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Phorbol ester markedly increases the sensitivity of intestinal epithelial cells to 1,25-dihydroxyvitamin D_3

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We have used a clonal intestinal epithelial cell line (IEC-18) to study the mechanism of action of 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2$D) in vitro. 1,25$(OH)_2$D (10^{-7} M) elevated by over 10-fold the mRNA levels for the cytochrome P450 component (P450cc24) of the 1,25$(OH)_2$D-24-hydroxylase. Increased P450cc24 mRNA levels were detectable at 6 h and peaked at 36 h. Below a concentration of 10^{-7} M, 1,25$(OH)_2$D had almost no effect. However, addition of phorbol ester for 2 h made the intestine responsive to 1,25$(OH)_2$D concentrations as low as 10^{-9} M.

1,25-Dihydroxyvitamin D; 24-Hydroxylase; Phorbol ester; Intestine; Rat

1. INTRODUCTION

1,25-Dihydroxyvitamin D_3 (1,25$(OH)_2$D), the active metabolite of vitamin D, acts on the intestine in vivo to increase calcium absorption and to increase 1,25$(OH)_2$D-24-hydroxylase (24-hydroxylase) activity. The 24-hydroxylase is found in tissues which are targets for 1,25$(OH)_2$D [1]. This enzyme may regulate intracellular concentrations of 1,25$(OH)_2$D by degrading it in target cells [2]. It has recently been shown that 1,25$(OH)_2$D elevates the mRNA levels for the cytochrome P450 component (P450cc24) of the intestinal 24-hydroxylase in the intact rat [3,4].

In order to study the induction of P450cc24 mRNA by 1,25$(OH)_2$D in vitro, we have used a clonal intestinal epithelial cell line (IEC-18). This cell line is derived from crypt cells [5] and has been shown to contain a receptor-like binding protein for 1,25$(OH)_2$D [6]. To monitor the action of 1,25$(OH)_2$D, we have measured mRNA levels for cytochrome P450cc24. We chose the IEC-18 cell line because it is a non-transformed cell line and because it is derived from the rat, the species from which the cytochrome P450cc24 was cloned [7].

In addition to the action of 1,25$(OH)_2$D, we were also interested in the interaction between 1,25$(OH)_2$D and phorbol esters. There have been numerous reports of an effect of 1,25$(OH)_2$D and phorbol esters on cell phenotype [8–10] but few studies on mechanisms. It has been demonstrated that 1,25$(OH)_2$D and phorbol esters modulate 24-hydroxylase activity in cultured renal cells [10]. Therefore, we were interested in the effects of 1,25$(OH)_2$D and phorbol esters on mRNA levels in intestinal cells.

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2. MATERIALS AND METHODS

The IEC-18 cell line was obtained from the American Type Culture Collection (Rockville, MD) at passage 14. This is an epithelioid-like cell line derived from the rat ileum [5]. Cells were cultured in T25 flasks (Costar, Cambridge, MA) with Dulbecco's modified Eagle's medium and F-12 nutrient medium (3:1) (Gibco, Grand Island, NY) containing 5% fetal calf serum (Hyclone, Logan, UT) and 10 μg/ml insulin. Cells reached confluency in 4 days, and were used between passages 17–28.

To study the effects of 1,25$(OH)_2$D, confluent cells were deprived of serum for 24 hours. The 1,25$(OH)_2$D, a kind gift of Dr. Milan Uskokovic (Hofmann-LaRoche, Nutley, NJ), was added in ethanol (0.1% final concentration). At the end of the experiment, cells were washed and stored frozen until isolation of RNA using RNAzol B (Tel-Test, Inc., Friendswood, TX).

The P450cc24 mRNA levels were measured by Northern and dot blot, as previously described [1]. mRNA was detected using the full-length clone for rat P450cc24 (p108, 3.2 kb), kindly supplied by Drs. Y. Ohyama and K. Okuda (Hiroshima University School of Dentistry, Hiroshima, Japan) [7]. Radiolabeled probe was prepared by random priming using a DECAprime labeling kit (Ambion, Inc., Austin, TX), and hybridization to the filter was performed at 42°C overnight. Filters were then washed extensively and exposed to X-ray film The dots on the X-ray film were quantitated by densitometry, using a linear region of the dilution curve.

Dot blots were routinely stripped and re-hybridized with a probe for β-actin (Oncor). This checked for uniformity of sample loading and the specificity of the 1,25$(OH)_2$D response. Based on actin rehybridization, sample loading was quite uniform. Therefore, data were routinely normalized to the amount of total RNA applied, which was determined spectrophotometrically.

Data are reported as the mean ± S.E. of the indicated number of determinations. Results of single experiments are shown, but findings were replicated at least three times. Two-tailed Student's *t*-test was used to determine statistical significance, and $P < 0.05$ was considered significant.

3. RESULTS

1,25(OH)$_2$D markedly increased cytochrome P450cc24 mRNA levels in IEC-18 cells. After a single dose of 1,25(OH)$_2$D (10^{-7} M), mRNA levels increased from almost undetectable levels, reached a maximum at 36 h, and returned to near normal by 60 h (Fig. 1A). Quantitation of the dot blot by densitometry showed that mRNA levels at 36 h were increased by over 10-fold compared to zero h (Fig. 1B). 1,25(OH)$_2$D had no effect on actin mRNA in the same experiment. A Northern blot of total RNA from cells treated with 1,25(OH)$_2$D for 36 h revealed a single major band with a size of about 3.7 kb (not shown). This is the same, within experimental error, as the 3.3 kb band reported in the intestine after 1,25(OH)$_2$D administration [1]. Using the 36 h time point, the effect of 1,25(OH)$_2$D concentration on P450cc24 mRNA levels was examined (Fig. 2). The cells showed only a small response to 1,25(OH)$_2$D below a concentration of 10^{-7} M.

The effect of the phorbol ester phorbol 12-myristate 13-acetate (TPA) on the action of 1,25(OH)$_2$D was examined in these cells. Since we wished to examine the rapid actions of phorbol esters, short incubation times with both phorbol ester and 1,25(OH)$_2$D were used. Cells were treated with 10^{-7} M 1,25(OH)$_2$D for 7 h and TPA was added during the final 2 h (Table I). Treatment of the cells with 1,25(OH)$_2$D alone for 7 h increased mRNA levels about 3-fold. TPA alone did not increase mRNA levels. However, TPA increased P450cc24 mRNA levels over 10-fold compared to 1,25(OH)$_2$D alone. This effect of TPA was abolished by H-7, a protein kinase C inhibitor (Table I).

Table I

Effect of 1,25(OH)$_2$D and phorbol ester on P450cc24 mRNA levels

Agonist	P450cc24 mRNA (OD/mg)	
	− H-7	+ H-7
None	89 ± 13	80 ± 7
1,25(OH)$_2$D	356 ± 26*	161 ± 13*
TPA	86 ± 14	111 ± 24
1,25(OH)$_2$D+TPA	4617 ± 57*	186 ± 17*

Table entries are the mean ± S E. of three determinations. Flasks were incubated in the presence or absence of 10^{-7} M 1,25(OH)$_2$D for 7 h. The phorbol ester TPA (200 nM) was added to half the flasks during the last 2 h of incubation. mRNA levels were determined by dot blot. Asterisks indicate that the entry is significantly different from corresponding None ($P < 0.05$, t-test).

Since TPA enhanced the effect of 10^{-7} M 1,25(OH)$_2$D, a dose–response study was performed to determine if TPA had an effect at lower concentrations of 1,25(OH)$_2$D. Using the same experimental design, 1,25(OH)$_2$D concentrations were varied from 10^{-10} to 10^{-7} M in the presence and absence of TPA. In the absence of TPA, 1,25(OH)$_2$D had a slight effect, but in the presence of TPA 1,25(OH)$_2$D markedly increased P450cc24 mRNA levels at concentrations as low as 10^{-9} M (Fig. 3A). Quantitation of the dot blot indicated that the presence of TPA increased mRNA levels more than

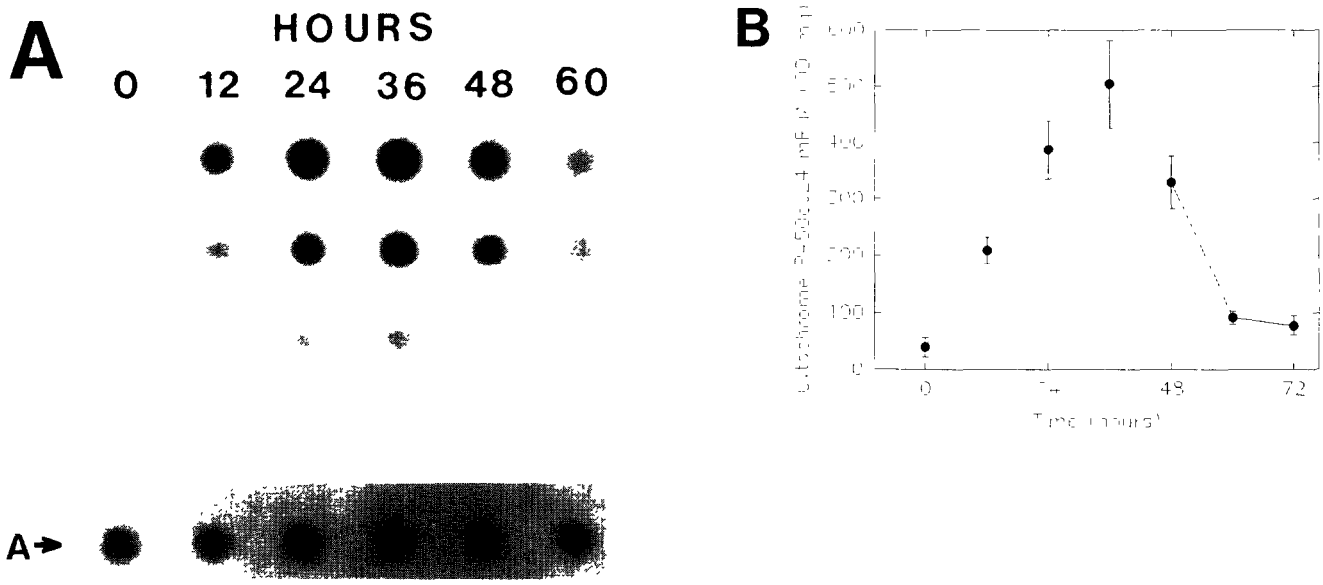

Fig. 1. Effect of 1,25(OH)$_2$D on cytochrome P450cc24 mRNA levels. Flasks were treated with 10^{-7} M 1,25(OH)$_2$D for the indicated time. For the dot blot (Fig. A), total RNA was serially diluted, dotted onto membrane, hybridized, and autoradiographed (shown). Rehybridization with the actin probe is shown at the bottom (A). The autoradiograph was quantitated by densitometry (Fig. 1B) Data is expressed as the mean ± S.E. of 3 determinations. mRNA levels of 1,25(OH)$_2$D-treated cells were significantly higher than the initial levels (zero time) at all time points (t-test, $P < 0.05$).

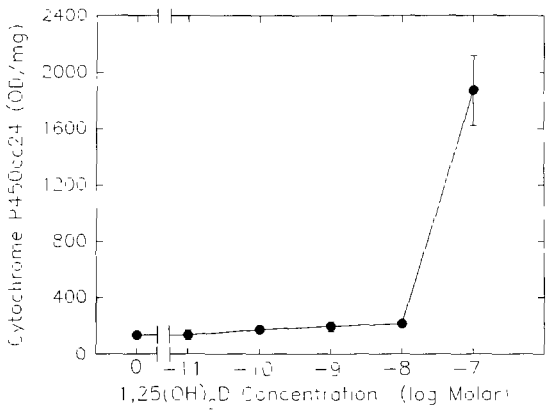

Fig. 2. Effect of dose of $1,25(OH)_2D$ on cytochrome P450cc24 mRNA levels. Flasks were treated with indicated concentration of $1,25(OH)_2D$ for 36 h, and mRNA levels were quantitated by dot blot. Data is expressed as the mean ± S.E. of 3 determinations. Only the mRNA levels of the cells treated with 10^{-7} M $1,25(OH)_2D$ were significantly higher than the control level (zero dose) (t-test, $P < 0.05$).

10-fold at 10^{-7} and 10^{-8} M $1,25(OH)_2D$ and about 7-fold at 10^{-9} M (Fig. 3B). Phorbol ester had no effect on actin mRNA levels.

4. DISCUSSION

These studies demonstrate that $1,25(OH)_2D$ acts directly on intestinal cells to increase cytochrome P450cc24 mRNA levels. This increase in P450cc24 expression probably accounts for the $1,25(OH)_2D$-dependent increase in 24-hydroxylase activity previously reported in intestinal CaCo-2 cells [11]. In both cases, the effects of $1,25(OH)_2D$ are seen as early as 6 h and are maximal at 24–36 h. In both cases, high concentrations of $1,25(OH)_2D$ (10^{-7} M) are needed to see maximal effects. This has led some investigators to conclude that the intestinal 24-hydroxylase may not be operative at physiological concentrations of $1,25(OH)_2D$ [11]. However, the experiments with phorbol esters (Fig. 3) demonstrate that it is possible to markedly increase intestinal cell responsiveness to $1,25(OH)_2D$ at physiological concentrations in vitro.

These studies also demonstrate that $1,25(OH)_2D$ and phorbol esters may interact with each other at the mRNA level. The large increase in P450cc24 mRNA in the presence of $1,25(OH)_2D$ and phorbol ester (Table I) may account for the additive effect of these two compounds on 24-hydroxylase activity in cultured renal cells [10]. An effect of $1,25(OH)_2D$ and phorbol ester on mRNA levels has been reported in one other cell type, U-937 mononuclear phagocytes [8]. In these studies, pretreatment with $1,25(OH)_2D$ followed by phorbol ester produced a marked increase in plasminogen activator inhibitor-2 mRNA levels. In all these studies, the phorbol ester was added after, rather than before, treatment with $1,25(OH)_2D$.

The mechanism by which phorbol ester increases intestinal sensitivity to $1,25(OH)_2D$ remains to be elucidated. Presumably, it involves protein kinase C, since the effect is blocked by H-7. A number of experimental findings suggest mechanisms by which $1,25(OH)_2D$ and phorbol esters could interact. These include the fact that phosphorylation of the vitamin D receptor by protein kinase C increases its activity [12] and that $1,25(OH)_2D$ itself increases phorbol ester binding sites [13]. Because of the magnitude of the phorbol ester effect, the IEC cell line should be useful in clarifying the relative contributions of these interactions.

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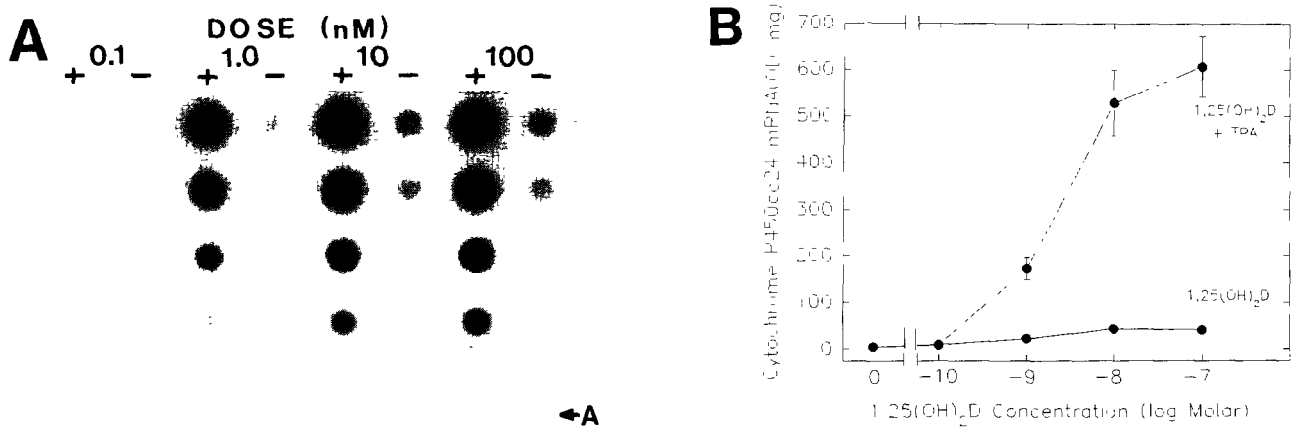

Fig. 3. Effect of phorbol ester and $1,25(OH)_2D$ on cytochrome P450cc24 mRNA levels. Flasks were treated with indicated concentration of $1,25(OH)_2D$ for a total of 7 h. During the last 2 h, 200 nM TPA (+) or vehicle only (−) was added. mRNA levels were quantitated by dot blot (Fig. A), and data is expressed as the mean ± S.E. of 3 determinations (Fig. B). mRNA levels of cells treated with TPA at 10^{-9}–10^{-7} M $1,25(OH)_2D$ were significantly higher than $1,25(OH)_2D$ alone (t-test, $P < 0.05$). Actin mRNA levels (A) are shown at bottom of Fig. A.

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