$1,25(OH)_2D_3$ Increases Calcium and Phosphatidylinositol Metabolism in Differentiating Cultured Human Keratinocytes

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The effect of $1,25(OH)_2D_3$ on the intracellular calcium, $(Ca^{+2})i$, in both cultured human keratinocytes and in cultured human dermal fibroblasts was investigated. When the intracellular calcium $(Ca^{+2})i$ in cultured human keratinocytes, grown in a serum-free medium containing 1.8 mM calcium, was measured by the fluorescent calcium-indicator, Fura-2, the $(Ca^{+2})i$ increased 154%, 202%, and 409% over the control value after incubation with $1,25(OH)_2D_3$ at 10^{-10} M, 10^{-8} M, and 10^{-6} M, respectively. This response was immediate (15 seconds), specific (no effect with either $25(OH)D_3$ at 10^{-8} M or vitamin D_3 at 10^{-8} M), and occurred with or without EGTA in the medium. In contrast, $1,25(OH)_2D_3$ did not increase the $(Ca^{2+})i$ in either cultured human keratinocytes that were grown in low calcium (0.05 mM), serum-free medium or in cultured human dermal fibroblasts that were grown in medium containing 0.05 mM calcium and 1% serum. The effect of $1,25(OH)_2D_3$ on the turnover of phosphatidylinositol was investigated as a possible cause for the observed increase in $(Ca^{+2})i$. Cultured human keratinocytes that were incubated with ^3H-inositol demonstrated a 50% ± 10% increase in the triphosphated, plasma membrane-bound metabolite of phosphatidylinositol, PIP_2, by 15 seconds, followed by a rapid decrease at 30 seconds, then a return toward basal levels by 1 minute. Lysophosphatidylinositol, which results from the sn-2 deacylation of phosphatidylinositol by phospholipase A_2, decreased 20% ± 8% within 30 seconds, then increased to 200% ± 10% of the control value by 5 minutes. The accumulation of IP_3 was increased 50% to 100% above the control value within 30 seconds and this increase was substained during the 5-minute incubation period. Stimulation of phosphatidylinositol turnover by $1,25(OH)_2D_3$ was not detected in either cultured human keratinocytes that were grown in serum-free, low calcium medium or in cultured human dermal fibroblasts that were grown in 1% serum.

Keywords: human skin; intracellular calcium; $1,25(OH)_2D_3$; phosphatidylinositol.

Introduction

The prevailing concept for the mechanism of action of $1,25(OH)_2D_3$ is that it acts on its target tissues—intestine, bone, and kidney—in a way analogous to that described for steroid hormones.[1] Specific cytosolic and nuclear receptors for this secosteroid hormone have been found in numerous organs and cell lines, including cutaneous cultured mouse keratinocytes[2] and both human epidermal cultured keratinocytes and dermal fibroblasts.[3] Incubation of cultured human dermal fibroblasts (CHDFs) with $1,25(OH)_2D_3$ (10^{-8} M) produced an inhibition of proliferation which was shown to be receptor-mediated and dose-dependent.[3] Furthermore, incubation of cultured human keratinocytes (CHKs) with $1,25(OH)_2D_3$ inhibited proliferation and stimulated differentiation.[4] The transglutaminase activity which was used as the marker for differentiation in these studies was stimulated by $1,25(OH)_2D_3$, and this stimulation was in-

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hibited by preincubation of the CHKs with actinomycin D.

Recent studies have shown that changes in cytosolic calcium mediate a number of hormonal processes and have a role in the regulation of cell proliferation and differentiation. Hepatocytes[5] and CHKs[6] rapidly respond to $1,25(OH)_2D_3$ by an increase in intracellular calcium. The rapidity of the response to $1,25(OH)_2D_3$ by these cells has provoked speculation that this action is not explained by a receptor-mediated nuclear event.[7] Suggested explanations for these rapid effects of $1,25(OH)_2D_3$ include a direct activation of some plasma membrane component resulting in a regulation of one or more plasma membrane lipids.[8] However, these explanations still remain speculative. On the other hand, Barsony and Marx recently reported a rapid increase in the cGMP after incubating CHDFs with $1,25(OH)_2D_3$. This increase in cGMP is not seen in $1,25(OH)_2D_3$ stimulated CHDFs derived from biopsies of patients characterized with defects for the $1,25(OH)_2D_3$ receptor.[9]

In the past 5 years, there has been rapid progress in understanding membrane receptors that generate intracellular signals from inositol phospholipids.[10] Several reports link calcium-mobilizing agonists with stimulated phosphoinositide turnover.[11] Terminal differentiation in murine keratinocytes has been shown to be a calcium-sensitive process.[12] Intracellular calcium has been shown to increase in CHKs in response to $1,25(OH)_2D_3$,[6] but the specificity of the response and a mechanism of action for $1,25(OH)_2D_3$ has yet to be discerned. Therefore, we investigated the specificity of $1,25(OH)_2D_3$ as a stimulator of intracellular calcium in human epidermal keratinocytes and examined a possible mechanism of action. Since $1,25(OH)_2D_3$ has been shown to stimulate phosphatidylinositol (PI) metabolism in cultured murine keratinocytes,[13] we studied this system in CHKs as a possible mechanism to increase intracellular calcium in CHKs.

Materials and methods

Keratinocyte culture

Keratinocytes were grown in culture using a modification of the method of Rheinwald and Green.[14] 3T3 cells (0.5×10^5/35 mm) were plated and were lethally irradiated 2 days later with a cobalt-60 source (5,000 rad). Keratinocytes were obtained from neonatal foreskin after overnight trypsinization at 4°C and treatment with 0.02% EDTA. Keratinocytes were plated in 2 ml of serum-free medium per culture on the lethally irradiated 3T3 cells. Each experiment was performed on primary or secondary keratinocyte cultures obtained from different skin samples. The serum-free medium consisted of Dulbecco's modified Eagle's medium (DMEM) with high (1.8 mM) or low (0.05 mM) calcium (M.A. Bioproducts, Walersville, MD, USA) containing seven growth factors: transferrin (5 μg/ml), epidermal growth factor (25 ng/ml), hydrocortisone (203 mg/ml), insulin (5 μg/ml), prostaglandin E-1 (50 ng/ml), cholera toxin (0.1 μg/ml; Sigma Chemical Co., St. Louis, MO, USA), and selenous acid (2 ng/ml; Collaborative Research, Lexington, MA, USA). Penicillin G (75 U/ml) and streptomycin (50 ng/ml) were also added to the cultures. At 1 week in culture, hydrocortisone and cholera toxin were removed from the medium, and the cells were washed with 0.02% EDTA to remove any remaining 3T3 cells. For the experiments studied, fresh medium containing vehicle alone (less than 0.1% absolute ethanol), one of the $1,25(OH)_2D_3$ (10^{-10}, 10^{-8}, and 10^{-6} M) (a gift of Dr. M. Uskokovic of Hoffmann-La Roche, Inc. Nutley, NJ, USA) or $25(OH)D_3$ (10^{-8} and 10^{-6} M) (a gift from J. Babcock of Upjohn Co., Kalamazoo, MI, USA) compounds or vitamin D_3 (Sigma Chemical Company) was added to each culture at a concentration of 10^{-8} or 10^{-6} M.

Human fibroblast cultures

Fibroblasts were isolated from human neonatal foreskin. The fibroblasts were isolated and cultured by placing the skin immediately into DMEM containing 10% fetal bovine serum (Microbiological Corp., Cambridge, MA, USA), penicillin G (75 U/ml), and streptomycin (50 ng/ml). Fibroblasts were grown from minced portions of dermis in 60 mm Falcon dishes containing DMEM, 1% newborn serum, and antibiotics as described above. The medium was changed twice weekly until the fibroblasts reached confluence. Cells were then harvested with 0.05% trypsin-0.02% EDTA in saline at 37°C and passed serially into larger (75 and 150 cm) culture flasks. Fibroblasts from passages 2 through 5 were used at 80% confluence in all experiments.

Incubation of keratinocytes with tritiated inositol

Tritiated inositol (10 μCi) (New England Nuclear, Boston, MA, USA) was added, in triplicate, for 36 hours at 37°C (representing a steady-state of uptake) to 35 mm culture plates that contained 40% to 80% confluent human keratinocytes. The labeled medium was removed and the cells were rinsed three times with DMEM. Cold, 15% TCA was added to the cultures and they were kept on ice for 30 minutes to extract the water-soluble inositol phosphates. The inositol phosphates were separated by Dowex anion-exchange column chromatography (Dowex 1 × 10 44350 Fluka Ag). The remaining inositol lipids were extracted with 2 ml of chloroform:methanol:HCl (200:100:1, vol/vol/vol). The cultures were rinsed with an additional 1 ml of the same solvent, and the two extractions were combined. Phase split was induced by adding 1 ml of chloroform and 1 ml of 0.1 N HCl. The sample was then mixed and briefly centrifuged to resolve the two phases. The lower (organic) phase was removed, dried under nitrogen, and analyzed by thin layer chromatography (TLC). Samples and standards for the inositides were spotted side-by-side on silica gel 60, thin layer plates (Merck) impregnated with 1% potassium oxalate, and separated in chloroform:methanol:4N

NaOH (9:7:2, vol/vol/vol). Spots were identified by comparison with [^{3}H]inositol-labeled standards and unlabeled standards were visualized by iodine vapor. For quantitation of the radioactive lipids, spots were visualized by autoradiography after treatment with Enhance (New England Nuclear). The spots were removed and the radioactivity was counted in a liquid scintillation counter.

Incubation of keratinocytes with ^{3}H-glycerol

Cultures of human keratinocytes at 40% to 80% confluence were incubated with 20 μCi ^{3}H-glycerol for 36 hours at 37°C, which represented a steady-state of uptake. Labeled medium was removed and the cells were rinsed twice with cold phosphate buffered saline (pH 7.4). One milliliter of 0.5N HCl was added to the culture, and the cells were scraped into 2 ml of cold chloroform. The cells were then rinsed with 1 ml, 1N HCl, then 2 ml of methanol. All the washes were combined with the cell extract. Samples were then vortexed, allowed to stand for 30 minutes, and briefly centrifuged to separate the two phases. The lower, organic phase was removed, and the aqueous phase was reextracted with 1 ml of chloroform. Pooled organic phases were then washed with 2 ml of 1N HCl:MeOH (1:1, vol/vol). The organic phase was dried under nitrogen, dissolved in CHCl:MeOH:H_2O (2:1:0:01, vol/vol/vol), and applied to TLC plates for isolation of the polyphosphoinositides, as previously stated.

For analysis of diacylglycerol (DAG), lipids were spotted on untreated plates and separated in benzene:ethyl acetate (60:40, vol/vol). Lipids were visualized by autoradiography after treatment of plates with Enhance. The radioactive spots were cut out and the radioactivity was quantitated by liquid scintillation counting.

Incubation of keratinocytes with 1,25(OH)$_2$D$_3$

Cultures of keratinocytes (35 mm) were incubated, in triplicate, with either 1,25(OH)$_2$D$_3$ (10^{-6}, 10^{-8}, and 10^{-10} M) or the vehicle (95% ETOH) in 2 ml of medium. At 0, 15 seconds, 30 seconds, 1 minute, 2 minutes, and 5 minutes, the incubation was stopped by adding 1 ml of 15% TCA and placing the cultures on ice for 20 minutes. Phosphatidylinositol and the lipid- and water-soluble inositides were extracted and quantitated as described above.

The results were expressed as proportions of inositol phosphate (IP), inositol diphosphate (IP$_2$), inositol triphosphate (IP$_3$), PI-4-phosphate (PIP), PI-4,5-biphosphate (PIP$_2$), and lysoPI to the zero time PI value to minimize errors due to variability in cell number and efficiency of extraction.

Measurement of intracellular calcium

Cultured human keratinocytes (5×10^7 cells) were incubated with 10 μM Fura-2 (Molecular Probes, Inc., Eugene, OR, USA, F-12101) for 30 minutes at 37°C.

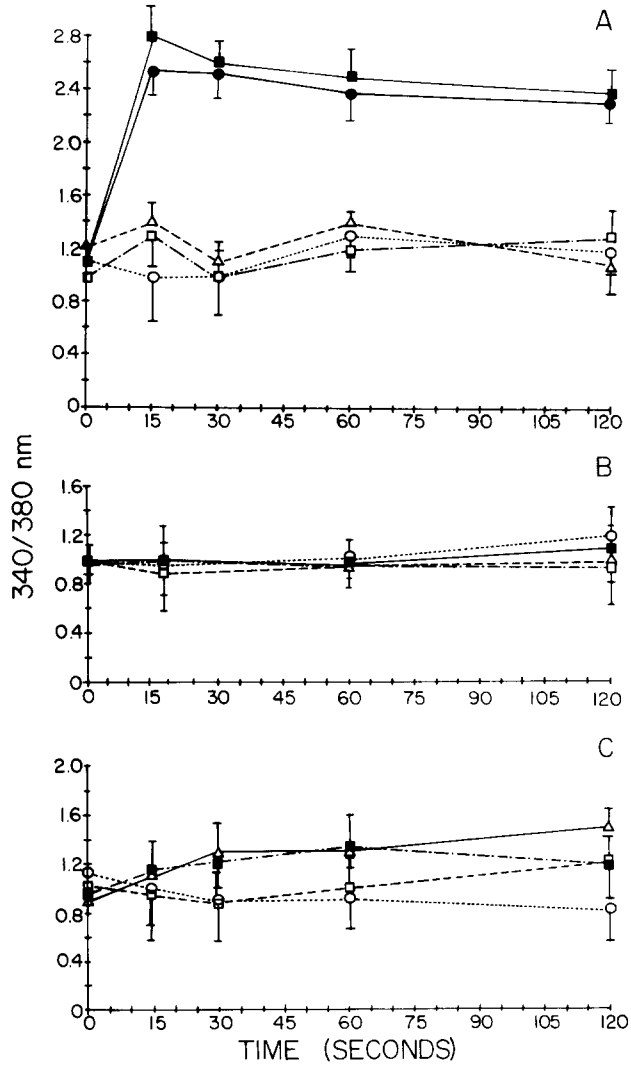

Figure 1 Representative 340- to 380-nm excitation curves monitored at 510 nm fluorescence of CHKs grown in 1.8 mM calcium (A), CHKs grown in 0.05 mM calcium (B), and CHDFs (C) loaded with 10 μM Fura-2, then incubated with ETOH 0.1%, (. . . . , O); vitamin D$_3$, 10^{-6} M (-·-·-, □), 25(OH)D$_3$, 10^{-8} M (---, Δ); or 1,25(OH)$_2$D$_3$, 10^{-8} M (with (●) and without (■) EGTA). The ratio changes in the same direction as the $(Ca^{2+})i$ and can be used to calculate the $(Ca^{2+})i$ from the equation $K(R - Ro/(Rs - R) = (Ca^{2+})i$, where Ro is the ratio at 0 calcium and Rs is the ratio at saturating calcium. K represents Kd (Fo/Fs), where Kd is the dissociation constant for Fura-2. Fo is the fluorescence at 380 nm in zero calcium and Fs is the fluorescence at 380 nm in saturating calcium concentration.

The cells were washed, spun, and counted. Cultured human keratinocytes (3×10^6 cells/0.9 ml buffered saline containing Ca^{2+} and Mg^{2+}) were mixed and, after sequential excitation at 340 nm and 380 nm, the fluorescence read at 510 nm. Digitonin (50 μM) was added and the readings at 340 and 380 nm were recorded. To the same cuvette, EGTA (1 mM) was added and the 340 and 380-nm readings were again recorded. This was repeated several times and averaged for the maximum and minimum fluorescence at 340 and 380 nm. Once the maximum and minimum fluorescence had been established for each experiment,

$1,25(OH)_2D_3$ (10^{-6}, 10^{-8}, and 10^{-10} M) was added to another sample of CHKs and, within seconds, the values at the excitation wavelengths, 340 and 380 nm, were recorded. The concentration of calcium was then calculated as described in the legend of *Figure 1*.

Results

The intracellular calcium was calculated from the curves shown in *Figure 1* that were generated from Fura-2 loaded CHKs or CHDFs and treated with vitamin D_3, $25(OH)D_3$, or $1,25(OH)_2D_3$. Cultured human keratinocytes were grown in medium that contained either 1.8 mM calcium (*Figure 1A*) or 0.05 mM calcium (*Figure 1B*). The CHDFs were grown in medium that contained 1% serum (*Figure 1C*). The cells were then incubated with $1,25(OH)_2D_3$ (10^{-8} M) without EGTA, and with EGTA, $25(OH)D_3$ (10^{-8} M), vitamin D_3 (10^{-6} M), or ETOH (0.1%). An increase of 230% ± 14% ($P < 0.01$) in the $(Ca^{2+})i$ in CHKs (*Figure 1A*) was observed after incubation with $1,25(OH)_2D_3$.

As demonstrated in *Figure 2*, the CHKs incubated with $1,25(OH)_2D_3$ exhibited a statistically significant ($P < 0.01$) dose-dependent increase in the intracellular calcium (154 ± 20 nM, 178 ± 10 nM, 242 ± 25 nm, for 10^{-10} M, 10^{-8} M, and 10^{-6} M, respectively). This dose dependency was not observed in the CHKs incubated with 25 $(OH)D_3$ at either 10^{-8} M (78 ± 10 nM) or 10^{-6} M (104 ± 18 nM), or with vitamin D_3 at 10^{-8} or 10^{-6} M. There was no significant increase in intracellular calcium over the control value in either CHKs ($P > 0.05$) grown in low calcium medium or in CHDFs ($P > 0.05$) grown in 1% serum.

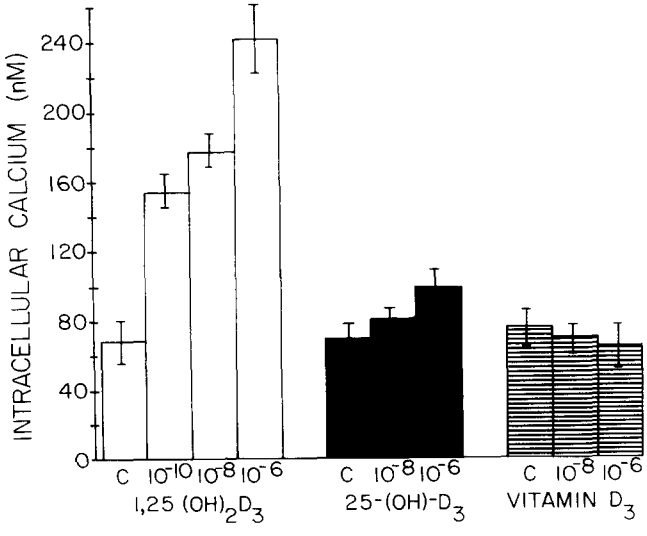

Figure 2 Intracellular calcium concentrations in CHKs after incubation with $1,25(OH)_2D_3$ (10^{-10}, 10^{-8}, and 10^{-6} M), □; 25-OH-D_3 (10^{-8}, and 10^{-6} M), ■; vitamin D_3 (10^{-8} and 10^{-6} M), ▤; or vehicle alone, (C). The amount of calcium (nM) is calculated as described in the legend for *Figure 1*. Each value represents the mean ± SEM of four different experiments, each done in triplicate.

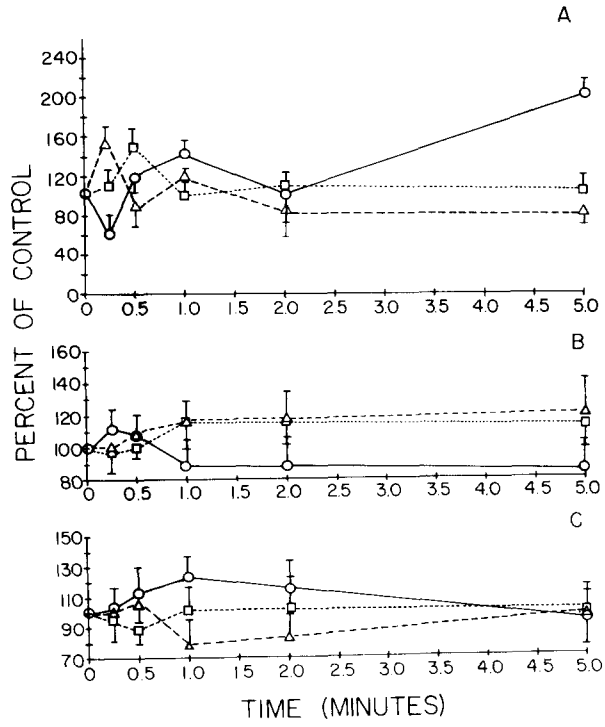

Figure 3 Lipid-soluble inositides (O, lysoPI; □, PIP; and △, PIP_2) isolated from CHKs grown in medium containing either 1.8 mM calcium (A) or 0.05 mM calcium (B), or from CHDFs grown in medium containing 1% fetal calf serum (C), incubated with $1,25(OH)_2D_3$ (10^{-8} M) for 0.25, 0.50, 1, 2, and 5 minutes. Each point is the mean ± SEM of four different experiments done in triplicate (A) and the mean ± SEM of two different experiments done in triplicate (B and C). Phosphatidylinositol at zero time = 254,000 cpm; PIP (. . .) at zero time = 17,000 cpm; PIP_2 (---) at zero time = 8,000 cpm; and LysoPI (——) at zero time = 27,000 cpm.

Incubation of cultured human keratinocytes with ^{3}H-inositol

There is now strong evidence that the regulation of intracellular calcium is due, at least in part, to a stimulation of the endoplasmic reticulum by a breakdown product of the membrane lipid, PI.[12] We therefore examined PI turnover in CHKs that were grown in medium containing either 1.8 mM calcium (*Figures 3A* and *4A*) or 0.05 mM calcium (*Figures 3A* and *4A*) and in CHDFs grown in medium containing 1% fetal calf serum (*Figures 3C* and *4C*) in response to 10^{-8} M $1,25(OH)_2D_3$. *Figures 3* and *4* represent data for 10^{-8} M $1,25(OH)_2D_3$. Incubation of CHKs with 10^{-10} M did not give a statistically significant response ($P > 0.05$). At 0, 15 seconds, 30 seconds, 1 minute, 2 minutes, and 5 minutes, the lipid- and water-soluble metabolites of PI were extracted and quantitated. Phosphatidylinositol, lysoPI, PIP_2, and PIP were quantitated as a proportion of all PIs and are represented as a percent of the control value. The values in *Figure 3A* represent the mean ± SEM of 12 dishes (four separate experiments, with each experiment containing triplicates for each time point) and *Figures 3B* and *3C* represent the mean ± SEM of six dishes (two different experiments performed in triplicate). In CHKs (*Figure 3A*) grown in 1.8 mM calcium, PIP_2 was increased within 15 seconds

(158% ± 15% of the control value), then returned to baseline levels by 5 minutes. Phosphatidylinositol-4-phosphate demonstrated an initial increase of 160% ± 15% ($P < 0.005$), and returned to control levels by 1 minute. Lysophosphatidylinositol immediately decreased to 40% ± 14% ($P < 0.01$) below the control value by 15 seconds, then increased to 140% ± 5% ($P < 0.001$) by 1 minute. By 5 minutes, lysoPI increased to 220% ± 11% ($P < 0.001$) of the control value. In experiments that were carried out to 20-minute incubations with 1,25(OH)$_2$D$_3$, lysoPI accumulated to 330% ± 8% ($P < 0.001$) of the control values (data not shown).

The water-soluble inositols, IP$_3$, IP$_2$, and IP, were calculated as a percentage of the zero time concentration of PI in each sample and are represented in *Figure 4* as a percent of the control value. Cultured human keratinocytes grown in 1.8 mM calcium (*Figure 4A*) were incubated with 1,25(OH)$_2$D$_3$ (10^{-8} M) for 0.25, .5, 1, 2, and 5 minutes, then IP, IP$_2$, and IP$_3$ were isolated and quantitated. The values are the average of triplicate dishes from four experiments. Inositol triphosphate increased to 170% ± 15% of the control value by 15 seconds and 200% ± 10% by 30 seconds, then decreased to 150% ± 5% ($P < 0.001$) of the control value by 5 minutes. Inositol triphosphate did not return to baseline levels during the course of the 5-minute incubation. Inositol diphosphate reached 185% ± 22% ($P < 0.001$) of the control value by 30 seconds and 200% ± 18% ($P < 0.001$) by 1 minute, then began to decline to 150% ± 17% ($P < 0.001$) and 144% ± 19% ($P < 0.05$) of the control value by 2 and 5 minutes, respectively. After 30 seconds, IP reached a plateau and was not significantly different from its 30-second level out to 5 minutes ($P > 0.05$). *Figures 4B* (CHKs grown in 0.05 mM calcium) and *4C* (CHDFs grown in 1% serum) show that 1,25(OH)$_2$D$_3$ has no effect on PI turnover. There was no significant difference in the treated CHDFs and CHKs above the ETOH control levels ($P > 0.05$).

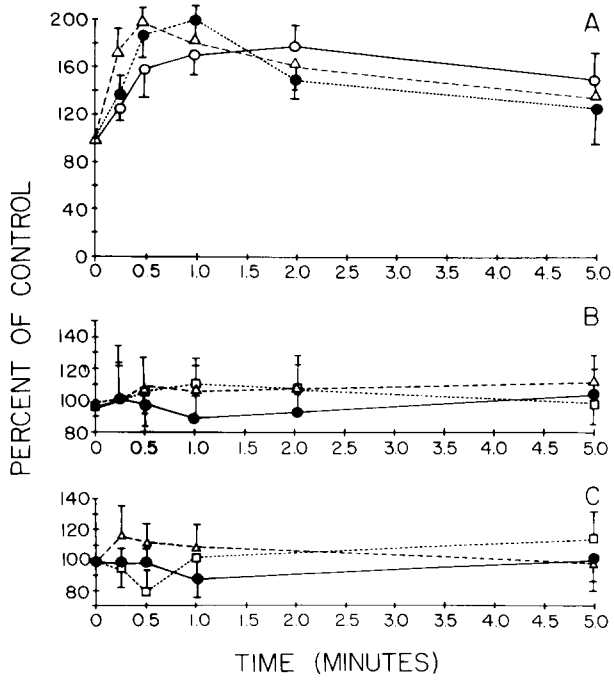

Figure 4 Water-soluble inositols (○, IP; ●, IP$_2$; and △, IP$_3$) isolated from CHKs grown in medium containing either 1.8 mM calcium (A) or 0.05 mM calcium (B), or CHDFs grown in medium that contains 1% fetal calf serum (C) after incubation with 1,25(OH)$_2$D$_3$ (10^{-8} M) for 0.25, 0.50, 1, 2, and 5 minutes. Each point in panel A represents the mean ± SEM of four different experiments done in triplicate, and each point in panels B and C represents the mean ± SEM of the two different experiments done in triplicate. Inositol triphosphate at zero time = 360 cpm; IP$_2$ at zero time = 1980 cpm; and IP at zero time = 6,600 to 9,800 cpm.

Table 1 Incubation of cultured human keratinocytes with ^{3}H-glycerol

Time (seconds)	DAG	TG	PI
0	303 ± 51	5120 ± 116	8888 ± 210
30	410 ± 49	5415 ± 269	9360 ± 316
60	425 ± 30	4998 ± 321	10,180 ± 319
90	450 ± 65	5556 ± 418	10,002 ± 211

Cultured human keratinocytes (1 × 10^6 M) were incubated with ^{3}H-glycerol for 72 hours, then stimulated with 1,25(OH)$_2$D$_3$ for 30, 60, and 90 seconds. Diacylglycerol, TG, and PI were isolated, and the incorporation of ^{3}H-glycerol was determined. The values represent the cpm from three different experiments that were done in triplicate ± SEM.

Incubation with ^{3}H-glycerol

The metabolism of PIP$_2$ results in the production of two second messengers, a water-soluble messenger (IP$_3$), and a membrane-bound diacylglycerol (DAG). Cultured human keratinocytes grown in medium containing 1.8 mM calcium were incubated with 20 μCI of ^{3}H-glycerol for 36 hours and then DAG, triglycerides (TGs), and PI were isolated. As shown in *Table 1*, analysis of DAG, TG, and PI by autoradiography demonstrated an 11% ± 4% ($P < 0.01$) increase in the ^{3}H-DAG isolated from CHKs incubated with 1,25(OH)$_2$D$_3$ (10^{-8} M). There was no significant difference in the incorporation of ^{3}H-glycerol in the PI or TGs after a 5-minute incubation with 1,25(OH)$_2$D$_3$ between the control and stimulated keratinocytes ($P > 0.01$).

Discussion

Although the factors that regulate intracellular calcium in CHKs remain unknown, there is no doubt that calcium regulation is critical in cutaneous keratinocyte differentiation. Yuspa et al.[15] have shown that mouse keratinocytes grown in medium containing low calcium (<0.1 mM) proliferate but do not differentiate, whereas those grown in a high-calcium medium (>1.0 mM) are a heterogeneous population of proliferating and differentiating cells. More recent studies by Yuspa[16] demonstrate that the calcium concentration and time of exposure to calcium is critical for the sequential appearance of the different molecular weight keratins that are markers for differentiation in cutaneous keratinocytes.

Calcium[17] and $1,25(OH)_2D_3$[13] have been reported to stimulate PI turnover in cultured murine keratinocytes; however, these experiments were done in the presence of 10% fetal calf serum, and the results differ considerably from the data we obtained in CHKs. Perhaps the growth factors contained in serum can account for the stimulation of PI turnover that was observed in the control cells.[13,17] Moreover, in our hands, CHKs grown in fetal calf serum tend to have a blunted response to $1,25(OH)_2D_3$ and may further explain the differences in our results.

Stages of cellular differentiation are often associated with differences in membrane composition, and plasma membrane phospholipid composition has been shown to be altered by $1,25(OH)_2D_3$.[8] When we examined the effects that $1,25(OH)_2D_3$ had on the hydrolysis of the membrane phospholipid PI, we found an increase in IP_3 (*Figure 3A*), a known stimulus of intracellular calcium release from the endoplasmic reticulum. Stimulation of $(Ca^{2+})i$ in CHKs may also be important for the activation of phospholipase A_2, which has been reported to regulate the concentration of aracadonic acid available to the cell.[18] Baran and Kelly demonstrated that $1,25(OH)_2D_3$ "induced increments" in cytosolic calcium are blocked by inhibition of phospholipase A and the Na/H+ exchange in cultured rat hepatocytes.[19] In an earlier report,[5] incubation of hepatocytes with EGTA inhibited an increase in intracellular calcium. However, incubation of CHKs with indomethecin (10 μg) before incubation with $1,25(OH)_2D_3$ did not inhibit the observed increase in intracellular calcium that we report here (data not shown), suggesting that $1,25(OH)_2D_3$ may increase influx of calcium in the hepatocyte but not in CHKs. Incubation of hepatocytes with LysoPI, however, stimulated a rapid increase in intracellular calcium which was not affected by inhibition of phospholipase A.[19] It may be that $1,25(OH)_2D_3$ has a different or additional mechanism with which to increase intracellular calcium in differentiating keratinocytes than in hepatocytes. Other investigators studying the effects of vasopressin in cultured rat hepatocytes[20] have shown that phospholipase C has a calcium concentration dependency only in hepatocytes that are cultured in medium containing below-physiologic concentration of calcium. The investigators suggest that the activation of the several kinases involved in PI turnover have different requirements for calcium; this may explain why no increase in intracellular calcium was seen in CHKs grown in medium containing 0.05 mM calcium. Furthermore, calcium has varying effects on the kinases that produce the IP_3 isomers.[21]

Recent evidence[22] demonstrates a role for lysoPI in culture carrot cells to increase the incorporation of inositol five- to seven-fold, into the plasma membrane and to increase the fusability between cells. Gordon et al.[23] demonstrated that CHKs grown in serum-free, normal calcium medium have a high requirement for inositol, and the increase in LysoPI may stimulate the replacement of inositol into the membranes. Therefore, the role of $1,25(OH)_2D_3$ in CHKs at the plasma membrane may have two effects: first, to directly stimulate PI turnover, resulting in an increase in the $(Ca^{2+})i$; and second, the increase in the $(Ca^{2+})i$ stimulates phospholipase A_2, resulting in an increased formation of lysoPI which would act to increase incorporation of inositol into the plasma membrane, subsequently increasing the availability of the substrate for PI production.

Incorporation of ^{3}H-glycerol into CHKs with subsequent incubation with $1,25(OH)_2D_3$ showed a 11% ± 4% increase in ^{3}H-DAG over the control level. Diacylglycerol is a known stimulator of protein kinase C which acts at the level of the plasma membrane, affecting cation transport and phosphorylation of several proteins.[11] More experiments are needed to explore this interesting observation.

Our data (*Figure 1A*) demonstrate a rapid increase in intracellular calcium in nonconfluent (there was no effect with CHKs 10 days past confluence, data not shown), differentiating CHKs in response to $1,25(OH)_2D_3$. Simultaneously, $1,25(OH)_2D_3$ increases the hydrolysis of the plasma membrane lipid, PI. These data suggest that $1,25(OH)_2D_3$ may be an important regulator of epidermal intracellular calcium but do not demonstrate which effect comes first, an increase in IP_3 or an increase in intracellular calcium. Further studies are needed to determine whether there is a causal effect between the stimulation of the PI pathway by $1,25(OH)_2D_3$ and the observed increase in intracellular calcium.

Acknowledgments

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