was sterile water for PDN and ethanol for U-74389G and U-83836E. Vitamin E (α-tocopherol acetate) was from Merck (Zürich, Switzerland). All compounds were of the purest grade available.

Culture treatment

Compounds (steroids, lazaroids, anti-oxidants and oxidants) were added to the cultures at the time of fusion, i.e. the third day in culture (unless specified otherwise) and Ca^{2+} influx was measured on day 7. 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. Oxidants were also tested acutely (i.e. within 1 h prior to the experiment).

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

The confluent monolayers of cells were washed twice 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 7.4, containing 0.12 mM $CaCl_2$. $^{45}Ca^{2+}$ influx was initiated by incubation with 0.2 ml PSS containing 1.2 mM $CaCl_2$ and 0.4 μCi of $^{45}Ca^{2+}$ (5–50 mCi/mg, Amersham-Rahn, Zürich, Switzerland) for 20 min (unless indicated otherwise) at 37°C. The cells were subsequently washed four times with ice-cold PSS containing no $CaCl_2$ but 0.2 mM EGTA (to remove extracellularly bound $^{45}Ca^{2+}$). Cells were then detached with 50 μl of trypsin (0.25%, wt/vol) per well and lysed with 250 μl of sodium dodecyl sulfate (SDS; 1%, wt/vol). The radioactivity of the lysate was measured by scintillation counting (Packard 460C, Zürich, Switzerland).

Expression of results and statistical analysis

Cultures were established in quadruplicate and experiments were performed in triplicate. Results are expressed as mean ± s.e.m. Statistical differences between control and treated cultures were evaluated by a two-tailed Student's *t*-test. IC_{50} values were calculated with GraphPad Prism Software (GraphPad Inc., U.S.A.).

Results

Time-dependent effects

C2C12 skeletal muscle cells were incubated with 10^{-6} M PDN for one to 6 h and for 1–4 days prior to the determination of $^{45}Ca^{2+}$ influx on the seventh day in culture. $^{45}Ca^{2+}$ influx was stimulated by increasing the Ca^{2+} concentration of the incubation buffer and cellular $^{45}Ca^{2+}$ accumulation was determined after 20 min (see Methods and Metzinger *et al*., 1995).

After a 24 h PDN treatment a reduction of approximately 25% in Ca^{2+} influx was observed, the effect becoming more pronounced after longer incubation times (Figure 1). Incubation periods shorter than 6 h were insufficient to elicit a decrease (Figure 1).

It has previously been reported that when glucocorticoids were added at the time of fusion of myoblasts (day 3 in culture) and subsequently washed out, only a short incubation time period (5 min to 1 h) was necessary to induce acetylcholine receptor expression in rat skeletal muscle cells (Braun *et al*., 1989). We examined whether such a short exposure to PDN during fusion was sufficient to reduce Ca^{2+} influx. PDN (10^{-6} M) was added for 1 min up to 1 h, washed out and influx was measured 4 days later. No effect was detected if PDN was present for up to 1 h at the time of fusion (Table 1).

Concentration-dependent modulation of $^{45}Ca^{2+}$ influx by corticosteroids

Several glucocorticoids and mineralocorticoids devoid of glucocorticoid activity were tested with respect to their ability to affect Ca^{2+} influx (Table 2). Compounds were added at day 3 and $^{45}Ca^{2+}$ influx was studied on the seventh day in culture. All four glucocorticoids tested were able to reduce $^{45}Ca^{2+}$ influx, dexamethasone being the most potent (Table 2 and Figure 2). Calculated IC_{50} values were $3.14 \pm 0.34 \times 10^{-8}$ M for dexamethasone, $1.42 \pm 0.18 \times 10^{-7}$ M for PDN, $1.62 \pm 0.03 \times 10^{-7}$ M for hydrocortisone, and $6.63 \pm 0.07 \times 10^{-7}$ M for prednisone. Comparison of maximal inhibition produced by these drugs show that PDN was the most efficacious (Table 2 and Figure 2). Surprisingly, the mineralocorticoids also potently reduced $^{45}Ca^{2+}$ influx. Both aldosterone and 11-deoxycorticosterone had IC_{50} values similar to those for dexamethasone; however, aldosterone exhibited a lower efficacy (Table 2 and Figure 2).

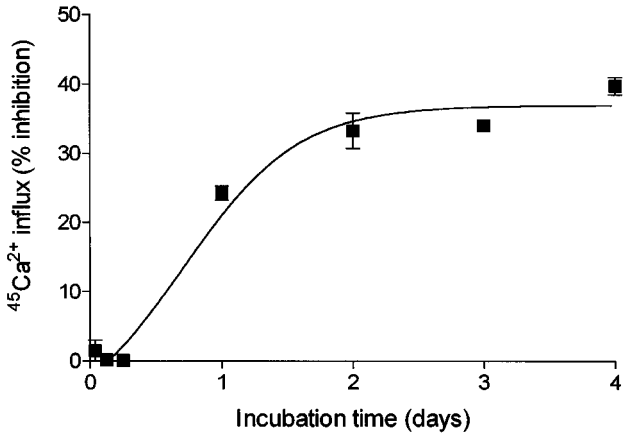

Figure 1 Time course of inhibition of $^{45}Ca^{2+}$ influx by α-methylprednisolone. C2C12 myocytes at day 3 in culture were exposed for various times to 10^{-6} M α-methylprednisolone and $^{45}Ca^{2+}$ influx was determined on the seventh day in culture. Results are expressed as the percentage of inhibition of cellular $^{45}Ca^{2+}$ influx ±s.e.m as compared to untreated cells. Two experiments were performed, each in quadruplicate.

Effect of lazaroids on calcium influx

The vitamin E-derived lazaroid U-83836E and the glucocorticoid-derived lazaroid U-74389G were added to C2C12 cells at the time of fusion (day 3) and at various concentrations in order to investigate their calcium-dependent effects as measured on day 7. Both compounds led to a decrease in $^{45}Ca^{2+}$ influx but at higher concentrations than the ones necessary for glucocorticoids (Figure 3). The lazaroid U-83836E which is devoid of glucocorticoid activity had a lower efficacy.

Effect of antioxidants and oxidants on calcium influx

Glucocorticoids display antioxidant properties (Uhler *et al.*, 1994). To test whether antioxidants were able to inhibit calcium influx, cells were pretreated with several antioxidants as described for glucocorticoids. Vitamin E, vitamin C, 2-*tert*.-butyl-4-methoxyphenol (BHA), 2,6-di-*tert*.-butyl-4-methylphenol (BHT) and, nordihydroguaiaretic acid (NDGA) were tested. Conversely to glucocorticoids, they did not affect calcium influx (Table 3).

Oxidants such as the sulfhydryl reagents, *tert*.-butyl hydroperoxide (TBHP) or thimerosal, have been shown to increase calcium influx into cardiac myocytes (Castro &

Table 1 Effect of exposure time with α-methylprednisolone (PDN) on $^{45}Ca^{2+}$ influx

	$^{45}Ca^{2+}$ uptake		*Protein content*	
Exposure time with PDN	*(c.p.m./well) (mean±s.d.)*	*(% of control) (mean±s.d.)*	*(μg/well) (mean±s.d.)*	*(% of control) (mean±s.d.)*
Control	448±31	100	68±2.6	100
1 min	443±15	98.8±3.3	66±0.9	97.0±1.3
10 min	452±15	100.8±3.3	70±0.4	102.9±0.6
30 min	461±16	102.9±3.6	66±2.5	102.9±3.9
1 h	474±33	105.8±7.4	71±2.5	104.4±3.7
4 days	293±9	65.4±2.0	49±0.2	72.0±0.3

PDN (10^{-6} M) was added on the third day of culture (time of fusion) and washed-out after short incubation times (1 min to 1 h). Influx was determined four days later.

Table 2 Inhibition of $^{45}Ca^{2+}$ influx into C2C12 myocytes by steroids and comparison with their relative affinities for glucocorticoid cytoplasmic receptors and *in vitro* activity

	Glucocorticoid activity		*Ca^{2+} influx*		
Agonists	**Relative receptor affinity*	**Approx. relative* in vivo *antiinflammatory activity*	*IC_{50} (nM)*	*Relative potency*	*Relative efficacy*
Hydrocortisone	1	1	162.5±2.7	1	1
α-methylprednisolone	11.9	4.5	142.2±1.8	1.14	1.47
Aldosterone	0.38	0	80.4±2.1	2.02	0.78
11deoxycorticosterone	0.39	0	54.4±1.3	2.99	1.02
Dexamethasone	7.10	30	31.4±3.4	5.18	1.21
Prednisone	0.05	3.5	663.1±7.0	0.25	0.67

*Data for glucocorticoid receptor affinity were obtained at 2°C and glucocorticoid activity refers to as anti-inflammatory activity (Ballard *et al.*, 1975).

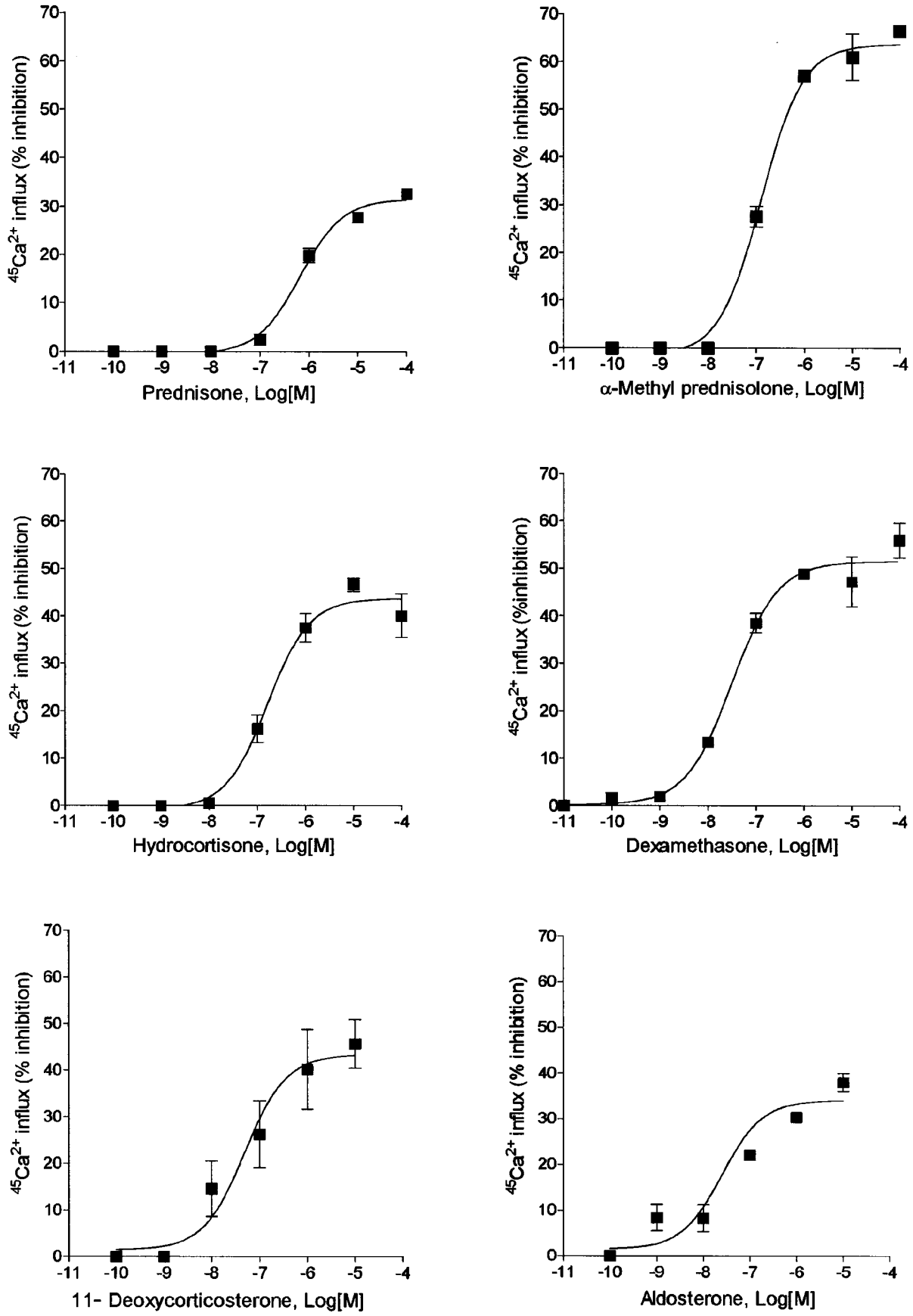

Figure 2 Concentration-response curves of four glucocorticoids and two mineralocorticoids. Steroids were added on the third day in culture at various concentrations. $^{45}Ca^{2+}$ influx was determined on the seventh day in culture. Results are expressed as the % of inhibition of cellular $^{45}Ca^{2+}$ influx $\pm$s.e.m. Three experiments were performed, each in quadruplicate.

Bhatnagar, 1993). We investigated whether oxidants could trigger such a calcium influx into C2C12 cells (either acutely or chronically). Neither TBHP nor thimerosal produced any response (Table 3). Other oxidants or radical producers (puromycin, pyrogallol, doxorubycin, paraquat and H_2O_2) were also tested in the same way and gave similar negative results (Table 3).

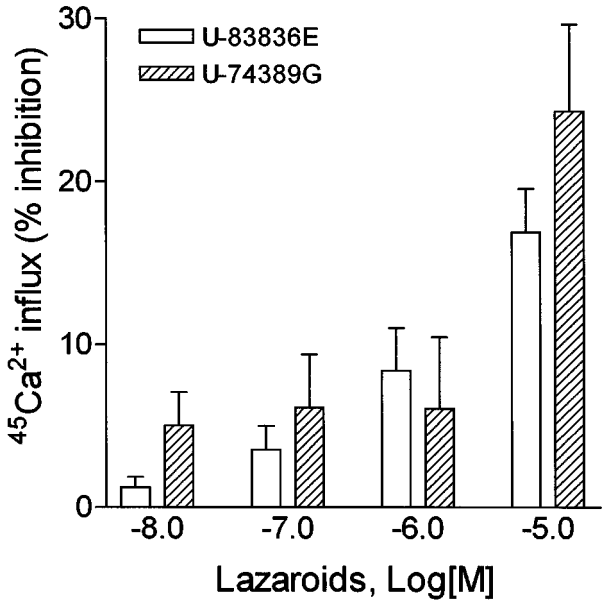

Figure 3 Concentration-response curves for two lazaroids. U74389G and U83836E were added on the third day in culture at various concentrations and $^{45}Ca^{2+}$ influx was determined on the seventh day in culture. Results are expressed as the % of inhibition of cellular $^{45}Ca^{2+}$ influx $\pm$s.e.m. Three experiments were performed and each expriment was done in quadruplicate.

Mifepristone modulation of PDN action

Mifepristone (RU38486) is a progesterone antagonist which has been reported to also exhibit antiglucocorticoid activity (Baulieu, 1989; Agarwai, 1996). To evaluate whether the inhibition of calcium influx is mediated *via* glucocorticoid receptors, this derivative was added 1 h prior to PDN (10^{-6} M) at day 3 and the effect was determined 4 days later. A concentration-dependent attenuation of the PDN-triggered inhibition of calcium influx was observed with an IC_{50} of $9.55 \pm 0.198 \times 10^{-8}$ M (Figure 4A). The concentration-response curve for PDN in the presence of RU38486 (10^{-6} M) was shifted to the right by about two orders of magnitude (Figure 4B). Concentrations of RU38486 up to 3×10^{-6} M prevented the decrease in total protein content due to PDN (at higher concentrations, RU38486 itself had a slight inhibitory effect on protein levels).

Effect of actinomycin D and cycloheximide

The transcriptional inhibitor actinomycin D and the translational inhibitor cycloheximide were used to investigate whether gene expression elicited by PDN was required for the inhibition of Ca^{2+} influx. Cells were treated with PDN in the presence of actinomycin D. Actinomycin D was not able to reverse the effect of PDN, although it did have an effect on the overall protein production (Figure 5). The inhibitory effect of PDN on the Ca^{2+} influx was also not altered by cycloheximide which was added on the same day as PDN at concentrations from 10^{-7} to 10^{-10} M (Figure 5).

Role of phospholipase A_2 and lipocortin

We have examined the possibility that inhibition of phospholipase A_2 (PLA_2) activity by glucocorticoid-induced lipocortin (Flower & Rothwell, 1994) could underly the effects

Table 3 Effects of treatments with several oxidant and antioxidant drugs on $^{45}Ca^{2+}$ influx. Data are expressed as percentage of control (100%)

Drug	*Drug conc. (mol/l)*	*$^{45}Ca^{2+}$ influx % control* Mean $\pm$ s.d.	*n*	*P*
α-Methylprednisolone	10^{-6}	48.07 $\pm$ 5.12	40	<0.001
Doxorubycine	10^{-5}	93.81 $\pm$ 5.42	4	ns
H_2O_2	10^{-5}	106.79 $\pm$ 6.55	16	ns
	10^{-4}	99.70 $\pm$ 3.16	16	ns
	10^{-3}	82.85 $\pm$ 5.17	4	ns
	10^{-2}	50.99 $\pm$ 9.09	4	toxic
Paraquat	10^{-4}	106.46 $\pm$ 4.91	4	ns
Puromycine	10^{-6}	100.45 $\pm$ 2.70	4	ns
Pyrogallol	10^{-4}	94.34 $\pm$ 3.02	4	ns
TBHP	10^{-4}	91.96 $\pm$ 5.57	4	ns
	10^{-5}	96.70 $\pm$ 3.36	4	ns
Thimerosal	10^{-8}	94.68 $\pm$ 7.97	4	ns
	10^{-7}	88.61 $\pm$ 3.93	4	ns
	10^{-6}	94.21 $\pm$ 0.83	4	ns
	10^{-5}	89.10 $\pm$ 2.03	8	ns
	10^{-4}	81.29 $\pm$ 1.36	8	ns
BHA	10^{-4}	82.14 $\pm$ 8.40	4	ns
BHT	10^{-5}	93.13 $\pm$ 8.13	8	ns
Vitamin E	5×10^{-4}	102.69 $\pm$ 5.38	4	ns
	5×10^{-5}	114.74 $\pm$ 1.97	4	ns
	3×10^{-5}	105.81 $\pm$ 1.89	4	ns
	10^{-5}	100.42 $\pm$ 1.06	4	ns
Vitamin C	5×10^{-5}	102.69 $\pm$ 5.38	8	ns

All compounds were added at the time of myocyte fusion. Myocytes have also been treated acutely (for various time periods, e.g. 2, 5 and 10 min) and no differences were noticed (data not shown).

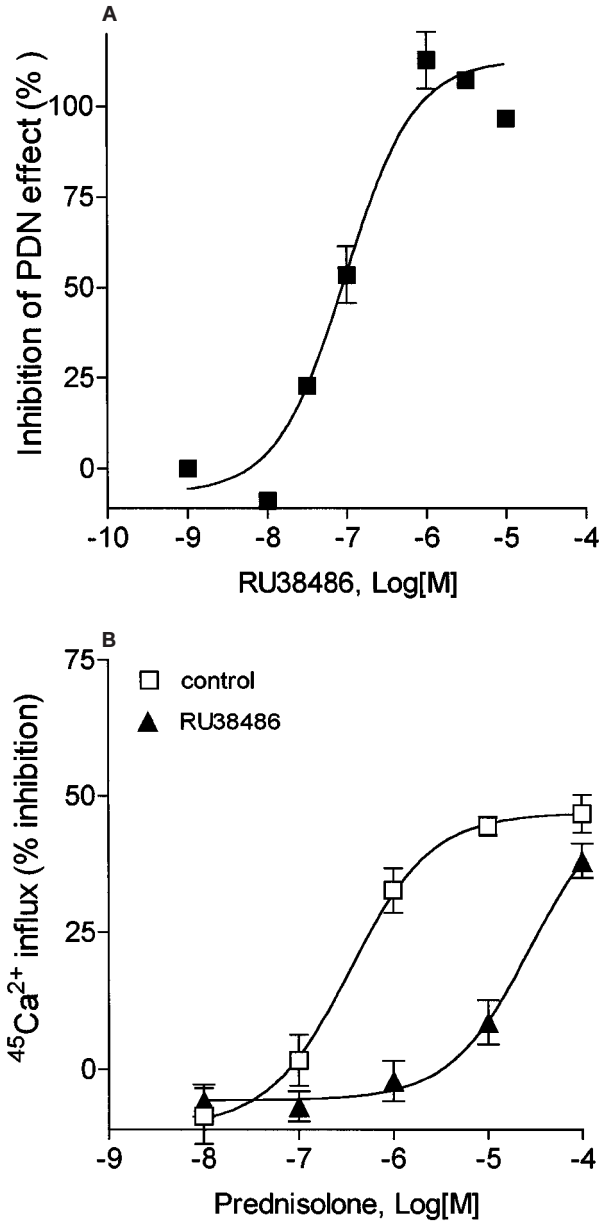

Figure 4 Effect of RU 38486 on the inhibition of $^{45}Ca^{2+}$ influx by α-methylprednisolone. (A) On the third day in culture, various concentrations of RU 38486 were added to the cells 1 h prior to addition of 10^{-6} M α-methylprednisolone. The effect of RU 38486 was determined on day 7 in culture. (B) Cells were either untreated or pretreated with 10^{-6} M RU 38486 1 h prior to the establishment of concentration-reponse curves for α-methylprednisolone.

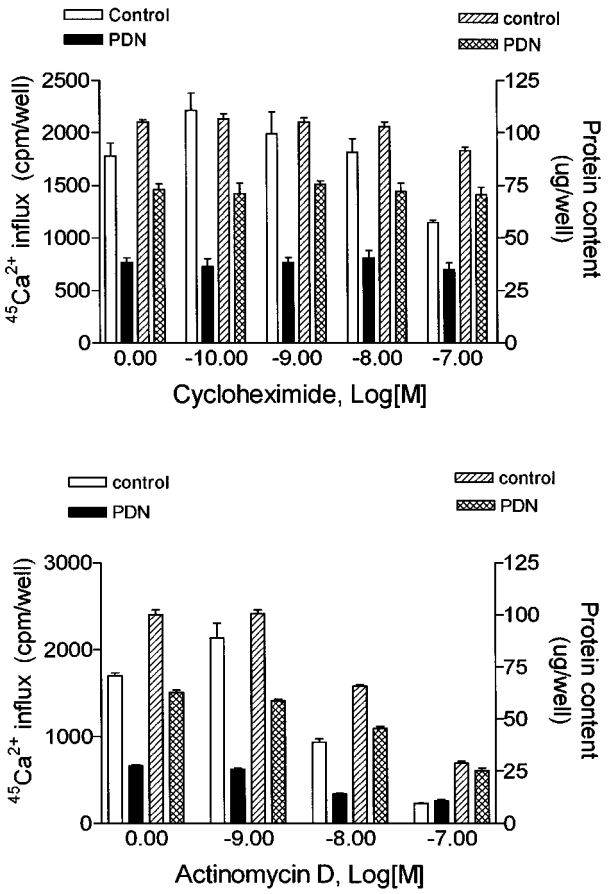

Figure 5 Effect of actinomycin D (ActD) and cycloheximide (CHX) on the PDN mediated inhibition of $^{45}Ca^{2+}$ influx and cellular protein content. C2C12 myocytes were treated with ActD or CHX from day 3 to 7 together with PDN (10^{-6} M). Results are means±s.e.m. of 2 experiments, each in quadruplicate.

seen on Ca^{2+}. The irreversible phospholipase A_2 inhibitor manoalide (Diamond *et al*., 1994; approximately IC_{50}, 10^{-7} to 10^{-8} M) was examined and compared to the effect of prednisolone. No effect was observed when confluent C2C12 cells were treated with manoalide at concentrations ranging from 10^{-8} to 10^{-5} M. However, a strong toxic effect was noticed at 10^{-5} M (data not shown).

Discussion

Even though anti-inflammatory glucocorticoids are well known for their muscle wasting adverse effects, they have been successfully used in clinical trials for palliative therapy of Duchenne muscular dystrophy (DMD) patients (for review, see Kahn, 1993; Dubowitz, 1996), in particular prednisolone (PDN) and deflazacort (Mendell *et al*., 1989; Fenichel *et al*., 1991; Angelini *et al*., 1994). Both the pathogenic mechanism responsible for the patients' severe muscle degeneration and the mechanism by which glucocorticoids increase muscle strength and function are unclear.

In an attempt to elucidate the mechanisms underlying the beneficial action of PDN, we have previously shown that it increased satellite cell proliferation and promoted myogenesis by increasing fusion indexes, number of myotubes and the expression of skeletal muscle cell markers of differentiation (Metzinger *et al*., 1993; Passaquin *et al*., 1993). Cellular calcium overload is an important step in the process of muscle damage in DMD and high $[Ca^{2+}]_c$ values in dystrophic cells have been demonstrated to result from increased calcium influx (Turner *et al*., 1988, 1993; Dunn & Radda, 1991; Fong *et al*., 1990; Leijendekker *et al*., 1996). Treatment of both normal and dystrophic cells with PDN resulted in a lowering of elevated $[Ca^{2+}]_c$ to normal levels (Metzinger *et al*., 1995; Leijendekker *et al*., 1996). This effect was correlated with a reduction in Ca^{2+} influx (Metzinger *et al*., 1995).

Most glucocorticoid effects are known to be mediated by hormone interaction with their cognate intracellular receptors which are ligand-regulated transcription factors acting at the genomic level (McEwan *et al*., 1997). Their non-genomic effects are less well documented and include membrane stabilization

and antioxidant properties (Massa *et al*., 1975; Hall *et al*., 1993). We address herein the question of the mechanism of glucocorticoid modulation of calcium influx in skeletal muscle cells in terms of genomic *versus* antioxidant effects.

All glucocorticoids tested were able to decrease calcium influx in C2C12 skeletal muscle cells although with different potencies and efficacies (Figure 2, Table 2). Prednisone, which has to be reduced to PDN in order to act on the glucocorticoid receptor, was also active. This suggests the presence of hydroxylase activity in C2C12 cells. Surprisingly, the mineralocorticoids, 11-deoxycorticosterone and aldosterone, which are devoid of glucocorticoid activities (Table 2), were also able to decrease calcium influx in C2C12 cells (Figure 2, Table 2). Likewise, the lazaroids displayed the same effects although at higher concentrations. Lazaroids are 21-aminosteroids developed to produce cerebroprotective therapeutic effects similar to those of PDN but with less dramatic side effects (Hall *et al*., 1993). We have previously shown that lazaroids were similarly myogenic (Metzinger *et al*., 1995). These 21-aminosteroids were developed due to their free radical scavenging properties for acute treatment of traumatic or ischemic CNS injury to replace glucocorticoids such as PDN (Hall *et al*., 1993). Since U-83836F is devoid of glucocorticoid activity (Hall *et al*., 1993), it is possible that its action could only be mediated *via* its antioxidant properties. Indeed, increased levels of free radicals have been implicated in dystrophic muscle and lipid peroxidation products have been found to be more elevated in plasma from DMD patients compared with controls (Jackson & O'Farrell, 1993; Haycock *et al*., 1996). High doses of glucocorticoids or lazaroids have been shown to be potent antioxidants, capable of mitigating the effects of oxygen radicals on lipid membranes *in vitro* (Uhler *et al*., 1994).

In order to investigate whether the antioxidant properties of glucocorticoids were responsible for reduction of Ca^{2+} influx, we attempted to elicit an oxidant-induced calcium influx but found that C2C12 cells did not respond to the oxidizing agents tested. Long-term treatments with a panel of antioxidants were performed. None of them was able to mimic effects similar to those observed for glucocorticoids (Table 3). In particular, dose-response curves established with vitamin E clearly showed that cellular calcium handling was not modified in C2C12 cells. In this context it might be noteworthy that a double blind clinical trial of vitamin E and penicillamine conducted for 18 months in 100 patients, revealed a lack of therapeutic effects (Fenichel *et al*., 1988).

Subsequently, we have investigated whether the effect of glucocorticoids could be mediated *via* the glucocorticoid receptor. Corticosteroids are pleiotropic substances acting by different mechanisms which can be subdivided into genomic (gene regulation at transcriptional or post-transcriptional levels) and non genomic (antioxidant, membrane stabilization) mechanisms, the former being the most extensively studied one (McEwan *et al*., 1997). Corticosteroids exert their genomic action by binding to two types of intracellular receptors known as mineralocorticoid receptors and glucocorticoid receptors, respectively (McEwan *et al*., 1997). Co-incubation of cells with equimolar concentrations of both prednisolone and RU38486, a progesterone receptor antagonist shown to have antiglucocorticoid properties (Baulieu, 1989; Agarwai, 1996), fully reversed the inhibitory effect of prednisolone on calcium influx. The dose-response curve of prednisolone was shifted to the right by approximately two orders of magnitude in the presence of 10^{-6} M of RU38486. The calculated IC_{50} for the inhibition of the prednisolone effect was $9.55 \pm 0.198 \times 10^{-8}$ M.

The mechanism of action for RU38486 is still not completely resolved and could occur at two levels: prevention of complete glucocorticosteroid receptor transformation and/or alteration of a step subsequent to DNA binding (Baulieu *et al*., 1989; Spitz *et al*., 1996). It was shown recently that RU38486 can display antioxidant properties (Behl *et al*., 1997). Moreover, the existence of an undefined G-protein coupled steroid receptor in the amphibian CNS has been reported (Orchinik *et al*., 1991). One explanation which could account for the inhibitory effect of RU38486 on PDN-mediated reduction of calcium influx is that RU38486 could impair the access of steroids to an as yet uncharacterized site of action. Apart from their interaction with their extensively studied cytoplasmic receptor, glucocorticoids may act through some yet undefined binding sites on the outer plasma membrane (Nurowska & Ruzzier, 1996).

From these results we conclude that genomic effects *via* the glucocorticoid receptor are not involved. Further reasons against involvement of transcription are as follows (1) Inhibitors of gene expression such as actinomycin D and cycloheximide did not modify the effect of PDN; (2) A few minutes of exposure to glucocorticoids has been shown to be sufficient to elicit gene expression changes in skeletal muscle cells (Braun *et al*., 1989) but this did not affect calcium influx; (3) The concentrations at which the steroids modulated calcium influx are well above those sufficient to activate the glucocorticoid or the mineralocorticoid receptor (Kd in the nM order, Table 2); and (4) There were no correlations between calculated steroid IC_{50}'s for Ca^{2+} influx and relative glucocorticoid activities or receptor affinities (Table 2). Furthermore, the lazaroid compounds devoid of glucocorticoid activity displayed the same effects on calcium influx as those bearing glucocorticoid activity.

We therefore propose that the mechanism involved belongs to the still poorly understood long-term non-genomic effects of glucocorticoids which, as detailed above, are not well deciphered. We have tested the hypothesis that the reduction of calcium influx could be mediated *via* the inhibition of phospholipase A_2 by the glucocorticoid-induced lipocortin. However, the results obtained with the phospholipase A_2 inhibitor manoalide (Diamond *et al*., 1994) were not in favour of such a hypothesis. The emerging field of glucocorticoid transporters could bring helpful insights (Kralli *et al*., 1996). One could speculate that at saturating glucocorticoid concentrations, such transporters, which may be localized at the plasma membrane or at the mitochondrial membrane, could modulate the activities of calcium pumps or channels. Alternatively, glucocorticoids have long been shown to enhance membrane fluidity, e.g. they can modulate membrane-bound enzymes and can change the overall glycosylation of membrane glycoproteins (Massa *et al*., 1975).

In summary, we conclude that various steroids are able to reduce Ca^{2+} influx in C2C12 cells. Some of them are quite potent (dexamathasone, PDN) and the effect can be antagonized by RU38486. The insensitivity of this effect to inhibitors of gene expression, however, indicates that a genomic pathway is unlikely. Antioxidant properties and lipocortin inhibition of phospholipase A2 were ruled out and our proposal that glucocorticoids act at the membrane level remains to be further investigated. These results may explain part of the beneficial effects observed in DMD patients treated with glucocorticoids.

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