Generation of inositol phosphates, diacylglycerol and calcium fluxes in myoblasts treated with 1,25-dihydroxyvitamin D₃

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We have examined the effects of the seco-steroid hormone 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] on membrane phosphoinositide metabolism, protein kinase C (PKC) activation and influx of extracellular Ca²⁺ in chick-embryo muscle-cell (myoblast) cultures. At physiological concentrations, the hormone induces a rapid (15 s) and transient release of inositol triphosphate (InsP₃) and diacylglycerol (DAG). InsP₃ release is maximal at 60 s (80% above controls) and then declines. The effects of 1,25(OH)₂D₃ on InsP₃ production exhibited specificity, as 25-hydroxy-vitamin D₃ and 24,25-dihydroxy-vitamin D₃ did not alter myoblast InsP₃ levels. The stimulation of DAG is biphasic, with peaks at 60 s (+105%) and 5 min (+700%). The second phase of DAG release is not associated with changes in InsP₃. 1,25(OH)₂D₃ induces a rapid

(within 60 s) accumulation of InsP₂, and its effect on InsP₃ is delayed (120 s). The hormone rapidly activates myoblast PKC, with maximal translocation of activity from the cytosol to the cell membrane occurring at 60 s. Myoblast ⁴⁵Ca uptake significantly increases within 30 s of exposure to 1,25(OH)₂D₃. The response is time- (0.5–10 min) and dose- (1 pM–10 nM) dependent. The effects of the hormone are mimicked by the Ca²⁺-channel agonist Bay K 8644 and are effectively suppressed by nifedipine and extracellular EGTA. The results suggest that the rapid non-genomic actions of 1,25(OH)₂D₃ in myoblasts involve second-messenger systems associated with the generation of InsP₃ and DAG and regulation of Ca²⁺ fluxes through voltage-operated channels.

INTRODUCTION

The hormonal form of vitamin D, 1,25-dihydroxy-vitamin D₃ [1,25(OH)₂D₃] exerts early physiological effects in several cell systems by activation of signal-transduction mechanisms similar to those elicited by peptide hormones after interaction with membrane receptors. Reported cellular effects of 1,25(OH)₂D₃ include alteration of membrane Ca²⁺ influx [1–8], release of intracellular Ca²⁺ stores [5,9], cyclic AMP [10,11], cyclic GMP [11–13], stimulation of protein kinase C activity [14–16] and protein phosphorylation [8,10,11].

In many of the target cells 1,25(OH)₂D₃ has been shown to induce degradation of inositol-containing phospholipids [15,17–22]. This reaction, which is catalysed by phospholipase C, leads to two putative second messengers, InsP₃ (which mobilizes Ca²⁺) and diacylglycerol (DAG, which stimulates protein phosphorylation by protein kinase C activation). Hydrolysis of polyphosphoinositides leads to a rise in the cytosolic Ca²⁺ concentration and is due to two separate mechanisms: a transient release of Ca²⁺ from intracellular stores mediated by InsP₃, accompanied by a more persistent increase in the Ca²⁺ influx across the plasma membrane [23]. The latter process has been suggested to be due to the opening of a Ca²⁺ channel [24], or to be triggered by intracellular second messengers such as Ca²⁺ [25], InsP₃ itself [26] or its phosphorylation product InsP₄ [27].

We have recently presented evidence indicating that the rapid actions of 1,25(OH)₂D₃ on muscle cells are mediated by the activation of the adenylate cyclase transducing system [10,11]. In continuing our search for the signalling systems activated by 1,25(OH)₂D₃, we have now examined the effects of the hormone on polyphosphoinositide metabolism and Ca²⁺ influx in cultured myoblasts.

MATERIALS AND METHODS

Materials

All radiochemicals were obtained from New England Nuclear (Chicago, IL, U.S.A.) and all chemicals from Sigma (St. Louis, MO, U.S.A.). Dowex AG1-X8 (formate form, 200–400 mesh) was obtained from Bio-Rad (Richmond, CA, U.S.A.). 1,25(OH)₂D₃ was kindly supplied by Dr. Milan Uskokovic (Hoffmann–La Roche, Nutley, NJ, U.S.A.).

Culture and incubation procedures

A myoblast suspension was prepared by controlled trypsin digestion of breast muscle from 12-day chick embryos essentially as described previously [28]. The freed cells were collected by centrifugation and resuspended in Eagle's minimum essential medium containing 10% fetal-calf serum, 10% chick-embryo extract and 1% antibiotic–antimycotic solution [29]. The suspension was dispersed by pipetting, filtered through nylon mesh and 'pre-plated' on gelatin-coated Petri dishes to remove contaminating fibroblasts. The unadsorbed cells were adjusted to a concentration of 2.5 × 10⁵ cells/ml and cultured as a monolayer or in suspension with mild shaking at 37 °C in a humidified atmosphere of CO₂/air (1:19). The medium was replaced after incubation for 24 h, with the concentration of chick-embryo extract lowered to 2%. To investigate the effects of 1,25(OH)₂D₃ on the hydrolysis of PtdIns(4,5)P₂ and the generation of InsP₃, the cells were incubated in inositol-free medium containing *myo*-[2-³H]inositol (2 μCi/ml; 67.9 Ci/mmol) for 2.5 h. To evaluate the action of the hormone on the formation of DAG, the cells were incubated with [³H]arachidonic acid (2 μCi/ml; 76 Ci/mmol) for 2.5 h. After the labelling period the cells were

Abbreviations used: 1,25(OH)₂D₃, 1,25-dihydroxyvitamin D₃; 25(OH)D₃, 25-dihydroxy-vitamin D₃; 24,25(OH)₂D₃, 24,25-dihydroxy-vitamin D₃; DAG, diacylglycerol; PKC, protein kinase C.

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washed three times with Krebs/Henseleit medium containing 0.2% glucose and incubated in the same medium, or in medium containing 30 mM LiCl for *myo*-[^3H]inositol-labelled cells, either with vehicle (0.01% ethanol) or $1,25(\text{OH})_2\text{D}_3$ (1 nM) for various incubation periods (from 15 s to 10 min).

Inositol phosphates

After treatment, extraction of the inositol phosphates was carried out by adding an equal volume of ice-cold 12% trichloroacetic acid and the precipitated proteins were removed by centrifugation. Trichloroacetic acid was removed from the supernatant by six extractions with diethyl ether. The final extract was adjusted to pH 7.5 with 0.2 M Tris and applied to a 1 ml column of anion-exchange Dowex AG1-X8 resin (formate form, 200–400 mesh). After washing with water to remove free *myo*-[^3H]inositol, InsP , InsP_2 and InsP_3 were separated by addition of increasing concentrations of ammonium formate as described by Berridge [30]. Radioactivity was counted by liquid scintillation with Aquasol.

DAG

After treatment, lipids were extracted with chloroform/methanol (2:1, v/v) as described by Folch et al. [31]. DAG was isolated by one-dimensional t.l.c. on silica gel G plates developed with hexane/diethyl ether/acetic acid (75:25:4, by vol.) as solvent. Lipids were detected by iodine staining and identified by comparison with standards. Labelled DAG was scraped off and counted for radioactivity by liquid scintillation with Aquasol.

Microsomal membrane preparation

Myoblasts exposed to vehicle (0.01% ethanol) or $1,25(\text{OH})_2\text{D}_3$ (1 nM) were homogenized in 20 mM Tris/HCl (pH 7.4)/1 mM EGTA/0.5 mM phenylmethanesulphonyl fluoride/leupeptin (40 $\mu\text{g}/\text{ml}$)/1 mM dithiothreitol/0.33 M sucrose. The homogenate was centrifuged at 120 000 g for 1 h in a Beckman L5-50B ultracentrifuge. 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 re-centrifugation at 120 000 g for 1 h. This supernatant (S2, solubilized membrane fraction) was collected. Fractions S1 and S2 were tested for protein kinase C (PKC) activity.

PKC activity

PKC activity was determined by a histone-phosphorylation assay, essentially as described previously [32]. Briefly, 60 μl samples of the cytosolic (S1) and solubilized membrane (S2) fractions were incubated in a reaction mixture (final volume 250 μl) containing (final concns.) 20 mM Tris/HCl, pH 7.4, 5 mM dithiothreitol, 10 mM 2-mercaptoethanol, 0.2 mM phenylmethanesulphonyl fluoride, 0.8 mg of BSA/ml, 50 μg of histone (type III-S), 10 mM MgCl_2 and 1 mM EGTA, with or without 1 mM CaCl_2 , 38 μg of phosphatidylserine/ml and 45 μg of 1,2-diolein/ml. Reactions were started by addition of 20 μl of 50 μM [γ - ^{32}P]ATP (1 μCi ; 3000 Ci/mmol) to 230 μl 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, centrifuged (2500 g, 15 min) and the pellet was washed four times with ice-cold trichloroacetic acid. The pellet was then dissolved in 1 M NaOH at 80 °C, and samples were taken for protein and radioactivity

measurements. PKC activity was calculated from the difference in phosphorylation assayed in the presence and the absence of Ca^{2+} , phosphatidylserine and 1,2-diolein. Enzyme activity (pmol of $^{32}\text{P}/\text{min}$ per mg of protein) was expressed as percentage of total PKC activity present in the cytosolic and membrane fractions.

^{45}Ca uptake measurements

Myoblasts were grown to 80% confluency, the medium was aspirated and the cells were preincubated 30 min with Krebs–Henseleit solution containing 0.2% glucose and 1 mM Ca^{2+} or 5 mM EGTA. The assay was initiated by aspirating the medium and adding the same medium containing $^{45}\text{CaCl}_2$ (2 $\mu\text{Ci}/\text{ml}$; 10–75 Ci/g) in the presence or absence of $1,25(\text{OH})_2\text{D}_3$, 25-hydroxy-vitamin D_3 [$25(\text{OH})\text{D}_3$] or 24,25-dihydroxy-vitamin D_3 [$24,25(\text{OH})_2\text{D}_3$]. When nifedipine was used, it was added to the cultures in the final 10 min of the preincubation period and throughout the assay. The assay was terminated after 5 min (or as indicated) of hormonal exposure by aspirating the labelled medium and washing three times with ice-cold 25 mM Tris (pH 7.4)/140 mM NaCl/1 mM LaCl_2 . The cells were then dissolved in 1 M NaOH, and samples were taken for measurement of protein content by the Lowry procedure [33] and radioactivity by liquid scintillation using Aquasol.

RESULTS

Incubation of *myo*-[^3H]inositol-labelled myoblasts with $1,25(\text{OH})_2\text{D}_3$ (1 nM) resulted in a rapid elevation of cellular inositol phosphate levels. As shown in Figure 1, increased levels of InsP_3 were observed within 15 s after addition of $1,25(\text{OH})_2\text{D}_3$, and maximal stimulation was reached at 60 s (80% above control). After the initial rise, InsP_3 levels decreased within 120 s (25% above control), and returned to near the control value after 3 min, reflecting rapid hydrolysis to InsP_2 . Maximal elevations in the levels of InsP_2 were seen between 60 and 120 s (52 and 75% above control, respectively) after hormone addition, followed by a decrease to a plateau level within 5 min that was 30% above control values. The effect of $1,25(\text{OH})_2\text{D}_3$ on InsP

Figure 1 Stimulation of inositol phosphate accumulation by $1,25(\text{OH})_2\text{D}_3$

Myoblasts prelabelled with *myo*-[^3H]inositol were incubated in Krebs–Henseleit/0.2% glucose/30 mM LiCl and exposed for 15 s–10 min to 1 nM $1,25(\text{OH})_2\text{D}_3$. At the indicated times, the incubation was terminated by addition of $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{HCl}$ (100:50:1, by vol.). Inositol phosphates were extracted from the neutralized aqueous phase and separated by ionic-exchange chromatography as described in the Materials and methods section. Results are expressed as percentages of control basal values (697 $\pm$ 31, 801 $\pm$ 38 and 1850 $\pm$ 101 c.p.m./mg of protein for InsP_3 , InsP_2 and InsP respectively). Values represent means $\pm$ S.D. of three separate experiments.

Figure 2 Specificity and dose-dependence of 1,25(OH)₂D₃ effects on InsP₃ accumulation

Myoblasts prelabelled with myo-[³H]inositol were treated with 1,25(OH)₂D₃, 25(OH)D₃ or 24,25(OH)₂D₃ for 1 min at the concentrations indicated. InsP₃ was isolated as described for Figure 1. Values are means of two experiments.

Figure 3 Stimulation of DAG accumulation by 1,25(OH)₂D₃

Embryonic-chick myoblasts prelabelled with [³H]arachidonic acid were incubated in Krebs–Henseleit/0.2% glucose and exposed for 15 s–10 min to 1,25(OH)₂D₃ (1 nM). At the indicated times, the incubation was terminated by adding CHCl₃/CH₃OH (2:1, v/v), and DAG was separated by t.l.c. as indicated in the Materials and methods section. Results are expressed as percentage of control values (370 c.p.m./mg of protein). Values represent means ± S.D. of three separate experiments.

accumulation was delayed, reaching a peak between 3 and 5 min and then returning near the control value (10% above).

As shown in Figure 2, the stimulation of InsP₃ accumulation by 1,25(OH)₂D₃ after 1 min was dose-dependent. Hormone concentrations as low as 10 pM produced a significant increase (+47%) in InsP₃ levels. The InsP₃ response increased up to 1 nM 1,25(OH)₂D₃. The effects of 1,25(OH)₂D₃ on InsP₃ production exhibited specificity, as physiological concentrations of 25(OH)D₃ and 24,25(OH)₂D₃, other major vitamin D₃ metabolites, did not alter myoblast InsP₃ levels (Figure 2).

Hydrolysis of polyphosphatidylinositols by phospholipase C results in the release of DAG. Addition of 1,25(OH)₂D₃ (1 nM) to myoblasts prelabelled with [³H]arachidonic acid stimulated the rapid formation of [³H]1,2-DAG (Figure 3). The effect of the hormone was biphasic; the early phase was rapid and transient, peaking at 60 s (95% above control). The late phase reached maximal level at 5 min (6-fold increase) and then declined to 90% above control value after 10 min of hormonal stimulation. The rapid increase in DAG in response to 1,25(OH)₂D₃ suggested that PKC might become activated. To test this possibility, PKC

Figure 4 Translocation of PKC induced by 1,25(OH)₂D₃

Embryonic-chick myoblasts preincubated in Krebs–Henseleit/0.2% glucose at 37 °C were treated with 1,25(OH)₂D₃ (1 nM) for 1–5 min. Cytosolic and membrane fractions were prepared as described in the Materials and methods section. 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. Data are expressed as percentages of total PKC activity (15 ± 0.98 pmol/min per mg of protein) present in cytosolic and membrane fractions. Values are means ± S.D. of four experiments: **P* < 0.001; ***P* < 0.005.

Table 1 Time and dose/response effects of 1,25(OH)₂D₃ on ⁴⁵Ca uptake by chick myoblasts

Myoblasts grown as a monolayer (80% confluency, 5–7 days after plating) were incubated in Krebs–Henseleit/0.2% glucose containing 1 mM Ca²⁺ in the absence or presence of 1 nM 1,25(OH)₂D₃ for 0.5–10 min. A 5 min treatment interval was used for dose (1 pM–10 nM)/response studies. The uptake of ⁴⁵CaCl₂ was measured as indicated in the Materials and methods section. Results are averages of three separate experiments + S.D.: **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

Time (min)	⁴⁵ Ca uptake (nmol/mg of protein)	[1,25(OH) ₂ D ₃] (M)	⁴⁵ Ca uptake (nmol/mg of protein)
0	4.10 ± 0.25	0	4.28 ± 0.31
0.5	4.81 ± 0.30*	10 ⁻¹²	5.30 ± 0.27*
1	4.93 ± 0.25*	10 ⁻¹¹	6.06 ± 0.42**
3	5.33 ± 0.31*	10 ⁻¹⁰	7.17 ± 0.35***
5	7.33 ± 0.52**	10 ⁻⁹	7.75 ± 0.62***
10	7.75 ± 0.47**	10 ⁻⁸	5.90 ± 0.35**

activity was measured in cytosolic and membrane fractions of myoblasts treated with 1 nM 1,25(OH)₂D₃. Short exposure of cells to the hormone resulted in activation of membrane-bound PKC (Figure 4). Of the total myoblast enzyme activity, about 68% was in the cytosol and 32% was membrane-associated in control cells; after a short treatment with the hormone, membrane PKC activity rose to 82% (*P* < 0.001), 73% (*P* < 0.005) and 75% (*P* < 0.001) at 1, 3 and 5 min respectively. Total myoblast PKC activity (cytosol + membrane: 15 ± 0.98 pmol of P_i/min per mg of protein) did not change with 1,25(OH)₂D₃ treatment.

In a separate set of experiments, we studied the rapid effects of 1,25(OH)₂D₃ on myoblast Ca²⁺ uptake. As shown in Table 1, in myoblasts grown to 80% confluency (5–7 days after plating) a 17% (*P* < 0.05) increase above control was observed after only 30 s of exposure to 1 nM 1,25(OH)₂D₃. The response varied with time of exposure, with 20% (*P* < 0.05), 30% (*P* < 0.05), 79% (*P* < 0.01) and 89% (*P* < 0.01) increases over control values after 1, 3, 5 and 10 min respectively. The stimulation of ⁴⁵Ca uptake elicited by 1,25(OH)₂D₃ was dose-dependent (Table 1): 1 pM 1,25(OH)₂D₃ produced a statistically significant increase

Table 2 Effects of Ca^{2+} -channel agonists and antagonists on myoblast ^{45}Ca uptake

Chick-embryo myoblasts were incubated in Krebs-Henseleit/0.2% glucose with or without (+5 mM EGTA) 1 mM Ca^{2+} in the absence or presence of $1,25(\text{OH})_2\text{D}_3$ (1 nM) or Bay K 8644 (2 μM) as described in the legend of Table 1. Nifedipine (1 μM) was added 5 min before hormone treatment. Results are averages of three experiments $\pm$ S.D.: * $P < 0.01$; ** $P < 0.001$.

	Ca^{2+} (nmol/mg of protein)
Control	4.20 ± 0.43
$1,25(\text{OH})_2\text{D}_3$	$7.25 \pm 0.65^*$
Bay K 8644	$7.56 \pm 0.58^*$
$1,25(\text{OH})_2\text{D}_3$ + Bay K 8644	$7.99 \pm 0.62^{**}$
Nifedipine	3.82 ± 0.47
$1,25(\text{OH})_2\text{D}_3$ + nifedipine	4.42 ± 0.48
EGTA	3.90 ± 0.32
$1,25(\text{OH})_2\text{D}_3$ + EGTA	4.11 ± 0.48

(+24%, $P < 0.05$). Cell Ca^{2+} -uptake responses increased up to 1 nM (+80%, $P < 0.001$), the higher concentration of hormone tested (10 nM) increasing Ca^{2+} uptake 45% ($P < 0.01$) above control. Physiological concentrations of $25(\text{OH})\text{D}_3$ (50 nM) increased ^{45}Ca incorporation into muscle cells by approx. 40%, whereas $24,25(\text{OH})_2\text{D}_3$ (5 nM) was without effects (results not shown). As shown in Table 2, treatment of myoblasts with the Ca^{2+} -channel activator Bay K 8644 (5 μM), resulted in ^{45}Ca uptake similar to those obtained with $1,25(\text{OH})_2\text{D}_3$. Treatment of myoblasts simultaneously with $1,25(\text{OH})_2\text{D}_3$ and Bay K 8644 did not additionally increase the stimulatory effect evoked by either agent. Exposure of myoblasts to 1 μM nifedipine, a Ca^{2+} -channel antagonist, blocked the elevation of ^{45}Ca uptake elicited by $1,25(\text{OH})_2\text{D}_3$. The effects of the hormone were also abolished in the absence of Ca^{2+} (+5 mM EGTA) in the medium (Table 2). These results strongly suggest the modulation of active Ca^{2+} -channels in embryonic muscle cells by $1,25(\text{OH})_2\text{D}_3$.

DISCUSSION

The present investigation demonstrates that the seco-steroid hormone $1,25(\text{OH})_2\text{D}_3$ stimulates *in vitro* the breakdown of membrane phosphoinositides in myoblasts isolated from 12-day chick embryos. The stimulation by $1,25(\text{OH})_2\text{D}_3$ of embryonic myoblasts leads to the formation of the two second messengers, InsP_3 and DAG, with subsequent activation of PKC. Neither $25(\text{OH})\text{D}_3$ nor $24,25(\text{OH})_2\text{D}_3$ induced changes in InsP_3 release, suggesting that $1,25(\text{OH})_2\text{D}_3$ activates myoblast phospholipase C in a specific fashion. These results are in agreement with previous observations showing that the seco-steroid hormone increases InsP_3 formation via hydrolysis of PtdInsP_2 in keratinocytes [17,18], enterocytes [15,21], osteoblasts [5,19] and parathyroid cells [22]. There are significant increases in the levels of InsP_2 and InsP , which could be derived either from dephosphorylation of InsP_3 or from the direct hydrolysis of PtdInsP or PtdIns . The generation of DAG is biphasic. The time courses of the early phase of DAG formation and the hydrolysis of polyphosphoinositides are almost the same in embryonic myoblasts stimulated by $1,25(\text{OH})_2\text{D}_3$. These findings suggest that the hormone-induced early transient phase of DAG formation results from the hydrolysis of PtdInsP_2 and is consistent with a role for phospholipase C in intracellular signal generation. The magnitude of the late phase of DAG formation is 6-fold higher than the transient phase, suggesting that phospholipase

C-mediated hydrolysis of polyphosphoinositides can not account for all of DAG formed during the action of $1,25(\text{OH})_2\text{D}_3$ and that DAG may also come from other phospholipids such as phosphatidylcholine, phosphatidic acid or phosphatidylethanolamine [34,35]. The sources of the $1,25(\text{OH})_2\text{D}_3$ -induced delayed phase of DAG formation, however, remain to be clarified. The short time sequence of PtdInsP_2 hydrolysis suggests that the effects of $1,25(\text{OH})_2\text{D}_3$ results from a direct interaction with the membrane. Whether these rapid non-genomic actions of the seco-steroid hormone are mediated by membrane receptors or reflect alterations in membrane lipid structure produced by the hormone remains to be elucidated. Activation of PKC, as indicated by translocation of the enzyme from the cytosol to the membrane fraction [36], temporally coincides with DAG formation. $1,25(\text{OH})_2\text{D}_3$ -dependent activation of PKC has also been demonstrated in intact skeletal muscle [16] and rat colonic epithelium [15]. Activation of the enzyme may play a role in the long-term cellular effects of $1,25(\text{OH})_2\text{D}_3$ in muscle cells [37,38], owing to the presence of PKC-response elements in the promoter regions of several genes [39], or, as discussed below, in fast stimulation of cell Ca^{2+} uptake by the hormone.

We have shown that $1,25(\text{OH})_2\text{D}_3$ rapidly (within 30 s) increases Ca^{2+} influx in embryonic myoblasts, presumably through voltage-gated Ca^{2+} channels, as the hormone-dependent influx is suppressed in the absence of external Ca^{2+} or by nifedipine, a well-known Ca^{2+} -channel blocker, and mimicked by Bay K 8644, a Ca^{2+} -channel agonist. The existence of Ca^{2+} channels in the early stages of chick muscle-cell development has been demonstrated [40]. These channels were of low and high threshold, the latter, or L-type, identifiable by their sensitivity to Ca^{2+} antagonists or entry-blocker drugs such as dihydropyridines, phenylalkylamines and benzothiazepines.

In agreement with our results in embryonic muscle cells, studies performed in skeletal [2] and cardiac muscle [8], as well as in diverse cell types [1,3–6], have also demonstrated enhancement of Ca^{2+} influx through Ca^{2+} channels by $1,25(\text{OH})_2\text{D}_3$.

Similarly to $1,25(\text{OH})_2\text{D}_3$, $25(\text{OH})\text{D}_3$ increased myoblast Ca^{2+} uptake, whereas $24,25(\text{OH})_2\text{D}_3$ was ineffective, in agreement with previous studies with osteoblastic cells [4,5]. It has been proposed that the 25-hydroxyl group may be involved in the activation of Ca^{2+} influx through the plasma membrane, whereas the 24-hydroxyl group would counteract this action [5].

The mechanisms of action of $1,25(\text{OH})_2\text{D}_3$ on Ca^{2+} gating remains speculative. DAG-induced activation of PKC may itself play a role in mediating Ca^{2+} influx during stimulation with $1,25(\text{OH})_2\text{D}_3$. Interestingly enough, PKC and protein kinase A have been shown to be involved in $1,25(\text{OH})_2\text{D}_3$ -dependent Ca^{2+} influx in skeletal muscle [10,16] and transcellular Ca^{2+} transport in the intestine [41]. In muscle, as a consequence of the seco-steroid hormone action, a complex interplay between the two major transmembrane signalling systems is observed [16]. Thus modulation of Ca^{2+} gating through phosphorylation is likely to be one of the primary mechanisms underlying $1,25(\text{OH})_2\text{D}_3$ -mediated regulation of intracellular calcium. In addition, InsP_3 , the other product arising from the action of phospholipase C on the phosphoinositides, or its derivative $\text{Ins}(1,3,4,5)\text{P}_4$, should be considered as possible agents responsible for the opening of Ca^{2+} channels [26,27].

Further work to explore the relationship between myoblast phosphoinositide metabolism and intracellular Ca^{2+} upon stimulation with the hormone is in progress.

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