Abstract
Non-transformed rat intestinal epithelial cell (IEC) lines were used to study the action of 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) in the intestine. The capacity of 1,25(OH)₂D₃ to increase the expression of the cytochrome P450 component of the vitamin D 24-hydroxylase (CYP24) was determined in IEC-6 and IEC-18 cell lines. In IEC-6 cells, which are derived from crypt cells isolated from the whole small intestine, 1,25(OH)₂D₃ markedly increased expression of CYP24 protein and mRNA within 12 h. In contrast, in IEC-18 cells, which are derived from crypt cells from the ileum only, 1,25(OH)₂D₃ did not increase expression of CYP24 until 24–48 h. The maximal levels of CYP24 mRNA seen in the IEC-18 cells were only 31% of the maximal levels seen in the IEC-6 cells. In the presence of 1,25(OH)₂D₃, phorbol esters rapidly increased CYP24 mRNA levels in IEC-18 cells from almost undetectable to levels seen in IEC-6 cells. Protein kinase inhibitors abolished the stimulation by 1,25(OH)₂D₃ and by phorbol esters in both cell lines. Stimulation of mRNA levels by phorbol esters required new protein synthesis but stimulation by 1,25(OH)₂D₃ did not. These studies demonstrated that the rapid action of 1,25(OH)₂D₃ in IEC-6 cells is related to the activation of protein kinase C, an event which is missing in the IEC-18 cells. This differential response to 1,25(OH)₂D₃ probably takes place at a post-receptor site, since the number of vitamin D receptors in each cell line was found to be similar.

Introduction
The small intestine is a major target of 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃), the active metabolite of vitamin D (DeLuca 1988, Minghetti & Norman 1988). 1,25(OH)₂D₃ stimulates the synthesis of intestinal calbindin and the plasma membrane Ca pump, proteins thought to be involved in calcium absorption (Wasserman & Fullmer 1995, Armbrecht et al. 1999). In general, these actions take several hours and are mediated by the cytosolic vitamin D receptor (VDR). In addition, 1,25(OH)₂D₃ has been shown to alter the proliferation and differentiation of cell lines, including colon cells (Pols et al. 1990). Most of these effects are mediated by the cytosolic VDR. However, some effects of 1,25(OH)₂D₃ are rapid, may involve a membrane-bound rather than a cytosolic VDR, and may be mediated by the protein kinase C (PKC) pathway (van Leeuwen et al. 1992).

An important action of 1,25(OH)₂D₃ in the intestine is to increase intestinal 1,25(OH)₂D₃-24-hydroxylase activity. The 24-hydroxylase enzyme is found in tissues which are targets for 1,25(OH)₂D₃, and hydroxylation of 1,25(OH)₂D₃ is thought to be the first step in the degradation of 1,25(OH)₂D₃ (Kumar 1984). Thus, the activity of the 24-hydroxylase may regulate the action of 1,25(OH)₂D₃ in the intestine. 1,25(OH)₂D₃ markedly increases the mRNA levels of the cytochrome P450 component of the 24-hydroxylase (CYP24) in the intestine of intact animals (Armbrecht & Boltz 1991) and in intestinal cell lines (Armbrecht et al. 1993, Koyama et al. 1994).

1,25(OH)₂D₃ has been reported to increase CYP24 mRNA levels in both intestinal epithelial cells (IEC-18 (Armbrecht et al. 1993) and IEC-6 (Koyama et al. 1994). These are non-transformed crypt-like cells isolated from the whole small intestine (IEC-6) (Quaroni et al. 1979) or the ileum only (IEC-18) (Quaroni & Isselbacher 1981). In both cell lines, PKC was implicated in the action of 1,25(OH)₂D₃. Interestingly, the IEC-6 cells appeared to respond much more rapidly and robustly to 1,25(OH)₂D₃
compared with the IEC-18 cells. However, the experiments were performed in different laboratories and not side-by-side.

The first purpose of these studies was to directly compare the capacity of 1,25(OH)\textsubscript{2}D to stimulate the expression of CYP24 in each cell line. The second purpose was to characterize the responses in the two cell lines in order to elucidate the mechanisms responsible for the differential sensitivity. The responses were characterized in terms of the involvement of the PKC pathway and the requirement for new protein synthesis. The VDR levels in terms of the involvement of the PKC pathway and the requirement for new protein synthesis. The VDR levels and the CYP24 mRNA stability were also compared in the two cell lines.

Materials and Methods

The IEC-6 and IEC-18 cell lines were obtained from the American Type Culture Collection (Rockville, MD, USA) at passage 14. Cells were cultured in T25 flasks (Costar, Cambridge, MA, USA) with Dulbecco’s modified Eagle’s medium and F-12 nutrient medium (3:1) (Gibco, Grand Island, NY, USA) containing 5% fetal calf serum (HyClone, Logan, UT, USA) and 10 µg/ml insulin. Cells reached confluence in 4–6 days. To study the effects of 1,25(OH)\textsubscript{2}D, confluent cells were deprived of serum for 24 h. The 1,25(OH)\textsubscript{2}D, a kind gift from Dr Milan Uskokovic (Hoffmann-LaRoche, Nutley, NJ, USA), was added in ethanol (0·1% final concentration) at the time and dose indicated. When present, the phorbol ester phorbol 12-myristate 13-acetate (TPA) was added during the last 2 h of incubation. This protocol was found to give the optimal response to TPA (Armbrecht et al. 1993).

For CYP24 mRNA studies, cells were washed and stored frozen until assayed for mRNA levels. Total RNA was isolated using RNAzol B (Tel-Test, Inc., Friendswood, TX, USA). CYP24 mRNA levels were determined by Northern and dot blots as previously described (Armbrecht et al. 1993). Dot blots were performed by random priming the full-length clone for rat CYP24, kindly supplied by Drs Y Ohyama and K Okuda (Ohyama et al. 1991). CYP24 mRNA levels were normalized to total RNA. In some initial studies, blots were stripped and rehybridized with β-actin. These studies showed that sample loading was quite uniform, so total RNA was routinely used for normalization.

CYP24 protein levels were measured by Western blotting as previously described (Armbrecht et al. 1998). Briefly, cells were rinsed, scraped, resuspended in isolation buffer, and homogenized. After low speed centrifugation (500 g for 12 min), the resulting pellet was subjected to 10% SDS-PAGE. Proteins were transferred electrophoretically to nitrocellulose membrane, which was then incubated with a rabbit polyclonal antibody raised against a synthetic peptide to the first 12 N-terminal amino acids of the mature rat CYP24 (Chiron, San Diego, CA, USA). Following treatment with anti-rabbit IgG linked to horseradish peroxidase, antigen–antibody complexes were visualized by chemiluminescence using the Amersham ECL kit and X-ray film (Amersham, Arlington Heights, IL, USA). Bands on X-ray film were quantitated by densitometry.

VDR protein was measured by Western blotting of whole cell extracts. Cells were rinsed, scraped and resuspended in 2 ml sonicating buffer (25 mM NaCl, 1 mM HEPES, and 0·2 mM phenylmethylsulfonyl fluoride (PMSF), pH 8·0). Cells were centrifuged at 1600 g for 5 min, and the pellet was resuspended in 100 µl sonicating buffer and sonicated on ice for 5 min at 50% power using a Tekmar (Cincinnati, OH, USA) Model TM50 sonicator. SDS was then added to a final concentration of 0·2%, and 12% SDS-PAGE was performed. After transfer to nitrocellulose, membranes were pre-blotted for 2 h with goat anti-rat IgG (Sigma, St Louis, MO, USA). Membranes were then washed and incubated overnight with a monoclonal anti-vitamin D receptor antibody (9A7) (Affinity Bioreagents, Inc., Golden, CO, USA). Antigen–antibody complexes were visualized using goat anti-rat IgG conjugated to horseradish peroxidase, followed by ECL detection and densitometry.

The PKC distribution in IEC cells was determined as previously described for Caco-2 cells (Bissonnette et al. 1995). Cells were rinsed, scraped and resuspended in a sonicating buffer consisting of 20 mM Tris–HCl (pH 7·5), 2 mM EGTA, 6 mM β-mercaptoethanol, 1 mM PMSF, and 50 µM leupeptin. The resuspended cells were sonicated for 30 s and then centrifuged at 140 000 g for 30 min. The supernatant was used for soluble PKC determination, and the pellet was used for particulate PKC determination. The PKC-α protein content of each fraction was determined by Western blotting, using a polyclonal antibody to PKC-α (Calbiochem, San Diego, CA, USA) and ECL detection.

Data are reported as the mean ± s.e. of the number of flasks indicated. 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.

Results

The effect of 1,25(OH)\textsubscript{2}D on CYP24 mRNA levels was determined in IEC cells by Northern and dot blotting (Fig. 1). Northern blotting revealed that 1,25(OH)\textsubscript{2}D induced a single band with a size of 3·5 kb in both cell lines (Fig. 1A). Since only a single band was induced, dot blots were used to quantitate the time-course and relative magnitude of 1,25(OH)\textsubscript{2}D induction (Fig. 1B). Dot blots were quantitated in the linear range as a function of time and cell type (Fig. 1C). 1,25(OH)\textsubscript{2}D rapidly increased CYP24 mRNA levels in IEC-6 cells with a maximum at
In IEC-18 cells, the response to 1,25(OH)$_2$D was much slower with little increase until 24 h. The CYP24 mRNA levels at 48 h in the IEC-18 cells were 31% of those seen in the IEC-6 cells at 12 h. In IEC-18 cells, maximal CYP24 mRNA levels are seen at 36–48 h (Armbrecht et al. 1993).

The shape of the 1,25(OH)$_2$D dose–response curve was compared between IEC-6 and IEC-18 cells (Fig. 2). Both cell lines showed a sigmoidal dose–response curve. There was a sharp increase in CYP24 mRNA levels between 10 and 100 nM. The EC$_{50}$ was about 30 nM for both the IEC-6 and the IEC-18 cells.

CYP24 mRNA stability was measured in the two cell lines in the presence of 1,25(OH)$_2$D (Fig. 3). There was no significant difference in the decay of CYP24 mRNA in the two cell lines. This suggests that the major effect of 1,25(OH)$_2$D is to increase gene transcription rather than to increase mRNA stability.

Since there were major differences in the induction of mRNA levels, the effect of 1,25(OH)$_2$D on CYP24 protein levels was determined in IEC cells (Fig. 4). 1,25(OH)$_2$D rapidly increased CYP24 protein levels in
IEC-6 cells with a maximum at 12–24 h. In IEC-18 cells, the response was much slower with little increase until 48 h. In general, the effect of 1,25(OH)\(_2\)D on protein levels paralleled that seen on mRNA levels in the two cell lines (Fig. 1C). This suggests that CYP24 protein levels in IEC cells largely reflect the mRNA levels.

To characterize the mechanism by which 1,25(OH)\(_2\)D increased CYP24 mRNA levels, the effects of phorbol esters and protein kinase inhibitors on 1,25(OH)\(_2\)D action were determined. The phorbol ester TPA, which activates PKC, potentiated the action of 1,25(OH)\(_2\)D in both IEC-6 and IEC-18 cells (Fig. 5). In the presence of 1,25(OH)\(_2\)D, TPA modestly increased CYP24 mRNA levels in the IEC-6 cells. In IEC-18 cells, TPA markedly increased CYP24 mRNA levels to levels comparable with those seen in IEC-6 cells. TPA alone had no effect on CYP24 mRNA levels (data not shown). The protein kinase inhibitor H-7 completely inhibited the effect of 1,25(OH)\(_2\)D and the effect of TPA in both cell lines. Since phorbol esters and H-7 modulate PKC, the PKC distribution in IEC cells was determined. In IEC-18 cells, 62 ± 3% of the PKC was in the particulate fraction. In the IEC-6 cells, 33 ± 5% was in the particulate fraction, which was significantly less (P<0.05, t-test).

To confirm the role of PKC in the action of 1,25(OH)\(_2\)D, the effects of several additional activators and inhibitors of PKC were studied (Table 1). In the first experiment, the effect of the PKC activator 1,2-dioctanoyl-sn-glycerol (DOG) was studied. DOG markedly increased CYP24 mRNA to levels similar to those seen with TPA in both cell lines (Table 1, top). In the second experiment, the effect of inhibitors of PKC were compared. Like H-7, the PKC inhibitors bisindolylmaleimide I (BIS) and staurosporin almost completely blocked the stimulatory effect of TPA (Table 1, bottom).
These results further implicate PKC in the action of 1,25(OH)₂D in both cell lines. The role of protein synthesis in the action of 1,25(OH)₂D and phorbol esters in IEC cells was determined using the protein synthesis inhibitor cycloheximide. Cycloheximide significantly increased CYP24 mRNA levels in both cell lines in the presence of 1,25(OH)₂D (Fig. 6). Cycloheximide and TPA independently increased CYP24 mRNA to similar levels in the presence of 1,25(OH)₂D. Interestingly, cycloheximide inhibited the effect of phorbol ester when the two were added together. However, there was no difference in the pattern of action of cycloheximide between the two cell lines.

Finally, the levels of VDR protein in the two cell lines were determined. Western blotting of nuclear extracts of the two cell lines showed a major band at 57 kDa (Fig. 7). This band co-migrated with the VDR band seen in MG-63 cells (not shown). Quantitation of the 57 kDa band yielded 5·65 ± 1·21 and 7·80 ± 3·17 OD/mg nuclear protein for IEC-6 and IEC-18 cells respectively (n=4 flasks). This was not significantly different.

Discussion

These studies demonstrated that the IEC-6 cells are much more responsive to 1,25(OH)₂D in terms of increasing the expression of CYP24 than the IEC-18 cells. The IEC-6 cells responded more rapidly and to a greater degree with regard to both CYP24 mRNA levels and protein levels.

Table 1 Effect of activators and inhibitors of PKC on 1,25(OH)₂D action. Values are means ± s.e. of four flasks

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<tr>
<th>Inhibitor</th>
<th>CYP24 mRNA levels (%)</th>
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<tr>
<td></td>
<td>IEC-6</td>
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<td>Activator</td>
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<td>—</td>
<td>100</td>
</tr>
<tr>
<td>DOG</td>
<td>280 ± 25*</td>
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<td>TPA</td>
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CYP24 mRNA levels are expressed as a percent of no activator (top experiment) or as a percent of TPA alone (bottom experiment). Cells were incubated with 100 nM 1,25(OH)₂D for 6 h under all conditions. The PKC activators TPA (200 nM) and DOG (10 μM) were added during the final 2 h. The PKC inhibitors H-7 (10 nM), BIS (1 μM), and staurosporin (200 nM) were present for the entire 6 h, following a 30-min preincubation. n.d., not detectable.

*Significantly different from no activator (P<0·05, t-test); **significantly different from TPA alone (P<0·05, t-test).
over the time-period studied (Figs 1 and 4). The increased protein levels have physiological significance in that 1,25(OH)_{2}D also stimulates 24-hydroxylase activity, as measured by the conversion of 25-hydroxyvitamin D (25(OH)D) to 24,25(OH)_{2}D, in a parallel fashion in these cells (authors’ unpublished studies). The difference in 1,25(OH)_{2}D response appears to be at the transcriptional level, since there is no difference in CYP24 mRNA stability between the two cell lines (Fig. 3). The CYP24 promoter has been previously shown to contain two active vitamin D response elements which make the gene highly responsive to 1,25(OH)_{2}D (Ohyama et al. 1994).

Several lines of evidence suggest that these effects of 1,25(OH)_{2}D are specific. First, 1,25(OH)_{2}D can increase CYP24 mRNA levels at concentrations as low as 1 nM when longer incubation times are used (Armbrecht et al. 1993, Koyama et al. 1994). In addition, in the presence of phorbol esters, 0·1 nM 1,25(OH)_{2}D is effective (Armbrecht et al. 1993). Finally, the effects of 25(OH)D and 24,25(OH)_{2}D at a concentration of 10^{-7}M are only 16% and 9% respectively of 1,25(OH)_{2}D in IEC-18 cells (authors’ unpublished studies).

These studies suggest that the differential responsiveness of the IEC cells is not due to differences in VDR content. The VDR content of the two cell lines is similar (Fig. 7) with the content of the IEC-18 cells being slightly higher, if anything. However, these studies do not rule out the possibility that the phosphorylation state of the VDR may be different in the two cell lines. Phosphorylation of VDR has been shown to alter its function (Hsieh et al. 1991).

Several lines of evidence suggest that differences in the PKC-dependent pathway may underlie the differential response of IEC-6 and IEC-18 cells to 1,25(OH)_{2}D. First, phorbol esters in the presence of 1,25(OH)_{2}D markedly increase CYP24 mRNA levels in IEC-18 cells to those seen in IEC-6 cells (Fig. 5). That both 1,25(OH)_{2}D and phorbol esters act through PKC-dependent pathways is suggested by the fact that specific PKC inhibitors block the action of both compounds (Table 1). Therefore, it may be that the capacity of 1,25(OH)_{2}D to activate the PKC-dependent pathway is decreased in IEC-18 cells. However, phorbol esters, which directly activate PKC pathways, can augment the action of 1,25(OH)_{2}D to the levels seen in IEC-6 cells.

Secondly, the percentage of PKC in the particulate fraction was almost twice as high in IEC-18 cells as in IEC-6 cells (62 ± 3 vs 33 ± 5%). In isolated rat colonocytes, 1,25(OH)_{2}D stimulates the movement of PKC-α from the soluble to the particulate fraction (Bissonnette et al. 1995). Movement of PKC from the cytosol to the membrane is associated with its activation as part of a signal transduction pathway. However, colonocytes isolated from vitamin D-deficient rats have a significantly higher percentage of PKC associated with the membrane. Addition of 1,25(OH)_{2}D to these colonocytes produces no further movement of PKC from the soluble to the particulate form. Based on these studies, the high percentage of PKC already associated with the membrane in IEC-18 cells could produce a refractoriness to 1,25(OH)_{2}D action in terms of further translocation of PKC.

The protein synthesis inhibitor cycloheximide had similar effects in both IEC-6 and IEC-18 cells. This suggests that the differences in the response of IEC cells to 1,25(OH)_{2}D are not due to differences in protein synthetic pathways. In both cell lines, cycloheximide significantly increased the effect of 1,25(OH)_{2}D on CYP24 mRNA levels (Fig. 6). This suggests that ongoing protein synthesis is not necessary for the action of 1,25(OH)_{2}D in these cell lines. The fact that CYP24 mRNA levels are increased in the presence of cycloheximide suggests that there is a rapidly turning over protein which normally down-regulates CYP24 mRNA levels. This is different from the effect of cycloheximide in other cell lines. In NRK-52E renal tubular cells (Armbrecht et al. 1997) and in UMR-106 osteoblastic cells (authors’ unpublished studies), cycloheximide inhibits the capacity of 1,25(OH)_{2}D to increase CYP24 mRNA levels. This suggests that ongoing protein synthesis is needed for the action of 1,25(OH)_{2}D in these cell lines. More studies are needed to determine whether the action of 1,25(OH)_{2}D is
actually tissue specific or whether these differences are reflections of the individual cell lines studied.

Although the action of 1,25(OH)\(_2\)D in IEC cells does not require new protein synthesis, it does require protein kinase activity, since the effect of 1,25(OH)\(_2\)D is blocked by H-7 (Fig. 5). In this way, IEC cells are similar to renal tubular cells (Armbrecht et al. 1997) but different from osteoblastic cells, in which phorbol esters have no effect (Armbrecht & Hodam 1994). Interestingly, cycloheximide inhibited the effect of phorbol esters in both cell lines (Fig. 6). This suggests that the pathway by which the phorbol esters potentiate the action of 1,25(OH)\(_2\)D is dependent on new protein synthesis. However, the action of 1,25(OH)\(_2\)D alone is not dependent on new protein synthesis.

It is tempting to speculate that the differential responsiveness of the IEC cells to 1,25(OH)\(_2\)D may reflect their origin. IEC-6 cells were derived from crypt cells isolated from the whole length of the small intestine (Quaroni et al. 1979). On the other hand, IEC-18 cells were derived from crypt cells isolated from the ileum only (Quaroni & Isselbacher 1981). In general, the ileum is much less responsive to 1,25(OH)\(_2\)D than the duodenum in terms of induction of calcium transport proteins (Armbrecht et al. 1999). Although some of this difference may be due to the decreased VDR content of the ileum, these present studies suggest that post-receptor differences in the intestine may also be important.

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