Interrelationship between signal transduction pathways and 1,25(OH)2D3 in UMR106 osteoblastic cells

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Yang, Wen, Sven Johan Hyllner, and Sylvia Christakos. Interrelationship between signal transduction pathways and 1,25-dihydroxyvitamin D3 [1,25(OH)2D3] action was examined in UMR106 osteoblastic cells. Treatment of these cells with 8-bromo-cAMP (1 mM) resulted in an upregulation of the vitamin D receptor (VDR) and an augmentation in the induction by 1,25(OH)2D3 of 25(OH)D3 24-hydroxylase [24(OH)ase] and osteopontin (OPN) mRNAs as well as gene transcription. Transfection with constructs containing the vitamin D response element devoid of other promoter regulatory elements did not alter the cAMP-mediated potentiation, suggesting that cAMP-enhanced transcription is due, at least in part, to upregulation of VDR. Treatment with phorbol ester [12-O-tetradecanoyl-phorbol-13-acetate (TPA) 100 nM], an activator of protein kinase C, significantly enhanced 1,25(OH)2D3-induced OPN mRNA and transcription but had no effect on VDR or on 24(OH)ase mRNA or transcription. Studies using OPN promoter constructs indicate that TPA-enhanced OPN transcription is mediated by an effect on the OPN promoter separate from an effect on VDR. Thus interactions with signal transduction pathways can enhance 1,25(OH)2D3 induction of 24(OH)ase and OPN gene expression, and, through different mechanisms, changes in cellular phosphorylation may play a significant role in determining the effectiveness of 1,25(OH)2D3 on transcriptional control in cells expressing skeletal phenotypic properties.

osteonopontin; 25-hydroxyvitamin D3 24-hydroxylase; vitamin D receptor; protein kinase A; protein kinase C

There is increasing evidence that the different pathways involved in steroid receptor action and second messenger signaling do not function independently. The action of steroid receptors can be influenced, for example, by an increase in the activity of cAMP-dependent protein kinase A (PKA) or phospholipase/protein kinase C (PKC). PKA has been reported to enhance steroid hormone-dependent transcriptional activation, to phosphorylate a number of steroid receptors, and to switch steroid receptor antagonists into agonists (5, 13, 21, 25, 26, 35). However, activation of PKC and activation protein (AP1) transcription factors has been reported to result in inhibitory or stimulatory effects on steroid receptor-mediated gene expression and transcriptional activation (1, 8, 21, 24, 40, 41). Although these findings suggest an interrelationship between signal transduction pathways and steroid hormone action, the mechanisms underlying these interactions are not well understood. They may involve an effect on the regulation of the steroid receptor, on the promoter of the target gene through AP1 sites or cAMP response elements (CREs), or an effect on other transcription factors.

With regard to the regulation of calcium homeostasis, cooperativity between second messenger systems and steroid hormone action is indicated by the interrelationship between parathyroid hormone (PTH) and 1,25-dihydroxyvitamin D3 [1,25(OH)2D3] action. PTH, a peptide hormone that can stimulate both PKA and PKC activity (19, 33, 45), and 1,25(OH)2D3, which acts analogously to the steroid hormones, are the major, physiologically important calcitropic hormones that act in an interrelated manner. Under conditions of hypercalcemia, PTH enhances the synthesis of 1,25(OH)2D3 in the kidney (18). In addition, 1,25(OH)2D3 potentiates PTH-dependent calcium transport in the distal tubule, and PTH has been reported to sensitize long bones to stimulation of resorption by 1,25(OH)2D3 (15, 46). Because the receptors for both hormones are found in osteoblasts rather than in osteoclasts, there is general agreement that 1,25(OH)2D3 and PTH regulate bone resorption via a primary action on the osteoblast that includes, in part, the stimulation of osteoclast-differentiating factor (or RANK ligand; Ref. 50). Cross talk between 1,25(OH)2D3 and signal transduction pathways is also noted by the modulation of the expression of 1,25(OH)2D3 target proteins by phorbol ester [12-O-tetradecanoyl-phorbol-13-acetate (TPA)], PTH, growth factors, and activation of PKA. For example, PKA has been reported to upregulate VDR, and fibroblast growth factor and activation of PKC have been reported to downregulate vitamin D receptor (VDNR) in NIH 3T3 mouse fibroblasts (24, 25). In addition, PTH, mainly due to the activation of PKA, has been reported.

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to enhance VDR abundance in osteoblastic cells (2, 23). Because up- or downregulation of VDR in these studies resulted in a corresponding alteration of a functional response, it was suggested that regulation of VDR by signal transduction pathways plays an important role in modulating cell responsiveness to 1,25(OH)2D3. However, opposite findings concerning the effect of PKA and PKC on VDR have also been reported (37, 38). Thus effects of signaling pathways not only on the VDR but also on endogenous VDR function and on the promoter of the 1,25(OH)2D3 target genes need to be considered.

To investigate the interaction between signaling pathways and 1,25(OH)2D3 action, we examined, in UMR106 osteoblastic cells, the effect of activation of PKA or PKC on the modulation of the expression of 24(OH)ase and osteopontin (OPN), target genes regulated by 1,25(OH)2D3 in bone. We addressed the mechanisms of these effects by use of transfected reporter constructs. Previous studies have suggested functions for 24(OH)ase and OPN in bone (44, 12). In bone, a major function of 24(OH)ase is to inactivate 1,25(OH)2D3, preventing elevated intracellular 1,25(OH)2D3 levels that may adversely affect mineralization (44). Thus 1,25(OH)2D3 self-induces its metabolism by inducing the 24(OH)ase enzyme. Recent studies by St-Arnaud et al. (44) indicated that mice deficient in 24(OH)ase have markedly elevated 1,25(OH)2D3 levels and impaired bone formation at specific sites. It was suggested that elevated 1,25(OH)2D3 levels, acting through VDR at specific sites, were responsible for the abnormalities in bone. OPN, also induced in response to 1,25(OH)2D3, is a highly phosphorylated, secreted protein, abundant in the bone matrix, which has been reported to modulate both resorption and mineralization (12). Our findings demonstrate for the first time that activation of PKA and PKC can enhance 1,25(OH)2D3 induction of OPN in cells expressing skeletal phenotypic properties and that the enhancement by PKC is due to an effect on the OPN promoter separate from an effect on VDR. Thus the effect of second messenger signaling pathways on 1,25(OH)2D3-regulated genes is not necessarily correlated with a similar change in VDR. However, the enhancement by cAMP of 1,25(OH)2D3-induced OPN and 24(OH)ase transcription was not due to an effect on a CRE in the promoter of these genes but rather may be due, at least in part, to upregulation of VDR. Thus our data indicate that interactions with signal transduction pathways can enhance 1,25(OH)2D3 induction of 24(OH)ase and OPN gene expression, and, through different mechanisms, cellular phosphorylation may play a significant physiological role in determining the effectiveness of 1,25(OH)2D3 action in mineral homeostasis.

MATERIALS AND METHODS

Materials. [14C]chloramphenicol (50 mCi/mmol) and [32P]dCTP (3,000 Ci/mmol, 370 MBq/ml) were obtained from Du Pont-New England Nuclear Products (Boston, MA). Oligonucleotides, RadPrime DNA labeling system, and all restriction enzymes were purchased from GIBCO-BRL-Life Technologies (Gaithersburg, MD). Biotrans nylon membranes were obtained from ICN Biochemicals (Costa Mesa, CA). Oligo(dT) cellulose was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). 8-Bromo-cAMP, acetyl-coenzyme A, and phosphor-12-myristate-13-acetate (PMA) were obtained from Sigma (St. Louis, MO). Phenol, formamide, and guanidinium thiocyanate were purchased from International Biotechnologies (IBI, New Haven, CT). Rat anti-vitamin D receptor antibody was from Affinity BioReagents (Neshanic Station, NJ). Chemically synthesized 1,25(OH)2D3 was provided by Dr. M. Uskokovic of Hoffmann-LaRoche (Nutley, NJ).

Northern blot analysis. Total RNA was prepared from UMR106–01 cells by the guanidium thiocyanate-phenol-chloroform method described by Chomczynski and Sacchi (9). Polyadenylated [poly(A+)] RNA was isolated by oligo(deoxythymidine)-cellulose chromatography. Northern blot analysis was performed as described previously (48). The blots were hybridized to specific 32P-labeled cDNA probes for 16 h at 42°C, washed, and subjected to autoradiography as previously described (48). Labeled probes were prepared using the RadPrime DNA labeling system according to the random primer method (4). To be normalized for sample variation, blots were probed with 32P-labeled β-actin cDNA. The blots were air dried and exposed to Kodak XAR-5 film at −80°C in the presence of intensifying screens. All autoradiograms were analyzed by densitometric scanning using the Dual-Wavelength Flying Spot Scanner (Shimadzu Scientific Instruments, Princeton, NJ). The relative optical densities obtained using the test probes were divided by the relative optical density obtained after probing with the control probe (β-actin) to normalize for sample variation.

Cell culture, cell transfection and assay of chloramphenicol acetyltransferase activity. UMR106–01 cells were obtained from the American Type Culture Collection. These cells were maintained in DMEM-F-12 (GIBCO-BRL-Life Technologies) supplemented with 5% heat-inactivated fetal bovine serum (FBS) (Gemini, Calabasas, CA) at 37°C in a 5% CO2 atmosphere. Cells were grown to 60–70% confluence in 100-mm tissue culture dishes, and 24 h before the start of experiments, medium was changed to 2% charcoal-stripped serum-containing medium. Cells were treated with vehicle or the compounds noted at the concentrations indicated for various times (1–24 h). Studies using different concentrations of TPA or 8-bromo-cAMP indicated that the enhancement of 1,25(OH)2D3 induction of OPN mRNA by TPA was maximal at 30 nM TPA (a plateau in responsiveness was observed between 25 and 100 nM) and that enhancement of 1,25(OH)2D3 induction of both OPN and 24(OH)ase mRNA was maximal at 1 mM 8-bromo-cAMP. Thus maximally effective concentrations of TPA (100 nM) and 8-bromo-cAMP (1 mM) were used. For transfections, cells were plated at a density of 1 × 106 cells/100-mm plate 24 h before transfection. UMR106–01 cells were cotransfected with reporter plasmid (8 μg) and the β-galactosidase expression vector pCH110 (4 μg; from Pharmacia), an internal control for transfection efficiency, by use of the calcium phosphate DNA precipitation method (4). Cells were transfected for 16 h, shocked for 1 min with 10% dimethyl sulfoxide in phosphate-buffered saline (PBS), washed with PBS, and treated for 24 h with vehicle (0.1% ethanol) or test compound at the concentrations indicated. After 24-h incubation, cells were harvested, and cell extracts were prepared by freeze-thaw (−80°C freeze, 37°C thaw; three times, 5 min each). The chloramphenicol acetyltransferase (CAT) assay was per-
formed at constant β-galactosidase activity with standard protocols (4, 16) and quantitated by densitometric scanning of TLC autoradiograms. For some experiments, several autoradiographic exposure times were needed for densitometric analysis. CAT activity was also quantitated by scanning TLC plates by means of the Packard Constant Image System (Packard Instrument, Meriden, CT).

**Complementary DNA probes, 24(OH)ase, and OPN-CAT constructs.** The 1-kb mouse OPN cDNA was generated by digestion with HindIII and was a gift from D. Denhardt (Rutgers University, Piscataway, NJ) (42). A 1.7-kb rat VDR cDNA was obtained by digestion of pIBI76 with EcoRI (34). The 3.2-kb rat 24(OH)ase cDNA was obtained by digestion of pLBI76 with EcoRI (34). The 24(OH)ase promoter (137) of the rat 24(OH)ase promoter with the CAT plasmid and was a gift from K. Okuda (Hiroshima University School of Dentistry, Hiroshima, Japan) (31). β-Actin cDNA was from Clontech (Palo Alto, CA).

For transfection studies, constructs of a chimeric gene in which the rat 24(OH)ase promoter (−671/+74; containing both vitamin D response elements (VDREs) at −258/−244 and −151/−137) was linked to the CAT gene, as previously described (22), were used. A proximal rat 24(OH)ase VDR thymidine kinase (tk) CAT reporter construct, prepared by introducing multiple copies of the proximal VDRE (−151/−137) of the rat 24(OH)ase promoter with the CAT plasmid (49), was also used. The mouse OPN promoter CAT construct (−777/+74; OPN VDRE at −757/−743) was obtained from D. Denhardt (10). The OPN VDRE tk-CAT construct, containing multiple copies of the OPN VDRE, was a gift of Dr. L. Freedman (Sloan Kettering Cancer Center, New York, NY).

**Western blot analysis.** For nuclear extracts for Western blot analysis of VDR, cells were harvested by trypsinization and washed with PBS. Each dish of cells was resuspended in 1 ml of sonication buffer [10 mM Tris, pH 7.4, 1.5 mM EDTA, and 1.0 mM dithiothreitol (TED), 10 mM sodium molybdate, 500 KIU/ml Trasylol, 300 μM phenylmethylsulfonylfluoride (PMSF), and 200 μg/ml soybean trypsin inhibitor] and sonicated. Cell extracts were centrifuged at 12,000 g for 10 min at 4°C. Crude nuclear pellets were resuspended in 5 ml of washing buffer (TED, 300 mM PMSF, and 0.5% Triton X-100), vortexed, and centrifuged at 13,000 g. After the third wash, the pellets were resuspended in 1 ml of resuspension buffer (0.3 M KCl, TED, 10 mM sodium molybdate, 500 KIU/ml Trasylol, 300 μM PMSF) and vortexed well. The protein concentration was determined by the method of Bradford (6). Protein (30 μg) from each sample was used for electrophoresis on a 12% SDS polyacrylamide gel. After electrophoresis, proteins were transferred electrophoretically to a nitrocellulose membrane (Hybond-ECL; Amersham) that was incubated with anti-VDR monoclonal antibody 9A7 (1:2,000 dilution) in Tris-buffered saline (TBS; pH 7.5) for 12 h at 4°C. After washing with TBS, the membrane was incubated with secondary antibody [goat anti-rat IgG conjugated to horseradish peroxidase (Sigma), 1:2,000 dilution] for 1 h at room temperature. After being washed with TBS, the antigen-antibody complex was detected using the enhanced chemiluminescent Western blotting detection system (Amerham) according to the manufacturer’s protocol.

**Statistical analysis.** Results are expressed as means ± SE, and significance was determined by Student’s t-test for two-group comparison or analysis of variance (ANOVA) for multiple group comparison. In conjunction with ANOVA, a post-test analysis by Dunnett’s multiple t-statistic was used with a significance level of 0.05.

**RESULTS**

To explore the mechanisms by which 1,25(OH)2D3 and signal transduction pathways coordinately regulate the expression of vitamin D3-responsive genes, we examined the effects of 1,25(OH)2D3, 8-bromo-cAMP (an activator of PKA), and TPA (an activator of PKC) on the mRNA expression of VDR, 24(OH)ase, and OPN in UMR106–01 cells (Fig. 1). UMR106–01 cells were treated with 1,25(OH)2D3 (10−8 M) in the presence or absence of 8-bromo-cAMP (1 mM) or TPA (100 nM). The treated cells were harvested at the indicated time points and mRNA was isolated. Results of Northern blot analyses are shown in Fig. 1. The first significant induction of VDR mRNA by 1,25(OH)2D3 was observed at 9 h. In the presence of both cAMP and 1,25(OH)2D3, VDR mRNA was induced as early as 3 h. Thus cAMP enhanced the rapidity of VDR mRNA expression induced by 1,25(OH)2D3. 8-Bromo-cAMP also enhanced the response of 24(OH)ase and OPN mRNA to 1,25(OH)2D3. The time course of response of VDR and 24(OH)ase mRNAs to 1,25(OH)2D3 was similar in the presence or absence of TPA, indicating that the PKC signaling pathway does not affect VDR and 24(OH)ase expression in UMR106 cells. However, TPA shifted the time course of response of OPN to 1,25(OH)2D3 to the left and resulted in an increase in the response of OPN to 1,25(OH)2D3 (Fig. 1).

The modulation of 1,25(OH)2D3 induction of VDR, 24(OH)ase, and OPN by PKA and PKC signaling pathways was further characterized in experiments performed using different concentrations of 1,25(OH)2D3 (Fig. 2, A, B, and D). Cells were treated for 9 h in these studies, because enhancement by cAMP of the 1,25(OH)2D3 response [VDR, OPN, and 24(OH)ase mRNAs] was observed at this time, and enhancement by TPA of 1,25(OH)2D3 induction of OPN mRNA was also observed after treatment for 9 h (Fig. 1). 1,25(OH)2D3 treatment (10−10−10−7 M) of UMR106 cells resulted in an upregulation of VDR mRNA, and cAMP enhanced the 1,25(OH)2D3 induction at all concentrations of 1,25(OH)2D3. 8-Bromo-cAMP also potentiated the induction of 24(OH)ase mRNA by 1,25(OH)2D3. In addition, the induction of OPN mRNA by 1,25(OH)2D3 was increased by cAMP but only in the presence of higher concentrations of 1,25(OH)2D3 [10−8−10−7 M]. In contrast to cAMP, TPA had no effect at any concentration of 1,25(OH)2D3 on VDR or 24(OH)ase gene expression. However, TPA enhanced OPN expression at all concentrations of 1,25(OH)2D3 (Fig. 2, A, B, and D). The effect of TPA in enhancing 1,25(OH)2D3-induced OPN expression appeared to be independent of the 1,25(OH)2D3 concentration. We also noted that 8-bromo-cAMP alone induced VDR mRNA levels but not 24(OH)ase or OPN mRNA levels (Fig. 2, C and D). TPA alone had no effect on VDR or 24(OH)ase gene expression. However, OPN mRNA is induced by TPA alone (Fig. 2, C and D).

Northern analysis of the time course of response indicated that, in the presence of cAMP, VDR mRNA is
induced by 1,25(OH)_{2}D_{3} before 24(OH)ase and OPN mRNAs, suggesting that cAMP may mediate the enhanced induction of these target genes by upregulating VDR levels. Therefore, we examined the effects of cAMP, TPA, and 1,25(OH)_{2}D_{3} on VDR protein expression (Fig. 3). We observed that 1,25(OH)_{2}D_{3} and cAMP increased VDR protein levels by 3.3- and 3.8-fold, respectively, and that TPA did not change VDR protein levels. These findings suggest that cAMP may enhance 1,25(OH)_{2}D_{3}-induced gene expression, at least in part, by upregulation of VDR levels.

To determine whether effects on the promoter, separate from effects on VDR, could account for at least part of the effects of the second messenger systems, UMR106 cells were transiently transfected with plasmids incorporating various regions of the rat 24(OH)ase or mouse OPN promoter linked to CAT. By use of the 24(OH)ase promoter construct −671/+74 phCAT that contains both VDREs and three consensus CREs (determined by computational analysis) (22, 49), cAMP (1 mM 8-bromo-cAMP) was found to significantly potentiate the dose-dependent activation of transcription by 1,25(OH)_{2}D_{3}, indicating that the 24(OH)ase modulation by cAMP of 24(OH)ase mRNA is at the transcriptional level (Fig. 4, top). Transfection of UMR106 cells with a construct containing multiple copies of the proximal 24(OH)ase VDRE devoid of other 24(OH)ase promoter regulatory elements did not alter the cAMP-mediated potentiation of 1,25(OH)_{2}D_{3}-dependent 24(OH)ase transcription (Fig. 4, bottom). In contrast, TPA had no significant effect on activation by 1,25(OH)_{2}D_{3} of 24(OH)ase transcription (Fig. 4, top and bottom). By use of the −777/+79 phCAT OPN promoter construct (VDRE at −757/−743 and AP1 sites at −9/−3 and −75/−69) (29, 10), both cAMP and TPA potentiated the activation of transcription of the OPN gene by 1,25(OH)_{2}D_{3} (Fig. 5, top). However, experiments done using an OPN VDRE tk-CAT construct indicated that this construct responds to 1,25(OH)_{2}D_{3} and cAMP but not to TPA (Fig. 5, bottom). Thus the VDRE of the mouse OPN gene, in the absence of other sequences in the OPN promoter, is sufficient to confer cAMP, but not TPA, enhancement.
DISCUSSION

Our results suggest that, through different mechanisms, changes in cellular phosphorylation mediated by activation of PKA or PKC play an important role in determining the effectiveness of 1,25(OH)₂D₃ action in osteoblastic cells. In our study, treatment with either cAMP or TPA resulted in an enhancement of 1,25(OH)₂D₃-induced OPN expression, although cAMP resulted in an upregulation of VDR but treatment with TPA did not. Thus cAMP and TPA do not necessarily have opposite effects on 1,25(OH)₂D₃ action, and the enhancement of VDR function by second messenger signaling pathways is not necessarily correlated with a similar increase in VDR levels. In previous studies, modulation by second messenger signaling pathways of 1,25(OH)₂D₃ induction of osteocalcin transcription was examined in NIH 3T3 mouse fibroblasts transfected with the human osteocalcin VDRE (−558/−338) fused to a tk-CAT construct. Up- or downregulation of VDR in NIH 3T3 cells by forskolin or PMA treatment respectively resulted in a corresponding enhancement or attenuation of 1,25(OH)₂D₃-induced transcription (24, 25). Although TPA treatment resulted in an inhibition of 1,25(OH)₂D₃-induced transcription with the human osteocalcin VDRE (24), in our study we found that 1,25(OH)₂D₃-induced OPN expression and transcription were enhanced by TPA in UMR106 cells. Previous studies have also noted an enhancement of OPN expression after TPA treatment, and functional AP1 sites in the OPN promoter have been identified.
Thus, in our study, promoter-specific effects contributed more significantly than receptor regulation to modulation of vitamin D-induced OPN transcription by PKC. The effect of TPA was gene specific, since 1,25(OH)2D3-induced 24(OH)ase expression and transcription were unaffected by TPA treatment.

Concerning the cAMP enhancement of 1,25(OH)2D3-induced 24(OH)ase transcription, the 24(OH)ase VDRE tk-CAT construct was able to confer cAMP enhancement, suggesting that regions in the 24(OH)ase promoter other than the VDRE are not primarily involved in the cAMP responsiveness. An OPN VDRE tk-CAT construct was also able to confer cAMP responsiveness (Fig. 5). Because 8-bromo-cAMP treatment resulted in an increase in VDR protein levels as well as an enhancement of 1,25(OH)2D3 induction of VDR mRNA levels in UMR cells, regulation of VDR levels by cAMP may be one mechanism by which cAMP modulates 1,25(OH)2D3-induced transcription of 24(OH)ase and OPN. Similar to our studies, Krishnan et al. (23) and Van Leeuwen et al. (47) also found that changes in VDR by agents that raise intracellular cAMP in UMR106 cells correspond to an enhanced functional response. In addition, preliminary studies in our laboratory using osteoblastic cells isolated from calvaria of 3- to 4-day-old mice indicate that upregulation of VDR mRNA by cAMP as well as the cAMP-mediated enhancement of 24(OH)ase and OPN mRNAs can be observed in primary osteoblasts as well as in UMR cells (K. Gengaro, M. Huening, and S. Christakos, unpublished observation). Because CREs have been identified by sequence homology in the promoter of the VDR gene (28), it will be of interest in future studies to determine the role of transcriptional regulation of VDR by cAMP in the interaction between the PKA signaling pathway and 1,25(OH)2D3. Because the time course and magnitude of the enhancement by cAMP differed for 24(OH)ase and OPN mRNAs (Figs. 1 and 2), more than one mechanism may be involved in the effect of cAMP. Phosphorylation of VDR or phosphorylation of other coactivators involved in VDR-mediated transcription may also play a role in the enhancement by cAMP of 1,25(OH)2D3-induced target gene expression. Because the enhancement by cAMP is observed even after 24 h of treatment for OPN gene expression but...
only at earlier times for 24(OH)ase gene expression, it is also possible that the PKA signaling pathway may have effects on the stability of OPN mRNA. Effects of PTH (mediated by cAMP) on the stability of osteocalcin mRNA in osteoblast-like cells have previously been reported (30). Thus the effect of PKA may involve not only an upregulation of VDR but also an effect on mRNA stability, an effect on the phosphorylation of the VDR or on the phosphorylation of another protein that is involved in VDR-mediated gene transcription.

In our studies in UMR106 cells, TPA treatment did not affect 1,25(OH)2D3-induced 24(OH)ase mRNA or transcription. However, in primary cultures of rat renal cells as well as in intestinal epithelial cells, treatment with phorbol ester enhances 1,25(OH)2D3-induced 24(OH)ase expression (1, 8). Thus cell type or tissue-specific factors can alter the effect of signal transduction pathways on VDR-mediated transcription. The lack of response to TPA of the 24(OH)ase promoter may be due to the absence of specific PKC-activated proteins in UMR cells, needed for enhanced 24(OH)ase transcription, that are present in intestinal and renal cells. Because the OPN promoter was sensitive to TPA, the response to TPA is not only cell type specific but is also gene specific. In future studies, it will be of interest to identify cell type-specific factors that are involved in the modulation by signal transduction pathways of the transcription of specific 1,25(OH)2D3-regulated genes.

The effect of second messenger signaling pathways on 24(OH)ase expression may have physiological importance. 24(OH)ase, an important enzyme involved in vitamin D metabolism, hydroxylates 25(OH)D3 and 1,25(OH)2D3 (32). The 24-hydroxylation of 1,25(OH)2D3 has been reported to be involved in the catabolism of 1,25(OH)2D3 (36). 1,25(OH)2D3 at high concentrations self-induces its deactivation by inducing the 24(OH)ase enzyme. Recent studies using 24(OH)ase-deficient mice have provided the first direct in vivo evidence for a role for 24(OH)ase in the catabolism of 1,25(OH)2D3 (44). Mice deficient in 24(OH)ase have not only impaired 1,25(OH)2D3 catabolism but also impaired bone formation at specific sites (calvaria, mandible, clavicle, and periosteum of long bones) (44). 24,25(OH)2D3 supplementation failed to correct most of the bone abnormalities, arguing against a direct role of 24,25(OH)2D3 on bone formation. Because crossing the 24(OH)ase-deficient mice with VDR-ablated mice totally rescued the bone phenotype, the authors suggested that elevated 1,25(OH)2D3 levels, acting through VDR at specific sites, were responsible for the abnormalities observed in bone development (44). Thus 24(OH)ase is an important modulator of 1,25(OH)2D3 action in bone cells, and its activation at lower, physiological concentrations of 1,25(OH)2D3 via cross talk with the PKA signaling pathway may be important for preventing elevated, intracellular 1,25(OH)2D3 levels that may adversely affect mineralization.

In contrast to the modulation of 1,25(OH)2D3 induction of 24(OH)ase expression only by PKA activation, both PKA and PKC are involved in the modulation of OPN expression in UMR cells. Although the specific functions of OPN have not been fully understood, OPN has been reported to modulate bone resorption and mineralization (7, 12, 39). OPN is a ligand for a subset of integrins including αvβ3, and the OPN-integrin interaction has been reported to be important in adherence of the osteoclast to bone and bone resorption (39). Studies in OPN-deficient mice indicate significantly fewer osteoclasts in the deficient mice, confirming the in vitro findings of a role for OPN in osteoclast recruitment (3). OPN is also believed to play a role in the process of matrix mineralization by influencing the rate of mineralization (43). In addition, mechanical stress has been reported to result in elevated OPN mRNA levels (17, 27), consistent with a function for
OPN in bone remodeling. Because 1,25(OH)2D3 affects bone resorption, it is possible that PKA and PKC are involved in enhancing the effect of 1,25(OH)2D3 by enhancing OPN expression and its reported effects more specifically on bone resorption. When considering the effects of kinases, it is of interest to note that OPN is a highly phosphorylated protein (12), and different forms of OPN (phosphorylated vs. dephosphorylated) have been reported to have different functional roles. For example, when OPN is dephosphorylated by tartrate-resistant acid phosphatase, which is secreted by osteoclasts, the dephosphorylated OPN is unable to bind to osteoclasts (14). On the other hand, phosphorylation increases the capacity of OPN to stimulate osteoclast attachment (20). Whether the different signal transduction pathways act by regulating not only the increase in the synthesis of OPN but also by regulating different forms of OPN remains to be determined.

In summary, it is becoming evident that signal transduction pathways and steroid receptors do not function independently in the cell and that understanding their interaction is critical to understanding the biological response. Our findings show that activation of either PKA or PKC can enhance 1,25(OH)2D3 induction of OPN in cells expressing skeletal phenotypic properties and that the effect of PKC is due to an effect on the OPN promoter separate from an effect on VDR. However, cAMP-mediated enhancement of 1,25(OH)2D3-induced OPN and 24(OH)ase transcription is not due to an effect on a CRE in the promoter of these genes but rather may be due, at least in part, to upregulation of VDR. Thus our findings suggest that, through different mechanisms, these transmembrane signaling pathways (that can be activated by PTH and by growth factors) can play an important role in modulating the responsiveness of osteoblastic cells to 1,25(OH)2D3.

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REFERENCES


