Journal of Cellular Biochemistry 55:230–240 (1994)

Protein Kinase C Is Involved in 24-Hydroxylase Gene Expression Induced by $1,25(OH)_2D_3$ in Rat Intestinal Epithelial Cells

Hidenori Koyama, Masaaki Inaba, Yoshiki Nishizawa, Shigeo Ohno, and Hirotoshi Morii

Second Department of Internal Medicine, Osaka City University Medical School, Osaka (H.K., M.I., Y.N., H.M.), and Department of Molecular Biology, Yokohama City University School of Medicine, Yokohama (S.O.), Japan

Abstract Effects of protein kinase C (PKC) inhibitor and activator on $1,25(OH)_2D_3$-induced gene expression were examined in rat intestinal epithelial cells, IEC-6 cells. A potent PKC inhibitor, H-7 (20 μM), completely abated $1,25(OH)_2D_3$-induced 24-hydroxylase gene expression at 3 and 6 h. The effect of H-7 was dose dependent with IC_{50} around 5 μM. Other protein kinase inhibitors, HA-1004 and H-89 (20 μM), had no effects. Furthermore, the activation of PKC by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) potentiated the effect of $1,25(OH)_2D_3$ by 1 h. TPA appeared to exert its effect at a transcriptional step, since mRNA stability was not affected by TPA treatment. At 3 h after the treatment of the cells with H-7 and TPA, vitamin D receptor (VDR) contents estimated by ^{3}H-$1,25(OH)_2D_3$ binding capacity were 72.4 and 63.2% of vehicle-treated cells without significant changes of binding affinities, suggesting that the effect of H-7 and TPA was not the result of changes in VDR content or its binding affinity. In conclusion, PKC is involved in $1,25(OH)_2D_3$-induced 24-hydroxylase gene expression in IEC-6 cells between $1,25(OH)_2D_3$-VDR binding and VDR-induced gene transactivation.

Key words: PKC, 1,25-dihydroxyvitamin D3, vitamin D receptor, VDR, rat

The active form of vitamin D_3, 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), binds to a high affinity vitamin D receptor (VDR) and acts in target cells in a manner analogous to the steroid hormones [Baker et al., 1988; Burmester et al., 1988; DeLuca et al., 1990; Haussler et al., 1988; McDonell et al., 1987; Pike 1991]. VDR is a member of the steroid hormone receptor superfamily and the occupied receptor is thought to act directly on the target genes. A number of vitamin D-responsive elements (VDRE) have been identified and characterized in the 5′-flanking region of various vitamin D-dependent genes [Darwish and DeLuca, 1992; Demay et al., 1990; Kerner et al., 1989; Morrison et al., 1989; Noda et al., 1990; Terpening et al., 1991]. All of these responsive elements are shown to bind the VDR and are able to mediate $1,25(OH)_2D_3$-dependent transcriptional activation.

Received November 29, 1993; revised January 27, 1994; accepted January 31, 1994.

Address reprint requests to Hidenori Koyama, Second Department of Internal Medicine, Osaka City University Medical School, 1-5-7, Asahi-machi, Abeno-ku, Osaka 545, Japan.

Evidence suggests that protein kinase C (PKC) plays a role in the action of $1,25(OH)_2D_3$. Firstly, $1,25(OH)_2D_3$ has been shown to increase PKC in HL-60 leukemic cells and kidney cells within 12–24 h [Martell et al., 1987; Simboli et al., 1992]. This regulation occurred in part at a transcriptional level [Obeid et al., 1990]. Furthermore, in skeletal muscle and in colon epithelium, $1,25(OH)_2D_3$ rapidly translocated PKC activity from cytosol to membranous fraction only within minutes [Massheimer and de Boland, 1992; Wali et al., 1990], indicating that $1,25(OH)_2D_3$ might regulate PKC activity at a posttranslational step as well as at a transcriptional level. Secondly, activation of PKC affected VDR content in a cell-specific manner [Krishnan and Feldman, 1991; Tahara et al., 1991; van Leeuwen et al., 1992b]. We showed that treatment of histiocytic lymphoma cells, U-937, with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) for 24 h augmented $1,25(OH)_2D_3$ action in inducing IL-1 β which was associated with an increase of VDR content [Tahara et al., 1991]. In contrast, TPA was shown to decrease VDR until 6 h in fibroblastic and osteoblast-like cells [Krishnan

and Feldman, 1991; van Leeuwen et al., 1992]. Thirdly, VDR might be a substrate for PKC β in a cell free system and in VDR transfected CV-1 cells [Hsieh et al., 1991], where phosphorylation of VDR is now considered to be essential for the transactivation of target genes by $1,25(OH)_2D_3$-bound VDR [Haussler et al., 1988; Hsieh et al., 1991; Brown and DeLuca, 1990; Jones et al., 1991]. Lastly, AP-1 transcription factor(s), which consists of Fos-Jun protooncogene families and can be regulated by PKC, cooperates with VDR in mediating transactivation of osteocalcin gene that is Cis-regulated by $1,25(OH)_2D_3$ [Owen et al., 1990; Ozono et al., 1990; Schule et al., 1990]. These observations raise intriguing possibilities that $1,25(OH)_2D_3$-induced activation of PKC is essential for $1,25(OH)_2D_3$-induced gene expression in intact cells. However, no evidence is available showing that PKC is essential for any of the $1,25(OH)_2D_3$-Cis-regulated gene activation in intact cells.

25-Hydroxyvitamin D-24-hydroxylase (24-hydroxylase) gene, recently cloned from rat kidney [Ohyama et al., 1991, 1993], was rapidly induced by $1,25(OH)_2D_3$ in rat intestine [Shinki et al., 1992; Armbrecht and Boltz, 1991]. 24-Hydroxylase gene expression seemed to be Cis-regulated by $1,25(OH)_2D_3$ because the gene expression occurred within 1 h after a treatment. Possible presence of VDRE-like sequence in the promoter of rat 24-hydroxylase gene supports this idea [Ohyama et al., 1993]. In this regard, we hypothesized that PKC mediates or modulates $1,25(OH)_2D_3$-induced 24-hydroxylase gene expression in intestinal cells. In addition to the observation that rat intestinal epithelial cell line, IEC-6 cells, contains VDR [Wiese et al., 1992], we showed in this paper that IEC-6 cells responded to $1,25(OH)_2D_3$ in inducing 24-hydroxylase gene expression. Furthermore, PKC isozymes expressed in these cells were identical to rat intestine; thus this cell line was a relevant model in examining the role of PKC in $1,25(OH)_2D_3$-induced 24-hydroxylase gene expression. We demonstrated for the first time in this study that PKC was essential in $1,25(OH)_2D_3$-dependent gene expression in intact cells.

METHODS

Cell Culture

Rat intestinal epithelial cell line, IEC-6, was purchased from American Tissue Culture Collection (Rockville, MD) and was used in all experiments. Cells were grown in Dulbecco's modified Eagle's medium (glucose: 4.5 g/L) supplemented with 10% fetal calf serum. After the cell confluency was achieved, medium was exchanged with the additions specified in each experiment. Cell viability after the treatment was determined by trypan-blue dye exclusion method, and was more than 95% in all experiments.

Additions

$1,25(OH)_2D_3$ was a generous gift from Chugai Pharmaceutical Company (Tokyo, Japan), and added in ethanol solution. 12-*O*-tetradecanoylphorbol-13-acetate (TPA), 1,2-dioctanoyl-sn-glycerol, and RNA synthesis inhibitor, 5,6,-dichlorobenzimidazole riboside (DRB) were purchased from Sigma Chemical Co. (St. Louis, MO), and were used from a stock solution in dimethylsulfoxide (DMSO). Protein kinase inhibitors H-7, HA-1004, and H-89 were purchased from Seikagaku Corporation (Tokyo, Japan), and staurosporine from Sigma. H-7 and HA-1004 were stocked in H_2O. H-89 and staurosporine were stocked in DMSO. Cycloheximide purchased from Sigma was freshly prepared in H_2O on a day of use.

Northern Blot Studies and Gene Probes

Total RNA was isolated by acid-guanidinium thiocyanate-phenol-chloroform extraction [Chomczynski and Sacchi, 1987]. Northern blot hybridization was performed as described previously [Koyama et al., in press]. Ten or twenty micrograms of RNA were electrophoresed in a 1% agarose containing formaldehyde, transferred to a nylon filter and hybridized with ^{32}P-labelled cDNA probes in 50% formamide, 3 × SSC (1 × SSC: 0.15 M NaCl, 0.015 M Na citrate, pH 7.4), 50 mM Tris-HCl, pH 7.5, 0.1% sodium dodecyl sulfate (SDS), 20 μg/ml tRNA, 20 μg/ml boiled salmon sperm DNA, 1 mM EDTA, 4 × Denhardt solution for 40 h at 37°C.

The mouse cDNAs for PKC α, β, γ, δ, ϵ, and ζ were isolated from mouse brain cDNA library as described [Mizuno et al., 1991]. PKC η cDNA was cloned from mouse skin cDNA library as reported [Osada et al., 1990]. The 24-hydroxylase cDNA fragment was cloned by reverse transcription-polymerase chain reaction (RT-PCR) by the use of duodenal RNA derived from $1,25(OH)_2D_3$-treated male Sprague-Dawley rat. Briefly, RT was done in 10 μl of reaction mixture each containing total RNA (1 μg), each deoxynucleotide triphosphate (10 nmol), ran-

dom hexamer (100 pmol), ribonuclease inhibitor (20 U), and MoMuLV-reverse transcriptase (100 U: GIBCO-BRL, Grand Island, NY) in 1 × RT buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 10 mM dithiothreitol, 3 mM $MgCl_2$). Reaction was carried out for 1 h at 37°C. Complementary DNA was amplified in 1 × PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.4, 1.5 mM $MgCl_2$, 0.1 mg/ml of gelatin), 0.1 mM of each deoxynucleotide triphosphate, 0.5 μM of each primer, and 25 U/ml of Taq polymerase (Takara Shuzo Co. Ltd., Kyoto, Japan). Amplification was carried out for 30 cycles of 60 s at 90°C, 60 s at 55°C, and 90 s at 72°C. Primers (5′-AACCTTGGAAAGCCTATCGCGA-3′, 5′-CGCTTGTCGAACTACACCTA-3′) for the reaction were derived from the sequence of the rat kidney 24-hydroxylase cDNA [Ohyama et al., 1991]. PCR product double-digested by Xba1-Pst1 was cloned into pBluescript SK(+) plasmid vector. The cloned DNA fragment was identified to be 24-hydroxylase cDNA by sequencing with the dideoxynucleotide method [Sanger et al., 1977].

Vitamin D Receptor Preparation and Determination

VDR was prepared as described previously [Wiese et al., 1992]. Confluent IEC-6 cells collected in PBS were lysed by three cycles of freeze-thawing method, and VDR extract was obtained by ultracentrifugation at 105,000*g* for 60 min. The protein concentration was determined by a described method [Bradford, 1976]. VDR content was determined by ^3H-$1,25(OH)_2D_3$ binding assay as previously reported [Inaba and DeLuca, 1989; Wecksler and Norman, 1979].

Densitometric Analysis

The density of each band was determined semi-quantitatively by a laser densitometer (Pharmacia LKB 2222 UltroScan XL, Uppsala, Sweden).

RESULTS

PKC Inhibitor H-7 Completely Abated the Expression of $1,25(OH)_2D_3$-Induced 24-Hydroxylase Gene

VDR in IEC-6 cells was recognized as identical to those in classical target tissues [Wiese et al., 1992], while it has not been known whether $1,25(OH)_2D_3$ has physiological effects on these cells. As shown in Figure 1A, 24-hydroxylase mRNA was induced as early as 1 h after the cells had been treated with 100 nM of $1,25(OH)_2D_3$, and the expression was increased linearly up to 12 h. Furthermore, 24-hydroxylase gene expression induced by $1,25(OH)_2D_3$ treatment for 12 h was observed in a dose-dependent fashion (1–100 nM; Fig. 1B). As little as 1 nM $1,25(OH)_2D_3$ induced detectable 24-hydroxylase mRNA, and the effect of $1,25(OH)_2D_3$ was not saturated even at the concentration of 100 nM. Furthermore, cycloheximide, a protein synthesis inhibitor, did not inhibit the $1,25(OH)_2D_3$-induced 24-hydroxylase gene expression at 1 h (not shown), suggesting that $1,25(OH)_2D_3$ activates the gene in a Cis-regulatory mechanism.

To investigate whether PKC signaling system is involved in the $1,25(OH)_2D_3$-induced 24-hydroxylase gene expression, effects of protein kinase inhibitors were examined (Fig. 2). Each inhibitor was simultaneously added with $1,25(OH)_2D_3$. A potent PKC inhibitor, H-7, at the concentration of 20 μM completely abolished the $1,25(OH)_2D_3$-induced 24-hydroxylase gene expression both at 3 and 6 h after the treatment (Fig. 2). However, neither HA-1004, a 10-fold less potent PKC inhibitor with similar inhibitory effects on other protein kinase compared with H-7, nor H-89, a specific inhibitor of cAMP dependent protein kinase (A-kinase), at the same concentration had any inhibitory effects. IC_{50} of H-7 for $1,25(OH)_2D_3$-induced 24-hydroxylase gene expression was observed at about 5.0 μM (Fig. 3A), which was almost identical to that of H-7 for PKC activity in vitro. Furthermore, another potent PKC inhibitor, staurosporine, had a similar inhibitory effect on $1,25(OH)_2D_3$-induced gene expression at 3 h with IC_{50} arround 0.5 nM (Fig. 3B). These data suggest that PKC signaling system is indispensable to $1,25(OH)_2D_3$-induced 24-hydroxylase gene expression.

PKC Activation Potentiated $1,25(OH)_2D_3$-Induced 24-Hydroxylase Gene Expression

To examine whether the activation of PKC influences the 24-hydroxylase gene expression induced by $1,25(OH)_2D_3$, 100 nM of $1,25(OH)_2D_3$ with or without TPA (10 ng/ml) was tested (Fig. 4A). Because TPA alone did not induce 24-hydroxylase mRNA at any time examined, PKC system does not seem sufficient for the induction of its mRNA. TPA significantly potentiated the $1,25(OH)_2D_3$-induced 24-hydroxylase gene expression as early as 1 h (Fig. 4A), with its

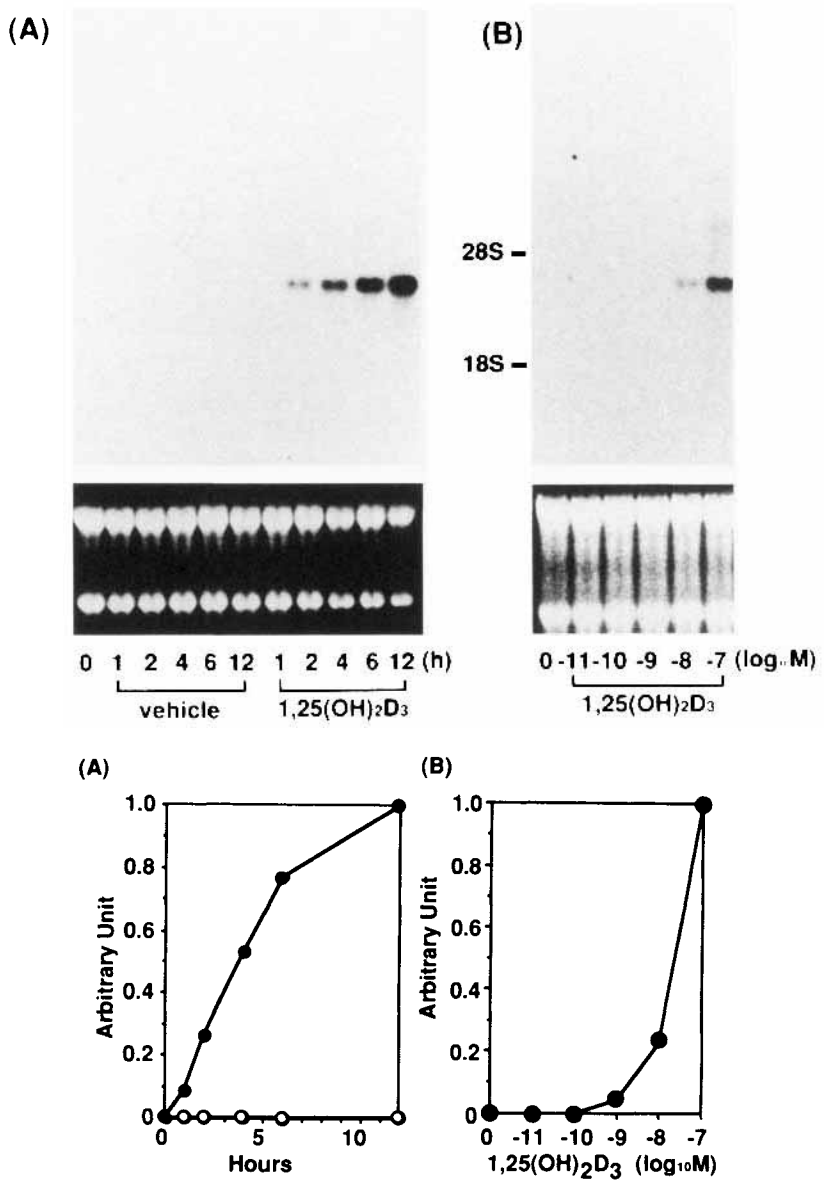

Fig. 1. **A:** $1,25(OH)_2D_3$-induced 24-hydroxylase gene expression in IEC-6 cells. Confluent cells were treated with 100 nM of $1,25(OH)_2D_3$ in ethanol or ethanol alone for indicated hours, followed by isolation of total RNA. Twenty micrograms of RNA were fractionated in a 1% agarose gel, transferred to a nylon filter and hybridized to labelled 24-hydroxylase cDNA probe. Corresponding ribosomal RNA stained with ethidium bromide of the samples prior to transfer is pictured below each radiograph. Results of densitometric analysis were shown in the lower panel, where closed circles represent $1,25(OH)_2D_3$ treatment, and open circles represent vehicle treatment. **B:** Dose dependent effect of $1,25(OH)_2D_3$ on 24-hydroxylase gene expression. Confluent cells were cultured for 12 h with indicated concentrations of $1,25(OH)_2D_3$. Northern analysis for 24-hydroxylase gene was done as described. Corresponding ribosomal RNA stained with ethidium bromide of the samples prior to transfer is pictured below each radiograph. Results of densitometric analysis were shown in the lower panel.

effect continued until 14 h after the treatment (not shown). The effect of TPA was dose-dependent from 1 to 100 ng/ml (not shown). $1,25(OH)_2D_3$-induced 24-hydroxylase gene expression was also enhanced by a physiological PKC activator, 1,2-dioctanoyl-sn-glycerol (Fig. 4B), indicating that activation of PKC potentiated the $1,25(OH)_2D_3$-induced 24-hydroxylase gene expression. To examine whether TPA affects the stability of 24-hydroxylase mRNA, transcription was arrested by adding 25 μg/ml of DRB after cells had been treated with $1,25(OH)_2D_3$ alone or in combination with TPA for 6 h. At indicated times after the addition of

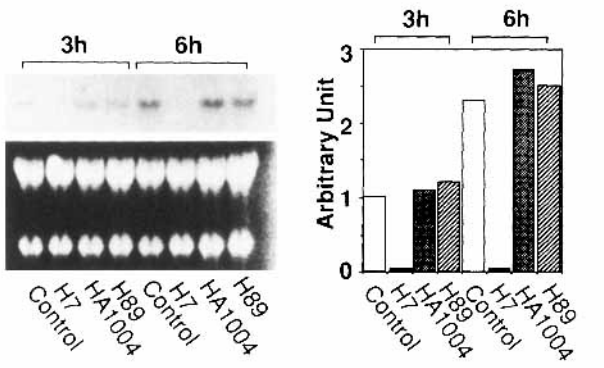

Fig. 2. Effect of inhibitors of protein kinase on $1,25(OH)_2D_3$-induced gene expression. Confluent IEC-6 cells were cotreated with 100 nM of $1,25(OH)_2D_3$ and 20 μM of vehicle, H-7, HA-1004, and H-89, for 3 and 6 h. Twenty micrograms of total RNA isolated were fractionated in a 1% agarose, transferred to a nylon filter, and hybridized with labelled probe for 24-hydroxylase. Corresponding ribosomal RNA stained with ethidium bromide of the samples prior to transfer is pictured below each radiograph. Results of densitometric analysis were shown in the right panel. This experiment was repeated twice and similar results were obtained.

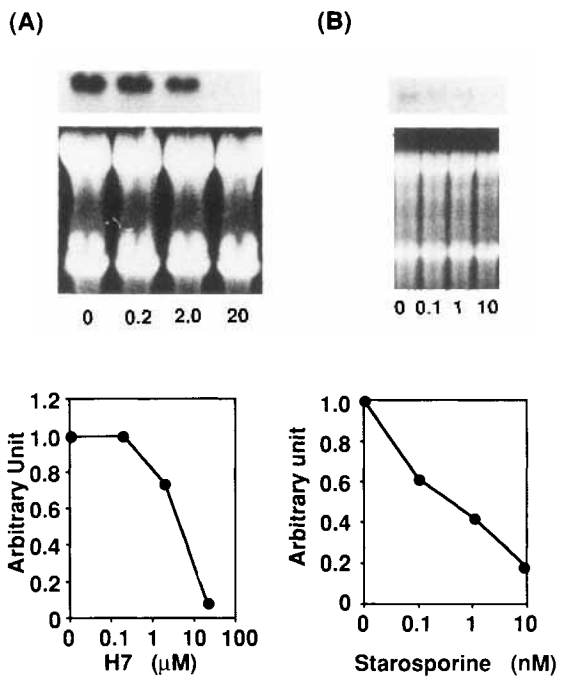

Fig. 3. **A:** Dose-dependent effects of H-7 on $1,25(OH)_2D_3$-induced 24-hydroxylase. Confluent IEC-6 cells were treated with 100 nM of $1,25(OH)_2D_3$ and indicated concentrations of H-7 for 6 h. Northern analysis of 24-hydroxylase gene was done as described. **B:** Effects of staurosporine on $1,25(OH)_2D_3$-induced 24-hydroxylase gene expression. Confluent IEC-6 cells were treated with 100 nM of $1,25(OH)_2D_3$ and indicated concentrations of staurosporine for 3 h. Northern blot analysis of 24-hydroxylase gene was done as described. Corresponding ribosomal RNA stained with ethidium bromide of the samples prior to transfer is pictured below each radiograph. Results of densitometric analysis were demonstrated in the lower panel. These experiments were repeated twice with similar results.

DRB, cells were harvested and 24-hydroxylase mRNA levels were determined by Northern blot analysis. In each group, 24-hydroxylase mRNA was rapidly degraded with its half life less than 3 h and the kinetics of the mRNAs were not significantly different within the groups (Fig. 5), suggesting that the effect of TPA occurred at a transcriptional step. Taken together, these results indicate that the PKC system is intimately involved but not sufficient for the transcriptional activation of 24-hydroxylase gene.

Effects of H-7 and TPA on VDR Content

To examine whether PKC affects $1,25(OH)_2D_3$ action by changing VDR content, effects of H-7 and TPA on cellular binding capacity and the affinity for ^3H-$1,25(OH)_2D_3$ were studied (Fig. 6). Inhibition of PKC by H-7 caused a decrease of binding sites at 3 h (72.4% of vehicle treated cells). Activation of PKC also down-regulated VDR at 3 h (63.2% of vehicle treated cells). Scatchard analysis revealed that binding affinities for $1,25(OH)_2D_3$ were not significantly altered both by H-7 and TPA (Kd of 42, 24, and 30 *pM for* vehicle, H-7, and TPA, respectively). At 3 h after the treatment of H-7, $1,25(OH)_2D_3$-induced 24-hydroxylase gene expression was completely abated (Fig. 2), while VDR content was decreased only by 27.6%. TPA potentiated $1,25(OH)_2D_3$-induced gene expression at 3 h (Fig. 4A). However, TPA did not increase ^3H-$1,25(OH)_2D_3$ binding sites at the same time, suggesting that TPA did not modulate $1,25(OH)_2D_3$ action by the change of VDR content. These results suggest that PKC is involved in $1,25(OH)_2D_3$-induced gene activation mainly at the step after $1,25(OH)_2D_3$-VDR binding.

Gene Expressions of PKC Isozymes in IEC-6 Cells

Next, we examined what types of PKC isozymes are expressed in IEC-6 cells, since PKC was revealed to be involved in $1,25(OH)_2D_3$-induced 24-hydroxylase gene expression. In IEC-6 cells, PKC δ and ζ were abundantly expressed. Lower amounts of mRNA for PKC α, ε, and η were also detected by Northern blot hybridization (Fig. 7). $1,25(OH)_2D_3$ did not affect the mRNA level of either PKC δ or ζ isozymes up to 12 h in these cells (not shown).

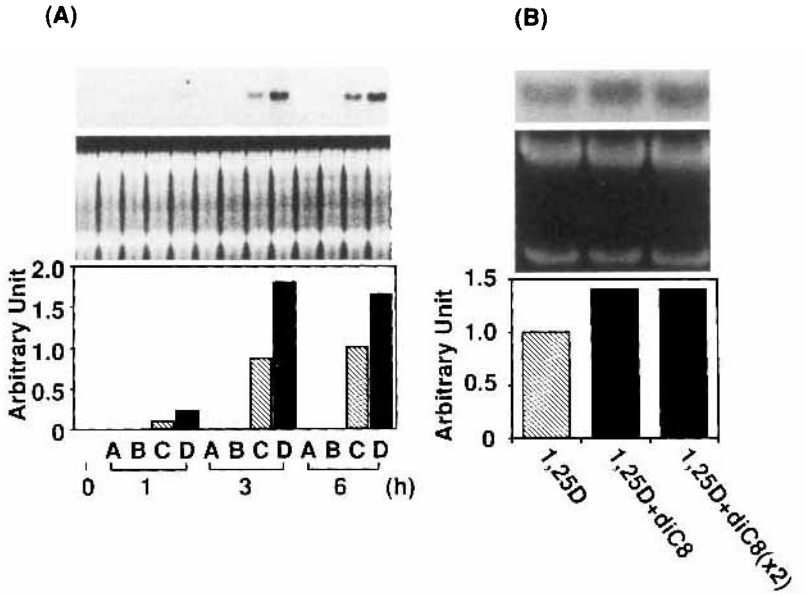

Fig. 4. **A:** Effects of PKC activator (TPA) on $1,25(OH)_2D_3$-induced 24-hydroxylase gene expression. Confluent IEC-6 cells were treated with vehicle (A), 10 ng/ml of TPA (B), 100 nM of $1,25(OH)_2D_3$ (C), and combinations of TPA and $1,25(OH)_2D_3$ (D) for indicated hours. Total RNA was isolated and Northern blot analysis for 24-hydroxylase was done as described in Figure 1. **B:** Effect of 1,2-dioctanoyl-sn-glycerol on $1,25(OH)_2D_3$-induced 24-hydroxylase gene expression. Confluent IEC-6 cells were treated with 100 nM of $1,25(OH)_2D_3$ with or without 1,2-dioctanoyl-sn-glycerol (20 μM of single treatment or 20 μM of initial dose with subsequent bihourly dose of 10 μM) for 3 h. Total RNA was isolated and Northern blot analysis for 24-hydroxylase was done as described in Figure 1. Corresponding ribosomal RNA stained with ethidium bromide of the samples prior to transfer is pictured below each radiograph. Results of densitometric analysis were demonstrated in the lower panels. These experiments were repeated twice and identical results were obtained.

DISCUSSION

It is now accepted that an active form of vitamin D, $1,25(OH)_2D_3$, acts via VDR-gene specific interaction commonly observed in steroid hormones. Our data gave a new insight into this area in that PKC signaling system plays an important role in $1,25(OH)_2D_3$-induced 24-hydroxylase gene transcription. This is the first report demonstrating that PKC is one of the major determinants in the magnitude of the effect of $1,25(OH)_2D_3$ on the expression of a gene which is Cis-regulated by $1,25(OH)_2D_3$ in intact cells.

Our data showed that IEC-6 cells were an ideal model for examining molecular mechanism of $1,25(OH)_2D_3$ action as well as PKC system in intact intestinal epithelial cells. PKC isozymes expressed in IEC-6 cells were identical to those in rat intestine (not shown), and the activation of PKC by TPA caused a rapid increase in expressions of c-fos and c-jun family. Concerning $1,25(OH)_2D_3$ action, $1,25(OH)_2D_3$ induced 24-hydroxylase gene expression in these cells by 1 h similar to the intestine in vivo [Shinki et al., 1992; Armbrecht and Boltz, 1991]. Furthermore, this action was not inhibited by cycloheximide, a protein synthesis inhibitor, indicating that $1,25(OH)_2D_3$ activates this gene in Cis-regulatory mechanism in IEC-6 cells. These observations support that $1,25(OH)_2D_3$-VDR complexes activate 24-hydroxylase gene through VDRE-like sequence in the promoter region [Ohyama et al., 1993], and this cell system is a relevant model in examining the mechanism of 24-hydroxylase gene transactivation.

Of note in our experiment is that the dose response for $1,25(OH)_2D_3$ in the induction of 24-hydroxylase gene does not correspond with regulated expressions of other gene products such as osteocalcin or parathyroid hormone. The significant gene expression of 24-hydroxylase was not observed until higher doses, 10 nM concentrations, of $1,25(OH)_2D_3$ were added. This specific gene regulation may be a cellular protective system to large amounts of $1,25(OH)_2D_3$, since 24-hydroxylase is a major enzyme responsible for $1,25(OH)_2D_3$ degradation. Similar dose-response induction of 24-hydroxylase was also observed in kidney epithelial cells [Chen et al., 1993], suggesting that the regulatory mecha-

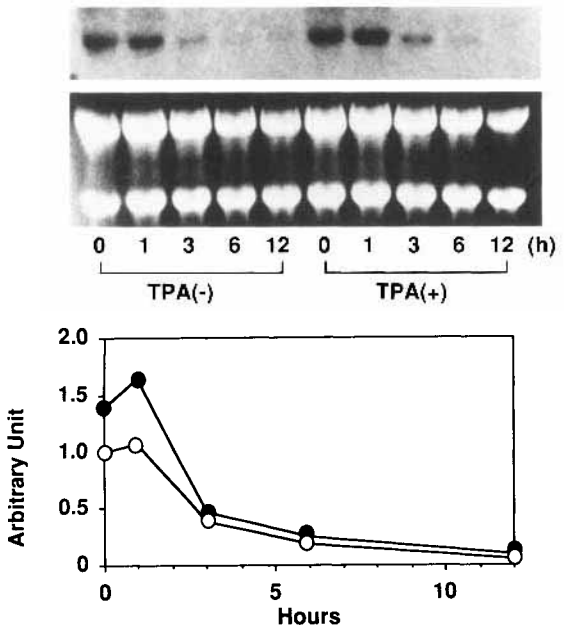

Fig. 5. TPA did not affect the stability of induced 24-hydroxylase mRNA. After confluent IEC-6 cells were treated with $1,25(OH)_2D_3$ alone or in combination with 10 ng/ml of TPA for 6 h, RNA synthesis was arrested by adding 25 μg/ml of DRB. At indicated times after the arrest, cells were harvested and 24-hydroxylase mRNA level was determined by Northern blot analysis as described. Corresponding ribosomal RNA stained with ethidium bromide of the samples prior to transfer is pictured below each radiograph. Results of densitometric analysis were shown at the bottom of the gel, where open circles represent treatment of $1,25(OH)_2D_3$ alone and closed circles represent $1,25(OH)_2D_3$ plus TPA. Essentially, identical results were obtained in another experiment.

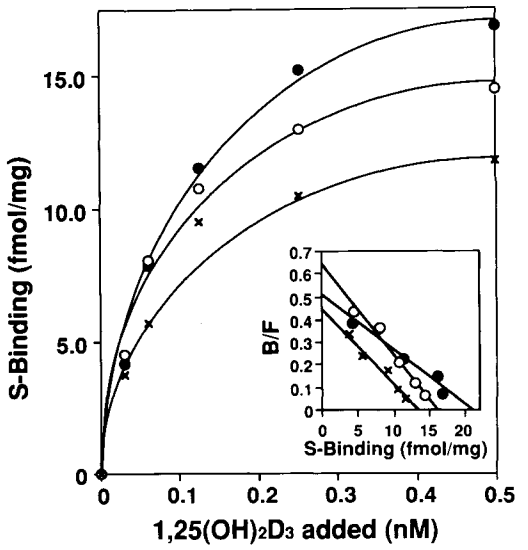

Fig. 6. Effect of H-7 and TPA on $1,25(OH)_2D_3$ binding capacity and affinity in IEC-6 cells. Confluent IEC-6 cells were cultured with vehicle, 20 μM of H-7 or 10 ng/ml of TPA, for 3 h. After cells were harvested, VDR extract was obtained by freeze-thawing method in $TEDK_{300}$ containing 5 mM of diisopropylfuorophosphate. VDR extract was incubated for 4 h with indicated concentrations of ^3H-$1,25(OH)_2D_3$ alone (total binding) or a combination with 100-fold excess of $1,25(OH)_2D_3$ (nonspecific binding). Bound VDR was separated by hydroxylapatite. Specific binding was calculated by subtraction of nonspecific binding from total binding. Each plot indicates the mean of 3 determinations of a sample. Vehicle (●), H-7 (○), and TPA (x). Scatchard analysis of ^3H-$1,25(OH)_2D_3$ binding sites is shown in the inset. Total binding sites, estimated from the x-intercept of the regression line, were 21.3, 15.4, and 13.6 fmol/mg protein for vehicle (●), H-7 (○), and TPA (x) with Kd of 42, 24, and 30 pM, respectively.

nism of 24-hydroxylase gene expression by $1,25(OH)_2D_3$ is somehow different from that of other $1,25(OH)_2D_3$-dependent genes. It is noteworthy that $1,25(OH)_2D_3$ increases the binding sites for phorbol ester in a similar dose-dependent manner [Obeid et al., 1990] as in the induction of 24-hydroxylase gene. These observations led us to investigate the role of PKC in the $1,25(OH)_2D_3$-induced 24-hydroxylase gene expression.

We drew conclusions from two lines of observations. First, a PKC inhibitor, H-7, completely abolished the $1,25(OH)_2D_3$-induced 24-hydroxylase gene expression. H-7 also has mild inhibitory effect on A-kinase. However, because neither H-89, a specific inhibitor of A-kinase, nor HA-1004, a 10-fold weaker PKC inhibitor with the same non-specific effects of H-7, affected this $1,25(OH)_2D_3$ action, the inhibitory effect of H-7 on $1,25(OH)_2D_3$-induced gene expression seems to be via inhibition of PKC. This was also supported by a dose-dependent analysis of H-7 action. We observed that H-7 was effective even at 2 μM which was comparable to IC_{50} for PKC in vitro. Furthermore, another potent PKC inhibitor, staurosporine, also inhibited $1,25(OH)_2D_3$-induced gene expression with IC_{50} arround 0.5 nM. The important question to be answered is whether H-7 abated $1,25(OH)_2D_3$ action through the alteration in VDR content in these cells. As far as we know, no reports are available concerning the effect of H-7 on VDR content. The binding capacity for $1,25(OH)_2D_3$ was slightly decreased with similar binding affinity at 3 h after the cells were treated with H7. However, the inhibitory effect of H-7 on $1,25(OH)_2D_3$-induced gene expression was not the results of changes in VDR binding sites or binding affinities, since the inhibitory effect of H-7 on $1,25(OH)_2D_3$ action preceded the effect on VDR content. Sec-

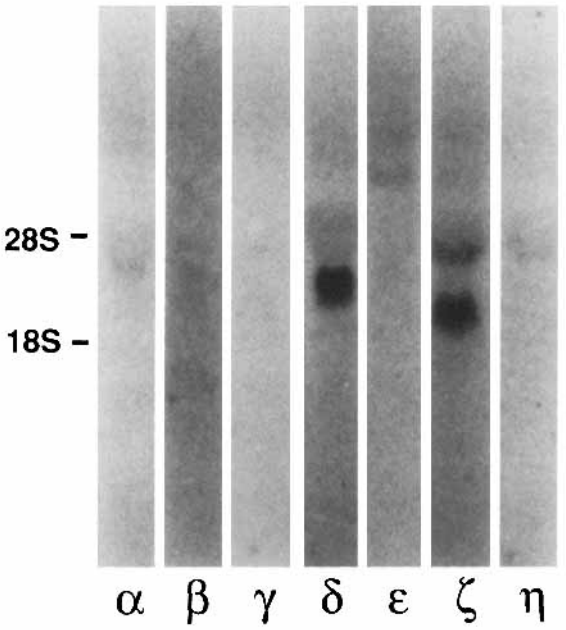

Fig. 7. Expression of the isozymes of PKC in IEC-6 cells. Twenty micrograms of total RNA isolated from the cells were fractionated in a 1% agarose gel electrophoresis containing formaldehyde, transferred to a nylon filter, and hybridized serially with labelled cDNAs for the isozymes of mouse PKC.

ondly, the activation of PKC by TPA potentiated the $1,25(OH)_2D_3$-induced 24-hydroxylase gene expression. Because the effect of TPA on 24-hydroxylase expression was also observed in kidney epithelial cells [Chen et al., 1993] and in HL-60 cells (Inaba et al., unpublished observations), the effect of TPA on 24-hydroxylase induction may be universal in all target tissues of $1,25(OH)_2D_3$. In IEC-6 cells, the effect of TPA was observed as early as 1 h after the treatment. This effect of TPA seemed to be a PKC-specific event because a physiological PKC activator, 1,2-dioctanoyl-sn-glycerol, had similar stimulatory activity. The involvement of PKC appears at a transcriptional step because TPA had no effects on the stability of 24-hydroxylase mRNA. Since TPA alone did not induce 24-hydroxylase gene expression, PKC is necessary but not sufficient for the gene transcription of 24-hydroxylase. When the cells were treated with TPA, VDR content was decreased with comparable binding affinity at 3 h, indicating that the effect of TPA was not mediated by changes in VDR content. Taken together, it is strongly suggested that PKC is involved in 24-hydroxylase gene activation between $1,25(OH)_2D_3$-VDR binding and VDR-induced gene transcription.

The previous studies demonstrated that $1,25(OH)_2D_3$ produces a stimulatory effect on calcium transport in the intestine, differentiation of leukemic cells, and bone resorption in organ culture at least in part through PKC system [de Boland and Norman, 1990; Martell et al., 1988; van Leeuwen et al., 1992a], because the actions induced by $1,25(OH)_2D_3$ were attenuated by a PKC inhibitor. However, it is not clear at which steps of $1,25(OH)_2D_3$-induced action PKC is involved. In the present paper, we could delimitate for the first time that PKC is involved in $1,25(OH)_2D_3$-induced action possibly between $1,25(OH)_2D_3$-VDR interaction and VDR-induced transactivation of the gene.

Understanding the molecular mechanism of roles of PKC in $1,25(OH)_2D_3$-induced 24-hydroxylase transcription is an important issue next to be elucidated. VDR-induced transactivation of a target gene may be regulated by PKC through induction of protooncogene products, FOS-JUN family (AP-1 transcription factor). In human osteocalcin promoter, AP-1 site juxtaposed to VDRE and AP-1 may interact with VDR in transactivating osteocalcin gene [Ozono et al., 1990; Schule et al., 1990]. In rat osteocalcin promoter, FOS-JUN complex could also be bound to VDRE [Owen et al., 1990] where no complete AP-1 site was found. It may be possible that AP-1 could also be functionally active in controlling 24-hydroxylase gene transcription. Although there is no complete consensus sequence for AP-1 site in the recently cloned promoter region of rat 24-hydroxylase [Ohyama et al., 1993], VDRE-like sequence or its juxtaposed sequence may serve as a functionally active AP-1 site. In a preliminary experiment, AP-1 factors such as c-fos, c-jun, fra-1, and Fos B were regulated by PKC system in IEC-6 cells (unpublished observation). Since these protooncogene products are so unstable, 3–6 h treatment of the cells with H-7 may be sufficient for the disappearance of these factors, which might be responsible for the inhibitory effect of H-7 on $1,25(OH)_2D_3$-induced gene expression. Recently, several reports revealed that VDR needs other nuclear protein, designated as nuclear accessory factor, in binding to its specific DNA (VDRE) in vitro [MacDonald et al., 1991; Ross et al., 1992; Sone et al., 1991]. The nuclear accessory factors, molecular weight of about 55 kD, has been shown to facilitate the binding of VDR to osteocalcin VDRE. This protein may be the retinoid X

receptor, which has shown to potentiate the receptor-DNA interaction for retinoic acid receptor, thyroid hormone receptor, as well as VDR [Yu et al., 1991]. It needs to be elucidated whether PKC regulates the synthesis and function of nuclear accessory protein or retinoid X receptor.

Another possible mechanism by PKC may be the phosphorylation of VDR. VDR is considered to be a substrate for PKC in a cell-free system and in VDR transfected CV-1 cells [Hsieh et al., 1991]. In VDR transfected CV-1 cells, the phosphorylation of VDR at Ser-51 was reported to be crucial for $1,25(OH)_2D_3$-dependent transactivation of osteocalcin gene. It may be possible that the magnitude of VDR-DNA interaction or VDR-induced gene transactivation is regulated by phosphorylation of VDR. Therefore, it is important to examine whether $1,25(OH)_2D_3$ treatment or PKC activation causes phosphorylation of VDR in IEC-6 cells.

Then what kinds of PKC isozymes are expressed and involved in $1,25(OH)_2D_3$-induced gene expression in IEC-6 cells? In our Northern blot analysis, major isozymes expressed were PKC δ and ζ, and small amounts of α, ϵ, and η were also detected. Although PKC β was reported to be responsible for VDR phosphorylation [Hsieh et al., 1991] and was transcriptionally regulated by $1,25(OH)_2D_3$ [Obeid et al., 1990], this isozyme was not detected in IEC-6 cells. Our data could not negate the possibility that, also in IEC-6 cells, undetectable amounts of PKC β are expressed and regulated by $1,25(OH)_2D_3$. Since PKC ζ is not activated by TPA in vitro [Nakanishi and Exton, 1992] and in IEC-6 cells (Koyama et al., unpublished observations), it is possible that the other PKC isozymes such as PKC α, δ, and ϵ, that were shown to be inhibited by H-7 (Mizuno et al., personal communication), might mediate $1,25(OH)_2D_3$-induced 24-hydroxylase gene expression. From our results, PKC δ mRNA level was not affected by $1,25(OH)_2D_3$. However, PKC isozymes may be activated post-translationally by $1,25(OH)_2D_3$ since the translocation of PKC activity induced by $1,25(OH)_2D_3$ was reported to occur only within minutes [Massheimer and de Boland, 1992; Wali et al., 1990].

In conclusion, PKC signaling system is indispensable to the $1,25(OH)_2D_3$-dependent transcription of 24-hydroxylase gene. Identification of the mechanisms of this phenomenon will provide important insights into understanding the molecular mechanism of $1,25(OH)_2D_3$ action.

ACKNOWLEDGMENTS

The authors thank Professor Shuzo Otani and Dr. Isao Matsui-Yuasa, Department of Biochemistry, Osaka City University Medical School, for fruitful discussion and critical comments.

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