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Evidence for the participation of protein kinase C and 3′,5′-cyclic AMP-dependent protein kinase in the stimulation of muscle cell proliferation by 1,25-dihydroxy-vitamin D_3

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Summary

Treatment with 1,25-dihydroxy-vitamin D_3 ($1,25(OH)_2D_3$) (1–12 h, 10^{-10} M) stimulates DNA synthesis in proliferating myoblasts, with an early response at 2–4 h of treatment followed by a maximal effect at 10 h. To investigate the mechanism involved in the mitogenic action of the hormone we studied the possible activation of intracellular messengers by $1,25(OH)_2D_3$. The initial phase of stimulation of [^{3}H]thymidine incorporation into DNA by the sterol was mimicked by the protein kinase C activator tetradecanoylphorbol acetate (TPA) in a manner which was dose dependent and specific as the inactive analog 4α-phorbol was without effect. Maximal responses to TPA (100 nM) were obtained at 4 h. Staurosporine, a protein kinase C inhibitor, blocked the effect of $1,25(OH)_2D_3$ on myoblast proliferation at 4 h. In addition, a fast (1–5 min) elevation of diacylglycerol levels and membrane-associated protein kinase C activity was observed in response to $1,25(OH)_2D_3$. The adenylate cyclase activator forskolin (20 μM) and dibutyryl-cAMP (50 μM) increased DNA synthesis reproducing the second $1,25(OH)_2D_3$-dependent stimulatory phase at 10 h. Inhibitors of protein kinase A blocked the increase in muscle cell DNA synthesis induced by $1,25(OH)_2D_3$ at 10 h. Significant increases in cyclic AMP levels were detected in myoblasts treated with the sterol for 1–10 h. The calcium channel antagonist nifedipine (5–10 μM) abolished both the effects of 4-h treatment with $1,25(OH)_2D_3$ or TPA and 10-h treatment with $1,25(OH)_2D_3$ or dibutyryl-cAMP. Similar to the calcium channel agonist Bay K8644, $1,25(OH)_2D_3$ stimulated myoblast ^{45}Ca uptake and its effects were blocked by nifedipine. Our results suggest that activation of calcium channels by phosphorylation via protein kinases C and A and is involved in the mitotic response of myoblasts to $1,25(OH)_2D_3$.

Introduction

Evidence accumulated during the last few years indicates that the hormonally active form of vitamin D_3 may play a role in regulating cell growth and differentiation in addition to its classical function of extracellular calcium homeostasis. The hormone either stimulates or inhibits proliferation in a variety of cells in vitro (Franceschi et al., 1985; Koh et al., 1988; Kremer et al., 1989; Okasaki et al., 1989). Myoblasts are mononucleated myogenic cells which proliferate actively in culture before undergoing differentiation into multinucleated myotubes (Wakelam, 1985). Both myoblasts and myotubes contain $1,25(OH)_2D_3$-receptors (Boland et al., 1985; Simpson et al., 1985; Costa et al., 1986). It has been reported that the sterol affects several myoblast functions that include calcium and phosphate metabolism (De Boland and Boland, 1985; Bellido and Boland, 1990), protein synthesis (Drittanti et al., 1989a) and lipid composition (Bellido et al., 1987), as well as morphological characteristics of myoblast cultures (Giuliani and Boland, 1984). Previous work has also shown that $1,25(OH)_2D_3$ increases [^{3}H]thymidine incorporation into DNA in proliferating myoblasts whereas it inhibits DNA synthesis in pre-fusion myoblasts (Drittanti et al., 1989b). Recent investigations have shown that the action of $1,25(OH)_2D_3$ on adult skeletal muscle may involve the activation of

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second messenger pathways (Fernández et al., 1990). In connection to these observations, we have investigated in the present study the participation of protein kinase C and A messenger systems in the mitogenic action of $1,25(OH)_2D_3$ in proliferative muscle cell cultures.

Materials and methods

Materials

$1,25(OH)_2D_3$ was provided by Hoffmann-LaRoche (Nutley, NJ, USA). Bovine pancreas trypsin, type III-s, Eagle's minimum essential medium (MEM), forskolin, dibutyryl-cAMP, 12-*O*-tetradecanoylphorbol 13-acetate (TPA), 4α-phorbol, staurosporine, compound H-7 (1-(5-isoquinolynsulfonyl)-2-methyl-piperazine), protein kinase A inhibitors from rabbit muscle (crude) and porcine heart (type III) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Methyl-[1,1′,2′-^{3}H]thymidine (3.60 TBq · mmol^{-1}), [5,6,8,9,11,12,14,15-^{3}H]-arachidonic acid (3.70 TBq · mmol^{-1}), $^{45}CaCl_2$, cyclic AMP and Omnifluor scintillation fluid were provided by New England Nuclear (Boston, MA, USA). All other reagents were of analytical grade.

Cell culture

Myoblasts were obtained from 12-day-old chick embryo breast muscle as previously described (Bellido and Boland, 1987). Fibroblast-free cells were cultured after isolation in MEM containing 10% chick serum, 10% chick embryo extract and 1% antibiotic-antimycotic solution (O'Neill and Stockdale, 1972) for 24 h under a constant flow of 95% air-5% CO_2 saturated with water. The medium was then replaced, lowering the concentration of chick embryo extract to 2%. $1,25(OH)_2D_3$ (10^{-10} M), TPA (10–200 nM), 4α-phorbol (100 and 200 nM), forskolin (20–50 μM) or dibutyryl-cAMP (50 and 100 μM) were added at this time and cells were cultured for up to 12 h. In the experiments in which nifedipine (5–10 μM), staurosporine (20–50 nM) or protein kinase A inhibitors (inhibitory capacity of 10 and 50 phosphorylating U/ml for the peptides from rabbit muscle and porcine heart, respectively) were used, they were added 1 h before addition of the agonists. Viability of cells was ensured by measurements of trypan blue dye exclusion. Chick serum and chick embryo extract samples used to supplement the cultures contributed 3.7 and 2 pg/ml medium of $1,25(OH)_2D_3$, respectively. The metabolite was assayed as described elsewhere (Bouillon et al., 1980).

Measurement of DNA synthesis

DNA synthesis was assessed by the accumulation of radioactivity into perchloric acid precipitable material (final concentration: 5%, w/v) following labeling of myoblasts with ^{3}H-thymidine (1 μCi/ml) in Krebs-Henseleit-0.2% glucose solution at 37°C under 95% O_2-5% CO_2 for 60 min, starting at designated intervals of culture under the indicated conditions. The resulting precipitate was further washed twice with 5% perchloric acid and dissolved in 0.1 M NaOH. Aliquots were taken for protein measurements (Lowry et al., 1951) and determination of radioactivity in a Beckman liquid scintillation spectrometer using Omnifluor as scintillation fluid.

Determination of cyclic AMP, diacylglycerol and protein kinase C

To determine cyclic AMP, myoblast samples were immediately frozen after treatment with $1,25(OH)_2D_3$ (10^{-10} M). The cells were homogenized 15 s in 5 volumes of cold 10% trichloroacetic acid using an Ultraturrax homogenizer (Jank and Kunkel, Staufen, Germany). The homogenate was centrifuged at $2500 \times g$ for 15 min and the supernatant was washed 5 times with 6 volumes of water-saturated diethyl ether. The washed extract was lyophilized and cyclic AMP was measured by a competitive protein binding technique (Tovey et al., 1974).

For evaluation of $1,25(OH)_2D_3$ effects on diacylglycerol (DG) production, myoblasts were labeled with [^{3}H]arachidonic acid for 2.5 h. Cells were then washed 3 times with Krebs-Henseleit-0.2% glucose and incubated in the same medium either with vehicle (ethanol 0.01%) or $1,25(OH)_2D_3$ (10^{-10} M). The radioactivity incorporated into DG was determined after its isolation from myoblast lipid extracts (Folch et al., 1957) by TLC on silica gel G plates developed with hexane/diethyl ether/acetic acid (75:25:4, v/v).

Studies on the effects of $1,25(OH)_2D_3$ on myoblast protein kinase C (PKC) were performed as follows. Myoblasts (approx. 60×10^6 cells) were washed twice with Krebs-Henseleit-0.2% glucose and resuspended in the same solution. The cells were preincubated at 37°C under 95% O_2:5% CO_2 for 30 min and then treated with 10^{-10} M $1,25(OH)_2D_3$ or ethanol for 1–5 min followed by centrifugation at $2000 \times g$. The pellet was homogenized twice for 30 s with 3 ml of 20 mM Tris-HCl (pH 7.4), 0.33 M sucrose, 1 mM EGTA, 40 μg/ml leupeptin, 0.3 mM phenylmethylsulfonyl fluoride, using an Ultraturrax homogenizer at maximum speed. The homogenate was centrifuged for 10 min at $1200 \times g$ in a Sorvall refrigerated centrifuge. Microsomal membranes were sedimented from this supernatant in a Beckman L5-50B ultracentrifuge at 100,000 $\times g$ for 1 h. The supernatant (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 $100,000 \times g$ for 1 h. PKC activity in myoblast cytosol and microsomal membrane fractions was determined from the differences in phosphorylation of histone (type III-S) mea-

sured with and without addition of $CaCl_2$, phosphatidyl-serine, and 1,2-diolein, as recently described (Massheimer and De Boland, 1992).

Measurement of calcium uptake

After treatment, myoblasts were preincubated 30 min at 25°C with Krebs-Henseleit-0.2% glucose. $^{45}CaCl_2$ (2 μCi/ml) was then added to the medium and the cells were further incubated for 4 min and the reaction was terminated by washing the myoblasts several times with cold medium. The cells were dissolved in 1 N NaOH and aliquots were taken for protein and radioactivity determination.

Statistical evaluation

Statistical evaluation of the results obtained was performed using the Student's t-test (Snedecor and Cochran, 1967).

Results

Treatment with physiological doses of $1,25(OH)_2D_3$ stimulates the incorporation of [^{3}H]thymidine into DNA in actively proliferating chick embryo myoblast cultures. As shown in Fig. 1, the effect is characterized by a phase of low responses during the first 1–4 h of treatment which is maintained for up to 8 h followed by a marked increase between 8 and 10 h of incubation with the sterol. In agreement with these observations a significant elevation in cell numbers has been previously observed in similar myoblast cultures incubated with $1,25(OH)_2D_3$ for 12 h (Drittanti et al., 1989b). To test whether changes in protein kinase C (PKC) would modify myoblast proliferation, DNA synthesis was measured in cells cultured for 1–13 h in the absence and presence of the phorbol ester TPA (50 and 100 nM), a known activator of PKC. Both concentrations of TPA stimulated [^{3}H]thymidine incorporation into DNA with maximal effects at 4 h, reproducing the early response of myoblasts to $1,25(OH)_2D_3$ (Fig. 2). Phorbol ester stimulation of myoblast DNA synthesis was dose dependent with 10, 25, 50 and 100 nM TPA, producing responses of 48, 61, 90 and 417% higher than controls, respectively, whereas 200 nM TPA exerted a lower stimulation of DNA precursor incorporation (94%). Treatment with PKC activator exhibited specificity as the inactive phorbol ester 4α-phorbol (100 and 200 nM) was, however, without effect (Table 1).

The possible participation of the cAMP signal pathway in the action of $1,25(OH)_2D_3$ on myoblast prolifer-

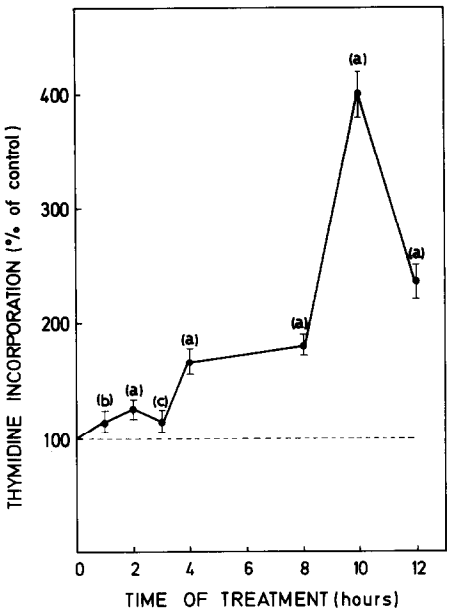

Fig. 1. Stimulation by 1,25-dihydroxy-vitamin D_3 of [^{3}H]thymidine incorporation into DNA by cultured myoblasts. Primary cultured (24 h) chick embryo myoblasts were incubated in minimum essential medium supplemented with 10% chick serum and 2% chick embryo extract in the absence and presence of $1,25(OH)_2D_3$ (10^{-10} M) for 1–12 h. The cells were then incubated with [^{3}H]thymidine in Krebs-Henseleit solution-0.2% glucose for 1 h and the acid-insoluble radioactivity was determined as indicated in Materials and methods. Results are expressed as percentage of the control values for each time and are means ± SD of four independent experiments. (Absolute values for control cells were 613 ± 20 and 1360 ± 107 dpm/mg protein for 1 and 12 h of culture, respectively). [a] $p < 0.0005$; [b] $p < 0.005$; [c] $p < 0.025$.

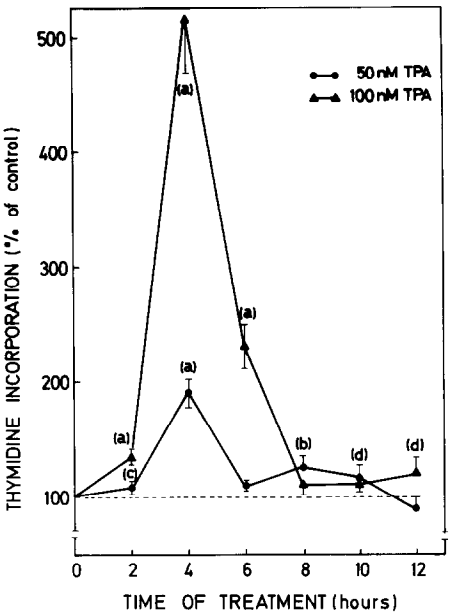

Fig. 2. Effect of phorbol ester TPA on myoblast DNA synthesis. Primary cultured (24 h) myoblasts were incubated in medium containing 2% chick embryo extract and 10% chick serum in the absence and presence of 50 nM (●——●) or 100 nM (▲——▲) TPA for 1–12 h. [^{3}H]Thymidine incorporation into myoblast DNA was then measured as indicated in the legend to Fig. 1. Data are expressed as percentage of the control values and are means ± SD of three independent experiments. [a] $p < 0.0005$; [b] $p < 0.0125$; [c] $p < 0.025$; [d] $p < 0.05$.

TABLE 1

DOSE–RESPONSE EFFECTS OF TPA ON MYOBLAST DNA SYNTHESIS

Primary cultured (24 h) chick embryo myoblasts were treated in medium containing 2% chick embryo extract and 10% chick serum with different doses of TPA (25–200 nM) or the inactive analog 4α-phorbol, for 4 h. [^{3}H]Thymidine incorporation into muscle cell DNA was measured as described in Materials and methods. Data are means ± SD of three independent determinations.

Treatment	Thymidine incorporation	
	dpm/mg protein	% of control
Control	977 ± 87	100 ± 9
+ TPA (nM)		
10	1,447 ± 117	148 ± 12 [b]
25	1,573 ± 147	161 ± 15 [b]
50	1,857 ± 157	190 ± 16
100	5,050 ± 440	517 ± 45 [a]
200	1,893 ± 187	194 ± 19 [a]
+ 4α-Phorbol		
100	1,043 ± 20	107 ± 2 [c]
200	918 ± 68	94 ± 7 [c]

[a] $p < 0.0005$; [b] $p < 0.0025$; [c] $p < 0.2$.

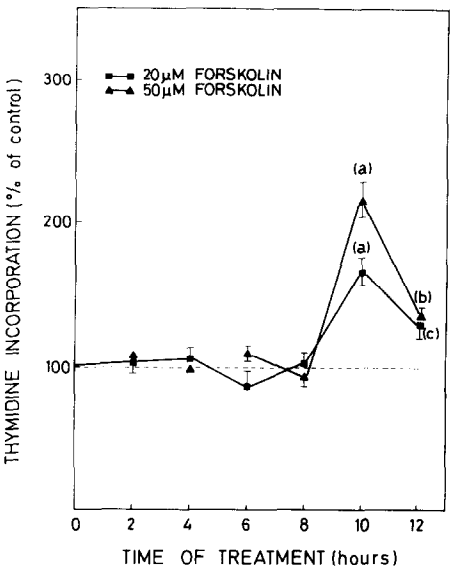

Fig. 3. Effects of forskolin on myoblast DNA synthesis. Proliferating myoblasts cultured as described in Fig. 1 were incubated for 1–12 h in the absence and presence of 20 μM (■ ——— ■) or 50 μM (▲ ——— ▲) forskolin. [^{3}H]Thymidine incorporation into DNA was then measured as indicated in the legend to Fig. 1. Data are expressed as percentage of the control values and are means ± SD of three independent experiments. [a] $p < 0.0005$; [b] $p < 0.0125$; [c] $p < 0.025$; [d] $p < 0.05$.

ation was also investigated. Cells were treated 1–12 h with 20 μM and 50 μM forskolin (Fig. 3) or with 50 μM and 100 μM dibutyryl-cAMP (Fig. 4). These concentrations of both agents have been shown to activate the cAMP-dependent pathway in cultured muscle cells (Sellés and Boland, 1992). The activator of adenylate cyclase and cAMP analogue produced a similar effect on myoblast DNA synthesis, mimicking the second $1,25(OH)_2D_3$-dependent stimulatory phase with maximal responses at 10 h of treatment. Both 20 μM and 50 μM forskolin increased [^{3}H]thymidine incorporation into DNA 68% and 115% over controls, respectively, at 10 h, the effects still being noticeable at 12 h of treatment (Fig. 3). The response of myoblasts to dibutyryl-cAMP was more rapid than to forskolin, with significant rises at 8 h (Fig. 4). Comparison of the time

TABLE 2

EFFECT OF PROTEIN KINASE C AND A INHIBITORS ON MYOBLAST DNA SYNTHESIS STIMULATION BY 1,25-DIHYDROXY-VITAMIN D_3

Myoblasts cultured as described in the legend to Fig. 1 were treated with $1,25(OH)_2D_3$ (10^{-10} M), in the absence and presence of the protein kinase C inhibitor staurosporine (20 nM and 50 nM) for 4 h or the protein kinase A inhibitors from rabbit skeletal muscle (inhibitory capacity of 10 phosphorylating U/ml) and from porcine heart (inhibitory capacity of 50 phosphorylating U/ml) for 10 h. The inhibitors were added 1 h before the vitamin D_3 metabolite. [^{3}H]Thymidine incorporation into DNA was measured as described in the legend to Fig. 1. Data are means + SD of three independent determinations.

Treatment	Thymidine incorporation dpm/mg protein (% of control)			
	Staurosporine		Protein kinase A inhibitor	
	20 nM	50 nM	Rabbit muscle (10 U/ml)	Porcine heart (50 U/ml)
Control	693 ± 30 (100 ± 4)		750 ± 63 (100 ± 8)	
$1,25(OH)_2D_3$	1,163 ± 83 (168 ± 12) [a]		2,970 ± 167 (396 ± 22) [a]	
C + inhibitor	743 ± 86 (100 ± 12)	689 ± 21 (100 ± 3)	827 ± 60 (100 ± 7)	797 ± 37 (100 ± 5)
$1,25(OH)_2D_3$ + inhibitor	667 ± 37 (90 ± 5) [c]	703 ± 34 (102 ± 5) [d]	1,844 ± 141 (223 ± 17) [a]	667 ± 67 (84 ± 8) [b]

[a] $p < 0.0005$; [b] $p < 0.025$; [c] $p < 0.1$; [d] n.s.

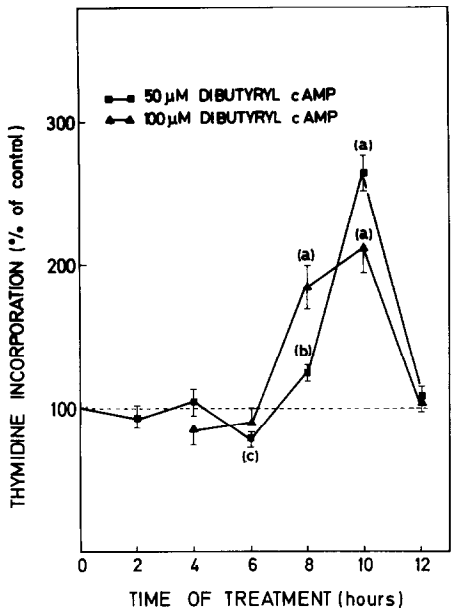

Fig. 4. Effect of dibutyryl-cAMP on [^{3}H]thymidine incorporation into DNA by cultured muscle cells. Proliferating (24 h) myoblasts were incubated in the absence and presence of 50 μM (■ ——— ■) or 100 μM (▲ ——— ▲) dibutyryl-cAMP for 1–12 h. [^{3}H]Thymidine incorporation into acid-insoluble material was then measured as indicated in the legend to Fig. 1. Data are expressed as percentage of the control values and are means ± SD of three independent experiments. [a] $p < 0.0005$; [b] $p < 0.005$; [c] $p < 0.0125$.

courses of $1,25(OH)_2D_3$-, TPA-, forskolin- and dibutyryl-cAMP-induced changes in myoblast DNA synthesis (Figs. 1–4) suggests that the stimulatory effect of the sterol would be the result of the time-dependent activation of PKC (2–6 h) and protein kinase A (PKA) (8–12 h). To experimentally verify this interpretation, we measured DNA synthesis in myoblasts treated 4 h and 10 h with $1,25(OH)_2D_3$ in the absence and presence of staurosporine (20 and 50 μM), a PKC inhibitor, and two different inhibitory peptides of cAMP-dependent kinases, respectively (Table 2). Both staurosporine concentrations were able to block the effect of 4-h treatment with $1,25(OH)_2D_3$ on DNA synthesis. Compound H-7 (15–30 μM), another PKC inhibitor, also suppressed the $1,25(OH)_2D_3$-dependent stimulation of [^{3}H]thymidine incorporation at 4 h (data not given). Similarly, a PKA inhibitor from porcine heart with an inhibitory capacity to block 50 PKA-dependent phosphorylating U/ml completely abolished the response generated by 10-h treatment with the vitamin D_3 metabolite (Table 2). Under similar conditions, this inhibitor has been shown in previous studies to effectively block $1,25(OH)_2D_3$ actions mediated by protein kinase A. Due to its peptide nature, the compound is possibly incorporated into the cell through endocytosis (De Boland and Norman, 1990; Sellés and Boland, 1992). In line with these results, an early elevation in myoblast DG levels was observed in response to $1,25(OH)_2D_3$ (Table 3). The hormone markedly increased DG after only 1 and 5 min of treatment (105% and 590%, respectively). At 10 min the effects were less pronounced. These changes were accompanied by a significant increase in protein kinase C activity associated with myoblast microsomal membranes at the expense of a decrease of cytosolic PKC activity (Table 3). As shown in Fig. 5, $1,25(OH)_2D_3$ increased myoblast cyclic AMP levels (75, 113, 504, 573 and 840% over controls after 1-, 2-, 4-, 6- and 10-h treatment, respectively). Forskolin (20 μM) also produced a marked elevation of myoblast cAMP levels (data not shown). As there is evidence indicating that the activation of protein kinases modulates via phosphorylation calcium channels (Curtis and Catteral, 1985; Levitan, 1985; Catteral, 1988), we tested whether

TABLE 3

EFFECT OF 1,25-DIHYDROXY-VITAMIN D_3 ON MYOBLAST DIACYLGLYCEROL LEVELS AND PROTEIN KINASE C ACTIVITY

Primary cultured (24 h) myoblasts were incubated in the absence and presence of $1,25(OH)_2D_3$ (10^{-10} M) for 1–5 min. For determination of diacylglycerol, cells were prelabeled with [^{3}H]arachidonic acid. DG was isolated by TLC as described under Materials and methods. PKC activity was determined in myoblast cytosolic and microsomal membrane fractions using a histone phosphorylation assay (Massheimer and De Boland, 1992). Data are means ± SD of three independent determinations.

Treatment time (min)	Diacylglycerol (% of control)	Protein kinase C (pmol Pi/min per mg protein)	
		Cytosol	Membrane
0	100	9.4 ± 0.8 (68%)	4.4 ± 0.8 (32%)
1	205 ± 17	2.6 ± 0.7 (18%)	11.6 ± 0.7 (82%)
3	–	3.7 ± 0.9 (27%)	9.5 ± 0.9 (73%)
5	690 ± 35	3.6 ± 0.7 (25%)	10.8 ± 0.6 (75%)
10	175 ± 15	–	–

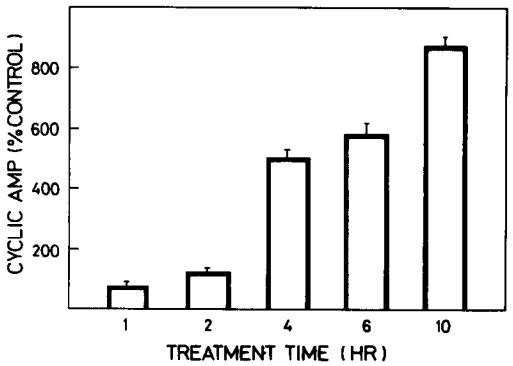

Fig. 5. Time course of effects of $1,25(OH)_2D_3$ on myoblast cyclic AMP content. Primary cultured (24 h) myoblasts were treated with 10^{-10} M $1,25(OH)_2D_3$ at the times indicated. Cell cAMP levels were measured as indicated in Materials and methods. Results are expressed as percentage of controls. Control values ranged between 1.07 ± 0.13 and 1.70 ± 0.08 pmol/mg protein at 1 h and 10 h, respectively. Values are the average of two experiments ± SD.

nifedipine, a calcium channel antagonist, would block the increases caused by $1,25(OH)_2D_3$, phorbol ester and dibutyryl-cAMP in myoblast DNA synthesis. The dihydropyridine compound at a concentration of 10 μM completely abolished both the effects of 4 h treatment with $1,25(OH)_2D_3$ or TPA and 10-h treatment with the sterol or dibutyryl-cAMP on [^{3}H]thymidine incorporation into DNA (Table 4). At a concentration of 2 μM, nifedipine inhibited 70% of the effect of $1,25(OH)_2D_3$ (10^{-10} M) on myoblast [^{3}H]thymidine incorporation. These concentrations of dihydropiridine are required to block L-type calcium channels in muscle cells (Fernández et al., 1990). In agreement with these results, $1,25(OH)_2D_3$ (10^{-10} M, 10 h), similar to the calcium channel agonist Bay K8644 (1 μM), increased myoblast ^{45}Ca uptake and its effect could be abolished by 2 μM nifedipine (3.97 ± 0.21 vs. 5.02 ± 0.26 vs. 4.85 ± 0.23 vs. 3.94 ± 0.48 nmol Ca/mg protein for control, Bay K8644, $1,25(OH)_2D_3$ and $1,25(OH)_2D_3$ + nifedipine, respectively).

Discussion

The hormonally active metabolite, $1,25(OH)_2D_3$, has been implicated in the regulation of cell growth and differentiation. Changes in cell proliferation in response to the hormone have been reported for different cell types including skeletal muscle cells (Franceschi et al., 1985; Koh et al., 1988; Drittanti et al., 1989b; Kremer et al., 1989). However, there is only scarce information concerning the mechanism involved in the mitogenic effect of $1,25(OH)_2D_3$. The results of the present work indicate that intracellular second messengers mediate the stimulation of DNA synthesis in proliferating myoblasts by $1,25(OH)_2D_3$. The employment of the tumor-promoting phorbol ester TPA, a substitute for the natural PKC activator diacylglycerol (Kraft and Andersen, 1983), and forskolin and dibutyryl-cAMP, useful tools to study cAMP-dependent cellular functions (Daly, 1984), suggested the sequential involvement of protein kinase A and C in the effects of the sterol, as the early (2–6 h) and subsequent maximal (8–12 h) $1,25(OH)_2D_3$ responses in [^{3}H]thymidine incorporation into myoblast DNA were temporally correlated to the responses elicited by TPA and cAMP pathway activators, respectively (Figs. 1–4). This interpretation is also supported by the fact that staurosporine and compound H-7, inhibitors of PKC activity (Tamaoki et al., 1986), abolished the effects of a 4-h treatment interval with $1,25(OH)_2D_3$ on myoblast DNA synthesis, whereas inhibitory peptides of PKA (Welsh, 1971) suppressed the response caused by 10-h incubation with the hormone (Table 2).

The mediation of $1,25(OH)_2D_3$ effects by these intracellular messengers was further suggested by the fast rise in myoblast diacylglycerol concentration and redistribution of PKC activity from cytosol to membranes induced by the hormone (Table 3). Additional evidence of the participation of the PKA pathway was provided by the marked increase in cAMP content of muscle cells observed between 1 h and 10 h of treatment with $1,25(OH)_2D_3$ (Fig. 5). It is likely that this elevation of cAMP levels is the result of a stimulation of adenylate cyclase and not of an inhibition of cAMP catabolism by $1,25(OH)_2D_3$, which occurs independently of changes in cell Ca^{2+}. Recent studies in the laboratory have shown that $1,25(OH)_2D_3$-induced increase of cAMP concentration and stimulation of dihydropyridine-sensitive calcium influx in muscle cells is blocked by the specific adenylate cyclase inhibitor 9-

TABLE 4

EFFECT OF CALCIUM CHANNEL ANTAGONIST NIFEDIPINE ON 1,25-DIHYDROXY-VITAMIN D_3-, TPA- AND DIBUTYRYL-cAMP-INDUCED CHANGES ON MYOBLAST DNA SYNTHESIS

Proliferating (24 h) myoblasts cultured as described in the legend to Fig. 1 were treated with $1,25(OH)_2D_3$ (10^{-10} M), TPA (100 nM) or dibutyryl-cAMP (50 μM), in the absence and presence of nifedipine (10 μM) added 1 h before as described in Materials and methods. [^{3}H]Thymidine incorporation into cell DNA was measured after 4 or 10 h of treatment. Data are means ± SD of three independent experiments.

Treatment	Nifedipine	Thymidine incorporation	
		dpm/mg protein (% of control)	
		4 h	10 h
Control	(−)	693 ± 30 (100 ± 4)	750 ± 63 (100 ± 8)
$1,25(OH)_2D_3$	(−)	1,163 ± 83 (168 ± 12) [a]	2,970 ± 167 (396 ± 22) [a]
TPA	(−)	3,583 ± 313 (517 ± 45) [a]	–
Dibutyryl-cAMP	(−)	–	1,583 ± 120 (211 ± 16) [a]
Control	(+)	720 ± 67 (100 ± 9)	833 ± 50 (100 ± 6)
$1,25(OH)_2D_3$	(+)	717 ± 7 (100 ± 1) [d]	883 ± 60 (106 ± 7) [c]
TPA	(+)	653 ± 50 (91 ± 7) [b]	–
Dibutyryl-cAMP	(+)	–	900 ± 50 (108 ± 6) [b]

[a] $p < 0.0005$; [b] $p < 0.1$; [c] $p < 0.2$; [d] n.s.

(tetrahydro-2-furyl) adenine (Sellés, J. and Boland, R., unpublished). These results are in agreement with various lines of evidence indicating that protein kinase C and cAMP may play a role in the production of a mitogenic response (Rozengurt, 1986). It has been reported that $1,25(OH)_2D_3$ may also induce elevations in PKC levels/activity in human HL-60 leukemic and U937 monoblastoid cells (Martel et al., 1987; Ways et al., 1987). Moreover, evidence on a direct stimulation of muscle cell protein kinase A by $1,25(OH)_2D_3$ has been recently obtained (Massheimer et al., 1992). The hormone has also been shown to increase the concentration of cAMP in intestine (Walling et al., 1967; Corradino, 1977) and skeletal muscle (Fernández et al., 1990). The possibility that $1,25(OH)_2D_3$ may also increase cGMP in muscle cells as reported for human skin fibroblasts cannot be excluded (Barsony and Marx, 1988).

Recent studies have indicated that $1,25(OH)_2D_3$ exerts biological actions in skeletal muscle (De Boland and Boland, 1987) as well as in other tissues/cells (Caffrey and Farach-Carson, 1989; Tornquist and Tashjian, 1989) through an activation of calcium channels. Phosphorylation is a key metabolic event in the modulation of calcium channel activity and this process can be mediated by the activation of PKC and PKA messenger pathways (Curtis and Catteral, 1985; De Reimer et al., 1985; Levitan, 1985; Catteral, 1988). It is then possible that stimulation of protein kinases C and A by $1,25(OH)_2D_3$ in myoblasts results in increased calcium fluxes through calcium channels as an earlier step in its mitogenic action. The participation of PKC and PKA in the activation of intestinal basolateral membrane calcium channels by $1,25(OH)_2D_3$ has been recently suggested (De Boland and Norman, 1990). Data from the present investigation support this hypothesis as the dihydropyridine nifedipine, a calcium channel antagonist, blocked the stimulation of [^{3}H]-thymidine incorporation into myoblast DNA in response to 4-h and 10-h treatment with $1,25(OH)_2D_3$ and also the increase in DNA synthesis induced by the incubation of muscle cells during 4 h with TPA and 10 h with dibutyryl-cAMP (Table 4). The presence of L-type calcium channels in the myoblast preparations used was evidenced by the stimulation of myoblast ^{45}Ca uptake by the calcium channel agonist Bay K8644. In agreement with these observations, $1,25(OH)_2D_3$ also stimulated a dihydropyridine-sensitive myoblast calcium uptake pathway. Similar observations have been made with TPA and forskolin at the same concentrations used in the present study (Fernández et al., 1990; Massheimer and De Boland, 1992).

It is conceivable that $1,25(OH)_2D_3$-dependent changes in intracellular Ca^{2+} involving activation of calcium channels may play a role, at least in part, in the stimulation of myoblast proliferation by the hormone in view that Ca^{2+} is an important factor in the regulation of the cell cycle (Whitfield et al., 1985). However, the mediation by cAMP-regulated reactions, independently of changes in Ca^{2+}, cannot be excluded. It has been shown that calcium ionophores mimic only partially the profile of modifications in DNA synthesis caused by $1,25(OH)_2D_3$ (Drittanti et al., 1889b). This, as well as other mechanistic aspects related to the transduction of the secosteroid hormone signal in myoblasts, deserve further study.

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