Modulation of 1,25-dihydroxyvitamin D₃-dependent Ca²⁺ uptake in skeletal muscle by protein kinase C

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In vitro studies have shown that short exposure (1–10 min) of vitamin D-deficient chick soleus muscle to 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] causes an acute stimulation of tissue ⁴⁵Ca uptake through voltage-gated Ca²⁺ channels, with parallel increases in cyclic AMP levels, adenylate cyclase activity and membrane protein phosphorylation. We further investigated the involvement of protein kinases in the rapid effects of 1,25(OH)₂D₃ on skeletal muscle. The hormone was found to stimulate the protein kinase C (PKC) activity of muscle membranes. The PKC activator phorbol 12-myristate 13-acetate (PMA, 100 nM) was found to rapidly stimulate muscle ⁴⁵Ca uptake, mimicking 1,25(OH)₂D₃. Increases of 68 % and 46 % were observed at 1 and 15 min of exposure to PMA respectively. The effects of PMA were dose-dependent (50–200 nM) and were specific, since the inactive analogue 4 α -phorbol was without effect. Analogously to the effects of the sterol, PMA-enhanced ⁴⁵Ca uptake was abolished by the Ca²⁺ channel antagonists nifedipine (30 μ M) and verapamil (50 μ M). Staurosporine (10 nM), a PKC inhibitor, surprisingly potentiated 1,25(OH)₂D₃-dependent stimulation of ⁴⁵Ca uptake. Exposure of skeletal muscle to PMA (100 nM) plus 1,25(OH)₂D₃ (1 nM) produced a less pronounced effect on ⁴⁵Ca uptake than either agent alone. PMA also decreased muscle cyclic AMP levels. These results suggest a regulatory link between the two major transmembrane signalling systems in the mechanism of action of 1,25(OH)₂D₃ in skeletal muscle.

INTRODUCTION

Vitamin D₃, acting through its daughter metabolite 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃], is an important regulator of skeletal muscle function [1]. Studies both *in vivo* and *in vitro* have shown that this sterol plays a role in the regulation of intracellular Ca²⁺ in skeletal muscle [2–5]. The sterol acts, in part, via a receptor-mediated genomic mechanism [6–8]. In addition, 1,25(OH)₂D₃ affects muscle Ca²⁺ fluxes by a non-genomic mechanism. Exposure of chick soleus muscle to 1,25(OH)₂D₃ *in vitro* causes a rapid stimulation of Ca²⁺ uptake, independently of new protein synthesis, which can be suppressed by Ca²⁺ channel blockers [9]. These effects are mimicked by forskolin [10], an activator of adenylate cyclase, and by dibutyryl cyclic AMP (V. Massheimer, L. Fernandez, R. Boland & A. R. de Boland, unpublished work). Recent evidence indicates that 1,25(OH)₂D₃ elevates muscle cyclic AMP levels, increases adenylate cyclase activity and results in a similar profile of muscle protein phosphorylation as does forskolin [11]. Since it is well established that voltage-dependent Ca²⁺ channels in skeletal muscle are subject to regulation by cyclic AMP-dependent phosphorylation of the channel itself or of associated regulatory proteins [12,13], these events suggest that phosphorylation through the cyclic AMP-dependent pathway is part of the mechanism by which 1,25(OH)₂D₃ affects Ca²⁺ channel activity. Changes in protein kinase C (PKC) activity in response to 1,25(OH)₂D₃ have been described [14]. PKC has also been shown to be involved in Ca²⁺ channel modulation [15–17].

In the present work, we examined the possible role of PKC in the mechanism of action of 1,25(OH)₂D₃ in chick skeletal muscle.

MATERIALS AND METHODS

Materials

Histone type III-S, leupeptin, dithiothreitol (DTT), phenylmethanesulphonyl fluoride (PMSF), phorbol 12-myristate 13-

acetate (PMA), 4 β -phorbol dibutyrate, 4 α -phorbol, staurosporine, nifedipine, verapamil, 1,2-diolein and phosphatidylserine were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). [³²P]ATP and ⁴⁵CaCl₂ were from New England Nuclear. The cyclic AMP assay kit was from Diagnostic Products Co. (Los Angeles, CA, U.S.A.). 1,25(OH)₂D₃ was a gift from Dr. Milan Uskokovic (Hoffman-La Roche, Rahway, NJ, U.S.A.).

Animals

Chicks were raised from 1 day of age on a vitamin D-deficient diet containing 1.6 % calcium and 1.0 % phosphorous [18] for 5 weeks in an environment deprived of light.

Treatment of muscle preparations *in vitro*

Soleus muscles dissected from both legs of vitamin D-deficient chicks were preincubated in Krebs/Henseleit buffer containing 2 % glucose [19] for 30 min at 37 °C under O₂/CO₂ (19:1) with constant shaking. Either 1,25(OH)₂D₃ (1 nM, selected on the basis of dose-response studies) or PMA (50–200 nM) (both dissolved in ethanol) was then added for 1–15 min. Ethanol (< 0.1 %) alone was added to the control samples. When nifedipine (30 μ M [9]), verapamil (50 μ M [9]) or staurosporine (10–100 nM) was used, they were added 5 min before the addition of PMA or 1,25(OH)₂D₃.

Measurement of ⁴⁵Ca uptake

Determinations of ⁴⁵Ca uptake were carried out using a modification of a procedure previously described [4,5]. Muscle samples prepared and treated as described above were incubated in Krebs/Henseleit/glucose containing ⁴⁵CaCl₂ (2 μ Ci/ml) at 37 °C for 5 min. The tissue was then quickly washed with ice-cold medium, blotted on to filter papers and dissolved in 1 M-NaOH at 80 °C. Aliquots were taken for determination of protein content by the Lowry procedure [20], and radioactivity was measured using Aquasol as the scintillation fluid.

Abbreviations used: 1,25(OH)₂D₃, 1,25-dihydroxyvitamin D₃; PKC, protein kinase C; DTT, dithiothreitol; PMSF, phenylmethanesulphonyl fluoride; PMA, phorbol 12-myristate 13-acetate.

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Microsomal membrane preparation

Control and treated soleus muscles were homogenized in 20 mM-Tris/HCl, pH 7.4, 1 mM-EGTA, 0.5 mM-PMSF, 40 μ g of leupeptin/ml, 1 mM-DTT and 0.33 M-sucrose. The homogenate was centrifuged for 10 min at 1200 g in a Sorvall refrigerated centrifuge. The supernatant was filtered through a triple layer of cheesecloth and centrifuged for 15 min at 11 300 g. Microsomal membranes were sedimented from this supernatant in a Beckman L5-50B ultracentrifuge at 120 000 g for 1 h. The supernatant (S1, cytosolic fraction) was saved and the pellet was resuspended in homogenization buffer containing 1% Triton X-100, and left on ice for 30 min before recentrifugation at 120 000 g for 1 h. This supernatant (S2, solubilized membrane fraction) was collected. Fractions S1 and S2 were tested for PKC activity.

Assay of PKC

PKC activity was determined using a histone phosphorylation assay, essentially as described previously [21]. Briefly, 60 μ l of the cytosolic (S1) and solubilized membrane (S2) fractions was incubated in a reaction mixture (final volume 250 μ l) containing (final concentrations) 20 mM-Tris/HCl, pH 7.4, 5 mM-DTT, 10 mM-2-mercaptoethanol, 0.2 mM-PMSF, 0.8 mg of BSA/ml, 50 μ g of histone (type III-S), 10 mM-MgCl₂ and 1 mM-EGTA, with or without 1 mM-CaCl₂, 38 μ g of phosphatidylserine/ml and 45 μ g of 1,2-diolein/ml. Reactions were started by addition of 20 μ l of 50 μ M- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (1 μ Ci) to 230 μ l of the assay mixture, and the incubations were carried out for 5 min at 30 °C. The reaction was stopped by addition of 1 ml of ice-cold 25% trichloroacetic acid, left on ice for 10 min and centrifuged (2500 g, 10 min), and the pellet was washed four times with ice-cold 5% trichloroacetic acid. The pellet was then dissolved in 1 M-NaOH at 80 °C and aliquots were taken for protein [20] and radioactivity measurements. PKC activity was calculated from the difference in phosphorylation assayed in the presence and the absence of Ca²⁺, phosphatidylserine and 1,2-diolein.

Enzyme activity (pmol of ³²P/min per mg of protein) was expressed as percentage of total PKC activity present in the cytosolic and membrane fractions.

Determination of muscle tissue cyclic AMP levels

Soleus muscle samples were immediately frozen in liquid nitrogen after 3 min of treatment with PMA (100 nM) or 1,25(OH)₂D₃ (1 nM). The tissue was homogenized for 15 s in 5 vol. of ice-cold 4% HClO₄ (4 ml/g of tissue) with an Ultra-Turrax homogenizer (Jank and Kunkel, Staufen, Germany). The homogenate was brought to pH 6.0 with 30% KHCO₃ and then centrifuged at 2500 g for 15 min. The supernatant was used for measurement of cyclic AMP by a protein-binding technique [22] using a commercial available kit (Diagnostic Products).

RESULTS

As shown in Table 1, short exposure of skeletal muscle from vitamin D-deficient chicks to 10 nM-1,25(OH)₂D₃ resulted in activation of membrane-bound PKC. Of total muscle PKC activity, 66% was in the cytosol and 34% was membrane-associated in the control tissue. After 1 min of exposure to the steroid about 50% of the cytosolic PKC had been translocated to the membrane fraction. Total muscle PKC activity (cytosol + membrane: 130 $\pm$ 9.5 pmol of P_i/min per mg of protein) did not change with 1,25(OH)₂D₃ treatment. Similarly, as with the sterol, treatment of muscle with the phorbol ester PMA, as well-known PKC activator, also resulted in increased activity of membrane PKC. To determine whether PKC has any role in skeletal muscle

Ca²⁺ uptake, the effects of PMA were evaluated. Treatment of chick soleus muscle with PMA *in vitro* rapidly increased ⁴⁵Ca uptake (Fig. 1). Within 1–3 min the phorbol ester increased ⁴⁵Ca uptake by 68% with respect to controls; this then declined to 55%, 36% and 48% after 5, 10 and 15 min of treatment respectively. Muscle ⁴⁵Ca uptake steadily increased with 1,25(OH)₂D₃ exposure (34% and 66% above controls at 1 and 15 min respectively).

A dose-response study of the rapid effects of PMA on muscle Ca²⁺ uptake was performed using a phorbol ester concentration range of 50–200 nM. Treatment for 5 min with 50 nM-PMA stimulated muscle ⁴⁵Ca uptake by 50%. The response to the phorbol ester was further increased at concentrations of 100 nM (+64%) and 150 nM (+85%); the highest concentration tested (200 nM) resulted in a submaximal response (+54%). As shown in Table 2, 4 β -phorbol dibutyrate (100 nM) also potentiated muscle ⁴⁵Ca uptake (+60% and +46% after 5 and 15 min of treatment respectively), whereas the inactive phorbol, 4 α -phorbol (100 nM), was without effect. These results indicate that the effects of PMA are due to its interaction with PKC, and not to general modifications of the cell membrane.

As with 1,25(OH)₂D₃, the PMA-dependent increase in muscle ⁴⁵Ca uptake was effectively suppressed by the Ca²⁺ channel

Table 1. 1,25(OH)₂D₃-induced activation of PKC

Soleus muscles from vitamin D-deficient chicks were incubated in Krebs/Henseleit/glucose at 37 °C and treated with 1,25(OH)₂D₃ (1 nM) or PMA (100 nM) for 1 min. Cytosolic and membrane fractions were prepared as described in the Materials and methods section. The PKC activity in each fraction was determined from the differences in phosphorylation of histone (type III-S) measured with and without addition of CaCl₂, phosphatidylserine and 1,2-diolein. Values are means $\pm$ S.D. for 9 determinations in separate experiments. **P* < 0.001; ***P* < 0.005 versus control.

Fraction	PKC activity (pmol of P _i /min per mg of protein)		
	Control	1,25(OH) ₂ D ₃	PMA
Cytosol (S1)	87.1 $\pm$ 6.7	54.6 $\pm$ 5.2*	32.5 $\pm$ 4.5*
Membrane (S2)	44.2 $\pm$ 3.8	80.6 $\pm$ 6.7**	94.9 $\pm$ 3.9**

Fig. 1. Time course of the effects of PMA and 1,25(OH)₂D₃ on skeletal muscle Ca²⁺ uptake

Vitamin D-deficient chick soleus muscles were incubated as described in the legend to Table 1, in the presence or the absence of PMA (100 nM, ●) or 1,25(OH)₂D₃ (1 nM, ○) for 1–15 min. The uptake of ⁴⁵CaCl₂ was then measured as indicated in the Materials and methods section. Results are means $\pm$ S.D. of four separate experiments. **P* < 0.001 versus control.

Table 2. Effects of phorbol esters on skeletal muscle ^{45}Ca uptake

Soleus muscles from vitamin D-deficient chicks were placed in Krebs/Henseleit/glucose and were treated with 4β -phorbol dibutyrate (100 nM) or 4α -phorbol (100 nM) for 5–15 min. The uptake of ^{45}Ca was then measured as indicated in the Materials and methods section. Results are the means $\pm$ S.D. of four experiments. * $P < 0.001$ with respect to control.

Incubation time (min)...	^{45}Ca uptake (% of control)	
	5	15
Control	100	100
4β -Phorbol	160 $\pm$ 8*	146 $\pm$ 10*
dibutyrate		
4α -Phorbol	104 $\pm$ 9	102 $\pm$ 11

Table 3. Rapid stimulation of muscle Ca^{2+} uptake by PMA and $1,25(\text{OH})_2\text{D}_3$ and its suppression by nifedipine and verapamil

Soleus muscles were incubated in Krebs/Henseleit/glucose solution in the absence or the presence of nifedipine (30 μM) or verapamil (50 μM) for 5 min. PMA (100 nM) or $1,25(\text{OH})_2\text{D}_3$ (1 nM) was then added, and the muscles were further incubated for 5 min. ^{45}Ca uptake was then measured as indicated in the Materials and methods section. Values are means $\pm$ S.D. of four independent experiments. * $P < 0.001$; ** $P < 0.005$ versus controls.

Addition	^{45}Ca uptake (nmol/mg of protein)		
	Control	$1,25(\text{OH})_2\text{D}_3$	PMA
None	36.9 $\pm$ 2.3	57.7 $\pm$ 3.8*	56.1 $\pm$ 2.3**
Verapamil	39.2 $\pm$ 3.1	38.5 $\pm$ 3.2	38.3 $\pm$ 3.5
Nifedipine	36.1 $\pm$ 2.5	37.3 $\pm$ 1.9	37.3 $\pm$ 3.2

antagonists nifedipine and verapamil (Table 3). In addition, the stimulation of ^{45}Ca uptake induced by the phorbol ester (100 nM; 5 min) was abolished by the PKC inhibitor staurosporine at a concentration of 10 nM (42% and 8% above controls in the absence and presence of staurosporine respectively). Unexpectedly, staurosporine potentiated $1,25(\text{OH})_2\text{D}_3$ -dependent muscle ^{45}Ca uptake (38% and 60% above controls in the absence and presence of staurosporine respectively). The potentiation by staurosporine of the effects of $1,25(\text{OH})_2\text{D}_3$ was dose-dependent. Fig. 2 shows that treatment of soleus muscle from vitamin D-deficient chicks with $1,25(\text{OH})_2\text{D}_3$ *in vitro* (5 min, 1 nM) in the presence of 10 nM-staurosporine increased ^{45}Ca uptake values by 58%. Higher concentrations of the PKC inhibitor (50 and 100 nM) further increased (+140 and +170% respectively) sterol-dependent muscle ^{45}Ca uptake. The unexpected finding that the blockade of the PKC pathway potentiated $1,25(\text{OH})_2\text{D}_3$ stimulation of ^{45}Ca uptake was further substantiated by experiments showing that treatment of muscle simultaneously with $1,25(\text{OH})_2\text{D}_3$ (1 nM) and PMA (50 nM) reduced by 80% the stimulatory effects evoked by either agent alone (results not shown).

Since adenylate cyclase and its product cyclic AMP have been shown to mediate rapid effects of $1,25(\text{OH})_2\text{D}_3$ in skeletal muscle [11], we determined whether the PKC inhibitor also potentiates cyclic AMP stimulation of muscle ^{45}Ca uptake. As shown in Table 4, treatment of muscle for a short time (5 min) with the cyclic AMP analogue dibutyryl cyclic AMP (10 μM) in the presence of staurosporine (10 nM) substantially potentiated

Fig. 2. Dose-response profile of the effects of staurosporine on $1,25(\text{OH})_2\text{D}_3$ -dependent muscle Ca^{2+} uptake

Chick soleus muscles were incubated for 5 min with various concentrations of staurosporine. $1,25(\text{OH})_2\text{D}_3$ was then added and the muscles were further incubated for 5 min. ^{45}Ca uptake was measured as indicated in the Materials and methods section. Results are means $\pm$ S.D. of three independent experiments. * $P < 0.001$; ** $P < 0.005$ versus control (no staurosporine).

Table 4. Cyclic AMP-dependent stimulation of vitamin D-deficient skeletal muscle ^{45}Ca uptake: effects of staurosporine

Chick soleus muscles were incubated for 5 min in Krebs/Henseleit/glucose in the presence or absence of 50 nM-staurosporine. Dibutyryl cyclic AMP (10 μM) was then added and the muscles were further incubated for 5 min. ^{45}Ca uptake was measured as indicated in the Materials and methods section. Values are means $\pm$ S.D. of four separate experiments. * $P < 0.0025$; ** $P < 0.001$ versus controls.

	^{45}Ca uptake (nmol/mg of protein)	
	– Staurosporine	+ Staurosporine
Control	3.65 $\pm$ 0.43	4.12 $\pm$ 0.45
Dibutyryl cyclic AMP	5.18 $\pm$ 0.65*	7.90 $\pm$ 0.87**

Table 5. Cyclic AMP content of vitamin D-deficient skeletal muscle: effects of $1,25(\text{OH})_2\text{D}_3$ and PMA

Chick soleus muscles placed in Krebs/Henseleit/glucose were treated with $1,25(\text{OH})_2\text{D}_3$ (1 nM), PMA (150 nM) or both substances for 3 min. Tissue cyclic AMP levels were measured as indicated in the Materials and methods section. Results are means $\pm$ S.D. of four experiments. * $P < 0.001$; ** $P < 0.01$ with respect to controls.

	Cyclic AMP (pmol/g of tissue)
Control	149.1 $\pm$ 15.7
$1,25(\text{OH})_2\text{D}_3$	238.6 $\pm$ 14.3*
PMA	84.3 $\pm$ 18.5**
PMA + $1,25(\text{OH})_2\text{D}_3$	67.9 $\pm$ 16.3**

(+52%) dibutyryl cyclic AMP-dependent Ca^{2+} uptake. It was therefore of interest to ascertain whether PMA affected muscle levels of cyclic AMP. To that end, soleus muscles were treated *in vitro* in the presence or the absence of PMA (150 nM, a dose which produced maximum stimulation of ^{45}Ca uptake), $1,25(\text{OH})_2\text{D}_3$ (1 nM) or both, and subsequently assayed for cyclic AMP content (Table 5). $1,25(\text{OH})_2\text{D}_3$, in agreement with previous

observations [11], elevated cyclic AMP levels, whereas the phorbol ester PMA diminished cyclic AMP by 44%. Simultaneous exposure of muscle to $1,25(\text{OH})_2\text{D}_3$ and PMA further decreased cyclic AMP levels (-54%).

DISCUSSION

In the present study we examined whether activation of PKC is part of the mechanism by which $1,25(\text{OH})_2\text{D}_3$ rapidly stimulates skeletal muscle Ca^{2+} uptake. The question of whether $1,25(\text{OH})_2\text{D}_3$ activates PKC was addressed by analysing the effects of the sterol on cellular redistribution of PKC. Membrane association of PKC has been observed after stimulation of cells with hormones whose action is believed to be mediated by PKC [23–26]. $1,25(\text{OH})_2\text{D}_3$ caused translocation of PKC and greatly potentiated muscle membrane PKC activity. We have recently obtained evidence that $1,25(\text{OH})_2\text{D}_3$ affects muscle cell phosphatidylinositol metabolism with increased formation of diacylglycerol, a known activator of PKC (S. Morelli, M. Skliar, A. R. de Boland & R. Boland, unpublished work), which further supports a stimulatory effect of the sterol on PKC activity. In addition, $1,25(\text{OH})_2\text{D}_3$ has been shown to stimulate PKC in tissues other than muscle [14].

We employed tumour-promoting phorbol esters, which substitute for the naturally occurring diacylglycerol in stimulating PKC, both *in vivo* and *in vitro* [27] to elucidate whether activation of PKC is involved in the rapid effects of $1,25(\text{OH})_2\text{D}_3$ on muscle Ca^{2+} uptake. PMA was able to reproduce the rapid stimulation of muscle Ca^{2+} uptake by the sterol, although the time course of its effects was different. Similarly to that caused by $1,25(\text{OH})_2\text{D}_3$ [9], the acute rise in muscle ^{45}Ca uptake evoked by PMA was effectively suppressed by Ca^{2+} channel antagonists such as verapamil and nifedipine. This is in accordance with evidence indicating that PKC is also involved in the enhancement of Ca^{2+} channel activity by phosphorylating several membrane proteins related to the channels [15–17]. This interpretation is further supported by the fact that staurosporine, a specific inhibitor of PKC activity [28], abolished the rise in Ca^{2+} uptake induced by PMA in skeletal muscle. However, contrary to the observed effects with PMA, staurosporine surprisingly potentiated $1,25(\text{OH})_2\text{D}_3$ -dependent stimulation of Ca^{2+} uptake. In accordance with this observation, PMA attenuated the potentiatory effect of $1,25(\text{OH})_2\text{D}_3$ on Ca^{2+} uptake and muscle cyclic AMP levels. It is conceivable that PKC-dependent phosphorylation may affect in some way the activity of the adenylate cyclase system [29] and/or of cyclic AMP phosphodiesterases [30], thereby modulating transduction signalling of $1,25(\text{OH})_2\text{D}_3$. The interaction of PKC and other second messenger pathways such as cyclic AMP may modify PKC-activated responses [31]. There is evidence indicating that these pathways may act co-ordinately to alter the response observed when they are stimulated separately. This may occur by affecting the pathways themselves, such as increasing or decreasing the level of second messenger [32–36], or by acting in concert at the target of the response [37–39]. Our results may be an example of how one signalling pathway alters the effect of another to create a unified response towards a common goal; in this case, acute stimulation of muscle Ca^{2+} uptake by $1,25(\text{OH})_2\text{D}_3$.

The present study provides evidence to support the view that $1,25(\text{OH})_2\text{D}_3$ -dependent stimulation of skeletal muscle Ca^{2+} uptake through a cyclic AMP-dependent pathway also involves the PKC pathway, and that both kinases modulate the transduction signalling of $1,25(\text{OH})_2\text{D}_3$.

This work was supported by grants from the National Research Council of Argentina (CONICET), Nestle Nutrition (Vevey, Switzerland) and Volkswagen Stiftung (Hannover, Germany).

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