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Increased PKC Activity in Cultured Human Keratinocytes and Fibroblasts After Treatment With 1α,25-Dihydroxyvitamin D_3

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Abstract 1α,25-Dihydroxyvitamin D_3 (10^{-12} M to 10^{-8} M) caused a dose dependent increase in PKC activity in the solubilized membrane fractions of cultured human keratinocytes and in the cytosolic fractions of cultured human fibroblasts. Maximum activity was induced by 1α,25-dihydroxyvitamin D_3 at 24 h. Sphingosine, which is believed to inhibit PKC mediated biological responses, blunted $1\alpha,25(OH)_2D_3$'s inducement of PKC activity in both keratinocytes and fibroblasts. Identical hormone treatment of vitamin D receptor deficient fibroblasts did not increase PKC activity. Treatment of keratinocytes and fibroblasts with 1β,25-dihydroxyvitamin D_3, which is believed to be ineffective in inducing genomic responses, did not induce PKC activity.

Key words: PKC activity, keratinocytes, fibroblasts, 1α,25-dihydroxyvitamin D_3, sphingosine

The biologically active form of vitamin D_3, 1α,25-dihydroxyvitamin D_3 ($1\alpha,25(OH)_2D_3$), can inhibit cell proliferation and increase cellular differentiation in cultured human and murine cells [Hosomi et al., 1988; Smith et al., 1986; Koeffler et al., 1985]. Similarly, protein kinase C (PKC), a serine/threonine protein kinase, can also regulate proliferation and differentiation in many cell systems [Zyler-Katz and Glazer, 1985; Weinstein et al., 1988], including normal human keratinocytes, where PKC activation has been considered to be a crucial regulatory step for keratinocyte differentiation [Yuspa et al., 1983; Snoek et al., 1987]. Primarily, PKC has been classified as an important mediator in signal transduction pathways, which can be initiated by numerous extracellular stimuli, including growth factors, hormones, and neurotransmitters [Nishizuka, 1984, 1988]. But more recently, complementary DNA cloning and sequencing have revealed a gene family of kinases that have distinct cofactors, functions and localizations within cell types [Knopf et al., 1986; Ono et al., 1988; Osada et al., 1990]. There is growing evidence that $1\alpha,25(OH)_2D_3$ may affect PKC activity in many different cell types and through many different pathways. Khare et al. [1993] have shown that $1\alpha,25(OH)_2D_3$ rapidly but transiently stimulates rat colonic particulate guanylate cyclase activity via a PKC-dependent mechanism. Utilizing an immunocytological technique, Barsony and Merz [1991] reported that 10 nM $1\alpha,25(OH)_2D_3$ caused, within 15 s, clumping of normal VDR receptors and accumulation of cGMP around these receptors in human fibroblasts. Data from various laboratories confirm that $1\alpha,25(OH)_2D_3$ can produce rapid non-genomic actions affecting intracellular calcium and membrane phosphoinositide metabolism [Baran and Milne, 1986; Baran et al., 1989; Civitelli et al., 1990; MacLaughlin et al., 1990; Wali et al., 1992]. These actions can in turn affect protein kinase C localization and activity in several cell types [Yada et al., 1989; Wali et al., 1990; Simboli-Campbell et al., 1994].

Of particular interest to us, however, is the accumulating evidence that $1\alpha,25(OH)_2D_3$ may also affect PKC levels through a genomic pathway. In its classic role as a steroid hormone, $1\alpha,25(OH)_2D_3$ binds to its receptor and initiates genomic activity by binding to recognition elements on specific genes [Kerner et al., 1989;

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Brown and DeLuca, 1990]. Martell et al. [1987] demonstrated that $1\alpha,25(OH)_2D_3$-induced differentiation of HL-60 cells was inhibited by exposing the cells to the protein kinase inhibitor H-7. They also found that protein kinase activity was increased twofold after 24 h exposure to $1\alpha,25(OH)_2D_3$ and that this response could be blocked by the protein synthesis inhibitor cycloheximide. In experiments with these same cells, Simpson et al. [1989] demonstrated that $1\alpha,25(OH)_2D_3$'s primary effect is to regulate PKC levels and that $1\alpha,25(OH)_2D_3$ down regulates c-myc transcription by regulating PKC. Obeid et al. [1990] have recently demonstrated that treatment of HL-60 cells with $1\alpha,25(OH)_2D_3$ increased the expression of genes for both the α and β isozymes. They suggest that $1\alpha,25(OH)_2D_3$ can activate the PKC pathway by transcriptional upregulation of PKC genes.

Since this genomic data links $1\alpha,25(OH)_2D_3$ and PKC activity, we investigated whether exposure to $1\alpha,25(OH)_2D_3$ over longer time periods might increase PKC activity in cultured human epidermal cells. We examined the effect of $1\alpha,25(OH)_2D_3$ on PKC activity in both cytosolic and soluble membrane fractions of normal cultured human keratinocytes and fibroblasts as well as in vitamin D receptor (VDR) deficient cultured human fibroblasts.

MATERIALS AND METHODS

Chemicals

Leupeptin, phenylmethylsulfonyl fluoride, benzimide, β-mercaptoethanol, EDTA, EGTA, Tris-HCl, DTT, protein kinase catalytic subunit, DL-erythro-dihydrospingosine, and nonidet P-40 were purchased from Sigma Chemical Co. (St. Louis, MO). Acetic acid was purchased from EM Science (Cherry Hill, NJ). Protein assay components were purchased from Biorad (Richmond, CA). DEAE-cellulose was purchased from Whatman BioSystems Ltd. (Kent, United Kingdom). The protein kinase C enzyme assay system and [γ-^{32}P] ATP were purchased from Amersham (Arlington Heights, IL). Bio-Safe II scintillation fluid was purchased from Research Products International Corp. (Mount Prospect, IL). $1\alpha,25(OH)_2D_3$ was a gift of Dr. M. Uskokovic of Hoffmann-LaRoche, Inc. (Nutley, NJ). $1\beta,25(OH)_2D_3$ was synthesized in our laboratory by Dr. Rahul Ray.

Cell Culture

Keratinocytes were grown in culture using a modification of Rheinwald and Green [1975]. The cells were obtained from human foreskin and were grown in serum free media and without a 3T3 feeder layer at passage. The serum free media consisted of MCDB 153 with a calcium concentration of 0.15 mM (Sigma Chemical Co., St. Louis, MO). The following growth factors were also added: epidermal growth factor (25 ng/ml), hydrocortisone (406 ng/ml), insulin (5 μg/L), prostaglandin E (50 ng/ml), and cholera toxin (0.1 μg/ml) (Sigma Chemical Co., St. Louis, MO). At second passage, keratinocytes were plated in 10 ml of serum free medium per 100 mm dish. Cells were treated at 75% confluency with either $1\alpha,25(OH)_2D_3$, $1\beta,25(OH)_2D_3$, or medium plus vehicle in the presence or absence of 6 μM DL-erythro-dihydrosphingosine. Experimental concentrations of the hormone ranged from 10^{-12} M to 10^{-6} M and the vehicle was absolute ethanol ($< 0.1\%$).

Normal fibroblasts were grown from human foreskins. After 4 to 5 weeks, cell growth in DMEM and 5% fetal calf serum (Sigma Chemical Co., St. Louis, MO) was adequate for passage. The fibroblasts were rinsed several times with EDTA (0.02%) in Ca^{++}/Mg^{++} free Hank's balanced salt solution (Sigma Chemical Co., St. Louis, MO). The cells were detached using trypsin (0.25%) in the same balanced salt solution. At second passage foreskin fibroblasts were plated for experimentation. Vitamin D receptor deficient fibroblasts were obtained from a patient with vitamin D-dependent rickets, type II (DDR-II), as previously described [Clemens et al., 1983]. The patient was a 14-year-old female born to normal parents who were first cousins. Early in life she developed total alopecia, hypocalcemia, hypophosphatemia, muscle weakness, and severe rickets which were unresponsive to various forms of vitamin D therapy including 150 μg/day 1α-hydroxyvitamin D_3 and 5 μg/day $1\alpha,25(OH)_2D_3$. However, treatment with massive doses ($4–7 \times 10^6$ IU/day) of vitamin D_2 resulted in elevation of the serum $1\alpha,25(OH)_2D_3$ concentration to the nanogram range, improvement in rachitic lesions, and restoration of serum biochemical parameters to normal. These cells were grown and passed using the same media and conditions as with normal fibroblasts. In all cases, the cells had reached confluency at the initiation of experimentation.

PKC Assay

For each control and experimental point, three 100 mm dishes were treated with vehicle or hormone in the presence or absence of 6 μM DL-erythro-dihydrosphingosine and harvested at specified time points. Cells were washed three times with 10 ml ice cold PBS buffer and then gently scraped and harvested with 3 ml of the same buffer. The cells were pelleted by centrifugation (100*g*; 10 min) and resuspended in ice-cold homogenization buffer [50 mM Tris-HCl, pH 7.5; 10 mM EGTA; 2 mM EDTA; 20 mM phenylmethylsulfonylfluoride; 10 mM benzimide; leupeptin (1 mg/100 ml), and 0.3% w/v 2-mercaptoethanol]. The cells were homogenized on ice (40 strokes) with a motorized pestle fitted to a Dounce homogenizer (Thomas Co., Philadelphia, PA). The homogenates were centrifuged at 100,000*g* for 45 min. The cytosols were collected and kept on ice. The pellets, containing the membranes, were resuspended via sonication in homogenization buffer containing 0.5% Nonidet P-40, agitated on ice for 30 min, and then centrifuged (100,000*g*; 45 min). The supernatants with solubilized membranes were collected. Cytosol and solubilized membrane fractions were further purified by DEAE batch chromatography. The DEAE cellulose was equilibrated with buffer A (20 mM Tris-HCl, pH 7.5; 2 mM sodium EDTA; and 0.5 mM EGTA) and the protein was eluted from the resin with two washes of 0.15 M NaCl in buffer A. Samples were run in assay within hours of cell harvests to eliminate any freeze/thawing effects that can interfere with the accurate measurement of PKC activity. PKC activity in the eluates was measured by Amersham's PKC enzyme assay system, which utilizes a modification of a mixed micelle assay [Hannun et al., 1985]. Samples were run in triplicate and phosphorylated peptide was separated on individual binding papers. These were washed twice with 5% v/v acetic acid. The filters were dried, transferred to a scintillation vial, and counted in 10 ml scintillation fluid using a Taurus liquid scintillation counter (*ICN, Micromedics Systems, Huntsville, AL*). Appropriate blanks were run to correct for any non-specific effects of [γ^{32}P] ATP or its radiolytic decomposition products binding to papers. Protein concentrations in the sample preparations were determined by analyzing duplicate sample aliquots in a Biorad protein assay (Richmond, CA) which is based on the Bradford methodology [Bradford, 1976]. PKC activity was calculated based on the total phosphate transferred per μg protein in the assay tube. To assure activity values were not due to protein variations, DNA determinations [Burton, 1956] were also performed on selected samples and relative values did not change.

Statistical Analysis

Assay samples were run in triplicate and the mean ± SEM was determined for each triplicate group. *P* values were determined using the Student's *t*-test. The statistical significance between the means of specific data points is defined in the figure legends and in the Results. Dose and time course experiments were representable and selected dose and time points have been repeated three or more times for confirmation.

RESULTS

PKC Activity in Normal Human Cultured Keratinocytes

There was a dose dependent increase in PKC activity in the solubilized membrane fractions of human keratinocytes after treatment with 1α,25$(OH)_2D_3$ for 24 h. As shown in Figure 1A, PKC activity increased 17% ± 2%, 23% ± 1%, and 77% ± 2% as compared to the 24 h control (*P* values were <0.001) when keratinocytes were incubated with 10^{-12} M, 10^{-10} M, and 10^{-8} M 1α,25$(OH)_2D_3$, respectively. When keratinocytes were incubated with 10^{-6} M of 1α,25$(OH)_2D_3$, PKC activity decreased 21% ± 2% in the solubilized membrane fraction compared to the 24 h control ($P < 0.001$). In contrast to the significant activity changes seen in the solubilized membranes, three separate experiments demonstrated less than 20% of the PKC activity in the membrane fraction was measurable in the cytosolic fraction of the same cell. At all concentrations tested, 1α,25$(OH)_2D_3$ did not significantly alter PKC activity in the cytosolic fractions. For example, the 24 h control value for the cytosol fraction was 337 ± 25 cpm/μg protein versus 1,835 ± 39 cpm/μg protein for the membrane fraction for the experiment depicted in Figure 1A. Experimental values measured in the cytosolic fractions varied <5% from the 24 h control value.

Since sphingosine is believed to inhibit PKC mediated biological responses, keratinocytes were treated with 6 μM sphingosine, in the absence and presence of 1α,25$(OH)_2D_3$. PKC

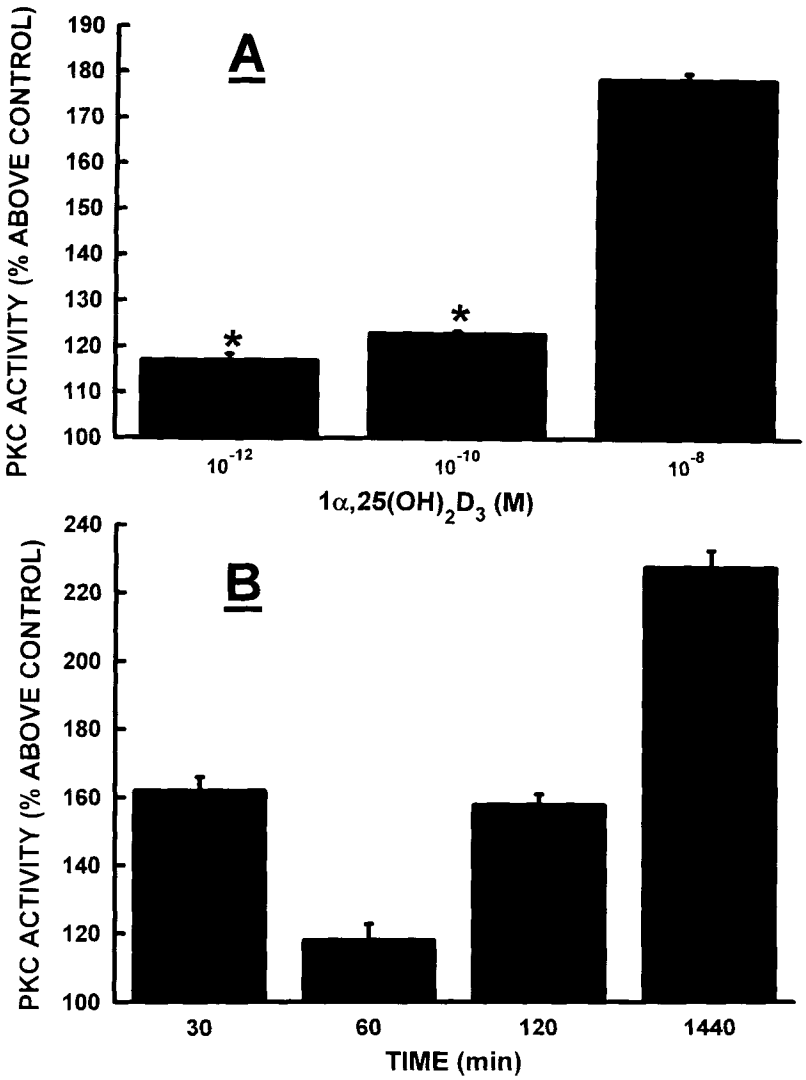

Fig. 1. **A:** Dose response of keratinocyte solubilized membrane PKC activity to $1\alpha,25(OH)_2D_3$. Data are expressed as % increase in PKC activity over the 24 h control. Values are mean ± SEM for triplicate determinations; $P < 0.001$ for all experimental values versus control. The * denote $P < 0.02$ between these values. **B:** Time course of human keratinocyte solubilized membrane PKC activity to 10^{-8} M $1\alpha,25(OH)_2D_3$. Data are expressed as % increase in PKC activity over individual control values at the designated time points. Values are means ± SEM for triplicate determinations; $P < 0.001$ for all experimental values versus control.

activity decreased in both control and $1\alpha,25(OH)_2D_3$-treated cells. In the control cells there was a 30% ± 2% decrease in endogenous PKC activity when keratinocytes were treated with 6 μM sphingosine for 24 h ($P < 0.001$). When keratinocytes were treated with 6 μM sphingosine and 10^{-12} M, 10^{-10} M, or 10^{-8} M $1\alpha,25(OH)_2D_3$, PKC activity decreased 38% ± 2%, 30% ± 2%, and 50% ± 1%, respectively, compared to cells treated with hormone alone (P values were <0.001). To assess the specificity of $1\alpha,25(OH)_2D_3$ on PKC activity, keratinocytes were treated with $1\beta,25(OH)_2D_3$ (10^{-8} M) for 24 h. $1\beta,25(OH)_2D_3$ had no effect on keratinocyte membrane activity (1,583 ± 13 cpm/μg protein in control cells and 1,610 ± 46 cpm/μg protein in treated cells; $P < 0.5$).

Figure 1B represents a time course for $1\alpha,25(OH)_2D_3$ at 10^{-8} M on PKC membrane activity. There was a biphasic response to $1\alpha,25(OH)_2D_3$ with 60% increased activity at 30 min followed by decreased activity at 60 min. The PKC activity again increased above control at 120 min and at 24 h there was a maximal inducement of membrane PKC activity that represented a 128% ± 5% increase over control. In

three separate experiments, PKC activity measured in membrane fractions of keratinocytes treated with $1\alpha,25(OH)_2D_3$ (10^{-8} M) for 24 h increased 93% ± 17% above 24 h control values. PKC activity at 48 h decreased from the 24 h control in both control and treated samples (data not shown).

PKC Activity in Normal Human Cultured *Fibroblasts*

PKC activity in the cytosolic fractions of normal human fibroblasts was increased in a dose-dependent manner when cells were treated with $1\alpha,25(OH)_2D_3$ for 24 h (Fig. 2A). PKC activity increased 25% ± 1%, 32% ± 2%, and 102% ± 3% as compared to the 24 h control ($P < 0.001$) when fibroblasts were incubated with 10^{-12} M, 10^{-10} M, and 10^{-8} M, respectively. As in the keratinocytes, maximal inducement (106% ± 3%) of PKC activity occurred when fibroblasts were treated with 10^{-8} M $1\alpha,25(OH)_2D_3$ for 24 h. When fibroblasts were incubated with 10^{-6} M $1\alpha,25(OH)_2D_3$, PKC activity in the cytosolic fraction decreased 13% ± 1% from the 24 h control ($P < 0.01$). In triplicate experiments, PKC activity in fibroblast solubilized membrane fractions was <15% of the activity in the cytosolic fractions of the same cells. For the experiment illustrated, 24 h control values were 5,212 ± 146 cpm/μg protein in the cytosol fraction as compared to 458 ± 8 cpm/μg protein in the solubilized membrane fraction. There were no significant differences (<5%) in the membrane PKC activity measured from fibroblasts treated with $1\alpha,25(OH)_2D_3$ versus those from control fibroblasts.

Fibroblasts were also treated with 6 μM sphingosine, in the absence and presence of $1\alpha,25(OH)_2D_3$. As was observed in keratinocytes, PKC activity was decreased in both control and experimental cells. Endogenous PKC activity decreased 23% ± 2 in sphingosine treated cells. Fibroblasts treated with $1\alpha,25(OH)_2D_3$ (10^{-12} M, 10^{-10} M, and 10^{-8} M) and sphingosine demonstrated 8% ± 2%, 13% ± 2%, and 23% ± 1% decreases, respectively, in $1\alpha,25(OH)_2D_3$ induced activity as compared to the 24 h control ($P < 0.001$). When fibroblasts were treated with $1\beta,25(OH)_2D_3$ there was no increase in PKC activity after 24 h treatment with 10^{-8} M (6,065 ± 56 cpm/μg protein in control cells and 6,015 ± 170 cpm/μg protein in treated cells; $P < 0.5$).

Figure 2B illustrates the time course for $1\alpha,25(OH)_2D_3$ (10^{-8} M) on PKC activity in fibroblast cytosolic fractions. Similar to the biphasic response seen in keratinocytes, there was a 48% increase in activity at 30 min followed by decreased activity at 60 min. Activity began to increase again at 120 min and maximal PKC activity (85% ± 5%) occurred after treatment with $1\alpha,25(OH)_2D_3$ for 24 h. In three separate experiments, PKC activity increased 95% ± 6% over control in the cytosolic fractions of fibroblasts treated with $1\alpha,25(OH)_2D_3$ (10^{-8} M) for 24 h. PKC activity in the control and in $1\alpha,25(OH)_2D_3$ treated cells at 48 hours decreased from 24 h control values (data not shown).

PKC Activity in Vitamin D Receptor Defective Human Cultured Fibroblasts

Human fibroblasts grown from a biopsy of a patient with vitamin D dependent rickets type II were treated with $1\alpha,25(OH)_2D_3$ (10^{-10} M, 10^{-8} M, or 10^{-6} M). Unlike the PKC activity changes seen in $1\alpha,25(OH)_2D_3$-treated normal keratinocytes and fibroblasts, there were no significant changes in PKC activity compared to control cells (Fig. 3). As a percent of control, cytosolic PKC activity was 95% ± 1%, 103% ± 4%, and 102% ± 1% in fibroblasts treated with 10^{-10} M, 10^{-8} M, and 10^{-6} M $1\alpha,25(OH)_2D_3$, respectively. The corresponding P values as compared to control were <0.4, <0.5, and <0.5. PKC activity levels in the solubilized membrane fractions were <15% of the activity in the cytosolic fractions of the same cells and $1\alpha,25(OH)_2D_3$ did not induce any significant changes in membrane fraction activity. Additionally, treatment of these cells for 24 h with $1\beta,25(OH)_2D_3$ (10^{-8} M) did not induce PKC activity (6,014 ± 140 cpm/μg protein in control cells and 6,245 ± 59 cpm/μg protein in treated cells; $P < 0.2$).

DISCUSSION

The data suggest that $1,25(OH)_2D_3$ stimulates PKC activity in normal keratinocytes and fibroblasts. PKC activity was induced in a dose dependent manner in keratinocyte solubilized membrane fractions and in fibroblast cytosolic fractions. PKC activity was maximally induced at 24 h after incubation with 10^{-8} M $1\alpha,25(OH)_2D_3$ in both cell types. However, treatment of these cells with 10^{-6} M $1\alpha,25(OH)_2D_3$ decreased PKC activity over a 24 h period. Prior viability studies in our laboratory established this concentration

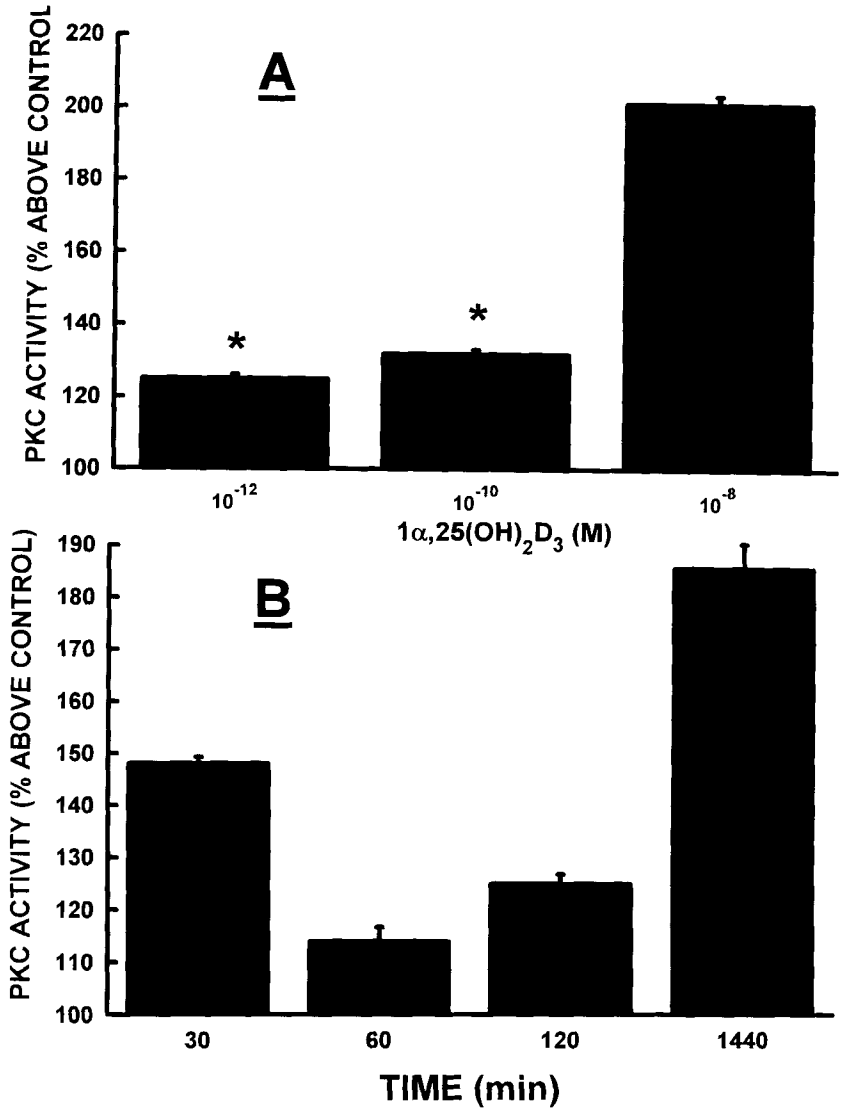

Fig. 2. **A:** Dose response of fibroblast cytosol PKC activity to $1\alpha,25(OH)_2D_3$. Data are expressed as % increase in PKC activity over the 24 h control. Values are mean ± SEM for triplicate determinations; $P < 0.001$ for all experimental values versus control. The * denote $P < 0.02$ between the data points. **B:** Time course of fibroblast cytosol PKC activity to $1\alpha,25(OH)_2D_3$. Data are expressed as % increase in PKC activity over individual control values at the designated time points. Values are mean ± SEM for triplicate determinations; $P < 0.001$ for all experimental values versus control.

is not toxic to the cells over this incubation period. Other investigators have found similar PKC activity increases in other cell types using physiological doses of $1\alpha,25(OH)_2D_3$. Ways et al. [1987] demonstrated that 10 nM $1\alpha,25(OH)_2D_3$ treatment of U937 human monoblastoid cells for 24 h increased PKC activity to 138% ± 7% and 187% ± 9% of vehicle treated cells in the cytosolic and solubilized particulate fractions, respectively. Martell et al. [1987] also reported that $1\alpha,25(OH)_2D_3$ (100 nM) treatment of HL-60 cells for 24 h increased PKC activity two-fold. Simboli-Campbell et al. [1994] found that $1\alpha,25(OH)_2D_3$ (100 nM) induced subcellular redistribution of both PKCα and PKCβ in renal epithelial cells. The increase in cytosolic rather than membrane PKC activity in fibroblasts suggests different localization patterns and perhaps different activation mechanisms for PKC activity in fibroblasts.

Sphingosine (6 μM) decreased PKC activity and when sphingosine was combined with $1\alpha,25(OH)_2D_3$, it inhibited the hormone's inducement of PKC activity. The extensive use of sphingosine in different cellular systems has proven that sphingosine inhibits most, if not all, PKC-

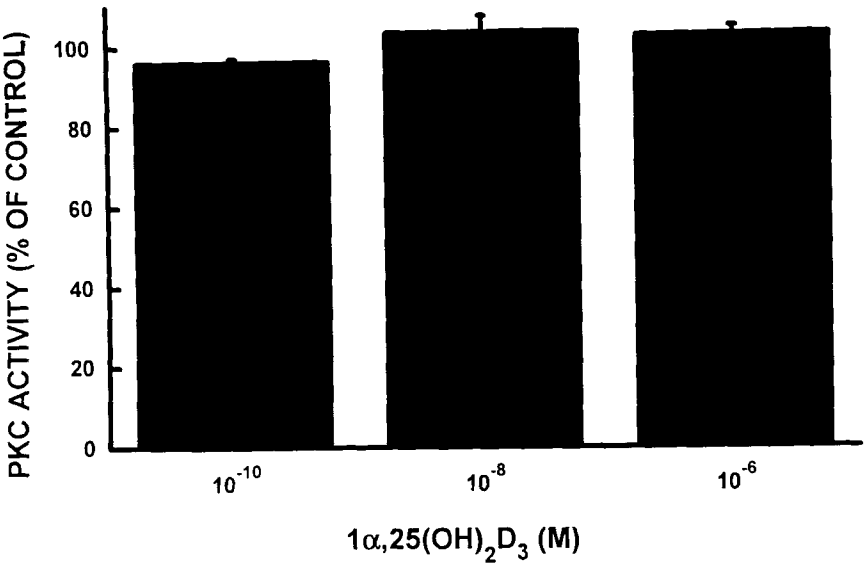

Fig. 3. Dose response of receptor defective fibroblast cytosol PKC activity to $1\alpha,25(OH)_2D_3$. Data are expressed as % of 24 h control PKC activity. Values are mean ± SEM for triplicate determinations; *P* values as compared to control were <0.4, <0.5, and <0.5, respectively.

mediated biological responses [Hannun et al., 1986; Hannun and Bell, 1989]. Additionally, sphingosine and other related long-chain bases inhibit differentiation in HL-60 cells [Simpson et al., 1989]. Interestingly, sphingosine inhibited $1\alpha,25(OH)_2D_3$ stimulation of PKC activity more in keratinocyte membrane fractions than in fibroblast cytosolic fractions. This response was expected since the fibroblast culture media contained fetal calf serum. The serum proteins most likely acted as a buffer to bind sphingosine, so as a result only a fraction of the sphingosine entered the cells and its effective cellular concentration was reduced. Others have suggested that an active $1\alpha,25(OH)_2D_3$ regulated sphingomyelin cycle exists in keratinocytes similar to that found in HL-60 cells [Okazaki et al., 1989] and that this can inhibit PKC activity. But even if this cycle exists, $1\alpha,25(OH)_2D_3$ has not been shown to increase sphingosine formation [Okazaki et al., 1990]. It is possible that in keratinocytes, as Kolesnick [1989] has demonstrated in HL-60 cells, that conversion of sphingomyelin to ceramide via sphingomyelinase could result in the generation of free sphingoid bases that could inactivate PKC. In either case, sphingosine's inhibitory effect on PKC activity in both keratinocytes and fibroblasts helps substantiate that PKC activity is an indicator of $1\alpha,25(OH)_2D_3$'s biological activity.

Additionally, when both keratinocytes and fibroblasts were treated with $1\beta,25(OH)_2D_3$, which is believed to be ineffective in inducing genomic responses [Norman et al., 1993], PKC activity levels in control and $1\alpha,25(OH)_2D_3$-treated cells were statistically the same. Moreover, treatment of VDR receptor negative fibroblasts with $1\alpha,25(OH)_2D_3$ did not induce any significant PKC activity changes in experimental versus control cells. These results suggest $1\alpha,25(OH)_2D_3$ acts through its receptor to induce PKC activity. As in HL-60 cells [Simpson et al., 1989], it may be that $1\alpha,25(OH)_2D_3$ can increase the amount of PKC by transcriptional regulation. Whatever the mechanism, the increase in PKC activity induced by $1\alpha,25(OH)_2D_3$ may facilitate the binding of ligands to specific hormone receptors and increase receptor phosphorylation. Hsieh et al. [1991] established that VDR is a substrate for PKCβ and demonstrated that ser-51 is a major phosphorylation site in hVDR *in vitro* and *in vivo*. $1\alpha,25(OH)_2D_3$ may regulate PKC transcription but in turn specific kinases may control VDR phosphorylation which is believed to be necessary for VDR to affect transcriptional control of target genes.

In summary, the data establish that PKC activity may be an indicator of $1\alpha,25(OH)_2D_3$'s biological activity in cultured human keratinocytes and fibroblasts. Further study is necessary to understand the mechanisms through which $1\alpha,25(OH)_2D_3$ exerts these longer term effects on PKC activity. Such studies should increase

our understanding of epidermal cell growth and differentiation.

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