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Acute effects of phorbol esters on the protein-synthetic rate and carbohydrate metabolism of normal and *mdx* mouse muscles

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1. *mdx* mice do not express dystrophin, the product of the gene which is defective in Duchenne and Becker muscular dystrophy. We have previously shown that protein-synthetic rates (k_s) are increased in *mdx* mouse muscles [MacLennan & Edwards (1990) Biochem. J. **268**, 795–797]. 2. The tumour-promoting stereoisomer of phorbol 12,13-didecanoate (4β-PDD) acutely increased the k_s of muscles from *mdx* and wild-type (C57BL/10) mice incubated *in vitro* in the absence of insulin. The effects of 4β-PDD are presumably mediated by activation of protein kinase C (PKC). 3. The muscle glycogen concentrations of *mdx* mice were higher than those of C57BL/10 mice. Studies performed *in vivo* and *in vitro* suggested that the effect might be at least partially due to increased rate of glycogen synthesis in *mdx* muscle. 4. 4β-PDD increased the glycogen-synthetic rates rates of C57BL/10, but not *mdx*, muscles incubated *in vitro* in the absence of insulin. 5. In muscles from both species incubated in the absence of insulin, treatment with 4β-PDD also induced increased rates of glucose uptake and lactate production. Kinetic studies of C57BL/10 and *mdx* muscles suggested that 4β-PDD raised the $V_{max.}$ of glucose uptake, but did not alter the K_m for the process. 6. The possible role of PKC in controlling the protein and carbohydrate metabolism of normal and *mdx* mouse muscles is discussed.

INTRODUCTION

Previous studies suggest that the Ca^{2+}- and phospholipid-dependent protein kinase C (PKC) may be involved in regulation of muscle carbohydrate and protein metabolism. Acute treatment with tumour-promoting phorbol esters *in vitro* brought about an increase in the fractional rate of protein synthesis (k_s) of cardiac myocytes (Fuller & Sugden, 1989) and a decrease in the degradative rate (k_d) of myofibrillar (but not of non-contractile) skeletal-muscle protein (Goodman, 1987). Experiments performed *in vivo* (Cleland *et al.*, 1989) and *in vitro* (Tanti *et al.*, 1989; Guma *et al.*, 1990) have implicated PKC in the regulation of skeletal-muscle glucose uptake. However, we are aware of no previous studies reporting the effects of phorbol esters on skeletal-muscle k_s; furthermore, the nature (Cleland *et al.*, 1990) and extent (Sowell *et al.*, 1988) of PKC involvement in skeletal-muscle carbohydrate metabolism are contentious (see the Discussion section for a more detailed examination of this issue).

mdx mice have a point mutation of the gene which encodes the protein dystrophin (Sicinski *et al.*, 1989). In humans, defective dystrophin expression results in the muscle-wasting diseases Duchenne and Becker muscular dystrophy (Hoffman *et al.*, 1987*a*), but, although the protein cannot be detected in *mdx* mouse tissues by Western blotting (e.g. Hoffman *et al.*, 1987*b*), their muscle function is essentially unimpaired (Dangain & Vrbova, 1984). We have previously shown that muscle protein-turnover rates are higher in *mdx* than in wild-type mice (MacLennan & Edwards, 1990), and the high glycogen concentrations observed in *mdx* mouse muscles (Cullen & Jaros, 1988) suggest an abnormality of carbohydrate metabolism.

The aims of the present study were three-fold: first, to determine whether or not skeletal-muscle k_s could be acutely changed by phorbol ester treatment; second, to re-examine the effects of phorbol esters on skeletal-muscle carbohydrate metabolism; third, to study *mdx* muscle carbohydrate metabolism and investigate the possibility that the *mdx* mutation might alter the response of mouse muscles to phorbol ester treatment.

EXPERIMENTAL

Materials

L-Phenyl[2,3-^{3}H]alanine and D-[U-^{14}C]glucose were purchased from Amersham International, Amersham, Bucks., U.K. Ethanol was removed from these radiochemicals by drying under a stream of nitrogen before use. 2-Deoxy-D-[1-^{3}H]glucose (in aqueous solution) was purchased from New England, Boston, MA, U.S.A. All other chemicals and biochemicals were from Sigma Chemical Co. or B.D.H., both of Poole, Dorset, U.K. The identification, source and maintenance of *mdx* and C57BL/10 mice have previously been described (MacLennan & Edwards, 1990). All studies were performed on fed mice weighing 23–32 g.

Studies *in vivo*

Tissue protein synthesis. For this, an adaptation (Jepson *et al.*, 1986) of the method described by Garlick *et al.* (1980) was employed, the protocol being essentially that previously described (MacLennan & Edwards, 1990), with extensor digitorum longus (EDL) and soleus muscles being removed in addition to gastrocnemius muscles.

Muscle glycogen concentration and synthetic rate. An adaptation (Leighton & Foot, 1990) of the method described by Rafaelsen (1964) was used. Briefly, mice were injected intraperitoneally with 0.3 ml of a solution containing [U-^{14}C]glucose (5 μCi) and 0.1% fat-free BSA in 150 mM-NaCl; 2 h later the animals were killed by cervical dislocation, and samples of gastrocnemius, soleus and EDL muscle and of liver were then rapidly removed and frozen in liquid nitrogen. The tissues were dissolved in 1 M-NaOH for 15 min at 100 °C. A sample of the digest was removed for assay of protein (Lowry *et al.*, 1951). Glycogen was precipitated, washed and assayed as previously described (Holness *et al.*, 1988); a portion of the washed precipitate was dissolved in water and used for liquid-scintillation counting.

Abbreviations used: DMSO, dimethyl sulphoxide; EDL, extensor digitorum longus; k_d, fractional rate of protein degradation; k_s, fractional rate of protein synthesis; 4β-PDD, 4β-phorbol 12,13-didecanoate; 4α-PDD, stereoisomer of 4β-PDD; PKC, protein kinase C.

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The recovery of glycogen isolated from muscle by this method was determined on gastrocnemius muscles obtained from six wild-type mice. Frozen muscles were powdered at liquid-nitrogen temperature, and one portion of the powder was used for determination of glycogen concentrations without prior precipitation (Keppler & Decker, 1974). Glycogen was precipitated from a known quantity of the remaining powder and was isolated by the method described by Holness *et al.* (1988). After digestion with amyloglucosidase, the hydrolysate was dried and dissolved in 1 ml of water. The recovery of glycosyl units was evaluated by determining the glucose concentration of a portion of this solution enzymically. The recovery was $85 \pm 4\%$ (mean $\pm$ S.D.; $n = 6$).

Studies *in vitro*

Mice were killed by cervical dislocation, and then both EDL muscles were rapidly and carefully removed and placed in bicarbonate-buffered medium (Close, 1964). Less than 5 min later, the muscles were tied on to special holders at approximately resting length and incubated at 37 °C in 4 ml of oxygenated medium which had been supplemented with 10 mM-glucose (except in studies of glucose uptake kinetics, in which a range of glucose concentrations was employed). Details of the apparatus used have been described previously (Jones *et al.*, 1983). The muscles were incubated for a period of some 10 min in this medium. Their subsequent treatment depended on whether they were destined for the study of carbohydrate metabolism or of protein-synthetic rates.

Protein synthesis *in vitro*. After the pre-incubation period, muscles were incubated for 15 min in a 3 ml of a medium which had been supplemented with 10 mM-glucose and with amino acids at the concentrations found in the plasma of fed rats (Williams *et al.*, 1980). The media were also supplemented either with 1 μM-4β-phorbol 12,13-didecanoate (4β-PDD) or with DMSO at an equivalent concentration (1 μl/ml). In some cases 1 m-unit of insulin per ml of medium was present. The effects of 4α-PDD (which does not stimulate PKC; Castagna *et al.*, 1982) on muscle k_s were also evaluated. Preliminary experiments showed that DMSO did not significantly affect muscle k_s in our system.

After the preincubation period, each muscle was incubated for a further 1 h in fresh medium which had a phenylalanine concentration of 2.5 mM and contained [^{3}H]phenylalanine at a nominal specific radioactivity of 1500 d.p.m./nmol, but which was in all other respects identical with the medium in which the muscle had been incubated for the previous 15 min period. At the end of the final 1 h incubation period, the muscles were placed in an ice/water mixture, then blotted dry and frozen in liquid nitrogen until analysis. The specific radioactivities of free (S_A) and protein-bound (S_B) phenylalanine were determined by previously described methods (Garlick *et al.*, 1980). Protein-synthetic rates (k_s) were calculated from the equation of Garlick *et al.* (1980):

$$k_s\ (\%/\text{day}) = \frac{S_B}{S_A} \times \frac{1}{t} \times 100\,\%$$

where t is time in days (in these experiments 0.0416 day). Muscle S_A reached a plateau after 2 min incubation in radioactive medium (results not shown); thus S_A measured at the end of the incubation was likely to be representative of S_A throughout the entire 1 h incubation in the presence of [^{3}H]phenylalanine.

Carbohydrate metabolism *in vitro*. The protocol used was similar to that employed for the protein-synthesis experiments, but amino acids were not included in the incubation media. After the 10 min preincubation period, the muscles were incubated for 15 min in media supplemented with either 4β-PDD or vehicle with or without insulin, then for a further 1 h in a final medium containing radioactive substrates. The effects of 4α-PDD on muscle carbohydrate metabolism, (in the absence of insulin) were also evaluated using this protocol. Preliminary experiments showed that DMSO did not appreciably influence muscle carbohydrate metabolism in our system.

The final media were supplemented with 2 μCi of [U-^{14}C]-glucose/ml for measurement of glycogen-synthetic rates. The specific radioactivity of each freshly prepared batch of incubation medium was measured by scintillation counting and spectrophotometric assay of glucose concentration (Lowry & Passonneau, 1972). At the end of the final incubation period the muscles were placed in ice-cold 150 mM-NaCl, blotted dry, frozen in liquid nitrogen and stored at -70 °C until analysed. The final incubation media were frozen at -20 °C for subsequent determination of lactate concentration (Gutman & Wahlefeld, 1974). The frozen muscles were dissolved in 1 M-NaOH at 100 °C for 15 min, and a sample of the digest was used for determining protein concentration (Lowry *et al.*, 1951). Glycogen was isolated from the remaining alkaline digest as described above (Holness *et al.*, 1988). Glycogen synthesis was estimated from the incorporation of ^{14}C into glycogen and from the specific radioactivity of [U-^{14}C]glucose in the medium.

Rates of glucose uptake and phosphorylation *in vitro* were determined, in separate experiments, by the method described by Ferré *et al.* (1985). A trace of 2-deoxy[^{3}H]glucose (2 μCi/ml; 66 nM) was added to the final incubation medium, with the 'specific' radioactivity of 2-deoxyglucose in freshly prepared media (i.e. 2-deoxyglucose radioactivity divided by glucose concentration) being determined as described above. Frozen muscles were dissolved in 230 μl of 1 M-NaOH for 45 min at 60 °C, a 20 μl sample was removed for protein determination, and 210 μl of 1 M-HCl was added to the remainder of the alkaline digest. A 200 μl sample of the neutral mixture was added to 1 ml of 6% (w/v) $HClO_4$, and a second 200 μl sample was deproteinized by the procedure of Somogyi (1945).

The resulting precipitates were removed by centrifugation, and the deproteinized $HClO_4$ and 'Somogyi' supernatants were scintillation-counted. The rates of glucose uptake (and phosphorylation) were calculated from the equation:

$$\text{Glucose uptake } (\mu\text{mol/g of protein}) = \frac{\left[\begin{matrix}\text{HClO}_4\text{ supernatant} \\ \text{(d.p.m./g of protein)}\end{matrix}\right] - \left[\begin{matrix}\text{'Somogyi' supernatant} \\ \text{(d.p.m./g of protein)}\end{matrix}\right]}{\begin{matrix}\text{'specific' radioactivity of 2-deoxyglucose in the medium} \\ (\text{d.p.m.}/\mu\text{mol})\end{matrix}}$$

The accuracy of glucose-uptake rates calculated in this way is dependent on the following assumptions: (1) that both 2-deoxyglucose and 2-deoxyglucose 6-phosphate are soluble in 6% $HClO_4$, but only 2-deoxyglucose is soluble in the Somogyi reagent (Ferré *et al.*, 1985); (2) that in skeletal muscle there is no substantial difference between the transport rates of glucose and 2-deoxyglucose [see Rennie *et al.* (1983) and Ferré *et al.* (1985) for evidence to support this contention]; (3) that 2-deoxyglucose 6-phosphate is not appreciably metabolized.

The kinetic characteristics of glucose uptake and

phosphorylation in the absence and presence of 4β-PDD were determined by measuring 2-deoxy[^{3}H]glucose 6-phosphate accumulation in muscle at 1 mM, 5 mM, 10 mM 25 mM and 50 mM extracellular glucose.

Data analysis

All data are presented as means ± S.D. Statistical analyses were by Student's unpaired t test, with a value of $P < 0.05$ being considered significant. For the purpose of kinetic analysis, it was assumed that glucose uptake in skeletal muscle obeys Michaelis–Menten kinetics (Chaudry & Gould, 1969). Values of K_m and $V_{max.}$ were derived without transformation of the data by computerized curve-fitting.

RESULTS

Protein-synthetic rates *in vitro*

In gastrocnemius, soleus and EDL muscles from *mdx* mice, k_s was higher than in C57BL/10 mice (Table 1). The soleus muscle is composed mainly of slow-twitch oxidative fibres, whereas in the EDL fast-twitch glycolytic fibres predominate (Ariano *et al.*, 1973; Nesher *et al.*, 1980). Thus the increased *mdx*-mouse muscle k_s which we have previously reported (MacLennan *et al.*, 1990) is not confined to a single group of muscles or to muscles of a single fibre type. The data also showed that the response of EDL k_s to the *mdx* mutation was typical of mouse skeletal musculature and provided a basis for subsequently evaluating how closely the k_s measured in EDL muscles *in vitro* approximated to the situation *in vivo*.

Glycogen concentrations and ^{14}C incorporation *in vivo*

In all skeletal muscles studied, the glycogen concentrations of *mdx* mouse muscles were higher than those of C57BL/10 muscles (Table 2), supporting the previous suggestion by Cullen & Jaros (1988). Liver glycogen concentrations were similar in *mdx* and C57BL/10 mice.

The incorporation of ^{14}C into glycogen was also increased in *mdx* mouse skeletal muscles (Table 2). The relationship between ^{14}C incorporation and glycogenic rate is uncertain in these experiments, because the extent of precursor labelling was not assessed in either species. Nevertheless the data are consistent with the idea that the elevated *mdx* muscle glycogen concentrations are the consequence of increased glycogen-synthesis rates, although the possibility that decreased glycogen utilization is a contributory factor, or even the sole factor, cannot be excluded.

Protein synthesis *in vitro*

When EDL muscles were incubated in the absence of insulin, k_s was significantly higher in *mdx* than in C57BL/10 mice (Table 3), reflecting the situation *in vivo* (Table 1). Inclusion of 4β-PDD in the medium significantly increased the k_s of both *mdx* and C57BL/10 muscles, implying that activation of PKC stimulates k_s in skeletal muscle.

The EDL-muscle k_s was increased to a similar extent in both species by the presence of 1 m-unit of insulin/ml. However, as previously described in cardiac myocytes (Fuller & Sugden, 1989), the effects of insulin and 4β-PDD on muscle k_s were not additive in either species (Table 3). The biologically inactive phorbol ester 4α-PDD did not influence the rate of protein synthesis in incubated muscles from *mdx* or wild-type mice (Table 7).

Table 1. Protein-synthetic rates in different skeletal muscles of C57BL/10 and *mdx* mice *in vivo*

The k_s of different muscles in *mdx* and C57BL/10 mice was measured *in vivo* with a flooding dose of [^{3}H]phenylalanine; further details are given in the text. ***($p < 0.001$) indicates a statistically significant difference between C57BL/10 and *mdx* mice; n = 4–5.

	Muscle protein-synthetic rate (%/day)		
Mice	Gastrocnemius	Soleus	EDL
C57BL/10	5.1 ± 0.7	7.8 ± 1.6	4.6 ± 1.0
mdx	9.4 ± 1.1***	12.4 ± 1.3***	8.2 ± 1.3***

Table 3. Effects of 4β-PDD and insulin on the protein-synthetic rates of C57BL/10 and *mdx* mouse muscles *in vitro*

EDL muscles from C57BL/10 and *mdx* mice were incubated as described in the text in the absence or presence of 4β-PDD and/or insulin as indicated below. Muscle k_s was evaluated from incorporation of ^{3}H into protein. Values with the same superscript letters are not significantly different; n = 4–5.

		Muscle protein-synthetic rate (%/day)	
Incubation conditions	Mice...	C57BL/10	*mdx*
0 insulin, DMSO		2.71 ± 0.43[a]	5.12 ± 0.62[c]
0 insulin, 4β-PDD		3.92 ± 0.46[b]	6.34 ± 0.61[d]
Insulin (1 m-unit/ml), DMSO		4.29 ± 0.61[b]	7.15 ± 0.73[d]
Insulin (1 m-unit/ml), 4β-PDD		4.06 ± 0.21[b]	7.64 ± 1.02[d]

Table 2. Glycogen concentrations and incorporation of ^{14}C into glycogen in skeletal muscle and liver of C57BL/10 and *mdx* mice *in vivo*

C57BL/10 and *mdx* mice were injected intraperitoneally with a tracer dose of [U-^{14}C]glucose. The animals were killed 2 h later and tissue glycogen concentrations and incorporation of radioactivity into glycogen were determined; further details are given in the text. *($P < 0.05$), **($P < 0.01$) and ***($P < 0.001$) indicate statistically significant differences between C57BL/10 and *mdx* mice; n = 3–4. Abbreviation: n.d., not determined.

	Gastrocnemius		Soleus		EDL		Liver	
Mice...	C57BL/10	*mdx*	C57BL/10	*mdx*	C57BL/10	*mdx*	C57BL/10	*mdx*
Glycogen concn. (μmol of glycosyl units/g wet wt.)	16.2 ± 2.4	23.9 ± 3.7*	10.4 ± 1.3	15.5 ± 3.2*	18.9 ± 1.7	28.2 ± 1.9***	341 ± 56	327 ± 63
Incorporation of ^{14}C into glycogen (d.p.m./2 h per mg of protein)	509 ± 161	734 ± 191**	821 ± 114	1247 ± 243**	129 ± 16	204 ± 52**	n.d.	n.d.

Table 4. Effects of 4β-PDD and insulin on the carbohydrate metabolism of C57BL/10 and *mdx* mouse muscles *in vitro*

EDL muscles from C57BL/10 and *mdx* mice were incubated as described in the text in the absence or presence of 4β-PDD and/or insulin as indicated below. Rates of glucose uptake and glycogen synthesis were measured radiochemically, with lactate production rates and glycogen concentrations being assayed spectrophotometrically; further details are given in the text. Values with the same superscript letter are not significantly different; $n = 4–5$.

Incubation conditions	Mice...	Glucose uptake (μmol/h per g of protein)		Glycogen synthesis (μmol/h per g of protein)		Lactate production (μmol/h per g of protein)		Glycogen concn. (μmol of glycosyl units/g of protein)	
		C57BL/10	*mdx*	C57BL/10	*mdx*	C57BL/10	*mdx*	C57BL/10	*mdx*
0 insulin, DMSO		25.6±6.4[a]	20.3±4.7[a]	4.1±0.9[a]	7.9±2.2[b]	46.2±12.4[a]	42.6±14.4[a]	70±16[a]	101±22[b]
0 insulin 4β-PDD		39.1±4.2[b]	34.2±6.9[b]	7.4±1.7[b]	7.3±2.6[b]	76.4±12.7[b]	71.5±20.2[b]	79±12[a]	96±25[b]
Insulin (1 m-unit/ml), DMSO		82.9±7.9[c]	75.9±18.6[c]	15.3±2.9[c]	13.4±3.9[c]	138.6±21.6[c]	141.7±25.7[c]	132±22[c]	169±37[c]
Insulin (1 m-unit/ml), 4β-PDD		74.0±19.9[c]	66.1±13.8[c]	14.7±3.2[c]	16.2±4.0[c]	152.3±16.4[c]	129.5±24.8[c]	125±39[b]	160±27[c]

Table 5. Effect of extracellular glucose concentration and 4β-PDD on rates of glucose uptake in C57BL/10 and *mdx* mouse muscles *in vitro*

Rates of glucose uptake at different glucose concentrations in the absence and presence of 4β-PDD were determined as described in the text. **($P < 0.01$) and ***($P < 0.001$) indicate statistically significant effects of the presence of 4β-PDD; $n = 3–5$.

Glucose concn. (mM)	Mice...	Rate of glucose uptake (μmol/h per g of protein)			
		C57BL/10		*mdx*	
		DMSO	4β-PDD	DMSO	4β-PDD
1		4.6±0.5	8.1±1.4***	3.9±0.6	6.8±2.1***
5		12.8±2.3	19.7±1.6***	10.8±3.7	20.5±4.2***
10		25.6±6.4	39.1±4.2**	20.3±4.7	34.2±6.9**
25		31.8±3.8	66.9±10.5***	33.1±6.2	55.6±15.3***
50		40.5±6.1	73.4±14.6***	38.2±4.1	63.8±20.2**

Table 6. Kinetic analysis of the effects of 4β-PDD on glucose uptake in muscles from C57BL/10 mice *in vitro*

The values of K_m and $V_{max.}$ were derived from the data in Table 5 by curve-fitting. Values with the same superscript letter were not significantly different.

Mice...	C57BL/10		*mdx*	
	DMSO	4β-PDD	DMSO	4β-PDD
K_m (mM)	11.4±2.8[a]	15.7±4.2[a]	15.6±2.6[a]	14.0±1.8[a]
$V_{max.}$ (μmol/h per g of protein)	48.9±4.2[a]	100.4±10.5[b]	51.3±3.4[a]	83.2±3.9[b]

Carbohydrate metabolism *in vitro*

At a glucose concentration of 10 mM, in the absence of insulin, glucose-uptake rates were similar in *mdx* and C57BL/10 mice (Table 4). The rate of this process was increased to a similar extent in both species by inclusion of 4β-PDD in the incubation medium. Kinetic studies showed that in both *mdx* and wild-type mice the presence of 4β-PDD induced an increase in the $V_{max.}$ of glucose uptake, but did not influence the K_m of this process (Tables 5 and 6). The K_m and $V_{max.}$ of glucose uptake were not significantly altered by the *mdx* mutation in either the absence or presence of 4β-PDD (Table 6).

Rates of lactate production were also increased by 4β-PDD in muscles from *mdx* and C57BL/10 mice incubated in the absence of insulin (Table 4). It seems unlikely that increased rates of glycogenolysis made an appreciable contribution to the latter effect, because the glycogen concentrations of muscles incubated in the presence of 4β-PDD tended to be higher than those of muscles incubated in its absence (Table 4).

In *mdx* mouse muscles incubated in the absence of insulin or 4β-PDD, rates of glycogen synthesis were higher than in muscles from C57BL/10 mice incubated under these conditions (Table 4). The presence of 4β-PDD induced an increase in the glycogen-synthetic rate of C57BL/10, but not of *mdx*, muscles. This finding was surprising, because a decreased rate of muscle glycogen synthesis in response to phorbol ester treatment has previously been reported (Sowell *et al.*, 1988).

The carbohydrate metabolism of muscles from C57BL/10 and *mdx* mice incubated in the absence of insulin at an extracellular glucose concentration of 10 mM was unaltered by 4α-PDD (Table 7). It therefore seems most unlikely that the effects of 4β-PDD on glucose uptake, lactate production and glycogen synthesis (Table 4) were the consequence of non-specific effects of phorbol esters.

At 10 mM-glucose, the presence of insulin significantly increased rates of glucose uptake, lactate production and glycogenesis in both species. The rates of these processes were unaltered by 4β-PDD when insulin was included in the incubation medium.

DISCUSSION

Validity of isolated muscle preparations

The use of incubated muscles to study protein and carbohydrate metabolism has been criticised on the grounds that such preparations may contain hypoxic 'cores' in which k_s (Maltin & Harris, 1985) and glycogen concentrations (Maltin & Harris, 1985; Breda *et al.*, 1990) are low. It is likely that the incubated muscles used in the present study developed such cores, because they were incubated at 37 °C for periods of up to 90 min. However, because phorbol esters cause acute constriction of blood vessels, their use in perfused muscle systems yields data

Table 7. Effects of 4α-PDD on the protein-synthetic rate and carbohydrate metabolism of muscles from C57BL/10 and *mdx* mice incubated *in vitro* in the absence of insulin

EDL muscles from C57BL/10 and *mdx* mice were incubated as described in the text in the absence or presence of 4α-PDD as indicated below. Muscle k_s was evaluated from the incorporation of ^3H into protein; rates of glucose uptake and glycogenesis were also determined radiochemically, with rates of lactate release being assayed spectrophotometrically. There were no statistically significant differences between incubations in the absence and presence of 4α-PDD performed on animals of the same strain.

Mice...	C57BL/10		*mdx*	
	DMSO	4α-PDD	DMSO	4α-PDD
Muscle k_s (%/day)	3.04±0.57	2.83±0.60	4.93±0.73	5.04±0.36
Glucose uptake (μmol/h per g of protein)	28.6±7.5	24.8±3.7	23.9±3.8	25.6±4.9
Glycogen synthesis (μmol/h per g of protein)	4.7±1.1	4.2±0.8	6.3±0.9	6.8±1.8
Lactate production (μmol/h per g of protein)	57.3±14.3	56.8±13.2	47.5±12.6	49.3±8.7

which are difficult to interpret (H. S. Hundal, personal communication). Thus, although our preparations are not ideal, we cannot conceive of another system in which the effects of phorbol esters on muscle metabolism could be studied.

The suggestion (Breda *et al.*, 1990) that incubated mouse muscles were unsuitable for measuring rates of glycogen synthesis was based on studies of unrestrained muscles. It has previously been reported that rat soleus and EDL muscles spontaneously shorten when incubated in an unrestrained state and that a Ca^{2+}-dependent activation of proteolysis takes place (Furuno & Goldberg, 1986). These processes might be related to the high variability of the glycogen-synthetic rates reported in the unrestrained muscles (Breda *et al.*, 1990) and the failure of 1m-unit of insulin/ml to stimulate significantly the rate of this process. Stimulation of muscle glycogen synthesis by insulin has been observed in the present study and in numerous others (e.g. Leighton & Foot, 1990) which employed muscles incubated at approximately resting length.

Effects of phorbol esters on carbohydrate and protein metabolism in normal mouse muscle

It seems likely that 4β-PDD stimulated muscle k_s by activating the translational phase of protein synthesis, firstly because the increased k_s was observed after treatment for only 75 min. Secondly, inhibition of transcription does not decrease the stimulation of k_s effected by phorbol ester treatment of cardiac myocytes (Fuller & Sugden, 1989), and it seems probable that these agents exert their effects on skeletal and cardiac-muscle k_s by similar mechanisms.

In the absence of insulin, we found that 4β-PDD stimulated muscle glucose uptake and phosphorylation at all extracellular glucose concentrations studied. This finding is in agreement with two previous studies which reported phorbol-ester-induced stimulation of glucose uptake (Tanti *et al.*, 1989; Guma *et al.*, 1990), but is contrary to two reports which found muscle glucose uptake to be unaltered by phorbol esters (Cleland *et al.*, 1990; Sowell *et al.*, 1988). The reason for the inconsistency is not obvious; however, both studies which found no effect of phorbol esters on muscle glucose uptake employed sucrose as an extracellular marker. It is conceivable that the presence of sucrose in the incubation media altered the muscles' responses to phorbol ester treatment, although this seems unlikely.

Sowell *et al.* (1988) also reported that muscle glycogen synthesis was inhibited by phorbol esters and suggested that the mechanisms involved might be analogous to those through which PKC activation inhibits glycogen synthesis in hepatocytes (e.g. Roach & Goldman, 1983). These data are contrary to our finding of a stimulation of glycogen-synthetic rate by phorbol esters in muscles incubated in the absence of insulin. The discrepancy may be related to the failure of phorbol esters to stimulate glucose uptake in the system of Sowell *et al.* (1988). It is possible that the increase in glucose uptake induced by 4β-PDD in our system brought about an increase in the concentration of an allosteric activator of glycogen synthase (such as glucose 6-phosphate), thereby increasing the rate of glycogen synthesis.

Our studies on the effects of phorbol esters on muscle glucose uptake differed from previous work in that the radioactive glucose analogue was present at a low concentration, with non-radioactive D-glucose serving as fuel for the muscle. We used this approach because it approximated to the circumstances *in vivo* and enabled measurement of glucose uptake, lactate production and glycogen synthesis to be carried out under identical experimental conditions. The rates of all of these processes (and of muscle k_s) were influenced by 4β-PDD, but were unaltered by 4α-PDD (which does not stimulate PKC; Castagna *et al.*, 1982); these findings lend considerable support to the idea that PKC participates in the regulation of muscle metabolism *in vivo*.

Protein and carbohydrate metabolism in *mdx* mice: effects of phorbol esters

In *mdx*-mouse EDL muscles incubated in media supplemented with insulin at supra-physiological concentrations, k_s was some 20% lower than *in vivo*. However because *mdx*-muscle k_s was significantly higher than C57BL/10-muscle k_s under all conditions studied *in vitro*, it may be inferred that the increased rate of this process observed in *mdx* mice *in vivo* is not solely due to the presence of a circulating factor. The k_s of *mdx* EDL muscles incubated without insulin was increased by 4β-PDD. Thus, although k_s was greater than that of C57BL/10 muscles, it was not operating at the maximum rate which was possible *in vitro* in the absence of insulin.

Our experiments *in vivo* and *in vitro* suggest that the increased glycogen concentration of *mdx* mouse muscles may be the result of increased glycogen synthesis. Because the rates of glucose uptake were similar in *mdx* and C57BL/10 mice *in vitro*, it is also implied that *mdx* mice utilize a greater proportion of glucose uptake for glycogenesis in muscle than do C57BL/10 mice. Similar rates of lactate release from incubated EDL muscles obtained from both species were observed (and the pentose phosphate pathway is negligible in muscle); thus it seems likely that a decreased proportion of glucose uptake was oxidized in muscles from *mdx* mice. However, direct measurements of glucose oxidation would be necessary to be certain of this.

The failure of 4β-PDD to stimulate glycogen synthesis in *mdx* muscle despite effecting an increased rate of glucose uptake

suggests that glycogen synthase might have been stimulated to the maximum degree possible in the absence of insulin. A second possibility is that dystrophin deficiency resulted in damage to the glycogen synthase enzyme molecules, which altered their regulatory properties.

Comments on the possible physiological role of PKC in regulating skeletal-muscle metabolism

The stimulation of muscle k_s by 4β-PDD suggests that there may be circumstances, *in vivo*, in which the activity state of PKC is a determinant of muscle k_s. It is possible that PKC might be involved in the stimulation of muscle k_s by insulin which is observed *in vivo* (Pain & Garlick, 1974) and *in vitro* (Flaim *et al.*, 1980). This suggestion is supported by the fact that insulin can increase tissue diacylglycerol concentrations (e.g. Farese *et al.*, 1984). Furthermore, phorbol esters can mimic some (Lee & Weinstein, 1979; O'Brien & Saladik, 1982; Saltid *et al.*, 1987), although not all (Cherqui *et al.*, 1986; Klip & Ramlal, 1987), of the actions of insulin.

Electrical stimulation of muscle induces diacylglycerol formation and activation of PKC (Cleland *et al.*, 1989), and, although muscular activity may cause acute decreases in k_s (see, e.g., Bylund-Fellenius *et al.*, 1984), many types of exercise are associated with increased muscle growth (reviewed by Babij & Booth, 1988). The data from the present study, in conjunction with the previous observation that phorbol esters inhibit the k_d of contractile proteins, but do not influence the rate of this process in non-contractile proteins (Goodman, 1987), indicate that the overall effect of PKC activation would be to induce net protein accretion. Activation of PKC might thus participate in exercise-induced muscle growth *in vivo*.

Similar arguments could also be used to suggest that PKC might partially mediate the increased glucose uptake and glycolytic rates which are associated with the action of insulin and muscular activity *in vivo*. Our finding that 4β-PDD increased the capacity, but not the affinity, of glucose uptake in muscle is consistent with this idea, because stimulation of the process by insulin affects glucose-uptake kinetics in a similar manner (Rennie *et al.*, 1983). The increased rate of glycogen synthesis induced by 4β-PDD might appear to be inconsistent with the idea that PKC is involved in the metabolic response of muscle to exercise. However, although muscle activity causes decreased glycogen synthesis and increased glycogenolysis in the short term, PKC might participate in a post-exercise anabolic phase in which glycogen is re-synthesized.

In *mdx*-mouse muscles incubated at normal extracellular Ca^{2+} concentrations, the intracellular Ca^{2+} concentrations are higher than those of C57BL/10 muscles (Turner *et al.*, 1988). It is therefore conceivable that, in *mdx* mice, the increased rates of k_s and glycogenesis are brought about by Ca^{2+}-induced activation of PKC. However, although the intracellular Ca^{2+} concentration of *mdx* mouse muscles may be decreased by incubating them at low extracellular Ca^{2+} concentrations (Turner *et al.*, 1988), this treatment does not acutely influence muscle k_s (MacLennan *et al.*, 1991) or glycogenic rate (results not shown). These data provide evidence against the idea that Ca^{2+} activation of PKC mediates the alterations in protein and carbohydrate metabolism found in *mdx* muscle. Nevertheless the possibility remains that PKC might have exerted a controlling influence through mechanisms which are not reversed acutely *in vitro* by lowering intracellular Ca^{2+} concentration.

The events through which activation of PKC might be linked to increased muscle k_s, glucose uptake, glycolysis and glycogen synthesis are unknown. It has, however, been suggested that PKC may phosphorylate and activate the Na^+/H^+ antiporter in cardiac muscle, thereby inducing intracellular alkalinization, which in turn causes increased k_s (Fuller *et al.*, 1990), and this sequence of events might also take place in skeletal muscle. It is conceivable that skeletal-muscle carbohydrate metabolism might also be influenced by PKC in this way, because an alkaline pH optimum for glucose uptake has been reported in this tissue (Chaudry & Gould, 1969); there is, however, no direct evidence that PKC exerts a regulatory influence on muscle carbohydrate and protein metabolism through a common mechanism.

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