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Promotion of HL-60 Cell Differentiation by 1,25-Dihydroxyvitamin D_3 Regulation of Protein Kinase C Levels and Activity

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ABSTRACT. The hormone 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] promotes differentiation of a number of cell types including HL-60 promyelocytic leukemia cells. It is now established that protein kinase Cβ (PKCβ) plays a critical role in HL-60 cell maturation to a monocyte/macrophage phenotype. In the present study, we investigated the importance of PKCβ levels and activation in 1,25-$(OH)_2D_3$-mediated differentiation of HL-60 cells. Cell differentiation promoted by 1,25-$(OH)_2D_3$ at 48 hr was 39 ± 3% (mean ± SEM) nitroblue tetrazolium (NBT) positive and at 72 hr it was 35 ± 2% NBT positive and 70% CD14 positive. Thus, promotion of cell differentiation by 20 nM 1,25-$(OH)_2D_3$ treatment was maximal at 48–72 hr. When PKCβ levels and cell differentiation were assayed at 72 hr, treatment with 20 nM 1,25-$(OH)_2D_3$ for the initial 6 hr increased PKCβ levels by 175% but had little effect on cell differentiation (7 ± 2% NBT positive; 11% CD14 positive). The effect of ionomycin, a calcium ionophore, on PKCβ levels and cell differentiation also was examined. Alone, 5 μM ionomycin promoted few cells (3% CD14 positive) to differentiate. In contrast, cells treated with 5 μM ionomycin for 66 hr after a 6-hr pretreatment with 20 nM 1,25-$(OH)_2D_3$ resulted in 34 ± 5% NBT positive cells and 73% CD14 positive cells. Quantitatively, this induction of differentiation was identical to that observed in cultures continuously treated with 1,25-$(OH)_2D_3$ (35 ± 2% NBT positive; 70% CD14 positive). Therefore, ionomycin seemed to replace the requirement for the continuous presence of 1,25-$(OH)_2D_3$. Chelerythrine chloride (3 μM), a specific PKC inhibitor, blocked differentiation promoted by 1,25-$(OH)_2D_3$ alone (82 ± 2% inhibition) or in sequence with ionomycin (86 ± 3% inhibition). Taken together, our data show that the capacity of 1,25-$(OH)_2D_3$ to both increase PKCβ levels and activate PKC is utilized to promote HL-60 cell differentiation. These data further suggest that 1,25-$(OH)_2D_3$ has a genomic action to increase PKCβ levels and also a nongenomic action requiring its continuous presence to promote HL-60 cell differentiation. BIOCHEM PHARMACOL **54**;8:909–915, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. 1,25-dihydroxyvitamin D_3; HL-60 promyelocytic cells; protein kinase C; cell differentiation; macrophages/monocytes; calcium

1,25-$(OH)_2D_3$[‖] regulates a variety of cell processes. In addition to its ability to regulate serum calcium and phosphate levels by actions on intestine, bone, and kidney [1], 1,25-$(OH)_2D_3$ affects the growth and differentiation of numerous cell types including keratinocytes [2], bone cells [3], leukemia cells [4, 5], and cardiac myocytes [6]. The HL-60 promyelocytic leukemia cell line has proven to be an excellent model for studying cell differentiation. 1,25-$(OH)_2D_3$ has been shown to induce HL-60 cells to differentiate along the monocytic/macrophage pathway [4]. Differentiation along the monocytic/macrophage pathway is usually assessed by measuring markers such as decreased DNA synthesis, decreased total transcription, modulation of cell surface antigens, reduction of NBT dye, and increases in nonspecific esterase (NSE) activity [7–11]. The appearance of these differentiation markers has been shown to require about 36–48 hr of continuous 1,25-$(OH)_2D_3$ treatment [7–11].

Previous studies have shown that 1,25-$(OH)_2D_3$ generates biological responses through two distinct mechanisms. Genomic pathways signal through the vitamin D receptor to modulate gene transcription [1, 12]. In addition to this pathway, increasing evidence exists to support a nongenomic mechanism of action. Thus, processes localized at the plasma membrane have also been demonstrated to be important for 1,25-$(OH)_2D_3$ actions. Studies have shown

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‖ *Abbreviations:* 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C; NBT, nitroblue tetrazolium; PMSF, phenylmethylsulfonyl fluoride; and DTT, dithiothreitol.

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that in several cell lines including osteoblast-like cells [13–15], keratinocytes [16], intestinal cells [17, 18], and HL-60 cells [19], 1,25-$(OH)_2D_3$ increases lipid turnover and intracellular calcium levels. These events, which would subsequently activate PKC and other enzymes, may be employed by 1,25-$(OH)_2D_3$ to regulate cellular processes including cell differentiation. There is also evidence that 1,25-$(OH)_2D_3$ can directly activate several PKC isozymes *in vitro*, suggesting that PKCs may be a receptor for this hormone [20].

Our group and others have shown that 1,25-$(OH)_2D_3$ increases PKC levels and activity [21–28]. Moreover, activation of PKC has been shown to be necessary for differentiation of HL-60 cells along the monocytic/macrophage pathway [21, 25, 26]. Recently, PKCβ has been suggested as the critical isozyme required for 1,25-$(OH)_2D_3$ induction of HL-60 cell differentiation [26–28]. However, the overall mechanism of action of 1,25-$(OH)_2D_3$ to induce HL-60 cell differentiation is not completely understood. In the present study we showed that 1,25-$(OH)_2D_3$ up-regulation of PKCβ levels by itself is insufficient to fully promote HL-60 cell differentiation. Thus, our data suggest that 1,25-$(OH)_2D_3$ has a genomic action to increase PKCβ levels and a nongenomic action to activate PKC in order to promote HL-60 cell differentiation.

MATERIALS AND METHODS

Chemicals

1,25-$(OH)_2D_3$ was purchased from the Du Phor Co. (Amsterdam, The Netherlands). The purity and structural integrity of vitamin D_3 metabolites were confirmed by high performance liquid chromatography and UV spectroscopy. Ionomycin was purchased from CalBiochem (La Jolla, CA). All other reagents were reagent grade or better.

Cell Culture

HL-60 promyelocytic leukemia cells were obtained from the American Type Culture Collection (Rockville, MD) and cultured in RPMI 1640 medium supplemented with 10% horse serum, 1000 U/mL penicillin G and 0.5 mg/mL streptomycin. Cells were incubated at 37° in a humidified atmosphere with 5% CO_2. Cells used in this study were from passages 14 to 40. Experiments were performed with cells in log phase growth at 2×10^5 cells/mL. Cells were allowed to equilibrate in growth medium for 24 hr prior to any treatment. Cell differentiation was assessed by the NBT dye reduction assay and CD14 surface marker expression.

NBT Dye Reduction Assay

Assessment of cell differentiation was measured by NBT dye reduction as previously described [25, 29]. Cells were washed two times with PBS and incubated for 30 min at 37° with an equal volume of NBT (1 mg/mL) and glucose (1 mg/mL) solution intracellular blue-black formazan deposits was determined by microscopic examination immediately after NBT incubation. Triplicate determinations of at least 50 cells were performed for each treatment condition.

CD14 Surface Antigen Expression

HL-60 cells were washed with PBS and incubated with fluorescein-labeled anti-CD14 antibody or isotype control and analyzed by fluorescence-activated cell sorting. Measurement of fluorescence was performed by the Flow Cytometer Laboratory at the University of Michigan with an Epics C cytometer (Coulter Electronics, Hialeah, FL). Data analysis of immunofluorescence was performed using Scat-Pak software.

Western Blot Analysis of PKCβ Protein Levels

Cells were washed twice with PBS, then were resuspended in lysis buffer (0.2 M Tris, 0.5 mM EGTA, 0.5 mM EDTA, 0.5% Triton × -100, 100 μM leupeptin, 0.4 mM PMSF, pH 7.5), and homogenized using a Dounce homogenizer. The protein content of the total cell homogenates was determined by the Bradford protein assay [30]. Equal amounts of protein from each condition were run on a 10% polyacrylamide gel, and the proteins were transferred subsequently to Immobilon paper (Millipore, Bedford, MA). The blot was blocked with buffer containing 1% bovine serum albumin (10 mM Tris, 0.1% Tween-20, and 1% bovine serum albumin, pH 7.4). The blot was probed for 2 hr with the primary antibody (PKCβ antibody, Life Technologies Inc., Gaithersburg, MD), then washed three times with blocking buffer, and incubated for 1.5 hr with a secondary antibody conjugated with horseradish peroxidase (Sigma Chemical Co., St. Louis, MO). The blot was then washed five times with Tween-TBS (10 mM Tris and 0.2% Tween-20, pH 7.4). Finally, the blot was developed using enhanced chemiluminescence (Amersham, Arlington Heights, IL) and exposed to X-ray film. PKCβ protein levels were quantified by densitometry (Beckman Appraise Scanning Fluorescence Densitometer, Beckman Instruments, Brea, CA).

PKC Activity Assay

PKC activity was determined using a commercially available kit (Amersham). Methods used were exactly as described in this kit. Briefly, HL-60 cells were homogenized in buffer containing 25 mM Tris (pH 7.4), 0.5 mM EDTA, 0.5 mM EGTA, 10 mM β-mercaptoethanol, 1 μg/mL leupeptin, 1 μg/mL aprotinin, and 50 μg/mL PMSF and incubated with calcium, lipid, peptide, DTT, and [^{32}P]ATP buffer for 15 min at 25°. Subsequently, termination buffer consisting of 7.5 M guanidine–HCl was added to stop the reaction. The terminated reaction mixture was pipetted onto binding paper and quantified by scintillation counting.

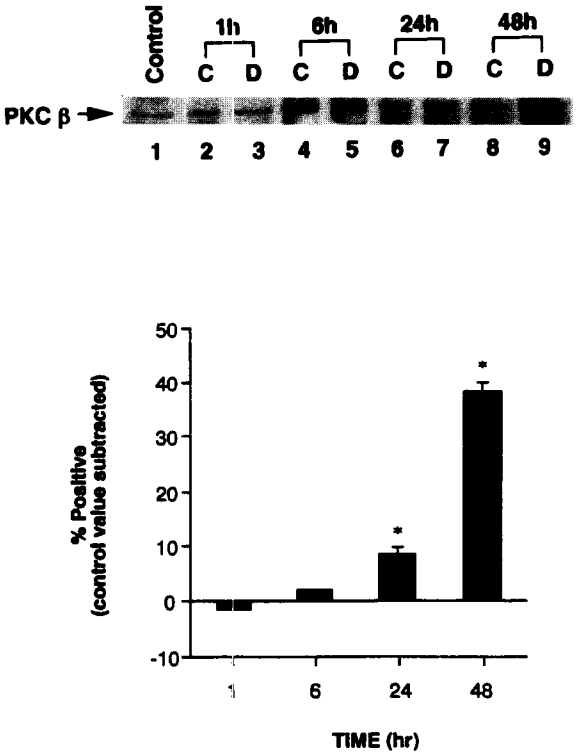

FIG. 1. (A) Effect of 1,25-$(OH)_2D_3$ on PKCβ protein levels in HL-60 cells. Cells were treated for 1 (lanes 2 and 3), 6 (lanes 4 and 5), 24 (lanes 6 and 7), and 48 (lanes 8 and 9) hr with control or 20 nM 1,25-$(OH)_2D_3$. Cells shown in lane 1 were not treated and were harvested on day 1. All other cells were harvested at each indicated time point, and PKCβ protein levels were visualized by western blot analysis. This panel is representative of three experiments. (B) Effect of 1,25-$(OH)_2D_3$ on HL-60 cell differentiation. Cells were treated for 1, 6, 24, or 48 hr with control or 20 nM 1,25-$(OH)_2D_3$. Cellular differentiation was assessed using the NBT reduction assay. Data are presented as a percentage of differentiated cells with the control value subtracted and represent the means ± SEM of three determinations. Key: (*) $P < 0.05$.

RESULTS

Effect of 1,25-$(OH)_2D_3$ on PKCβ Expression and Cell Differentiation

It has been shown by our laboratory and others that PKC plays a critical role in 1,25-$(OH)_2D_3$ differentiation of HL-60 cells [21–28]. We chose to use 20 nM 1,25-$(OH)_2D_3$ because it is in the middle of the concentration-response curve for promotion of cell differentiation. HL-60 cells were treated with vehicle or 20 nM 1,25-$(OH)_2D_3$ for various time periods (1, 6, 24, and 48 hr). 1,25-$(OH)_2D_3$ induced a time-dependent increase in PKCβ levels (Fig. 1A). Cell differentiation promoted by 1,25-$(OH)_2D_3$ at 48 hr (Fig. 1B) or 72 hr (Fig. 2B) of treatment was statistically similar (39 ± 3 vs 35 ± 2%). Therefore, promotion of cell differentiation by 20 nM 1,25-$(OH)_2D_3$ was maximal at 48–72 hr.

Effect of Ionomycin on PKCβ Activation and Cell Differentiation

Calcium ionophores, such as ionomycin, have been shown to increase intracellular calcium and phospholipid turnover in various cell lines [31]. Ionomycin (5 μM) increased intracellular calcium levels from resting levels of 80 ± 21 to 940 ± 80 nM (mean ± SEM, N = 3). To determine the effect of ionomycin on PKCβ levels, western blot analysis was performed as shown in Fig. 2A. Exposure of HL-60 cells to 5 μM ionomycin for 72 hr resulted in decreased PKCβ levels, suggesting agonist-induced down-regulation (lane 3). A time–course of calcium and phospholipid-dependent PKC activity was also determined in HL-60 cells treated with 5 μM ionomycin or 20 nM 1,25-$(OH)_2D_3$. Total PKC activity increased from basal to 1.3 pmol of ^{32}P incorporation/min/mg protein after 1 min of ionomycin treatment. Furthermore, PKC activity in ionomycin-treated cells was absent at time points after 1 hr, coincident with the down-regulation of PKCβ levels. In contrast, 1,25-$(OH)2_2D_3$ increased PKC activity much later, inducing increases of 3.4 and 4.4 pmol of ^{32}P incorporation/min/mg protein at 24 and 48 hr of hormone treatment, respectively. The time–course of 1,25-$(OH)_2D_3$ enhancement of PKC activity is in agreement with previous reports [22, 24, 32]. Taken together, our data suggest that ionomycin is activating PKC by increasing factors such as intracellular calcium.

The effect of ionomycin on cell differentiation was assessed by CD14 expression (Fig. 3) and NBT reduction (Fig. 4). Cell differentiation promoted by ionomycin (72 hr) ranged from 2 ± 1% for 1 μM to 8 ± 3% for 5 μM as measured by NBT reduction. The effect of ionomycin on NBT reduction occurred immediately (within 15 min), suggesting that ionomycin has a small direct effect on this assay. Treatment with 5 μM ionomycin for 72 hr resulted in only 3% CD14 positive cells (Fig. 3). Importantly, a direct effect of ionomycin on CD14 expression was not observed. Ionomycin was toxic to cells at concentrations greater than 5 μM. To determine if there is a synergistic interaction between 1,25-$(OH)_2D_3$ and ionomycin in the promotion of cell differentiation, HL-60 cells were treated continuously with 1,25-$(OH)_2D_3$ and ionomycin for 72 hr (Fig. 4). Alone, 2 nM 1,25-$(OH)_2D_3$ promoted 19 ± 2% of the cells to differentiate. When cells were treated with both 1 or 2.5 μM ionomycin and 2 nM 1,25-$(OH)_2D_3$ the number of NBT positive cells decreased to 13 ± 1 and 15 ± 2%, respectively. This lack of synergy between 1,25-$(OH)_2D_3$ and ionomycin in the promotion of cell differentiation was also observed with 20 and 200 nM 1,25-$(OH)_2D_3$ (Fig. 4). Cells continuously treated with both 1,25-$(OH)_2D_3$ (2, 20, or 200 nM) and 5 μM ionomycin were not viable after 72 hr.

Effect of Ionomycin on 1,25-$(OH)_2D_3$-Pretreated Cells

In a different protocol, cells were treated with 1,25-$(OH)_2D_3$ for 6 hr and then transferred to fresh medium in

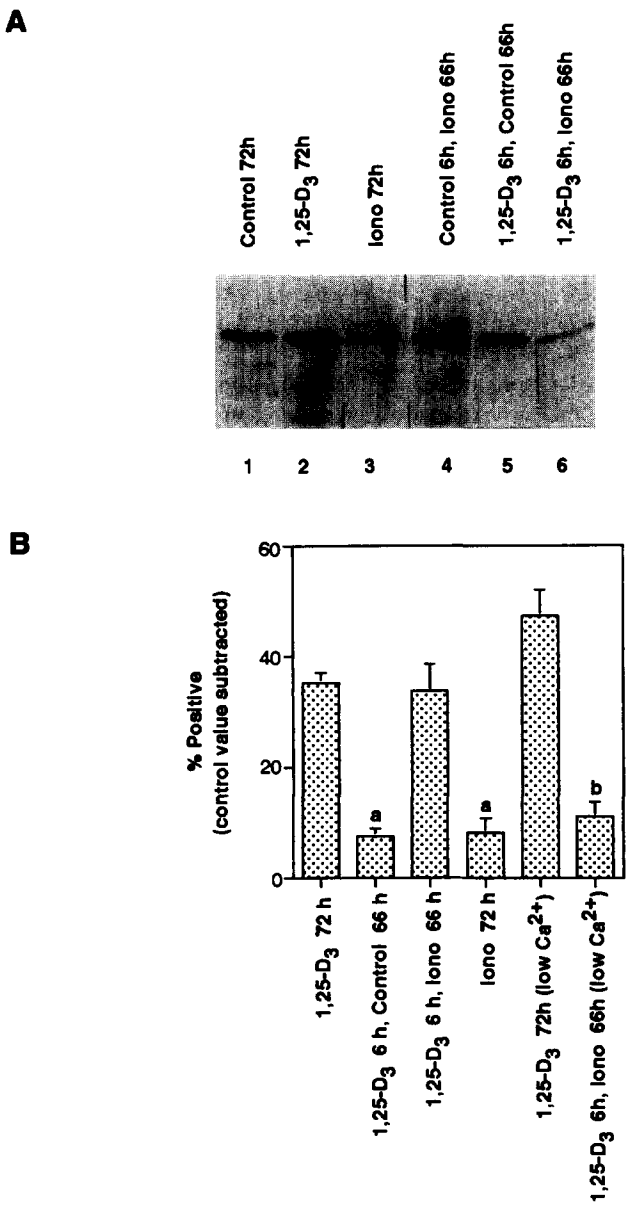

FIG. 2. (A) Effect of ionomycin on PKCβ protein levels in HL-60 cells. Cells were treated with control for 72 hr (lane 1), 20 nM 1,25-$(OH)_2D_3$ for 72 hr (lane 2), 5 μM ionomycin for 72 hr (lane 3), control for 6 hr followed by control for 66 hr (lane 5), or 20 nM 1,25-$(OH)_2D_3$ for 6 hr followed by 5 μM ionomycin for 66 hr (lane 6). Cells were harvested, and PKCβ protein levels were visualized by western blot analysis. This panel is representative of three experiments. (B) Cell differentiation promoted by ionomycin on 1,25-$(OH)_2D_3$-pretreated HL-60 cells. Cells were treated with 20 nM 1,25-$(OH)_2D_3$ for 72 hr (bar 1), 20 nM 1,25-$(OH)_2D_3$ for 6 hr followed by control for 66 hr (bar 2), 20 nM 1,25-$(OH)_2D_3$ for 6 hr followed by 5 μM ionomycin for 66 hr (bar 3), or 5 μM ionomycin for 72 hr (bar 4). Additionally, cells were grown and treated in low calcium medium with 20 nM 1,25-$(OH)_2D_3$ for 72 hr (bar 5), or 20 nM 1,25-$(OH)_2D_3$ for 6 hr followed by 5 μM ionomycin for 66 hr (bar 6). Cellular differentiation was assessed using NBT reduction assay. Data are presented as a percentage of differentiated cells with the control value subtracted and represent the means ± SEM of three determinations. Key: (a) $P < 0.05$ compared with 1,25-$(OH)_2D_3$ 72 hr treatment group (bar 1); and (b) $P < 0.05$ compared with 1,25-$(OH)_2D_3$ 6 hr, ionomycin 66 hr treatment group (bar 3).

the absence of 1,25-$(OH)_2D_3$ for the remainder of the 72-hr protocol (Fig. 2). With this protocol, PKCβ levels were increased by 175% of control (Fig. 2A, lane 5). However, promotion of cell differentiation was only 7 ± 2% as assessed by NBT reduction (Fig. 2B, bar 2) and 11% as assessed by CD14 expression. These results suggest that even though 1,25-$(OH)_2D_3$ can increase PKCβ levels by a relatively short treatment period (6 hr), this action by itself is not sufficient to fully promote cell differentiation. Therefore, we determined if activation of increased PKCβ levels by 6 hr of 1,25-$(OH)_2D_3$ pretreatment was sufficient to promote differentiation to the extent observed with continuous hormone treatment. Ionomycin was used to activate the increased levels of PKCβ in 1,25-$(OH)_2D_3$-pretreated cells. As shown in Fig. 2A, ionomycin was able to down-regulate PKCβ levels elevated by 6 hr of 1,25-$(OH)_2D_3$ pretreatment (lane 5 vs lane 6). Interestingly, when cells were exposed to ionomycin for 66 hr following a 6-hr pretreatment with 1,25-$(OH)_2D_3$, the extent of cell differentiation was comparable to that in cells treated continuously for 72 hr with 1,25-$(OH)_2D_3$ (34 ± 5 vs 35 ± 2% NBT positive; 73 vs 70% CD14 positive). This is consistent with the suggestion that PKCβ levels present in cells after 6 hr of 1,25-$(OH)_2D_3$ treatment are sufficient to promote cell differentiation to the extent noted with continuous hormone treatment. The ability of ionomycin to promote differentiation in cells pretreated with 1,25-$(OH)_2D_3$ was blocked (86 ± 3% inhibition) in the presence of chelerythrine chloride (3 μM), a specific PKC inhibitor. Chelerythrine chloride (3 μM) also significantly inhibited differentiation (82 ± 2% inhibition) promoted by 72 hr of 20 nM 1,25-$(OH)_2D_3$ treatment. Multiple concentrations (5 μM every 12 hr) of 1,2-dioctanoylpropanediol (deoxy-diC_8), a membrane permeable diacylglycerol, acted like ionomycin to promote differentiation only in HL-60 cells pretreated with 1,25-$(OH)_2D_3$ (20 nM) for 6 hr (data not shown). To determine that differentiation promoted by ionomycin on 1,25-$(OH)_2D_3$- pretreated HL-60 cells was through a calcium-dependent mechanism, cell were grown and treated in low calcium (0.04 μM) medium (Fig. 2B). In low calcium medium, differentiation promoted by ionomycin on 1,25-$(OH)_2D_3$-pretreated cells significantly decreased to 13 ± 3% NBT positive (Fig. 2B, bar 6). In contrast, 1,25-$(OH)_2D_3$ was still able to promote cell differentiation in low calcium medium. Moreover, the extent of cell differentiation promoted by 1,25-$(OH)_2D_3$ was greater when HL-60 cells were grown in low calcium than in normal calcium medium (0.36 μM) (Fig. 2, bar 5 vs bar 1).

DISCUSSION

The importance of PKC in 1,25-$(OH)_2D_3$ promotion of HL-60 cell differentiation is now generally accepted. 1,25-$(OH)_2D_3$ has been demonstrated to induce an increase in PKC levels [21–24]. Additionally, it has been shown that classical inhibitors of PKC, H7 and sphinganine, blocked

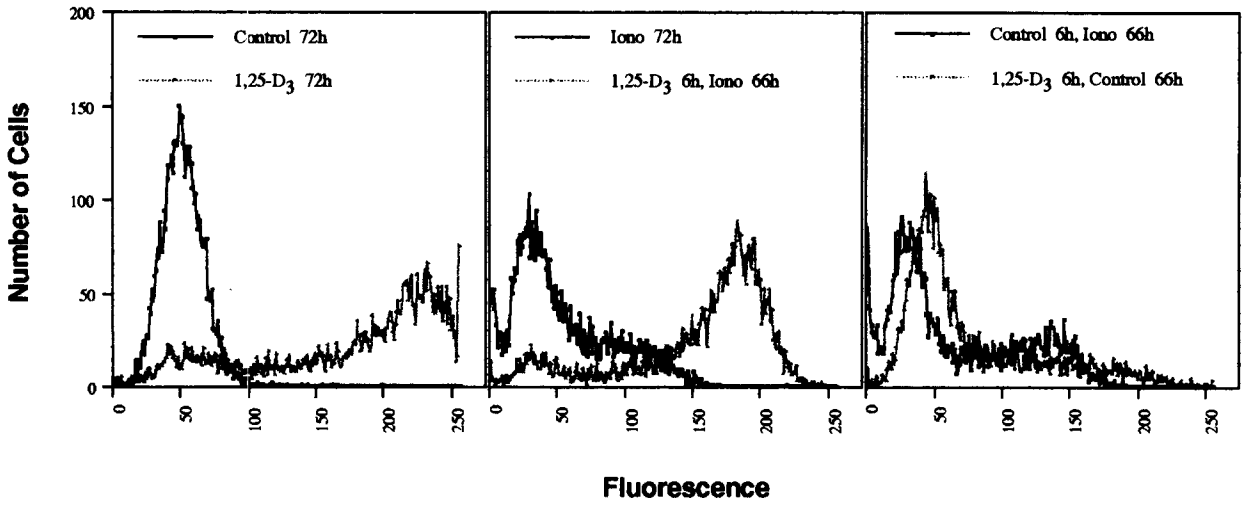

FIG. 3. Cell differentiation promoted by ionomycin on 1,25-$(OH)_2D_3$-pretreated HL-60 cells as assessed by CD14 surface antigen expression. Cells were treated with the same protocol as described in Fig. 2 and incubated with fluorescein-labeled anti-CD14 antibody. Data were analyzed by flow cytometry and presented as histograms. This figure is representative of three experiments. % CD14 positive cells with control value subtracted: 70% 1,25-$(OH)_2D_3$ 72 hr, 3% Iono 72 hr, 78% 1,25-$(OH)_2D_3$ 6 hr, Iono 66 hr, 5% Control 6 hr, Iono 66 hr, and 11% 1,25-D_3 6 hr, Control 66 hr.

1,25-$(OH)_2D_3$ promotion of cell differentiation [21, 25]. Furthermore, a PKCβ antisense construct partially inhibited 1,25-$(OH)_2D_3$ promotion of HL-60 cell differentiation as measured by NBT dye reduction [28]. Also, a phorbol ester-tolerant (PET) HL-60 cell line, lacking basal PKCβ expression, could not be induced to differentiate with phorbol esters [26]. Interestingly, these PET cells differentiated normally in response to phorbol esters after they were first induced to express PKCβ by 1,25-$(OH)_2D_3$ [26]. These studies provide evidence that PKCβ is the critical isozyme required for monocytic/macrophage differentiation of HL-60 cells.

This present study showed that continuous treatment of HL-60 cells with 1,25-$(OH)_2D_3$ increases PKCβ levels and promotes cell differentiation (Fig. 1). In addition, 6 hr of 1,25-$(OH)_2D_3$ treatment followed by hormone removal for 66 hr resulted in increased PKCβ levels (Fig. 2A). However, with this shorter hormone treatment protocol, promotion of cell differentiation was diminished greatly (by 79% as assessed by NBT reduction; by 84% as assessed by CD14 expression) relative to continuous hormone treatment (Figs. 2B and 3). Thus, the genomic action of 1,25-$(OH)_2D_3$ to increase PKCβ levels is not in itself sufficient to promote HL-60 cell differentiation to the levels noted with continuous hormone treatment. Others have shown that overexpression (4–5 times HL-60 cells) of $PKC\beta_I$ or $PKC\beta_{II}$ in HL-525 cells, an HL-60 variant deficient in PKCβ, did not promote differentiation to mature monocytes/macrophages [27]. However, these $PKC\beta_I/PKC\beta_{II}$ overexpressing clones differentiate into mature monocytes/macrophages in response to phorbol esters [27]. Therefore, 1,25-$(OH)_2D_3$ may also provide additional factors to activate PKCβ, and this action would explain the need for continuous hormone treatment.

In addition to increasing PKCβ levels, 1,25-$(OH)_2D_3$ has been shown to have nongenomic actions [17, 18, 31,

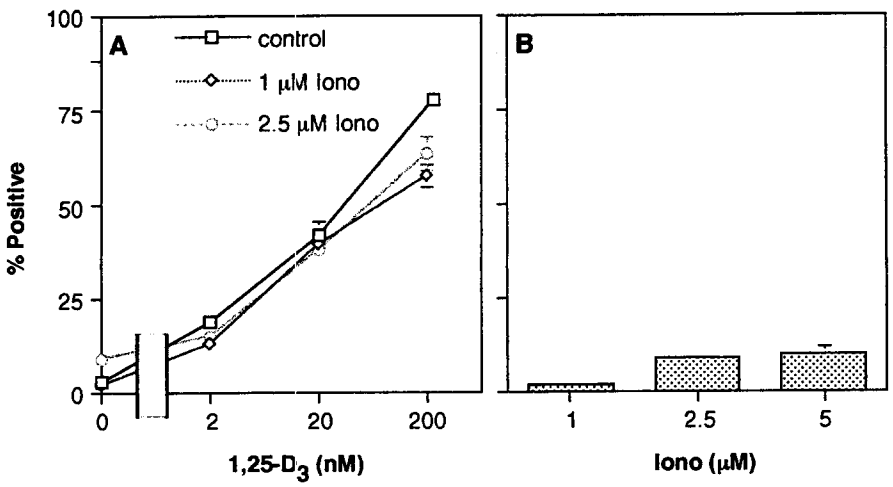

FIG. 4. (A) Cell differentiation promoted by 1,25-$(OH)_2D_3$ and ionomycin. Cells were treated with 2, 20, or 200 nM 1,25-$(OH)_2D_3$ alone or in combination with ionomycin (1 or 2.5 μM) for 72 hr. (B) Cell differentiation promoted by ionomycin. Cells were treated with 1, 2.5, or 5 μM ionomycin for 72 hr. Cellular differentiation was assessed using NBT reduction assay. Data are presented as a percentage of differentiated cells and represent the means ± SEM of three determinations.

33]. Calcium spikes induced by 1,25-$(OH)_2D_3$ have been linked to classical calcium-dependent processes [31]. Furthermore, phospholipid turnover and rapid increases in intracellular calcium following 1,25-$(OH)_2D_3$ treatment have been reported [17, 18]. Recently, 1,25-$(OH)_2D_3$ has been shown to increase phospholipid turnover in keratinocytes by increasing phospholipase C (PLC) levels [33]. These studies suggest that the breakdown of phosphatidylinositol 4,5-bisphosphate (PIP_2) to inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) by PLC may be the mechanism used by 1,25-$(OH)_2D_3$ to increase phospholipid turnover and thereby activate PKC. Alternatively, others have reported that 1,25-$(OH)_2D_3$ increases ceramide by enhancing sphingomyelin turnover [34].

In this study, a 1,25-$(OH)_2D_3$ pretreatment period of 6 hr was chosen for two reasons. First, PKCβ levels are increased significantly and, second, PKC activity is not enhanced at this time point. Several studies have reported that 1,25-$(OH)_2D_3$ requires 24 hr to significantly enhance calcium- and phospholipid-dependent PKC activity [22, 24, 32]. Ionomycin was used to increase intracellular calcium and, secondarily, phospholipid turnover. Ionomycin stimulated PKC activity and down-regulated PKCβ levels (Fig. 2A). However, ionomycin had little effect on cell differentiation in unstimulated HL-60 cells. This observation is consistent with the suggestion that threshold cellular levels of activated PKCβ are required for promotion of HL-60 cell differentiation. Differentiation promoted by ionomycin in 1,25-$(OH)_2D_3$-pretreated cells (6 hr) was identical to differentiation promoted by continuous 1,25-$(OH)_2D_3$ treatment (Figs. 2B and 3). Our data suggest that in 1,25-$(OH)_2D_3$-pretreated HL-60 cells, ionomycin promoted differentiation because these cells have significantly more PKCβ available for activation. This premise is further supported by the observation that chelerythrine chloride (3 μM), a selective PKC inhibitor, blocked differentiation (86 ± 3% inhibition) promoted by ionomycin in cells pretreated for 6 hr with 1,25-$(OH)_2D_3$. Similarly, chelerythrine chloride blocked differentiation (82 ± 2% inhibition) of HL-60 cells continuously treated with 1,25-$(OH)_2D_3$ alone. These results show that there is enough PKCβ present, if activated by ionomycin, in these cells after 6 hr of 1,25-$(OH)_2D_3$ treatment to promote cell differentiation to the extent noted with continuous hormone treatment. However, a prolonged period of 1,25-$(OH)_2D_3$ exposure is required (> 24 hr) before it significantly activates PKC and fully promotes cell differentiation. In low calcium medium, 1,25-$(OH)_2D_3$ is still able to promote cell differentiation. A possible explanation for this is that 1,25-$(OH)_2D_3$, unlike ionomycin, is increasing intracellular calcium levels by liberating calcium stored at the endoplasmic reticulum. In support of this hypothesis, Kim *et al.* [35] reported that the nongenomic actions of 1,25-$(OH)_2D_3$ to increase intracellular calcium and phospholipid turnover may be due to the association between 1,25-$(OH)_2D_3$-occupied vitamin D receptor (VDR) and a specific acceptor site at the endoplasmic reticulum. Another possibility is that 1,25-$(OH)_2D_3$ may be directly activating PKCs in a ligand-receptor manner as suggested by Slater *et al.* [20], who showed direct activation of PKCs by 1,25-$(OH)_2D_3$ *in vitro*. Alternatively, 1,25-$(OH)_2D_3$ may be activating PKCs through a membrane-bound VDR [36]. Our data do not exclude any of these possibilities but argue that if these processes are involved in differentiation and our experimental paradigm then they require chronic treatment with 1,25-$(OH)_2D_3$ and can be mimicked by increasing intracellular calcium levels, by increasing DAG, and by activating PKC.

In summary, our findings demonstrated that the 1,25-$(OH)_2D_3$-induced increase in PKCβ levels is not, in itself, sufficient to fully promote HL-60 cell differentiation. This information along with data showing that PKC inhibitors block 1,25-$(OH)_2D_3$ promotion of differentiation leads us to propose that chronic activation of PKC is the process that requires the continuous presence of this hormone to promote HL-60 cell differentiation.

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