# 1,25-Dihydroxyvitamin $D_3$ Upregulates the Phosphatidylinositol Signaling Pathway in Human Keratinocytes by Increasing Phospholipase C Levels

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## Abstract

**1,25-Dihydroxyvitamin $D_3$ ($1,25(OH)_2D_3$) induces the differentiation of normal human keratinocytes, in part by increasing their basal intracellular calcium levels ($Ca_i$) over a period of hours. Agonists such as ATP acting through membrane receptors cause an immediate but transient increase in $Ca_i$ accompanied by an increase in inositol trisphosphate ($IP_3$). Treatment of keratinocytes for 24 h with 1 nM $1,25(OH)_2D_3$ resulted in a two- to four-fold potentiation of the $Ca_i$ response of these cells to ATP. This potentiation was inhibitable with cycloheximide, unaccompanied by a change in total intracellular calcium pools, but associated with an increase in basal $IP_3$ levels and ATP-stimulated $IP_3$ production. Treatment with $1,25(OH)_2D_3$ raised the protein and mRNA levels of phospholipase C isoenzymes, particularly phospholipase C-$\beta$1 in a dose-dependent manner. These studies indicate that $1,25(OH)_2D_3$ modulates the keratinocyte signal transduction pathway by induction of phospholipase isoenzymes, a previously undescribed action for this hormone. (*J. Clin. Invest.* 1995. 96:602–609.) Key words: keratinocytes • calcium • differentiation • phospholipase C • inositol trisphosphate**

## Introduction

The differentiation of normal human keratinocytes in culture is induced by extracellular calcium ($Ca_o$)[1] and 1,25-dihydroxyvitamin $D_3$ ($1,25(OH)_2D_3$), the hormonally active form of vitamin D (1–6). Phorbol esters, presumably by regulating protein kinase C activity, are also capable of stimulating differentiation and modulating the prodifferentiating effects of calcium (7–9). The mechanism by which $1,25(OH)_2D_3$ induces keratinocyte differentiation, although not fully elucidated, is known to involve changes in intracellular calcium ($Ca_i$) and is likely to include the changes in phospholipase C (PLC) and protein kinase C activity that accompany calcium-induced differentiation (10, 11). The potential ability of protein kinase C to phosphorylate and inactivate PLC (12, 13) contributes to the complexity of the pathway mediating calcium- and $1,25(OH)_2D_3$-induced differentiation.

The classic role of vitamin D, namely the regulation of calcium transport in the intestine, bone, and kidney, is mediated by the steroid hormone–like action of vitamin D through its binding to specific nuclear receptors and regulation of specific gene expression (14–16). However, several recent observations support a role for $1,25(OH)_2D_3$ in other tissues not directly involved in plasma calcium homeostasis (reviewed in reference 10). Some of these effects involve genomic actions of $1,25(OH)_2D_3$, whereas other rapid effects appear to be nongenomic. Among the rapid effects described for $1,25(OH)_2D_3$ are the stimulation of phosphatidylinositol (PI) metabolism, $Ca_i$ mobilization, and protein kinase C activity. In keratinocytes, an acute increase in $Ca_i$ associated with increased PI turnover has been observed after $1,25(OH)_2D_3$ administration in some studies (17–20). The acute increase in $Ca_i$ in association with the increased levels of diacylglycerol produced from the PI turnover would be expected to activate protein kinase C and promote the differentiation of keratinocytes. However, vitamin D metabolites that do not induce differentiation ($1\alpha$(OH)D and $24,25(OH)_2D_3$) also acutely stimulate $Ca_i$ and PI metabolism of keratinocytes (17). In our studies, we failed to observe rapid mobilization of $Ca_i$ by $1,25(OH)_2D_3$ in keratinocytes despite its ability to stimulate differentiation (6, 21). Rather, we observed a gradual rise in $Ca_i$ in response to several hours of treatment with $1,25(OH)_2D_3$. A similar long-term effect of $1,25(OH)_2D_3$ on $Ca_i$ has been observed in other cell systems (22–24). In these other systems $1,25(OH)_2D_3$ appears to act by increasing agonist-induced PI turnover. For example, a 48-h treatment with $1,25(OH)_2D_3$ potentiates the response of HL-60 cells to formyl Met Leu Phe–induced PI turnover and $Ca_i$ transients, as part of the prodifferentiating effect of this hormone (25). Likewise, in pituitary cells, a 24-h treatment with $1,25(OH)_2D_3$ potentiates thyroid-releasing hormone (TRH)– and bombesin-induced PI turnover and $Ca_i$ transients resulting in enhanced prolactin secretion (26, 27).

Extracellular ATP triggers the rapid increase in $Ca_i$. This action of ATP is mediated by cell surface receptors coupled to G protein and phospholipase C-$\beta_1$ (PLC-$\beta_1$) in a variety of cells (28, 29). In keratinocytes, for example, ATP stimulates PI metabolism, mobilizes $Ca_i$, and increases DNA synthesis (30, 31). In this study we evaluated whether the long-term effects of $1,25(OH)_2D_3$ on $Ca_i$ in keratinocytes include upregulation of agonist (ATP)–induced PI metabolism and $Ca_i$ transients similar to that seen in HL-60 and pituitary cells. Our results indicate that treatment of keratinocytes with $1,25(OH)_2D_3$ potentiates the $Ca_i$ and inositol trisphosphate ($IP_3$) response of the cells to ATP. This potentiation required time and protein synthesis. It was not due to an increase in

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1. *Abbreviations used in this paper:* $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D; $Ca_i$, $Ca_o$, intra- and extracellular calcium; Indo-1 AM, indo-1 acetoxymethylester; $IP_3$, inositol triphosphate; KGM, keratinocyte growth medium; PI, phosphatidyl inositol; $PIP_2$, phosphatidyl inositol biphosphate; PLC, phospholipase C; TRH, thyroid-releasing hormone.

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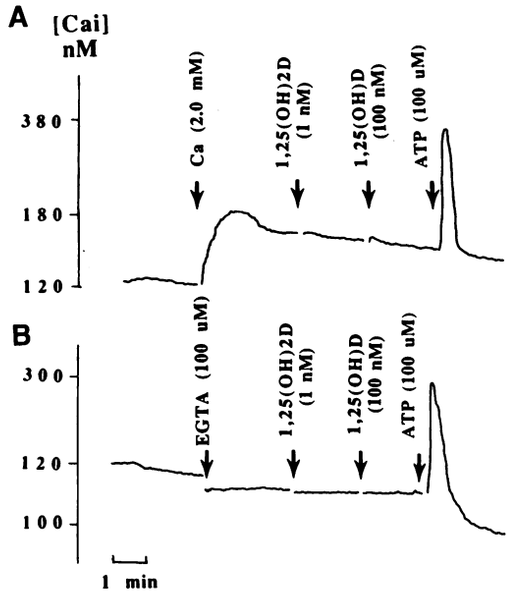


*Figure 1.* Acute effects of $1,25(OH)_2D_3$ and ATP on $Ca_i$ levels of keratinocytes in the presence of $Ca_o$ (*A*) or in its absence (*B*). Keratinocytes were grown to confluence in 0.07 mM calcium, loaded with 2 $\mu$M indo-1 AM, and resuspended in buffer A, and $Ca_i$ was measured as described in Methods. The concentrations of $Ca_o$, $1,25(OH)_2D_3$, and ATP shown are the final concentrations after each addition. Each trace is representative of experiments repeated at least three times. ATP stimulates an acute and transient increase in $Ca_i$ both in the presence and absence of $Ca_o$. Note the absence of an acute effect by $1,25(OH)_2D_3$.

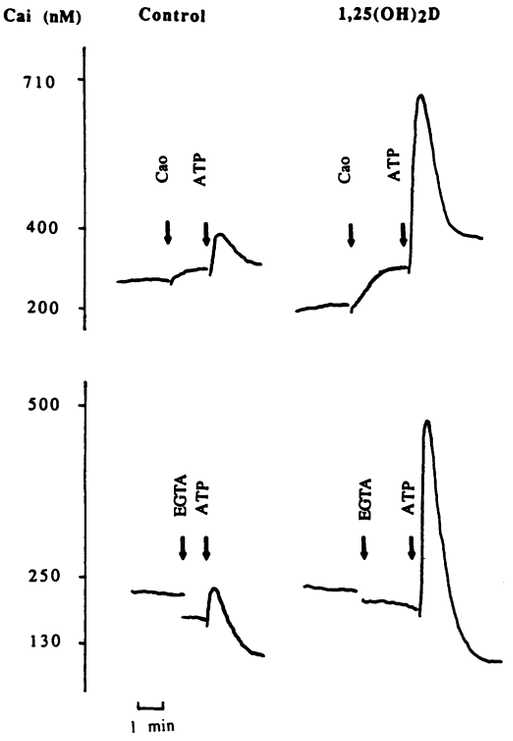


*Figure 2.* The acute response of $Ca_i$ to ATP in cells preincubated with 1 nM $1,25(OH)_2D_3$. The experimental conditions were as described in Fig. 1 except 1 nM $1,25(OH)_2D_3$ or vehicle was added to cultures 24 h before measurement of $Ca_i$. In the top panels the response to 100 $\mu$M ATP was determined after the acute addition of 2 mM calcium; in the bottom panels the response was determined after the acute addition of 100 $\mu$M EGTA. Cells studied in the left panels were grown in the absence of $1,25(OH)_2D_3$; cells studied in right panels were grown in the presence of 1 nM $1,25(OH)_2D_3$. $1,25(OH)_2D_3$ enhanced the $Ca_i$ response to ATP in the presence and absence of $Ca_o$.

releasable calcium pools but to an increase in PLC-$\beta_1$ synthesis, as indicated by an increase in the protein and mRNA levels for PLC-$\beta_1$. We then extended these studies and found that the production of other PLC isoenzymes was likewise increased by $1,25(OH)_2D_3$. These studies suggest that $1,25(OH)_2D_3$ modulates the keratinocyte signal transduction pathway by increasing PLC activity at the level of transcriptional regulation.

## Methods

*Materials.* Indo-1 acetoxymethylester (Indo-1 AM) was purchased from Molecular Probes, Inc. (Junction City, OR). Ionomycin was purchased from Calbiochem Corp. (San Diego, CA). $1,25(OH)_2D_3$ was a gift from Hoffmann La-Roche (Nutley, NJ). ATP, histamine, bradykinin, and all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

*Cell culture.* Normal human keratinocytes were isolated from neonatal human foreskins and grown in serum-free keratinocyte growth medium (KGM; Clonetics Corp., San Diego, CA) as described previously (32). Briefly, keratinocytes were isolated from newborn human foreskins by trypsinization (0.25% trypsin, 4°C, overnight), and primary cultures were established in KGM containing 0.07 mM calcium. Second-passage keratinocytes plated in KGM plus 0.07 mM calcium were grown to 50% confluence (48 h) before switching to KGM containing different calcium concentrations for the experiments. Details of each experiment are given in the figure legends.

*Measurement of $Ca_i$.* $Ca_i$ of keratinocytes was measured as previously described (30). Briefly, keratinocytes dispersed with trypsin plus EDTA were loaded with 2 $\mu$M Indo-1 AM in buffer A (20 mM Hepes buffer, pH 7.4, containing 120 mM sodium chloride, 5 mM potassium chloride, 1 mM magnesium chloride, 1 mg/ml pyruvate, 1 mg/ml glucose, and 0.03 mM calcium chloride) at 37°C for 1 h. Cells were then washed and resuspended in buffer A at a cell concentration of $1-1.5 \times 10^6$ cells per ml. Cells were washed and resuspended in fresh buffer A before each measurement, and all of the measurements were completed within 2–2.5 h after loading to minimize Indo-1 AM leakage. Fluorescence was recorded with a fluorimeter (model 650-40; Perkin-Elmer Corp., Norwalk, CT) using 350 and 405 nm for excitation and emission wavelengths, respectively. A thermostatted cuvette (37°C) and a magnetic stirrer were used. Fluorescence signals were recorded after each addition and calibrated for each sample by the addition of ionomycin (10 $\mu$M final concentration) ($F_{max}$) followed by 0.1% Triton X-100 and 10 mM EGTA, Tris, pH 8.3 ($F_{min}$). $Ca_i$ was calculated from the following formula: $Ca_i = K_d (F - F_{min})/(F_{max} - F)$, where $K_d$ for Indo-1 AM for calcium is 250 nM (33).

*PI metabolism of keratinocytes.* Turnover of inositol phosphates was measured by a modification of the method by Berridge et al. (34). Briefly, confluent cultures of keratinocytes in 6-well multi-well plates were prelabeled with 1 $\mu$Ci/ml [$^3$H]myoinositol (sp act 18 Ci/mmol; Amersham Corp., Arlington Heights, IL) in inositol-free KGM for 48 h. After washing, the cells were preequilibrated for 5 min with 1 ml of myoinositol-free KGM containing 20 mM Hepes, pH 7.4, and 10 mM lithium chloride and then treated with 100 $\mu$M ATP for the various time periods indicated. The reaction was stopped by aspiration of the medium and addition of ice-cold 10% TCA. Cells were scraped from the dish, TCA was extracted with diethyl ether, and inositol phosphates were separated using Dowex 1-X8 (Sigma Chemical Co., St. Louis, MO) columns eluted with stepwise gradients of ammonium formate buffers. $IP_1$, $IP_2$, and $IP_3$ levels were quantitated by counting the appropriate column fractions. Inositol phosphate levels of control cells and ATP-treated cells were determined in parallel, and the results were plotted

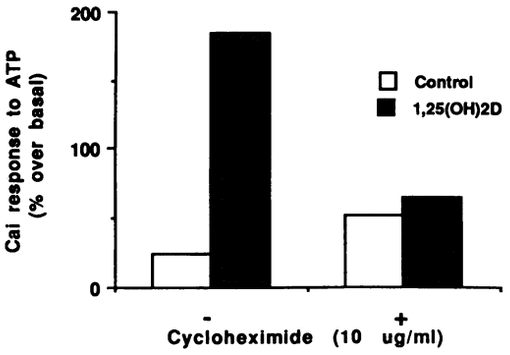


*Figure 3.* The effect of cycloheximide on the ability of $1,25(OH)_2D_3$ to increase ATP-stimulated $Ca_i$. Cycloheximide (10 $\mu$g/ml) was added to some of the cultures 1 h before addition of $1,25(OH)_2D_3$. 4 h after addition of $1,25(OH)_2D_3$, the ability of ATP to raise $Ca_i$ was tested. The results are shown as the percent rise in $Ca_i$ over baseline for each of the four conditions. Cycloheximide blocked the ability of $1,25(OH)_2D_3$ to enhance the stimulation of $Ca_i$ by ATP.

as the percent change over control for each time point. In addition, the $IP_3$ levels were quantitated using the protein binding assay system obtained from Amersham Corp. The competition between unlabeled $IP_3$ in the sample with a fixed quantity of tritium-labeled $IP_3$ for a limited number of binding sites on a bovine adrenal binding protein was quantitated by separating the bound $IP_3$ by centrifugation. The amount of radioactivity bound to the protein pellet was determined by scintillation counting. The amount of $IP_3$ in the sample was determined by interpolation from a standard curve.

*Protein levels of PLC isoenzymes.* Immunodetection of PLC-$\beta$1, -$\gamma$1, and -$\delta$1 isoenzymes was performed using anti-PLC-$\beta$1 monoclonal antibody, anti-PLC-$\gamma$1 polyclonal antibody, and anti-PLC-$\delta$1 antibody obtained from Upstate Biotechnology Inc. (Lake Placid, NY). After SDS-PAGE of lysed cells in 7.5% gels, proteins were transblotted onto polyvinylidene difluoride membranes. Detection was performed using the Enhanced Chemiluminescence system obtained from Amersham Corp. In the data shown, the same blot was probed for all three isoenzymes.

*mRNA levels of PLC isoenzymes.* Total RNA was prepared according to the method of Chomczynski and Sacchi (35). In brief, cells were dispersed in lysis solution (4 M guanidine isothiocyanate, 25 mM sodium citrate, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol) and then extracted with phenol–chloroform followed by ethanol precipitation. For Northern analysis, RNA was electrophoresed through 1% agarose–formaldehyde gels and transferred to nylon membranes (Hybond-N+; Amersham Corp.). The blots were hybridized with $^{32}P$-labeled cDNA probes for PLC-$\beta$2 and PLC-$\gamma$1 (gifts from Dr. John Imboden, V. A. Medical Center, San Francisco, CA) and an 18S RNA probe for normalization. The probes for PLC isoenzymes $\beta$1 and $\delta$1 were oligonucleotides synthesized according to the published sequences available from GenBank. The probe for PLC-$\beta$1 was TCTTGGCGAGGCTGCTGTTGG and was the antisense region from 1306 to 1327 of the sequence published by Bahk et al. (36). The probe for PLC-$\delta$1 was ACGTGAGTTGAAGGTGGTGTT, which is the antisense region from 1901 to 1922 of the sequence published by Yagisawa et al. (37). The results were quantitated by densitometry of the resulting autoradiogram.

## Results

The acute effects of $Ca_o$, $1,25(OH)_2D_3$, and ATP on $Ca_i$ are shown in Fig. 1. $Ca_o$ increased $Ca_i$ in an acute and sustained manner, possibly as a result of both calcium influx and calcium release from intracellular stores. ATP increased $Ca_i$ in an acute but transient manner and only by release of calcium from intracellular stores (i.e., EGTA did not block the acute response of $Ca_i$ to ATP). In contrast, $1,25(OH)_2D_3$ had no acute effect on $Ca_i$ levels either in the presence or absence of $Ca_o$. Similar results were obtained in cells attached to culture dishes in which $Ca_i$ was measured using fluorescence microscopy (data not shown). Consistent with these data, ATP stimulated $IP_3$ production in keratinocytes, but $1,25(OH)_2D_3$ did not (data not shown). Keratinocytes grown in the presence of 1 nM

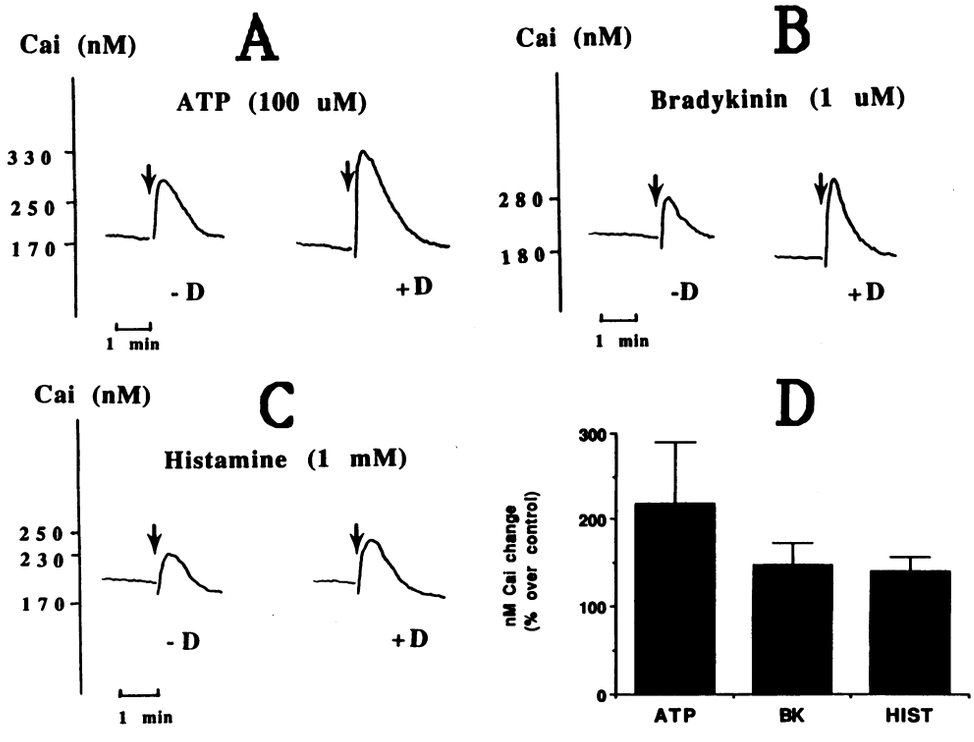


*Figure 4.* Effect of $1,25(OH)_2D_3$ on the $Ca_i$ response of keratinocytes to bradykinin and histamine. Keratinocytes were exposed to 10 nM $1,25(OH)_2D_3$ or vehicle for 24 h, and their $Ca_i$ responses to ATP (100 $\mu$M) bradykinin (1 mM), and histamine (1 mM) were determined. $Ca_i$ responses of keratinocytes to all of these calcium agonists were enhanced by $1,25(OH)_2D_3$. Representative experiments are shown in *A–C*, and mean±SD values of three experiments for each agonist are given in *D*.

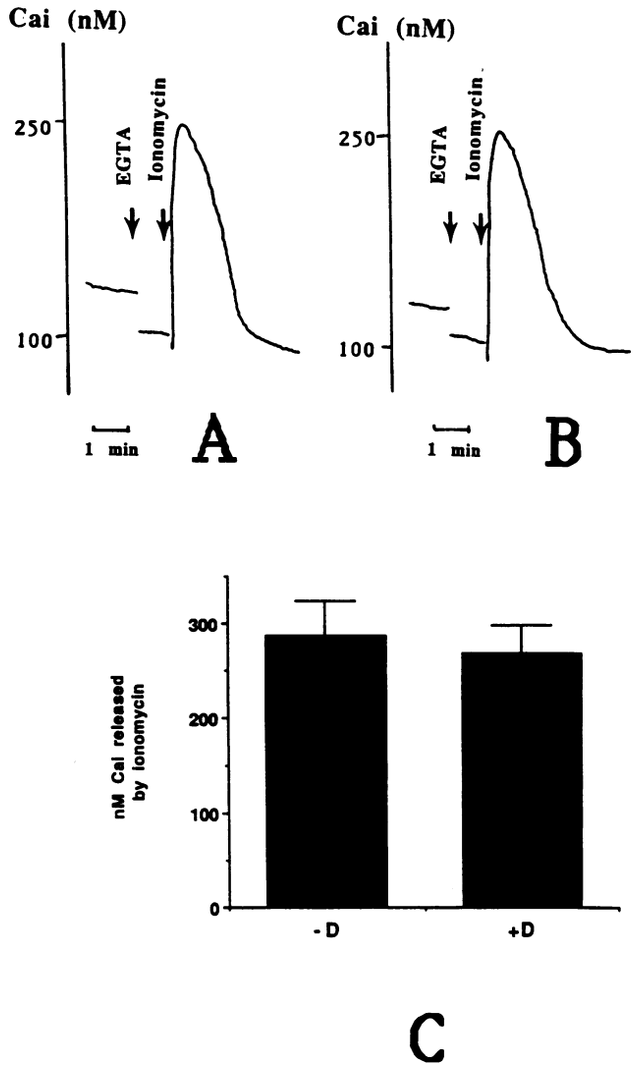


*Figure 5.* Effect of $1,25(OH)_2D_3$ on ionomycin-releasable total calcium pools of keratinocytes. Keratinocytes grown in 0.07 mM calcium to confluence were treated with 10 nM $1,25(OH)_2D_3$ or vehicle for 24 h. The $Ca_i$ response of these cells to ionomycin was measured in cells suspended in 100 $\mu$M EGTA. Ionomycin-releasable calcium pools of keratinocytes were similar in $1,25(OH)_2D_3$- and vehicle-treated cells. A representative experiment is shown. Values given in *C* are the mean±SD of three experiments.

$1,25(OH)_2D_3$ for 24 h demonstrated a greater $Ca_i$ response to $Ca_o$ and to ATP both in the presence and absence of $Ca_o$ (Fig. 2). This enhanced response to ATP required several hours of exposure to $1,25(OH)_2D_3$. Acute addition or short-term exposure (5 min) to $1,25(OH)_2D_3$ failed to enhance the $Ca_i$ response to ATP, but the enhancement was observed by 4 h. The effect was maximal after a 24-h incubation with $1,25(OH)_2D_3$. Cycloheximide (10 $\mu$g/ml) treatment alone had a minimal effect on the ATP-induced $Ca_i$ response, but completely blocked the $1,25(OH)_2D_3$-induced enhancement of the ATP response (Fig. 3).

To determine the generality of this action of $1,25(OH)_2D_3$, we performed the experiments shown in Fig. 4. Keratinocytes possess cell surface receptors for bradykinin and histamine as well as ATP (38). These agonists also lead to a transient increase in $Ca_i$ and $IP_3$ production. A 24-h pretreatment of keratinocytes with 10 nM $1,25(OH)_2D_3$ enhanced the $Ca_i$ response to bradykinin and histamine as well as to ATP. From this we infer that $1,25(OH)_2D_3$ potentiates the $Ca_i$ response to these agonists by a mechanism common to them all.

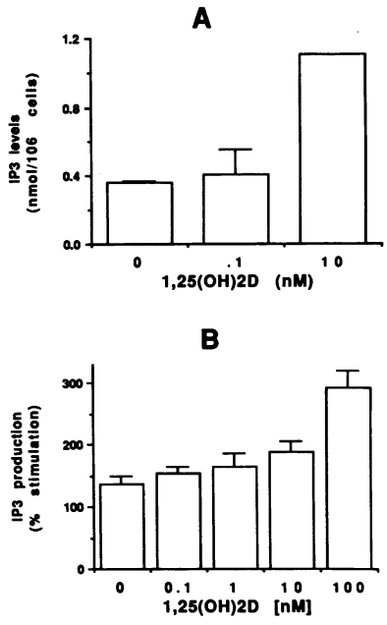


*Figure 6.* Effect of $1,25(OH)_2D_3$ on basal $IP_3$ levels and $IP_3$ turnover in response to ATP. (*A*) Basal $IP_3$ levels: Keratinocytes were treated with the indicated amounts of $1,25(OH)_2D_{33}$ for 24 h. Cells were precipitated with TCA, and $IP_3$ was extracted. The amount of $IP_3$ in the extract was determined using the protein binding assay. Each point represents the mean±range of duplicate determinations. (*B*) ATP-stimulated $IP_3$ turnover: Keratinocytes were exposed to the indicated concentrations of $1,25(OH)_2D_3$ for 24 h. $IP_3$ levels were measured using radiolabeled myo-inositol incorporation into phosphatidyl inositol biphosphate ($PIP_2$) and its subsequent hydrolysis to radiolabeled $IP_3$. $IP_3$ levels were measured before and after stimulation with 100 $\mu$M ATP for 2 min, and the data are expressed as percent stimulation over basal. $1,25(OH)_2D_3$ increased ATP-stimulated $IP_3$ production in a dose-dependent manner. Data shown are mean±SD of triplicate determinations.

Since $1,25(OH)_2D_3$ could act by increasing $Ca_i$ pools releasable by these agonists, we evaluated such pools by determining the rise in $Ca_i$ after exposure to the calcium ionophore ionomycin in the absence of $Ca_o$. As seen in Fig. 5, $1,25(OH)_2D_3$ did not alter the ionomycin response in cells incubated in the presence of 1 mM EGTA, indicating that $1,25(OH)_2D_3$ did not increase the calcium content of these pools. Conceivably, the response to ionomycin does not measure the pools specifically releasable by ATP and other agonists; but at this point we turned our attention to the possibility that $1,25(OH)_2D_3$ altered the $IP_3$ response to these agonists and thus the stimulus for $Ca_i$ release.

Basal levels of $IP_3$ were increased by a 24-h incubation with $1,25(OH)_2D_3$ (Fig. 6 *A*). Furthermore, ATP-stimulated $IP_3$ production was increased in keratinocytes incubated with 0.1–100 nM $1,25(OH)_2D_3$ for 24 h (Fig. 6 *B*). These data

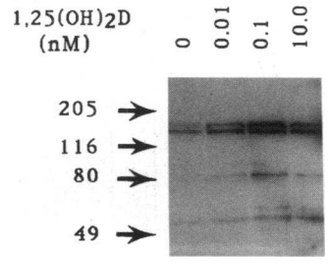


*Figure 7.* Effect of $1,25(OH)_2D_3$ on keratinocyte PLC-$\beta$1 protein levels. Cells were treated for 24 h with 0 or 0.01–10 nM $1,25(OH)_2D_3$ in the presence of 0.07 mM calcium for 24 h. Cells were lysed in the SDS/DTT lysis buffer, and 50 $\mu$g total protein was loaded on a 7.5% SDS-PAGE gel. Western blotting and detection of PLC-$\beta$1 protein were performed as described in Methods. $1,25(OH)_2D_3$ increased the 140- and 150-kD PLC-$\beta$ protein levels three- to four-fold.

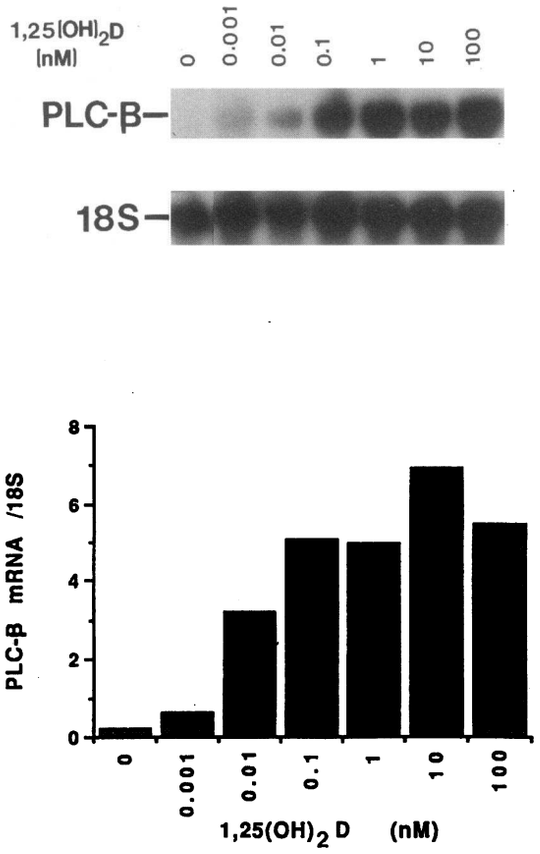


*Figure 8.* Effect of $1,25(OH)_2D_3$ on keratinocyte PLC-$\beta$1 mRNA levels. Keratinocytes were treated for 24 h with the indicated amounts of $1,25(OH)_2D_3$, and Northern blot analysis was performed to determine the PLC-$\beta$1 mRNA levels. Levels of PLC-$\beta$1 mRNA were normalized for equal loading with 18S RNA and plotted. 10 nM $1,25(OH)_2D_3$ enhanced the PLC-$\beta_1$ mRNA level by 80-fold.

indicate that increased production of $IP_3$ is a mechanism by which $1,25(OH)_2D_3$ could mediate the increased $Ca_i$ transients in response to ATP. To determine whether the stimulation by $1,25(OH)_2D_3$ of ATP-mediated $IP_3$ generation and $Ca_i$ transients is mediated by increased PLC-$\beta$1 mRNA and protein levels, we performed the experiments shown in Figs. 7 and 8. PLC-$\beta$1 of keratinocytes separated as a doublet in this experiment corresponding to 150- and 140-kD forms as described previously (12). In addition, two minor bands corresponding to 80 and 60 kD were visible in keratinocytes. Exposure of keratinocytes to 0.01–10 nM $1,25(OH)_2D_3$ for 24 h increased the PLC-$\beta_1$ protein levels by three- to four-fold (Fig. 7). PLC-$\beta$1 mRNA was even more responsive to $1,25(OH)_2D_3$ (Fig. 8). Exposure of keratinocytes to as little as 1 pM $1,25(OH)_2D_3$ induced an increase in the steady-state PLC-$\beta$1 mRNA levels, whereas 10 nM $1,25(OH)_2D_3$ increased the PLC-$\beta$1 mRNA levels by 80-fold. Thus, $1,25(OH)_2D_3$ appears to increase the ATP-stimulated $Ca_i$ and $IP_3$ production in keratinocytes by stimulating PLC-$\beta$1 production at the genomic level.

We then explored whether this effect of $1,25(OH)_2D_3$ was limited to PLC-$\beta$1, or whether other isoenzymes of PLC were also affected. The results of these studies are shown in Figs. 9 and 10. In Fig. 9, the ability of $1,25(OH)_2D_3$ to stimulate different isoenzymes as assessed by Western analysis is shown using the same blot to assess each isoenzyme in the same preparation. In this experiment, PLC-$\beta$1 was the most responsive and was stimulated fourfold by $10^{-12}$ M $1,25(OH)_2D_3$ and sevenfold by $10^{-8}$ M $1,25(OH)_2D_3$. In this experiment, only the higher (150 kD) band of PLC-$\beta$1 was observed. PLC-$\gamma$1 and -$\delta$1 were less responsive, being stimulated only 50 and 100%, respectively. In other experiments (data not shown) using cells grown in a higher calcium concentration (1.2 mM), severalfold stimulation of PLC-$\gamma$1 and -$\delta$1 by $1,25(OH)_2D_3$ was observed with lesser effect on PLC-$\beta$1. Thus, the effect of $1,25(OH)_2D_3$ on PLC isoenzyme production appears to be calcium regulated, as is seen for other proteins in the keratinocyte (39). Regardless of the variable stimulation of PLC isoenzyme proteins, $1,25(OH)_2D_3$ increased the mRNA levels of all PLC isoenzymes tested ($\beta$1, $\beta$2, $\gamma$1, and $\delta$1) in a coordinate fashion (Fig. 10). As for the Western analysis, the same blot was used to measure the mRNA levels for each isoenzyme in the same preparation for the data presented. We confirmed these findings using individual blots (data not shown). The effect of $1,25(OH)_2D_3$ was biphasic in this experiment, with peak stimulation of all mRNAs at $10^{-10}$ M $1,25(OH)_2D_3$.

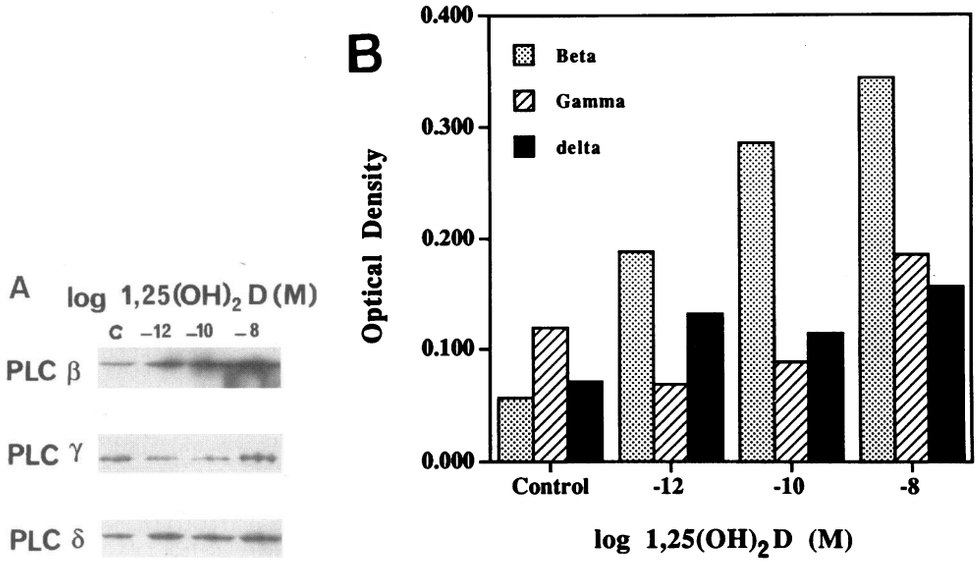


*Figure 9.* Effect of $1,25(OH)_2D_{33}$ on PLC $\beta$1, -$\gamma$1, and -$\delta$1 protein levels. The experiment was performed as described in the legend to Fig. 7. The blot was probed not only for PLC-$\beta$1, but for PLC-$\gamma$1 and $\delta$1. *A* shows the Western blots, which are plotted as a function of $1,25(OH)_2D_3$ concentration in *B*. $1,25(OH)_2D_3$ stimulated PLC-$\beta$1 more than the other isoenzymes in this experiment, although all three were increased.

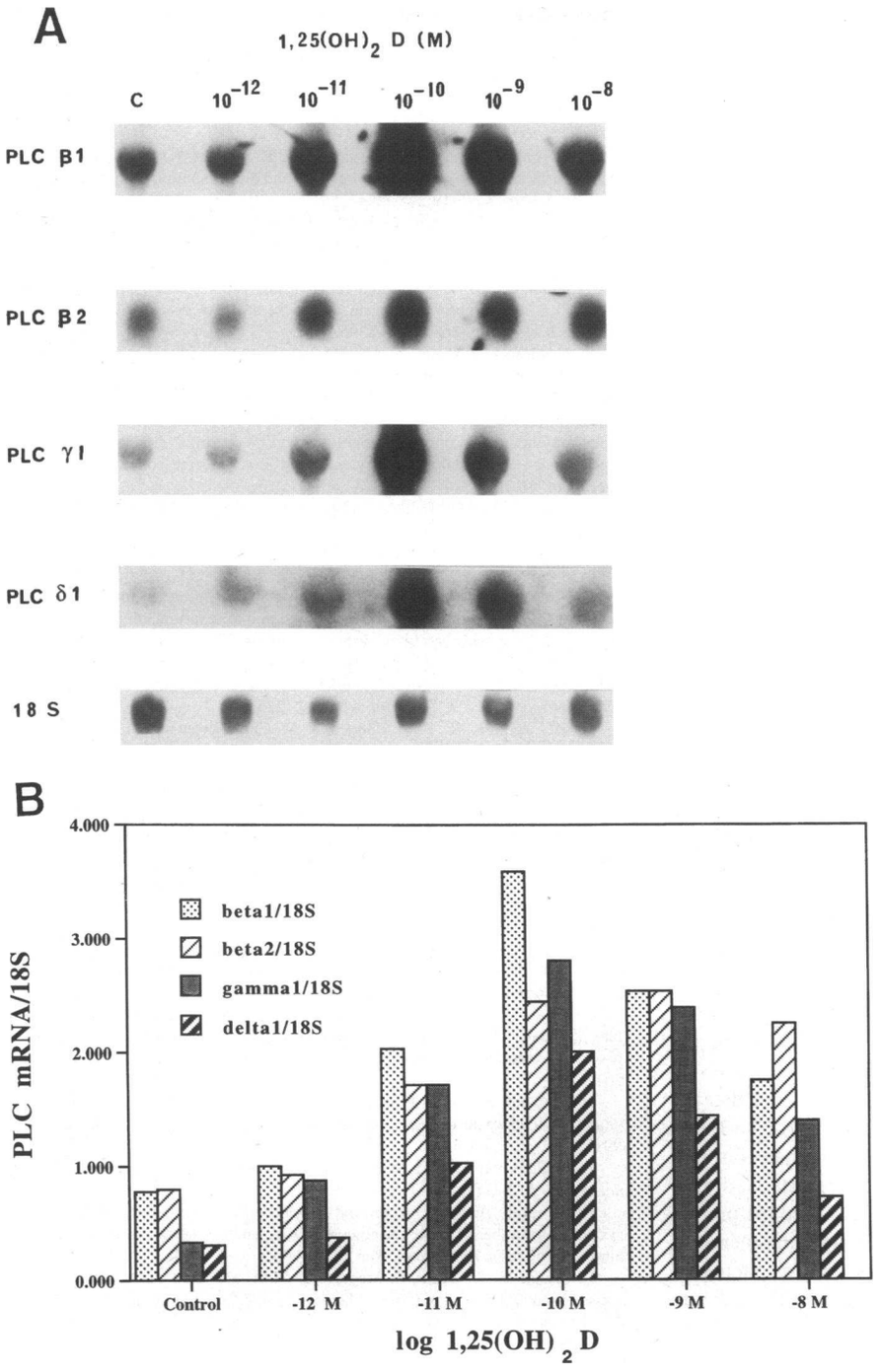


*Figure 10.* Effect of $1,25(OH)_2D_3$ on PLC $\beta 1$, $-\beta 2$, $-\gamma 1$, and $-\delta 1$ mRNA levels. This experiment was performed as described in the legend to Fig. 8. The same blot was hybridized with the five probes (including the 18S probe), and the probe was stripped off between hybridizations. The Northern results are shown in *A*, and are plotted as a function of $1,25(OH)_2D_3$ concentration in *B*, using normalization of the densitometric data to that for 18S.

## Discussion

In this report we provide evidence that $1,25(OH)_2D_3$ stimulates the PLC-mediated signal transduction pathway in keratinocytes. In particular, we noted that $1,25(OH)_2D_3$ treatment enhanced ATP-induced $Ca_i$ transients and $IP_3$ production and that this enhanced response was associated with an increase in PLC-$\beta 1$ protein and mRNA levels. However, other PLC isoenzymes were also increased by $1,25(OH)_2D_3$, indicating that this action of $1,25(OH)_2D_3$ is broader than that originally suspected and may contribute to the mechanism by which $1,25(OH)_2D_3$ regulates proliferation and differentiation of a number of cells in response to a variety of agonists.

$1,25(OH)_2D_3$, a regulator of calcium homeostasis, also stimulates calcium flux into a variety of cells, including bone, muscle, epithelial, monocytic, and pituitary cells (reviewed in reference 10). In most cell types this response demonstrates hormone specificity and is abolished by cycloheximide treatment. In pituitary cells, $1,25(OH)_2D_3$ enhances calcium entry by voltage-dependent calcium channels as well as by increasing their TRH- and bombesin-induced $Ca_i$ transients and prolactin secretion (26, 27). In HL-60 cells $1,25(OH)_2D_3$-induced calcium influx is accompanied by induction of fMLP-stimulated calcium transients and their differentiation into monocytes (25). The mechanism of action of $1,25(OH)_2D_3$ in the induction of agonist-mediated signaling events remains obscure, but in both

pituitary and HL-60 cells, hours of exposure to $1,25(OH)_2D_3$ are required for the effects to be seen, suggesting the requirement for new protein synthesis. Potential mechanisms include induction of voltage-dependent calcium channels in the case of pituitary cells (27) and increased calcium influx–mediated replenishment of $IP_3$-sensitive $Ca_i$ stores in the case of HL-60 cells (25). We have shown here that $1,25(OH)_2D_3$ increases the agonist-releasable $Ca_i$ in the absence of $Ca_o$ and without altering the total amount of releasable calcium pools in keratinocytes, suggesting that calcium channels and calcium stores are not altered. The effect of $1,25(OH)_2D_3$ is not limited to ATP, suggesting that $1,25(OH)_2D_3$ acts by stimulating a mechanism common but distal to the cell surface receptors for the $Ca_i$ agonists.

ATP, bradykinin, histamine, as well as a number of other agonists raise $Ca_i$ by stimulating PLC activity through a mechanism involving a GTP-binding protein (Gq) that couples the activated hormone receptor to PLC. PLC catalyzes the hydrolysis of phosphatidyl inositol biphosphate ($PIP_2$) to $IP_3$ and diacylglycerol. PLC is a family of enzymes, but the one that is regulated by Gq, and thus ATP, is PLC-$\beta$1. The effect of $1,25(OH)_2D_3$ on PLC-$\beta$1 mRNA levels was much greater than its effect on PLC-$\beta$1 protein or on basal and ATP-induced $IP_3$ production. The reason for this apparent discrepancy is unclear. Regulation at the level of translation of the PLC-$\beta_1$ mRNA may exist in keratinocytes to limit the PLC-$\beta_1$ enzyme activity in these cells. Kinases and phosphatases capable of rapidly metabolizing $IP_3$, limiting its rise, most certainly exist in the keratinocyte. Nevertheless, the physiological response of increased PLC-$\beta_1$ gene expression, namely increased $IP_3$ production and $Ca_i$ transients in response to ATP, is significantly stimulated in $1,25(OH)_2D_3$-treated cells, demonstrating its physiological relevance.

However, PLC-$\beta$1 is not the only PLC isoenzyme found in the keratinocyte (40). In fact, in murine keratinocytes, PLC-$\beta$1 is not detected, whereas PLC-1 and $\delta$1 are readily detected (40). Calcium raises the PLC-1 protein levels but not the mRNA levels (40). Therefore, we extended these studies to include several of these other isoenzymes. We found that the mRNA levels of all of the other isoenzymes tested ($\beta$2, -1, and $\delta$1) were increased by $1,25(OH)_2D_3$ in a coordinate fashion. Just as the rise in PLC-$\beta$1 protein level did not reflect the degree of increase in the mRNA, so too the PLC-$\beta$2, -1, and -$\delta$1 protein levels did not show a one-to-one correlation with the increases in their mRNAs. Although more investigation of this phenomenon is required, our preliminary data indicate that the calcium concentration of the medium in which the cells are grown regulates the extent to which the protein is produced, which is similar to the observations of Punnonen et al. (40). We (39) have made similar observations for involucrin and transglutaminase, two proteins induced by $1,25(OH)_2D_3$ that are involved in keratinocyte differentiation. Regardless of how $1,25(OH)_2D_3$ induction of PLC isoenzymes is controlled, these results indicate that the effect of $1,25(OH)_2D_3$ on this important signaling pathway is substantial and more general than indicated by the original observation (increased $Ca_i$ response to ATP) that led us into this study.

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## References

1. Hennings, H., D. Michael, C. Cheng, P. Steinert, K. Holbrook, and S. H. Yuspa. 1980. Calcium regulation of growth and differentiation of mouse epidermal cells in culture. *Cell.* 19:245–254.

2. Boyce, S. T., and R. G. Ham. 1983. Calcium-regulated differentiation of normal human epidermal keratinocytes in chemically defined clonal culture and serum-free serial culture. *J. Invest. Dermatol.* 81:33s–40s.

3. Pillai, S., D. D. Bikle, and P. M. Elias. 1988. 1,25-Dihydroxyvitamin D production and receptor binding in human keratinocytes varies with differentiation. *J. Biol. Chem.* 263:5390–5395.

4. Hosomi, J., J. Hosoi, E. Abe, T. Suda, and T. Kuroki. 1983. Regulation of terminal differentiation of cultured mouse epidermal cells by 1$\alpha$,25-dihydroxyvitamin $D_3$. *Endocrinology.* 113:1950–1957.

5. Smith, E. L., N. C. Walworth, and M. F. Holick. 1986. Effect of 1$\alpha$,25-dihydroxyvitamin $D_3$ on the morphologic and biochemical differentiation of cultured human epidermal keratinocytes grown in serum-free conditions. *J. Invest. Dermatol.* 86:709–714.

6. Pillai, S., and D. D. Bikle. 1991. Role of intracellular-free calcium in the cornified envelope formation of keratinocytes: differences in the mode of action of extracellular calcium and 1,25-dihydroxyvitamin $D_3$. *J. Cell. Physiol.* 146:94–100.

7. Jones, K. T., and G. R. Sharpe. 1994. Staurosporine, a non-specific PKC inhibitor, induces keratinocyte differentiation and raises intracellular calcium, but Ro31-8220, a specific inhibitor, does not. *J. Cell. Physiol.* 159:324–330.

8. Dlugosz, A. A., and S. H. Yuspa. 1993. Coordinate changes in gene expression which mark the spinous to granular cell transition in epidermis are regulated by protein kinase C. *J. Cell Biol.* 120:217–225.

9. O'Driscoll, K. R., P. V. Madden, K. M. Christiansen, A. Viage, T. J. Slaga, D. Fabbro, C. T. Powell, and I. B. Weinstein. 1994. Overexpression of protein kinase C beta I in a murine keratinocyte cell line produces effects on cellular growth, morphology and differentiation. *Cancer Lett.* 83:249–259.

10. Jaken, S., and S. H. Yuspa. 1988. Early signs for keratinocyte differentiation: role of $Ca^{2+}$-mediated inositol lipid metabolism in normal and neoplastic epidermal cells. *Carcinogenesis.* 9:1033–1038.

11. Lee, E., and S. H. Yuspa. 1991. Aluminum fluoride stimulates inositol phosphate metabolism and inhibits expression of differentiation markers in mouse keratinocytes. *J. Cell. Physiol.* 148:106–115.

12. Ryu, S. H., U.-H. Kim, M. I. Wahl, A. B. Brown, G. Carpenter, K.-P. Huang, and S. G. Rhee. 1990. Feedback regulation of phospholipase C-$\beta$ by protein kinase C. *J. Biol. Chem.* 265:17941–17945.

13. Park, D. J., H. K. Min, and S. G. Rhee. 1992. Inhibition of CD3-linked phospholipase C by phorbol ester and by cAMP is associated with decreased phosphotyrosine and increased phosphoserine contents of PLC-1. *J. Biol. Chem.* 267:1496–1501.

14. Reichel, H., H. P. Koeffler, and A. W. Norman. 1989. The role of the vitamin D endocrine system in health and disease. *N. Engl. J. Med.* 320:980–991.

15. Walters, M. R. 1985. Steroid hormone receptors and the nucleus. *Endocrinol. Rev.* 6:512–543.

16. Norman, A. W., J. Roth, and L. Orci. 1982. The vitamin D endocrine system: steroid metabolism, hormone receptors, and biological response (calcium binding proteins). *Endocrinol. Rev.* 3:331–366.

17. Yada, Y., T. Ozeki, S. Meguro, S. Mori, and Y. Nozawa. 1989. Signal transduction in the onset of terminal keratinocyte differentiation induced by 1$\alpha$,25-dihydroxyvitamin $D_3$: role of protein kinase C translocation. *Biochem. Biophys. Res. Commun.* 163:1517–1522.

18. Tang, W., V. A. Ziboh, R. Isseroff, and D. Martinez. 1987. Novel regulatory action of 1,25-dihydroxyvitamin D on the metabolism of polyphosphoinositides in murine epidermal keratinocytes. *J. Cell. Physiol.* 132:131–136.

19. McLaughlin, J. A., L. C. Cantley, and M. F. Holick. 1990. $1,25(OH)_2D_3$ increased calcium and phosphatidylinositol metabolism in differentiating cultured human keratinocytes. *J. Nutr. Biochem.* 1:81–87.

20. Bittner, B., S. S. Bleenhen, and S. MacNeil. 1991. $1,25(OH)_2D$ increased intracellular calcium in human keratinocytes. *Br. J. Dermatol.* 124:230–235.

21. Pillai, S., and D. D. Bikle. 1991. Epidermal vitamin D metabolism function and regulation. *Adv. Lipid Res.* 24:321–341.

22. Hruska, K. A., Z. Bar-Shavit, J. D. Malone, and S. Teitelbaum. 1988. $Ca^{2+}$ priming during vitamin D-induced monocytic differentiation of a human leukemia cell line. *J. Biol. Chem.* 263:16039–16044.

23. Mezzetti, G., M. G. Monti, L. P. Casolo, G. Piccinini, and M. S. Moruzzi. 1988. 1,25 Dihydroxycholecalciferol-dependent calcium uptake by mouse mammary gland in culture. *Endocrinology.* 122:389–394.

24. Bikle, D. D. 1990. Regulation of intestinal calcium transport by vitamin D [$1,25(OH)_2$]: role of membrane structure. *In* Membrane Transport and Information Storage. R. C. Aloia, C. C. Curtain, and L. M. Gordon, editors. Wiley-Liss, New York. 191–219.

25. Tanaka, H., K. A. Hruska, Y. Seino, J. D. Malone, Y. Nishii, and S. L. Teitelbaum. 1991. Disassociation of the macrophage-maturational effects of vitamin D from respiratory burst priming. *J. Biol. Chem.* 266:10888–10892.

26. Chisholm, J. C., S. Kim, and A. H. Tashjian, Jr. 1988. Modulation by $1,25(OH)_2D$ of the acute change in cytosolic free calcium induced by thyrotropin-releasing hormone in $GH_4C_1$ pituitary cells. *J. Clin. Invest.* 81:661–668.

27. Tornquist, K. 1991. $1,25(OH)_2D$ enhances both the bombesin-induced transient in intracellular free calcium and the bombesin-induced secretion of prolactin in $GH_4C_1$ pituitary cells. *Endocrinology.* 128:2175–2182.

28. Dubyak, G. R., D. S. Cowen, and L. M. Meuller. 1988. Activation of inositol phospholipid breakdown in HL 60 cells by P2 purinergic receptors for extracellular ATP. *J. Biol. Chem.* 263:18108–18117.

29. Cockroft, S., and J. Stutchfield. 1989. ATP stimulates secretion in human neutrophils and HL-60 cells via a pertussis toxin sensitive guanine nucleotide binding protein coupled to phospholipase C. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 245:25–29.

30. Pillai, S., and D. D. Bikle. 1992. ATP stimulates phosphoinositide metabolism, mobilizes intracellular calcium and inhibits terminal differentiation of human epidermal keratinocytes. *J. Clin. Invest.* 90:42–51.

31. Suter, M. M., F. M. Crameri, J. P. Slattery, P. J. Millard, and F. A. Gonzales. 1991. Extracellular ATP and some of its analogs induce transient rises in cytosolic free calcium in individual canine keratinocytes. *J. Invest. Dermatol.* 97:223–229.

32. Pillai, S., D. D. Bikle, M. Hincenbergs, and P. M. Elias. 1988. Biochemical and morphological characterization of growth and differentiation of normal human neonatal keratinocytes in a serum-free medium. *J. Cell. Physiol.* 134:229–237.

33. Grynkiewicz, G., M. Poenie, and R. Y. Tsien. 1985. A new generation of calcium indicators with greatly improved fluorescence properties. *J. Biol. Chem.* 260:3440–3447.

34. Berridge, M. J., R. M. C. Dawson, C. P. Downes, J. P. Heslop, and R. F. Irvine. 1983. Changes in the level of inositol phosphates after agonist-dependent hydrolysis of membrane phosphoinositides. *Biochem. J.* 212:473–482.

35. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156–159.

36. Bahk, Y. Y., Y. H. Lee, S. H. Ryu, P. G. Suh, T. G. Lee, and J. Seo. 1994. Two forms of phospholipase C-beta 1 generated by alternative splicing. *J. Biol. Chem.* 269:8240–8245.

37. Yagisawa, H., H. Tanase, and H. Nojima. 1991. Phospholipase C-delta gene of the spontaneously hypertensive rat harbors point mutations causing amino acid substitutions in a catalytic domain. *J. Hypertens.* 9:997–1004.

38. Talwar, H. S., G. J. Fisher, V. A. Harris, and J. J. Voorhees. 1989. Agonist-induced hydrolysis of phosphoinositides and formation of 1,2-diacylglycerol in adult human keratinocytes. *J. Invest. Dermatol.* 93:241–245.

39. Su, M.-J., D. D. Bikle, M. L. Mancianti, and S. Pillai. 1994. 1,25 Dihydroxyvitamin D potentiates the keratinocyte response to calcium. *J. Biol. Chem.* 269:14723–14729.

40. Punnonen, K., M. Denning, E. Lee, L. Li, S. G. Rhee, and S. H. Yuspa. 1993. Keratinocyte differentiation is associated with changes in the expression and regulation of phospholipase C isoenzymes. *J. Invest. Dermatol.* 101:719–726.