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Synergistic Enhancement by 12-*O*-Tetradecanoylphorbol-13-acetate and Dibutyryl cAMP of 1α,25-Dihydroxyvitamin D_3 Action in Human Promyelocytic Leukemic HL-60 Cells

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Abstract. We have reported that dibutyryl cAMP (dbcAMP), an activator of cAMP-dependent protein kinase (PKA), potentiated the effects of 1α,25-dihydroxyvitamin D_3(1,25-$(OH)_2D_3$)-induced 24-hydroxylation activity in HL-60 cells by increasing 1,25-$(OH)_2D_3$ receptor (VDR). The present study demonstrated that 12-*O*-tetradecanoylphorbol-13-acetate (TPA), a potent phorbol ester, also potentiated the effect of 1,25-$(OH)_2D_3$ on HL-60 cells and that TPA and dbcAMP acted in a synergistic manner to enhance the effect of 1,25-$(OH)_2D_3$. It is interesting that TPA induced 24-hydroxylation activity far more efficiently than dbcAMP, in addition to their effects in increasing VDR. TPA increased basal levels of *c-fos* mRNA to the maximum by 1 h after the treatment, whereas dbcAMP failed to affect *c-fos* gene expression. Together with the previous data indicating the presence of AP-1-like sequence in the promoter of 24-hydroxylase gene, it was suggested that TPA potentiated the effect of 1,25-$(OH)_2D_3$ through an activation of *c-fos* gene expression. This notion was further supported by the data showing that TPA and dbcAMP also acted in a synergistic manner to activate *c-fos* gene expression. Neither TPA nor dbcAMP affected *c-jun* early response gene in the HL-60 clone used in the present study.

The present study suggested that the activation of early *c-fos* response gene by TPA might be another mechanism to enhance the effect of 1,25-$(OH)_2D_3$, besides up-regulation of VDR.

Key Words: 1α,25-Dihydroxyvitamin D_3, 12-*O*-Tetradecanoylphorbol-13-acetate, Dibutyryl cAMP, HL-60 cells

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1α,25-DIHYDROXYVITAMIN D_3 (1,25-$(OH)_2D_3$) induces human promyelocytic leukemic HL-60 cells to differentiate into monocytes/macrophages [1–4] *via* 1,25-$(OH)_2D_3$ receptor (VDR) [5]. 1,25-$(OH)_2D_3$ has its specific effect in HL-60 cells, such as increasing VDR [6] and induction of 24-hydroxylase (24-OH-ase) [7]. 1,25-$(OH)_2D_3$ induces 24-OH-ase via VDR in *cis*-regulated fashion, because two vitamin D-responsive elements (VDRE) have been identified in the 5′-flanking region of the human 24-OH-ase gene [8]. Activator protein-1 (AP-1)-like sequence was also found in the upstream of 24-OH-ase gene [8].

We have shown that dibutyryl cyclic adenosine 3′:5′-monophosphate (dbcAMP), an activator of cAMP-dependent protein kinase (PKA), potentiated the effect of 1,25-$(OH)_2D_3$ to induce 24-OH-ase activity in HL-60 cells in part by increasing VDR [9].

12-*O*-Tetradecanoylphorbol-13-acetate (TPA), a potent phorbol ester to activate protein kinase C (PKC), affects VDR content in various cells [10–12]. We have shown recently that TPA potentiated 1,25-$(OH)_2D_3$-induced 24-OH-ase gene expression in rat intestinal epithelial IEC-6 cells but without causing an increasing in VDR [13].

AP-1, a heterodimer consisting of *fos* and *jun* families, modifies 1,25-$(OH)_2D_3$ action by binding to the specific AP-1-like sequence just upstream of or in

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the VDRE [14]. TPA is known to stimulate binding of AP-1 to DNA [15].

This background prompted us to examine how PKC and PKA systems interact with each other to modify 1,25-$(OH)_2D_3$-induced 24-OH-ase activity in HL-60 cells, particularly focusing on their effects on VDR content and on gene expression of *c-fos* and *c-jun*.

Materials and Methods

Materials

An HL-60 clone was provided by Dr. Hector F. DeLuca (Department of Biochemistry, University of Wisconsin-Madison). 1,25-$(OH)_2$[26,27-^{3}H]D_3 was purchased from Dupont/New England Nuclear (Boston, MA). Nonradioactive 1,25-$(OH)_2D_3$ was a gift from Chugai Pharmaceutical Co. (Tokyo, Japan). Diisopropylfluorophosphate (DFP), dbcAMP and TPA were purchased from Sigma (St Louis, MO), and sodium periodate from Aldrich (Milwaukee, WI). Buffers used were as follows: phosphate-buffered saline (PBS), 15 mM KH_2PO_4 (pH 8.0), 8.1 mM Na_2HPO_4 (pH 8.0), 137 mM NaCl, 2.7 mM KCl; $TEDK_{300}$, 50 mM Tris-HCl (pH 7.4), 1.5 mM EDTA, 5 mM DTT, 300 mM KCl; SSC, 0.15 M NaCl, 0.015 M Na citrate (pH 7.4).

Cells and cell culture

HL-60 cells were cultured essentially as described [3, 4]. Briefly, cells were cultured in RPMI 1640 medium (Flow Laboratories, Irvine, Scotland) with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY) in a humidified air at 37°C. Under these conditions, the doubling time was less than 48 h. Cell proliferation was determined with a hemocytometer and cell viability by trypan blue exclusion.

Preparation of HL-60 cell extract

HL-60 cells were processed essentially as described [12]. All operations were performed at 0–4°C unless otherwise indicated. HL-60 cells were washed three times with Ca^{2+}- and Mg^{2+}-free PBS. Cell pellets were suspended in $TEDK_{300}$ buffer with 5 mM DFP and sonicated in three 20-s cycles interrupted by 60-s pauses. The sonicate was centrifuged at 105,000 g for 60 min to yield the receptor preparation. The receptor preparation was frozen in liquid nitrogen and stored at −80°C until used. The protein concentration was determined by the method of Bradford [16] with bovine serum albumin (BSA) as the standard.

Measurement of VDR contents

VDR content was determined by saturation analysis as described previously [5]. Briefly, aliquots (180 μl) of HL-60 cell extract were incubated with varying concentrations (0.01–1.0 nM) of 1,25-$(OH)_2$[26,27-^{3}H]D_3. Nonspecific binding was measured in samples in which a 100-fold excess of nonradioactive 1,25-$(OH)_2D_3$ was added. The mixture was incubated at 4°C for 4 h.

Bound 1,25-$(OH)_2$[26,27-^{3}H]D_3 was determined by the hydroxyapatite assay [17]. Scatchard analysis [18] was performed to determine the equilibrium dissociation constant (Kd) and the number of binding sites (Nmax).

Measurement of the ability of HL-60 cells to hydroxylate 1,25-$(OH)_2$[26,27-^{3}H]D_3 to form 1,24,25-$(OH)_3$[26,27-^{3}H]D_3

The ability of HL-60 cells to convert 1,25-$(OH)_2$[26,27-^{3}H]D_3 to 1,24,25-$(OH)_3$[26,27-^{3}H]D_3 was determined as described [19] by periodate-based assay [7, 20]. Briefly, HL-60 cells (2.0×10^6 cells/ml) were treated with 10^{-7} M 1,25-$(OH)_2D_3$ with and without 10^{-7} M dbcAMP and/or 1.0 ng/ml TPA for 24 h. Then the cells were subjected to a 'washout' procedure to remove added 1,25-$(OH)_2D_3$ [21]. This consisted of replacing the culture medium containing 1,25-$(OH)_2D_3$ with RPMI 1640 containing 2% FBS but without 1,25-$(OH)_2D_3$ at 30 min intervals twice before cell harvest. Then HL-60 cells were washed twice with PBS and resuspended in RPMI 1640 medium containing 2% FBS at 1.0×10^7 cells/ml. Aliquots (200 μl) were incubated with 10 pmol of 1,25-$(OH)_2$[26,27-^{3}H]D_3 (sp. act. 6,000 dpm/pmol) at 37°C for 3 h. The reaction was stopped by the addition of 0.3 ml of 0.1 N acetic acid. The amounts of 1,24,25-$(OH)_3$[26,27-^{3}H]D_3 synthesized were measured by the periodate-based assay [20], which is validated by direct comparison with the well-estab-

lished HPLC assay [7, 21]. After the addition of 0.5 ml of saturated sodium periodate, the mixture was incubated on ice for 30 min. By incubation with periodate, the [^{3}H]acetone was generated by periodate cleavage at the bond between C-24 and C-25 of 1,25-(OH)$_2$[26,27-^{3}H]D$_3$. The samples were filtered through a 1 ml (C-18) solid-phase extraction cartridge (Supelco, Bellefonte, PA) followed by washing twice with 0.4 ml of water. The pooled filtrate, in which [^{3}H]acetone was collected, was counted for its radioactivity.

Isolation and hybridization of RNA

Total RNA was isolated from HL-60 cells by acid guanidinium thiocyanate-phenol chloroform extraction [22]. Twenty μg of total RNA was electrophoresed on a 1% agarose gel containing formaldehyde and transferred to a nylon filter (Hybond N, Amersham International plc., Buckinghamshire, UK). The nylon filter was prehybridyzed at 37°C for 16 h in a buffer containing 50% formaldehyde, 3 × SSC, 50 mM Tris-HCl (pH 7.5), 0.1% SDS, 20 μg/ml tRNA, 20 μg/ml boiled salmon sperm DNA, 1 mM EDTA and 1 × Denhardt solution, and then hybridized with [^{32}P]-labeled 24-OH-ase, *c-fos* and *c-jun* cDNA probe in the same buffer at 37°C for 40 h [23]. The density of each band was semiquantitated by a laser densitometer (Pharmacia LKB 2222 Ultrascan XL, Uppsala, Sweden) and the data were expressed in arbitrary units (AU).

Statistical analysis

The results were expressed as the mean ± SD. Significant differences between groups were determined by Student's t test.

Results

Effect of TPA or dbcAMP on the induction by 1,25-(OH)$_2$D$_3$ of the ability of HL-60 cells to hydroxylate C-24 of 1,25-(OH)$_2$[26,27-^{3}H]D$_3$

1,25-(OH)$_2$D$_3$ and TPA induce HL-60 cells to differentiate into monocytes/macrophages [24, 25], and dbcAMP into granulocytes [26]. Among these three differentiation inducers, only 1,25-(OH)$_2$D$_3$ specifically induced HL-60 cells to hydroxylate C-24 of 1,25-(OH)$_2$[26,27-^{3}H]D$_3$, but TPA and dbcAMP enhanced the effect of 1,25-(OH)$_2$D$_3$ on the induction of 1,25-(OH)$_2$D$_3$-24-hydroxylation activity by 14.6-fold and by 1.8-fold, respectively. Furthermore, TPA acted synergistically with dbcAMP in the enhancement of this effect of 1,25-(OH)$_2$D$_3$ by approximately 20-fold (Fig. 1). These data strongly suggest that PKC and PKA systems each may have a distinct pathway to enhance the effect of 1,25-(OH)$_2$D$_3$ in HL-60 cells.

To deny the positive regulation of 24-OH-ase activity by covalent modifications through cAMP- and TPA-mediated pathway, the direct effect of dbcAMP or TPA on 1,25-(OH)$_2$D$_3$-24-hydroxylation activity was measured by adding dbcAMP and TPA to the assay mixture containing the HL-60 cells which had been incubated for 24 h with 1,25-(OH)$_2$D$_3$. The results indicated that neither reagent affected 24-hydroxylation activity appreciably (data not shown).

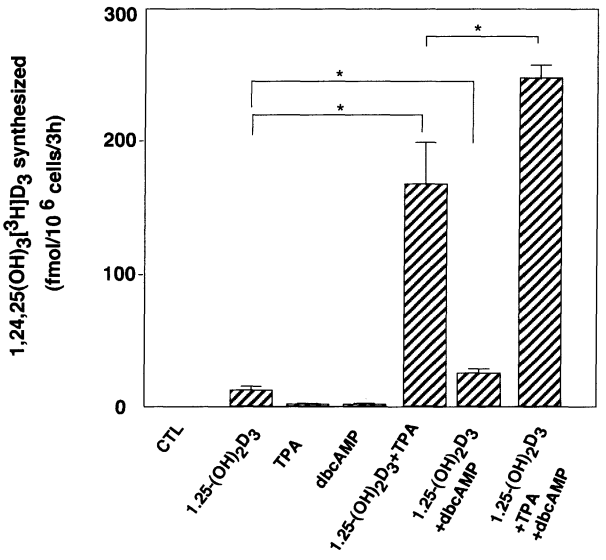

Fig. 1. Effect of TPA and/or dbcAMP on 1,25-(OH)$_2$D$_3$-induced HL-60 cell ability to hydroxylate C-24 of 1,25-(OH)$_2$[26,27-^{3}H]D$_3$ (24 h). HL-60 cells were incubated with 10^{-7} M 1,25-(OH)$_2$D$_3$ in the presence or absence of 10^{-7} M dbcAMP and/or 1.0 ng/ml TPA for 24 h. The cells' ability to hydroxylate 1,25-(OH)$_2$[26,27-^{3}H]D$_3$ to form 1,24,25-(OH)$_3$[26,27-^{3}H]D$_3$ was determined after 3 h of incubation with 1,25-(OH)$_2$[26,27-^{3}H]D$_3$ in the periodate-based assay as described in Materials and Methods. (*) Significant difference ($P < 0.05$). 1,24,25-(OH)$_3$[26,27-^{3}H]D$_3$ was not detected without 1,25-(OH)$_2$D$_3$.

Effect of TPA and dbcAMP on gene expressions of 24-OH-ase in HL-60 cells

We quantified the 24-OH-ase mRNAs.Synergism between TPA and dbcAMP effects was also observed at the message level, which seems relevant to the increase in 24-hydroxylase activities (Fig. 2).

Effect of TPA and dbcAMP on VDR abundance in HL-60 cells

We next studied the mechanisms by which TPA and dbcAMP enhanced the effect of 1,25-$(OH)_2D_3$. To determine the VDR abundance, 1,25-$(OH)_2D_3$ was excluded from the medium. The 1,25-$(OH)_2D_3$-induced increase in VDR was explained in most part by a posttranslational mechanism resulting from the stabilization of the VDR molecule by 1,25-$(OH)_2D_3$-binding [5], whereas the TPA and dbcAMP-induced increase in VDR was explained by a transcriptional mechanism [27, 9]. First, the effects of two reagents on VDR abundance in HL-60 cells were examined, since the magnitude of 1,25-$(OH)_2D_3$ action correlated well with intracellular VDR content [28]. As shown in Fig. 3, TPA and dbcAMP caused a significant heterologous increase in VDR by 1.7-fold and 2.3-fold, respectively. The two reagents also acted synergistically to increase VDR in HL-60 cells by 6.4-fold.

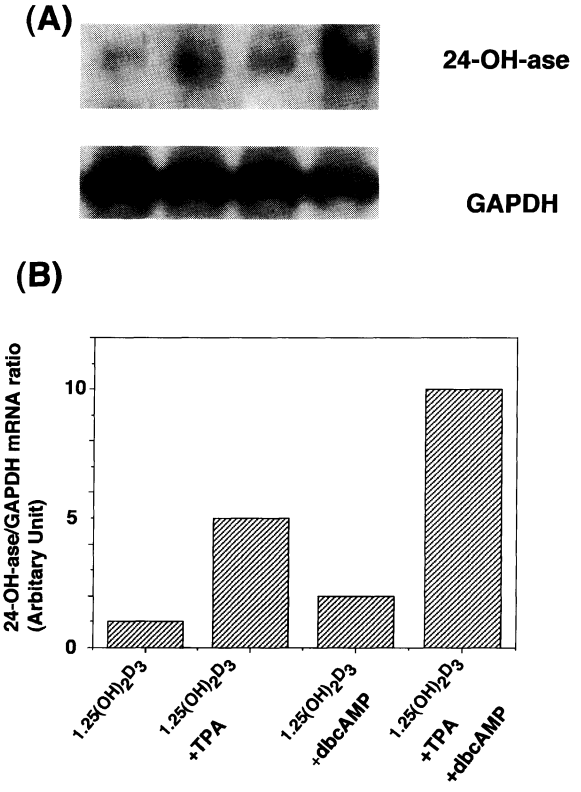

Fig. 2. Effect of TPA and/or dbcAMP on 24-OH-ase gene expression(A) and its densitometric analysis (B) in HL-60 cells. HL-60 cells were cultured for 3 h with 10^{-7} M 1,25-$(OH)_2D_3$ in the presence or absence of 1.0 ng/ml TPA and/or 10^{-7} M dbcAMP. 20 μg of total RNA was fractionated on a 1% agarose gel, transferred to a nylon filter and then hybridized to a labeled 24-OH-ase or GAPDH cDNA probe. Results of densitometric analysis are shown in (B). Results are expressed as the ratio of the control value designated as 1.

Effect of TPA or dbcAMP on gene expressions of c-fos and c-jun in HL-60 cells

We next examined whether potentiation by TPA and/or dbcAMP of 1,25-$(OH)_2D_3$ actions on HL-60 cells might be mediated by *c-fos* and/or *c-jun* expressions. As shown in Fig. 4, Northern blot study shows that TPA had significantly increased the stationary level of *c-fos* mRNA by approximately 1.9-fold at 1 h after the addition, but dbcAMP alone did

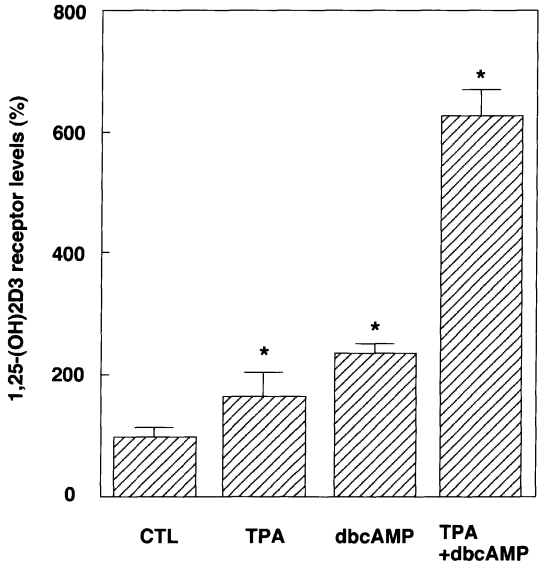

Fig. 3. Effect of TPA and/or dbcAMP on VDR in HL-60 cells. After HL-60 cells were incubated with and without 1.0 ng/ml TPA and/or 10^{-7} M dbcAMP for 24 h, VDR content were determined by ligand-binding assay as described in *Methods*. (*) Significant difference ($P<0.05$) vs control cells.

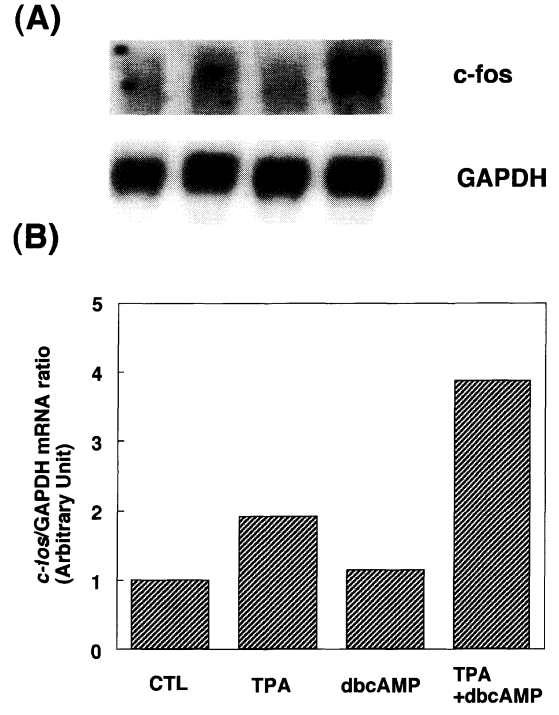

Fig. 4. Effect of TPA and/or dbcAMP on *c-fos* mRNA expression (A) and its densitometric analysis (B). HL-60 cells were cultured for 1 h with or without 1.0 ng/ml TPA and/or 10^{-7} M dbcAMP. 20 μg of total RNA was fractionated on a 1% agarose gel, transferred to a nylon filter and then hybridized to a labeled *c-fos* or GAPDH cDNA probe. Results of densitometric analysis are shown in (B). Results are expressed as the ratio of the control value designated as 1.

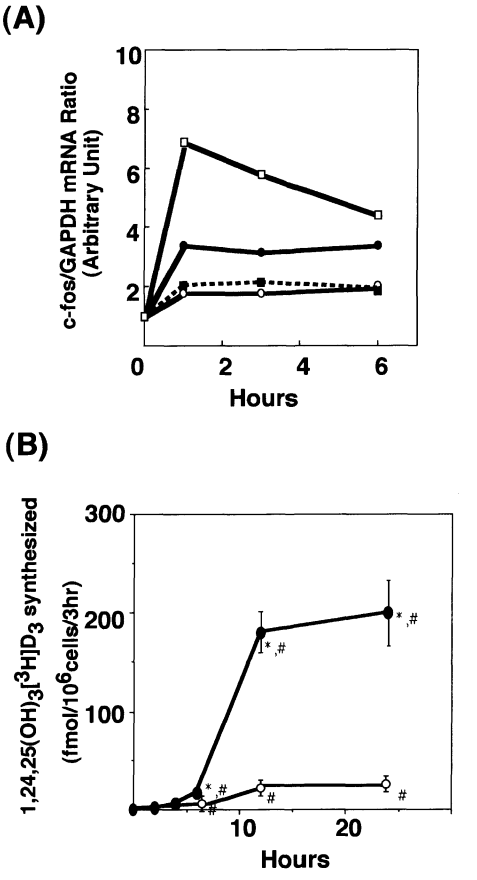

Fig. 5. Time course of the effect of TPA and/or dbcAMP on *c-fos* mRNA expression(A) and of the effect of TPA on 1,25-(OH)$_2$D$_3$-induced 24-hydroxylation activity (B) in HL-60 cells. (A) HL-60 cells were cultured with or without 1.0 ng/ml TPA and/or 10^{-7} M dbcAMP for the times indicated. Northern blot analysis for *c-fos* was then done as described. The autoradiogram obtained was scanned for the determination of relative levels of HL-60 *c-fos* transcript. The level of HL-60 *c-fos* mRNA was determined relative to that of GAPDH mRNA. Key: (○) control cells; (■) 10^{-7} M dbcAMP-treated cells; (●) 1.0 ng/ml TPA-treated cells; (□) dbcAMP plus TPA-treated cells. (B) HL-60 cells were incubated for the times indicated with 10^{-7} M 1,25-(OH)$_2$D$_3$ in the presence or absence of 1.0 ng/ml TPA. The cells were examined for their 24-hydroxylation activities by using the periodate-based assay. Key: (○) 1,25-(OH)$_2$D$_3$-treated cells; (●) TPA plus 1,25-(OH)$_2$D$_3$-treated cells. (#) Significant difference ($p<0.05$) vs 0h; (*) Significant difference ($p<0.05$) vs cells treated without TPA.

not appreciably affect the expression of *c-fos* gene. It is also interesting concerning this effect that dbcAMP and TPA acted in a synergistic manner to increase the levels of *c-fos* mRNA by approximately 3.9-fold. A kinetic study shows that *c-fos* mRNA levels had increased rapidly to the maximum by as early as 1 h after the addition of TPA, whereas a significant potentiation by TPA of 1,25-(OH)$_2$D$_3$-24-OH-ase activity was firstly detected at 4 h and increased until 24 h (Fig. 5), thus indicating that the stimulation of *c-fos* gene expression by TPA is preceded by TPA-induced potentiation of 1,25-(OH)$_2$D$_3$ actions. Furthermore, at 1, 3 or 6 h after the addition, dbcAMP and TPA acted synergistically in an activation of *c-fos* gene expression in HL-60 cells. It is interesting that gene expression of *c-jun* was not affected by the treatment with those reagents in the HL-60 clone which we used (data not shown), which contrasts with previous reports [29–31].

Discussion

Whereas dbcAMP alone induces HL-60 cells to differentiate into granulocytes [26], 1,25-$(OH)_2D_3$ and TPA induce HL-60 cell differentiation along a monocyte and macrophage pathway [24, 25]. We have previously reported that dbcAMP plus 1,25-$(OH)_2D_3$ induces HL-60 cell differentiation into monocytes and macrophages more efficiently than 1,25-$(OH)_2D_3$ alone, indicating that dbcAMP enhances the effect of 1,25-$(OH)_2D_3$ to induce HL-60 cells to differentiate into monocytes and macrophages [9]. The enhancement by dbcAMP of 1,25-$(OH)_2D_3$ action in HL-60 cells was also supported when assessed by the induction of 1,25-$(OH)_2[^3H]D_3$-24-hydroxylation activity [9]. DbcAMP increased VDR content by approximately 2.0-fold, which was similar to a 1.8-fold augmentation of 1,25-$(OH)_2D_3$-induced 24-hydroxylation activity, suggesting that an increase in VDR may be a major mechanism by which dbcAMP enhances the effect of 1,25-$(OH)_2D_3$ in HL-60 cells [9].

In addition to the PKA system, the PKC system is also important in modifying the effect of 1,25-$(OH)_2D_3$ in various cells including HL-60 cells [31, 26], intestinal epithelium [13] and osteoblast-like cells [32]. Although cooperative interaction between PKC and PKA systems is reported in the process of HL-60 cell differentiation [33], the present study demonstrated for the first time that PKA and PKC systems act together in a synergistic manner to potentiate the effect of 1,25-$(OH)_2D_3$ in HL-60 cells. While the enhancement by dbcAMP of 1,25-$(OH)_2D_3$-induced 24-hydroxylation activity correlated well with the degree to which dbcAMP increases VDR, TPA enhanced the effect of 1,25-$(OH)_2D_3$ to induce 24-hydroxylation activity by approximately 14.6-fold, much more efficiently than a 1.7-fold increase in VDR caused by TPA. TPA is reported to increase VDR in human histiocytic lymphoma U937 cells [10], to counter the infuluence of VDR in rat intestinal IEC-6 cells [13] and to reduce VDR in NIH-3T3 mouse fibroblast [34] and osteoblast-like UMR 106 and ROS 17/2.8 cells [32]. We have reported that TPA significantly enhanced the expression of 24-OH-ase gene by 1hr in rat intestinal IEC-6 cells without causing an increase in VDR[13]. These data suggest the presence of another mechanism, besides an increase in VDR, by which TPA enhanced the effect of 1,25-$(OH)_2D_3$ in HL-60 cells.

The *c-fos* proto-oncogene complexes with the proto-oncogene, *c-jun*, to form the cellular transcription factor, AP-1, which binds to its specific DNA sequence just upstream or within the region of vitamin D-responsive element (VDRE) [8] to affect the magnitude of 1,25-$(OH)_2D_3$-induced expressions of osteocalcin gene [14]. TPA, which increased the stationary level of *c-fos* mRNA, enhanced 1,25-$(OH)_2D_3$-stimulated 24-OH-ase gene expression. Previous studies demonstrated that *c-fos* mRNA expression is induced in HL-60 cells very rapidly by TPA, which is consistent with the notion that *c-fos* gene is an important early response gene which alters the pattern of cellular gene expression [35, 36]. The present study shows that the levels of *c-fos* mRNAs peaked by 1 h after the addition of TPA, although *c-jun* expression remained unchanged (Fig. 5). The proto-oncogene, *c-fos*, is important in determining DNA binding affinity of AP-1 complex [37]. Furthermore, TPA by itself stimulates the binding of *fos-jun* complex to the AP-1 sequence [15]. A kinetic study of the induction of 24-OH-ase activity by 1,25-$(OH)_2D_3$ (Fig. 5), together with the previous report of ours showing that the gradual increase of 24-OH-ase gene expression caused by 1,25-$(OH)_2D_3$ until 12 h in IEC-6 cells, suggests that increased *c-fos* expression may lead to an increase in AP-1 activity, resulting in the enhancement of the effect of 1,25-$(OH)_2D_3$ in the induction of 24-hydroxylation activity. This possibility was also supported by previous reports demonstrating that AP-1-like sequence is present in the upstream of rat 24-OH-ase gene [18] and that TPA enhanced 1,25-$(OH)_2D_3$-induced 24-OH-ase gene expression in primary-cultured rat renal cells along with a transient expression of *c-fos* gene which peaked as early as 30 min after the addition of TPA [38]. Furthermore, supportive of this notion are the data showing that TPA and/or dbcAMP enhanced both 1,25-$(OH)_2D_3$-induced 24-OH-ase gene expression (Fig. 2) and the expression of *c-fos* gene (Fig. 4) in a synergistic manner. Since dbcAMP did not increase *c-fos* gene expression, the magnitude of the enhancement of 1,25-$(OH)_2D_3$-induced 24-OH-ase activity correlated exactly with its effect on VDR increase. Therefore, the reason why TPA induced 24-hydroxylation activity much more efficiently than dbcAMP on the basis of their effect in increasing

VDR, might be accounted for by the different effects of TPA and dbcAMP on *c-fos* expression.

Previous reports demonstrated that TPA, but not $1,25\text{-}(OH)_2D_3$, induces a rapid increase in *c-jun* mRNA in HL-60 cells,suggesting that an early response of *c-jun* gene is not obligatory for macrophage differentiation [29–31]. Nevertheless, TPA failed to induce a significant change in *c-jun* expression in the HL-60 clone we used in the present study. Since TPA has a similar magnitude of differentiation-inducing effect in our HL-60 clone to those reported in our previous studies (data not shown), the present study also supported the notion that an early response of *c-jun* gene to TPA does not seem to play an important role in the diffentiation-inducing effect of TPA in HL-60 cells.

In summary, it was demonstrated that TPA and dbcAMP each shows the enhancement of the effect of $1,25\text{-}(OH)_2D_3$ on HL-60 cells and that TPA and dbcAMP act in a synergistic manner to enhance the effect of $1,25\text{-}(OH)_2D_3$, suggesting that each reagent exerts its effect through a distinct pathway. Furthermore, more efficient induction of 24-hydroxylation activity by TPA than by dbcAMP seems to be explained by TPA-induced activation of *c-fos* gene expression.

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