Vitamin D₃ treatment of the human promyelocytic cell line, HL-60, is accompanied by an increase in phorbol ester receptor number (Martell, R. E., Simpson, R. U., and Taylor, J. M. (1987) J. Biol. Chem. 262, 5570-5575). In this study, the mechanism and significance of vitamin D₃-induced changes in protein kinase C levels were investigated. Treatment of HL-60 cells with 1,25-dihydroxyvitamin D₃ (1,25-(OH)₂D₃) resulted in a 2-3-fold increase in phorbol dibutyrate binding at 24 h. This was accompanied by a 4.2-fold increase in steady state mRNA levels for the β isoenzyme of protein kinase C by a 3.8-fold increase in its transcriptional rate as determined from nuclear run-off studies. Protein kinase C α mRNA, which was approximately 15% of the β isoenzyme levels, showed similar increases in mRNA and transcriptional rates in response to 1,25-(OH)₂D₃. Protein kinase C γ mRNA was not detected. The increases in protein kinase C levels were accompanied by increases in activation of the enzyme by phorbol esters. More importantly, 1,25-(OH)₂D₃ caused a 1.5-2.0-fold increase in the endogenous phosphorylation of protein kinase C substrates independent of exogenous activators or endogenous formation of diacylglycerol. This is the first report of transcriptional activation of protein kinase C. This mechanism of up-regulation of protein kinase C may explain the increased activity of protein kinase C in vitamin D₃-treated HL-60 cells, and may constitute an important mechanism for the long term modulation of the protein kinase C pathway of cell regulation independent of diacylglycerol generation.

EXPERIMENTAL PROCEDURES

Materials

RPMI 1640 and fetal bovine serum were purchased from GIBCO. [³²P]Phosphate, [α-³²P]dCTP, and [α-³²P]UTP, from Du Pont-New England Nuclear. HL-60 cells were obtained from Dr. James Niedel (Department of Medicine, Duke University). 1,25-Dihydroxyvitamin D₃ was kindly provided by Dr. M. Uskokovich (Hoffmann-LaRoche, Nutley, NJ). PMA, PDBu, insulin/transferrin, and salmon sperm DNA were from Sigma. Human protein kinase C α, β, and γ cDNA probes were from American Type Culture Collection. An additional 550-base pair genomic probe
was used which corresponds to the first coding exon of protein kinase C β and flanking 5′ untranslated and 3′ intervening sequences. This genomic probe was cloned from a human leukocyte genomic EMBL3 library (Clontech) using protein kinase C β CDNA as a probe at high stringency. Escherichia coli DAG kinase was a kind gift of Drs. Carson Loomis and Robert Bell (Department of Biochemistry, Duke University).

**Methods**

**Cell Culture** Human promyelocytic leukemia HL-60 cells were grown in plastic tissue culture flasks at 37 °C and 5% CO2 in RPMI 1640 supplemented with 10% fetal bovine serum (14). Cells were grown at a density of 1.0 × 106/ml.

**Phorbol Binding to HL-60 Cells**—For phorbol binding studies, cells were suspended in serum-free media at 1 × 106 cells/ml before treatment with 1,25-(OH)2D3. Phorbol binding to intact HL-60 cells was performed as described (12, 15).

**Northern Blot Analysis**—Cells were collected at the appropriate time points and washed three times with cold phosphate-buffered saline. Total cellular RNA was collected by the method of Chirgwin et al. (16). RNA was denatured and fractionated by electrophoresis on 1% agarose gels containing formaldehyde as described (17). RNA was then transferred to a nitrocellulose membrane and baked at 80 °C in a vacuum oven for 2 h. The membrane was prehybridized for 16 h at 42 °C in 50% formamide, 2 X SSC, 50 mM NaPO4, 0.1% SDS, 10 × Denhardt’s solution, and 400 µg/ml sheared salmon sperm DNA. Hybridization to human α, β, and γ protein kinase C Isozymes and chicken β-actin CDNA probes was done in the same solution at the same temperature (18) using 2 × 106 counts/ml of probe labeled by nick translation with [α-32P]dCTP to a specific activity of 6 × 106 cpm/µg DNA as recommended by the manufacturer (Bethesda Research Laboratories). Following hybridization for 16 h, the filters were washed twice with 0.2 × SSC and 0.1% SDS at room temperature for 20 min and then twice at 65 °C for 1 h. The filters were then exposed to Kodak X-AR films at −70 °C using intensifying screens for the indicated time intervals. Autoradiographic bands were quantitated by densitometry using an LKB laser scanning densitometer. RNA band sizes were estimated by comparison with an RNA ladder (Bethesda Research Laboratories).

**Nuclear Run-off Assays**—HL-60 cells (at 1 × 106 cells/ml) were treated with 100 nM 1,25-(OH)2D3 and cells were collected at appropriate time points. Nuclei were extracted by the method of Bitter and Roeder (19), resuspended in 40% glycerol, 20 mM Hepes, pH 7.6, and 2 mM MgCl2, and stored frozen at −70 °C. Transcription reactions were carried out as described (14). Sodium hydroxide-denatured plasmid (8 µg of DNA) containing β-actin, protein kinase C α, β, and γ, or control plasmid were slot blotted on nitrocellulose using a Schleicher and Schuell slot blotter. The filters were baked and prehybridized as described for the Northern blots. Labeled RNA from the transcription reactions was extracted three times with phenol/chloroform, precipitated in ethanol, and reprecipitated in 100% tri-chloroacetic acid. The labeled RNA was then hybridized to the denatured plasmids in the same solutions as the prehybridization. Hybridization was carried out at 42 °C for 4 days (20) using 2 × 106 cpm/ml of fluid. The filters were washed twice at room temperature, twice at 65 °C in 0.2 × SSC and 0.2% SDS, and then washed in 10 µg/ml RNase at 37 °C for 30 min. The filters were then exposed to Kodak X-AR film for 3–7 days.

**Phorbol Ester-induced Phosphorylations in Intact Cells**—These studies were performed essentially as described (21). Briefly, HL-60 cells were harvested, washed with phosphate-free RPMI 1640, and resuspended in the same media at 1 × 106 cells/ml for 30 min. [32P]were then added to the cells at 0.1 µCi/ml for 2 h at 37 °C. The cells were then centrifuged at 1500 × g, unincorporated label removed, and cells resuspended in fresh phosphate-free RPMI 1640 at a concentration of 3.3 × 106 cells/ml. PMA (100 nM) or vehicle was then added to 150-µl aliquots of cells at a concentration of 106 cells/ml. Incubations were continued for 1 h at 37 °C after which the reaction was terminated by adding one-third volume of a 10% SDS stop buffer (10% SDS, bromphenol blue, and 2-mercaptoethanol). The samples were boiled for 5 min and then loaded on a 12.5% SDS-polyacrylamide gel (33). The gels were stained with Coomassie Blue, destained, fixed, and exposed to Kodak XAR-5 film for 3 h at −70 °C. Proteins whose phosphorylation was prominently increased by PMA treatment were excised and counted in 10 ml of scintillation fluid in an LKB β-scintillation counter.

**Phosphorylation of Endogenous Protein Kinase C Substrates in Response to 1,25-(OH)2D3 Treatment**—To study the effects of 1,25-(OH)2D3 on endogenous protein kinase C substrates, cells were treated with either 1,25-(OH)2D3 or vehicle. The cells were then harvested at indicated time points, labeled with [32P] as described above for 2 h, reactions terminated with SDS stop buffer, and samples loaded on SDS-polyacrylamide gel electrophoresis. Three protein kinase C substrates identified from PMA-induced phosphorylation studies (namely proteins of Mr, of 17,000, 27,000, and 62,000) were excised, and incorporated radioactivity was counted in 10 ml of scintillation fluid.

**DAG Quantitation**—DAG mass was quantitated using E. coli DAG kinase as described (22). Briefly, lipids were extracted by the method of Bligh and Dyer (23), dried down under N2, and resuspended in 100 ml of chloroform. 40 µl were used to measure phospholipid phosphate (24), and 40 µl were used to quantitate DAG mass.

**Transcriptional Regulation of Protein Kinase C**

![Graph](http://www.jbc.org/.../fig1.html)

**Fig. 1.** Effects of vitamin D3 on [3H]PDBu binding in HL-60 cells. A, dose-response of [3H]PDBu binding to vitamin D3 treatment. Cells were treated with variable concentrations of 1,25-(OH)2D3 for 18 h, rinsed twice with phosphate-buffered saline, and harvested in serum-free medium. Each data point represents 5 × 106 cells. B, time course of induction of [3H]PDBu binding by 1,25-(OH)2D3. HL-60 cells were treated with 100 nM 1,25-(OH)2D3 for the indicated time periods. Each point is the mean ± S.D. of two determinations. Data shown is for specific binding after subtracting nonspecific binding from total binding. The data are representative of three different experiments.
**RESULTS**

1,25-(OH)_{2}D_{3} Induces an Increase in Phorbol Dibutyrate Binding—As shown in Fig. 1A, phorbol dibutyrate-specific binding increased in response to 1,25-(OH)_{2}D_{3} with a maximal response achieved at 100-500 nM 1,25-(OH)_{2}D_{3}. These results are in agreement with those of Martell et al. (12) and correspond to concentrations of 1,25-(OH)_{2}D_{3} that induce maximal differentiation of HL-60 cells (25). 100 nM 1,25-(OH)_{2}D_{3} was, therefore, used for all subsequent experiments. We next studied the time course of a 1,25-(OH)_{2}D_{3}-induced increase in phorbol ester binding to intact HL-60 cells. 1,25-(OH)_{2}D_{3} caused a progressive increase in phorbol dibutyrate binding (Fig. 1B). The increase was detected as early as 4-8 h after stimulation with 100 nM 1,25-(OH)_{2}D_{3} and peaked at 24 h, well before the onset of the differentiated phenotype in response to 1,25-(OH)_{2}D_{3}, which occurs over 2-6 days following treatment of cells with the hormone. These results suggest that a predominant effect of 1,25-(OH)_{2}D_{3} on the DAG/protein kinase C pathway may be the up-regulation of protein kinase C.

Effects of 1,25-(OH)_{2}D_{3} on Steady State mRNA of Protein Kinase C Isoenzymes—To investigate the mechanism of the increase in phorbol ester binding and receptor number, we next examined the levels of protein kinase C mRNA in 1,25-(OH)_{2}D_{3}-treated HL-60 cells using Northern blot analysis. 1,25-(OH)_{2}D_{3} treatment resulted in a progressive increase in mRNA for protein kinase C β isoenzyme (Fig. 2A). A similar increase in protein kinase C α message was detected although the level of expression of protein kinase C α mRNA was approximately 15% that of the β isoenzyme (data not shown).

No message for protein kinase C γ isoenzyme was detected in resting or 1,25-(OH)_{2}D_{3}-stimulated HL-60 cells, consistent with the brain specificity of this isoenzyme (7). When the increase in protein kinase C β mRNA was quantitated by densitometry and expressed relative to actin message (26), there was a 4-5-fold increase in β mRNA at 24-48 h (Fig. 2B). This increase in steady state mRNA appears sufficient to account for the observed increase in phorbol ester receptor number.

1,25-(OH)_{2}D_{3} Induces Protein Kinase C β Gene Transcription—Since increases in mRNA levels could result from either induction of gene expression or from post-transcriptional effects, we next examined the rate of transcription of protein kinase C α, β, and γ genes using nuclear run-off assays. Nuclei from control and 1,25-(OH)_{2}D_{3}-treated cells were isolated, transcription reactions continued, and RNA hybridized to plasmids bearing α, β, and γ protein kinase C inserts in addition to β-actin and control plasmids. Control plasmid showed no hybridization to nuclear RNA, and, as previously shown (26), there was little variation in the transcriptional rate of the β-actin gene with 1,25-(OH)_{2}D_{3} (Fig. 3A). Increases in transcriptional rates for β protein kinase C were detected as early as 4 h and peaked at 12 h. When quantitated by densitometry and corrected for β-actin, 1,25-(OH)_{2}D_{3} caused a 3.8-fold increase in protein kinase C β transcriptional rates at 12 h (Fig. 3B). Similarly, transcription of protein kinase C α increased with vitamin D_{3} treatment. As with Northern blots, there was no detectable signal with protein kinase C γ.

**Fig. 3.** Induction of protein kinase C (PKC) transcription by 1,25-(OH)_{2}D_{3}. HL-60 cells were treated with 100 nM 1,25-(OH)_{2}D_{3}. Nuclei were collected and transcription rates were determined by nuclear run-off studies as described under “Experimental Procedures.” A, representative autoradiogram of nuclear run-off analysis of protein kinase C β, control PBR plasmid, and β-actin. B, autoradiograms were quantitated by densitometric scanning and transcriptional rates of protein kinase C β were corrected for β-actin and expressed relative to control untreated cells.

*Untreated cells showed no detectable signal with protein kinase C γ.*

*Since other protein kinase C isoenzymes have not been described in human tissue, these were not evaluated in this study. While possible transcriptional activation of these isoenzymes may occur in response to 1,25-(OH)_{2}D_{3}, this would not alter the conclusions of this study.*
Transcriptional Regulation of Protein Kinase C

with 1,25-(OH)$_2$D$_3$ show a time-dependent enhancement of PMA-induced phosphorylation of these substrates (Fig. 4). These studies show that the increase in protein kinase C levels are accompanied by an increase in the activation of this enzyme by exogenous activators and are consistent with previous results showing an increase in Ca$^{2+}$- and phosphatidylinositol-dependent phosphorylation in 1,25-(OH)$_2$D$_3$-treated HL-60 cells (11). They also resulted in the identification of protein substrates of protein kinase C that were used to monitor the endogenous level of activation of the enzyme in response to 1,25-(OH)$_2$D$_3$ (see below).

The demonstrations of increases in protein kinase C levels and activation by exogenous activators (PMA) suggest, but do not prove, that 1,25-(OH)$_2$D$_3$ causes an increase in the intrinsic activity of protein kinase C. The effects of 1,25-(OH)$_2$D$_3$ on the intrinsic phosphorylation of endogenous substrates of protein kinase C were investigated. 1,25-(OH)$_2$D$_3$ treatment of HL-60 cells resulted in a time-dependent increase in phosphorylation of protein kinase C substrates (pp17, pp27, pp62) in the absence of added activators of the enzyme (Fig. 5A). This 1.5–2.0-fold enhancement of substrate

![Substrate Phosphorylation](image)

**Fig. 4.** Effects of 1,25-(OH)$_2$D$_3$ on PMA-induced phosphorylation in intact HL-60 cells. HL-60 cells were treated with 100 nM 1,25-(OH)$_2$D$_3$ and harvested at the indicated time points. Endogenous protein phosphorylations in response to 100 nM PMA or vehicle (dimethyl sulfoxide) were determined as described under "Experimental Procedures" (autoradiogram). Three proteins of Mr values of 17,000, 27,000, and 62,000 were prominently phosphorylated in response to PMA. (Similar proteins were loaded on the different lanes as judged by Coomassie Blue staining, data not shown.) Phosphorylation in response to vehicle was subtracted from PMA-induced phosphorylation, and the results are plotted as fold increase of PMA-induced phosphorylation/base-line untreated cells. Asterisks indicate statistically significant increases as judged by one sided t tests. Results represent duplicate measurements and are representative of two independent experiments.

Therefore, the increase in mRNA levels appears to be a result of increases in transcription rates, predominantly of protein kinase C $\beta$, with a minor contribution by protein kinase C $\alpha$.

**Effects of 1,25-(OH)$_2$D$_3$ on the DAG/Protein Kinase C Pathway**—Since transcriptional activation of protein kinase C by 1,25-(OH)$_2$D$_3$ results in increased levels of protein kinase C (measured as phorbol ester receptors), we next investigated the effects of 1,25-(OH)$_2$D$_3$ on protein kinase C activity. The ability of 1,25-(OH)$_2$D$_3$ to induce protein kinase C was assayed by examining the effects of PMA on the phosphorylation of endogenous protein kinase C substrates. Treatment of HL-60 cells with PMA results in the phosphorylation of a number of proteins. The most prominent were of Mr values of 17,000, 27,000, and 62,000 (Ref. 27 and Fig. 4). HL-60 cells pretreated

![DAG Levels](image)

**Fig. 5.** Effects of 1,25-(OH)$_2$D$_3$ on the DAG/protein kinase C pathway. A, effects of 1,25-(OH)$_2$D$_3$ on endogenous phosphorylation of protein kinase C substrates. HL-60 cells were treated with 100 nM 1,25-(OH)$_2$D$_3$ or with vehicle (ethanol). At the indicated time points, cells were harvested and endogenous protein phosphorylation was assessed as described under "Experimental Procedures." The endogenous phosphorylation of the three protein kinase C substrates (pp17, pp27, and pp62) that were identified from PMA-induced phosphorylations were then quantitated. The increase in phosphorylation of these substrates was plotted relative to cells treated with vehicle. Asterisks indicate statistically significant increases as judged by one sided t tests. B, effects of 1,25-(OH)$_2$D$_3$ on DAG levels. HL-60 cells were treated with either 100 nM 1,25-(OH)$_2$D$_3$ or vehicle (ethanol). DAG levels were measured using E. coli DAG kinase. DAG levels were corrected for phospholipid phosphate and are plotted as changes relative to cells treated with vehicle. Results represent averages of triplicate measurements and are representative of two independent experiments.

The previous identification of pp17 and pp27 as protein kinase C substrates in HL-60 cells (27) allowed us to study the phosphorylation of these substrates on one-dimensional SDS-polyacrylamide gel electrophoresis. 
phosphorylation occurred in the absence of increases in DAG levels (Fig. 5B). These results suggest that 1,25-(OH)_{2}D_{3} causes activation of the protein kinase C pathway, not through activation of the phospholipid cycle, but by induction of protein kinase C isoenzymes.

**Discussion**

An important pathway of cell regulation involves the activation of protein kinase C which causes the phosphorylation of a number of protein substrates resulting in short term (e.g. platelet activation) and long term cellular responses (e.g. cell differentiation). Cell agonists that operate through protein kinase C-dependent pathways activate the enzyme by causing the generation of DAG second messengers from the hydrolysis of phosphatidylinositol bisphosphate and other membrane phospholipids (29). DAG then causes transient activation of protein kinase C.

In this report we show that 1,25-(OH)_{2}D_{3} induces the transcription of the gene for protein kinase C beta causing an increase in steady state mRNA levels. A similar, but lesser effect is observed with protein kinase C alpha. The increase in mRNA appears sufficient to explain the observed elevation in phorbol ester binding, phorbol ester receptor number, and protein kinase C activity. This results in an increase in protein kinase C activity as judged by the increase in phosphorylation of endogenous protein kinase C substrates in response to 1,25-(OH)_{2}D_{3}.

Transcriptional activation of the protein kinase C beta gene by 1,25-(OH)_{2}D_{3} may, therefore, constitute a significant mechanism for regulating the DAG/protein kinase C pathway independent of phospholipid hydrolysis and diacylglycerol generation. This mechanism may operate in mediating long term effects of protein kinase C such as cell differentiation and tumor promotion. Transcriptional regulation of protein kinase C may be particularly important in the transduction of effects of hormones that lack cell surface receptors and that may not be coupled to phospholipid turnover. According to this hypothesis, base-line DAG levels (Fig. 5B) may be sufficient to increase the activity of protein kinase C if the levels of the enzyme increase. In HL-60 cells, the increase in transcription of protein kinase C results in increased phorbol ester binding and protein kinase C activity. The modest 2-4-fold increase in protein kinase C levels and activity causes significant increases in the phosphorylation of protein kinase C substrates. These changes may be sufficient for transducing protein kinase C-dependent effects in the absence of changes in DAG levels. This mechanism may be important for mediating the differentiation effects of 1,25-(OH)_{2}D_{3} as activation of protein kinase C by phorbol esters results in similar differentiation of HL-60 cells.

This hypothesis is further supported by studies showing that a 1.5-2.0-fold increase in protein kinase C levels and activity induced by transfection of fibroblasts with cDNA for protein kinase C alpha results in cell transformation and tumor formation in nude mice (30). These studies, therefore, suggest that modest changes in protein kinase C levels may alter cell growth and differentiation.

Further studies are required to determine the precise role of protein kinase C in HL-60 differentiation and the role of transcriptional regulation of protein kinase C as a mechanism for transducing long term cellular activities.

These studies also raise questions as to the mechanism of transcriptional activation of protein kinase C by 1,25-(OH)_{2}D_{3}. 1,25-(OH)_{2}D_{3} belongs to the steroid family of hormones that share similar mechanisms of action (31, 32). Steroid hormones bind to high affinity intracellular receptors (32). These receptors share similar structural organization with distinct ligand and DNA-binding domains. Ligand-bound receptors interact with specific enhancer/suppressor DNA elements resulting in modulation of gene expression (31). 1,25-(OH)_{2}D_{3} modulates the expression of a number of proteins. Only a few of these, however, have been studied at a transcriptional level and little is known about the molecular mechanisms of gene regulation by 1,25-(OH)_{2}D_{3}. Recently, 1,25-(OH)_{2}D_{3} responsive elements have been defined for the osteocalcin gene (34). Our results demonstrate that protein kinase C beta is an additional gene subject to up-regulation by 1,25-(OH)_{2}D_{3}. Preliminary studies show that cycloheximide fails to block the effects of 1,25-(OH)_{2}D_{3} on protein kinase C transcription, raising the possibility that protein kinase C gene(s) harbor vitamin D3 responsive elements. Current experiments are directed at determining the responsible cis-DNA regions in the gene for protein kinase C beta.

Acknowledgment—We thank Dr. Elizabeth Weiss for helpful discussions.

REFERENCES

Transcriptional regulation of protein kinase C by 1,25-dihydroxyvitamin D3 in HL-60 cells.

L M Obeid, T Okazaki, L A Karolak and Y A Hannun