



Evidence for coordinated regulation of osteoblast function by 1,25-dihydroxyvitamin D₃ and parathyroid hormone

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Abstract

From several animal studies and clinical observations it became evident that at target tissue level 1,25-dihydroxyvitamin D₃ (1,25-(OH)₂D₃) and parathyroid hormone (PTH) must act in an interrelated manner. In the present study we examined the interaction between 1,25-(OH)₂D₃ and PTH in the target cell of these hormones in bone, the osteoblast. In addition we studied the role of PTH-activated signal pathways. The three osteoblastic cell lines UMR 106, ROS 17/2.8 and MG-63 were used as model systems. In UMR 106 cells 1,25-(OH)₂D₃ and PTH caused a synergistic up-regulation of the vitamin D receptor (VDR) which was accompanied by a synergistic induction of VDR mRNA expression whereas in both ROS 17/2.8 and MG-63 cells no interaction was observed. In UMR 106 cells the effect of PTH on homologous up-regulation of VDR could be mimicked by the cAMP agonist forskolin and by dibutyrylic-cAMP. Phorbol ester activation of protein kinase C reduced basal as well as 1,25-(OH)₂D₃-induced up-regulation of VDR. 1,25-(OH)₂D₃ induced 24-hydroxylase activity in UMR 106 and MG 63 cells and, in contrast to VDR regulation, in both cell lines PTH and 1,25-(OH)₂D₃ synergistically induce 24-hydroxylase activity. Similar to VDR regulation the effect of PTH was mimicked by activation of cAMP production whereas protein kinase C activation reduced the induction by 1,25-(OH)₂D₃. Finally, we examined the interaction with respect to osteocalcin synthesis. In ROS 17/2.8 and MG-63 cells 1,25-(OH)₂D₃ stimulated osteocalcin production. In ROS 17/2.8 cells PTH as well as stimulation of cAMP production by forskolin enhanced 1,25-(OH)₂D₃-induced osteocalcin production whereas, as we have shown previously, activation of protein kinase C does not change 1,25-(OH)₂D₃-stimulated osteocalcin production. In MG-63 cells neither PTH nor forskolin significantly changed 1,25-(OH)₂D₃ induction of osteocalcin synthesis. From the present study it can be concluded that indeed at target cell level 1,25-(OH)₂D₃ and PTH act in a coordinated manner. On basis of the potentiation of 1,25-(OH)₂D₃ action by PTH in osteoblasts together with the previously reported inhibition of PTH-stimulated cAMP production by 1,25-(OH)₂D₃ we postulate a negative feedback-loop at target cell level. The activation of the cAMP pathway results in an enhancement of the 1,25-(OH)₂D₃ action whereas the protein kinase C pathway attenuates the 1,25-(OH)₂D₃ action. Finally, the present study provides a basis for the indications from *in vivo* observations about an interrelated action of 1,25-(OH)₂D₃ and PTH at the target cell. More generally it demonstrates on the basis of analyses of endogenous cellular responses evidence for an interplay between receptor-activated pathways of peptide and steroid hormones.

Keywords: Vitamin D₃; Parathyroid hormone; Bone; Osteoblast; Interaction; Second messenger

1. Introduction

1,25-Dihydroxyvitamin D₃ (1,25(OH)₂D₃) and parathyroid hormone (PTH) are two important hormones involved in the regulation of calcium homeostasis. From *in vitro* as well as *in vivo* studies evidence has been obtained demonstrating that these calcitropic hormones mutually regulate

each others synthesis and/or secretion. Their actions on the kidneys and parathyroids are regulated via a feed-back loop. PTH has been shown to induce an increase in renal 1 α -hydroxylase activity [1,2] and can also modulate 1,25(OH)₂D₃ receptor (VDR) level in kidney [3]. In return, 1,25(OH)₂D₃ lowers preproPTH mRNA levels [4,5] with a subsequent reduction in PTH secretion [6]. Moreover, at osteoblast level several interactions have been observed. 1,25-(OH)₂D₃ reduces PTH-stimulated cAMP production [7,8] and PTH increases the VDR number [9] via an increase in VDR mRNA in UMR 106 cells [10]

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whereas a decrease in VDR was observed in ROS 17/2.8 cells [11].

In calcium homeostasis one of the target tissues for $1,25(\text{OH})_2\text{D}_3$ and PTH is bone. For both hormones the receptors in bone are located on the osteoblast [12–14]. Although $1,25(\text{OH})_2\text{D}_3$ receptors have been demonstrated in osteoclast progenitors [15] it is generally accepted that $1,25(\text{OH})_2\text{D}_3$ and PTH stimulate osteoclastic bone resorption via a primary action on the osteoblast [16]. At the tissue level interaction between vitamin D and PTH with respect to bone resorption was already suggested by Raisz in 1965 [17]. This was supported by *in vivo* studies which indicated that PTH and $1,25(\text{OH})_2\text{D}_3$ work in concert to mobilize bone mineral [18]. In a recent study we have demonstrated that pretreatment with PTH potentiates the $1,25(\text{OH})_2\text{D}_3$ -stimulated bone resorption *in vitro* [19].

Besides the above mentioned indications for interactions between $1,25(\text{OH})_2\text{D}_3$ and PTH the background of the current study is formed by the knowledge of the cascade of events following a serum calcium disturbance. First response, within minutes, to a reduction in serum calcium concentration is a rise in PTH levels. Subsequently, within hours, stimulation of renal $25(\text{OH})\text{D}_3$ -1 α -hydroxylase by PTH occurs which eventually results in an increased serum $1,25(\text{OH})_2\text{D}_3$ concentration [1]. Under these circumstances also bone will first be exposed to increased PTH levels before increased $1,25(\text{OH})_2\text{D}_3$ levels. However, a positive interaction between PTH and $1,25(\text{OH})_2\text{D}_3$ in osteoblasts might also result in an early enhancement of $1,25(\text{OH})_2\text{D}_3$ responses independent of an increase in $1,25(\text{OH})_2\text{D}_3$ concentration. Moreover, the observation that in patients with hypoparathyroidism normally higher doses of $1,25(\text{OH})_2\text{D}_3$ than produced endogenously under normal circumstances are needed to obtain normocalcemia suggests a relative resistance to $1,25(\text{OH})_2\text{D}_3$ in the absence of PTH [20,21].

In the present study we focussed on the interaction between PTH and $1,25(\text{OH})_2\text{D}_3$ in the regulation of bone cell function by using three different osteoblastic cell lines: UMR 106, ROS 17/2.8 and MG-63. In addition the role of intracellular signal pathways known to be activated by PTH have been studied with respect to interaction with $1,25(\text{OH})_2\text{D}_3$.

2. Materials and methods

2.1. Materials

Bovine fragment 1-34 PTH (bPTH(1-34), dibutyl cAMP (Bt_2cAMP), phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma (St. Louis, MO, USA). Forskolin was purchased from Calbiochem-Behring (La Jolla, CA, USA) and Bay K 8644 was a generous gift of Dr. Garthoff of Bayer AG (Wuppertal, Germany). [$^{23,24}\text{-}^3\text{H}$]- $1,25(\text{OH})_2\text{D}_3$ (105 Ci/mmol), [$^{26,27}\text{-}^3\text{H}$]- $25(\text{OH})\text{D}_3$ (18.71

Ci/mmol), and [^{32}P]dATP were obtained from Amersham (Aylesbury, Buckinghamshire, UK). Non-radioactive $1,25(\text{OH})_2\text{D}_3$ and $24,25(\text{OH})_2\text{D}_3$ were generously provided by LEO Pharmaceuticals, Weesp, The Netherlands. GeneScreen filters were from New England Nuclear (Boston, MA, USA) and X-AR x-ray films from Eastman Kodak (Rochester, NY, USA). The VDR cDNA probe was generously provided by Dr. J.W. Pike. Fetal calf serum (FCS), α -Minimum Essential Medium (α MEM), penicillin, streptomycin, and glutamine were purchased from Flow Laboratories (Rockville, NY, USA). Chromosphere SI HPLC columns were from Chrompack International (Middelburg, The Netherlands).

2.2. Cell culture

UMR 106, ROS 17/2.8 (rat), and MG-63 (human) cells were seeded at 50000 cells/cm² and cultured for 24 h with α MEM supplemented with 2 mM glutamine, 0.1% glucose, 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, and 10% FCS. Next, medium was replaced by α MEM with 2% charcoal-treated FCS and cells were cultured for a 24 h or 48 h period during which the cells reached confluence. During this period, cells were incubated with the agents to be tested.

2.3. Preparation of cell extracts and $1,25(\text{OH})_2\text{D}_3$ binding assay

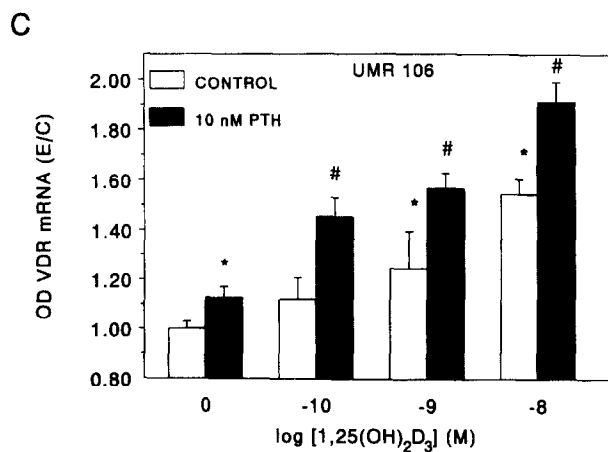
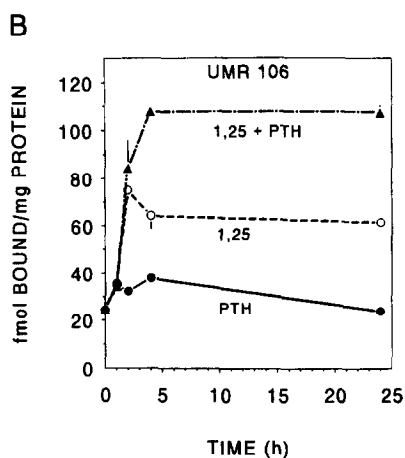
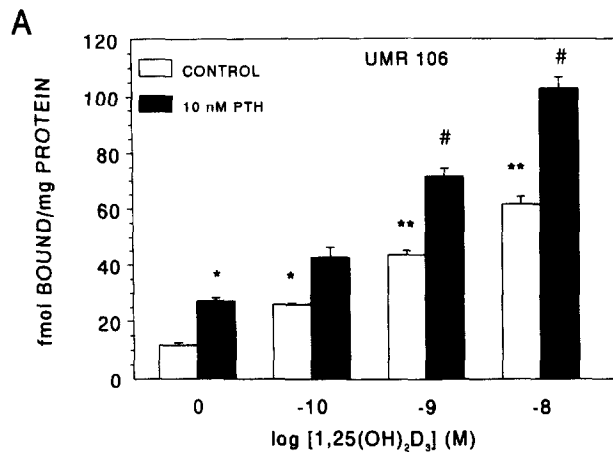
For single point assays, conditions were employed which were previously shown to provide valid estimates of total receptor content in cytosolic extracts [22]. The cells were incubated and cultured as described above. Before preparation of cell extracts, media were aspirated and the cells were incubated for an additional 2 h with MEM + 10% FCS at 37°C to promote dissociation of hormone from the receptor. Next, the cell pellet was extracted on ice in a hypertonic buffer consisting of 300 mM KCl, 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 5 mM dithiothreitol, 10 mM sodium molybdate and 0.1% Triton X-100. High-speed supernatants were obtained and 200 μl aliquots were incubated overnight at 0°C with 0.5 nM [^3H]- $1,25(\text{OH})_2\text{D}_3$ in the absence or presence of a 200-fold molar excess of unlabeled hormone. Receptor bound $1,25(\text{OH})_2\text{D}_3$ was separated from unbound sterol by charcoal adsorption [23].

2.4. 24-Hydroxylase activity

After incubation with $1,25(\text{OH})_2\text{D}_3$ in the absence or presence of the other agents the cells were washed for 2 h with α -MEM + 2% BSA followed by an incubation for 1 h with 10 nM [^3H]- $25(\text{OH})\text{D}_3$ in the presence of 0.1% BSA. Subsequently, the formation of $24,25(\text{OH})_2\text{D}_3$ was assessed with HPLC using a Silica column and a mobile phase of hexane/isopropanol/methanol (95:4:1) to elute the metabolites as described previously [24].

2.5. Osteocalcin measurements

Osteocalcin measurement in medium of MG-63 cells was performed by use of the INCSTAR (Stillwater, MN, USA) RIA kit for osteocalcin. Before measurements, medium was freeze-dried and reconstituted in assay buffer, so that medium was concentrated 6-fold. Osteocalcin measurements in cells and medium of ROS 17/2.8 cells were performed according to the method described by Verhaeghe et al. [25].



2.6. RNA isolation and hybridization

RNA isolation was performed according to the method of Chomczynski [26] and used for dot blot analysis. Blots were prehybridized for 2 h at 42°C in a buffer containing 50% formamide, 0.2% SDS, 1 × Denhardt's solution, 5 × SSC (1 × SSC = 150 mM NaCl and 15 mM Na citrate, pH 7.0), 20 mM NaH₂PO₄, 6% dextran sulfate, 1 μg/ml herring sperm DNA and then hybridized for 16–24 h at 42°C with ³²P-labeled VDR cDNA probe (1.7 kb). Next, after washing the filters several times they were exposed to X-ray films. For normalization filters were washed for 2 h at 65°C with 5 mM Tris-HCl (pH 8.0), 0.2 mM Na₂EDTA (pH 8.0), 0.05% sodium pyrophosphate and 0.1 × Denhardt's solution and rehybridized with a 0.8 kb human GAPDH fragment.

2.7. Protein and DNA measurement

The protein concentration was measured according to the method of Bradford [27]. DNA content was assessed by the fluorimetric method of Johnson-Wint and Hollis [28].

2.8. Data analyses

Interactions between various drugs were evaluated using analysis of variance for two-way design. This statistical method enables evaluation of the significance of interaction between different factors and is applied in all cases when two drugs are added together. Other statistical analyses were performed using Student's *t*-test.

3. Results

3.1. Effect of 1,25-(OH)₂D₃ in combination with PTH on the VDR

Firstly, we studied the effect of 1,25-(OH)₂D₃ in combination with PTH on the VDR in UMR 106 cells. Both

Fig. 1. Effect of PTH on 1,25-(OH)₂D₃-induced up-regulation of VDR and VDR mRNA expression in UMR 106 cells. (A) Cells were incubated for 4 h with several concentrations 1,25-(OH)₂D₃ and 10 nM PTH. (B) Time-course of coordinated up-regulation of VDR by 1,25-(OH)₂D₃ and PTH in UMR 106 cells. Cells were incubated for various periods of time with 10 nM 1,25-(OH)₂D₃ and 10 nM PTH. VDR content was assayed in cytosolic extracts as described in Section 2. Data are expressed as fmol of [³H]1,25-(OH)₂D₃ bound per mg protein ± S.D. for at least four different cultures. (C) Effect on VDR mRNA expression. Cells were incubated for 4 h with several concentrations 1,25-(OH)₂D₃ and 10 nM PTH. Next, total RNA was isolated and used for dot-blot analysis. The autoradiogram was quantitated by densitometric scanning and the ratio of VDR/GAPDH is expressed as experiment over control. * *P* < 0.05 and ** *P* < 0.001 versus no addition of 1,25-(OH)₂D₃ and PTH; # *P* < 0.005 calculated as the the significance of interaction between 1,25-(OH)₂D₃ and PTH.

1,25-(OH)₂D₃ and PTH cause an up-regulation in these cells with a different magnitude and time-course (Refs. [9,19], and Fig. 1). Incubation for 4 h of 10 nM PTH together with a dose-range of 1,25-(OH)₂D₃ resulted in synergistic up-regulation of the VDR in UMR 106 cells (Fig. 1A). Time-course studies revealed that the effect of PTH alone was transient whereas up to 24 h synergistic up-regulation of VDR by 1,25-(OH)₂D₃ and PTH was observed (Fig. 1B). Next, we examined the effect of coincubation on the VDR mRNA level in UMR 106 cells. As shown in Fig. 1C, coincubation with 1,25-(OH)₂D₃ and PTH also synergistically induced VDR mRNA expression.

Secondly, the combined actions of 1,25-(OH)₂D₃ and PTH on VDR level in ROS 17/2.8 and MG 63 cells were studied. As in UMR 106 cells 1,25-(OH)₂D₃ caused up-regulation of VDR in ROS 17/2.8 cells whereas 10 nM PTH did not affect VDR in these cells (Fig. 2). Both 1,25-(OH)₂D₃ and PTH had no effect on VDR in MG 63 cells (Fig. 2). In contrast to UMR 106 cells, in ROS 17/2.8 as well as MG 63 cells 1,25-(OH)₂D₃ and PTH had no synergistic effect on VDR (Fig. 2).

3.2. Effect of second messengers on homologous up-regulation of VDR

PTH has been shown to activate several intracellular signal pathways [29–34]. In order to assess a role for cAMP in the potentiation of homologous up-regulation of VDR we tried in UMR 106 cells to mimic the PTH effect by the cAMP agonist forskolin. As shown in Fig. 3, forskolin (10 μM) and 1,25-(OH)₂D₃ synergistically up-regulate VDR level in UMR 106 cells. With the cAMP analogue Bt₂cAMP a similar effect was observed (Fig.

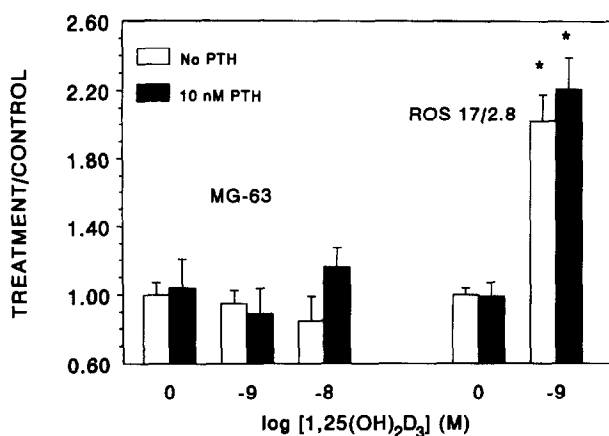


Fig. 2. Effect of PTH and 1,25-(OH)₂D₃ on VDR level in ROS 17/2.8 and MG-63 cells. Cells were cultured as described in Section 2 and after incubation with PTH and 1,25-(OH)₂D₃ the VDR content was assayed in cytosolic extracts. For reasons of clarity data are expressed as treatment over control ROS 17/2.8 or control MG 63 cells. Control VDR levels were 42.89 ± 1.57 and 12.57 ± 0.89 fmol/mg protein for ROS 17/2.8 and MG 63 cells, respectively. * *P* < 0.001 versus no addition of 1,25-(OH)₂D₃ and PTH.

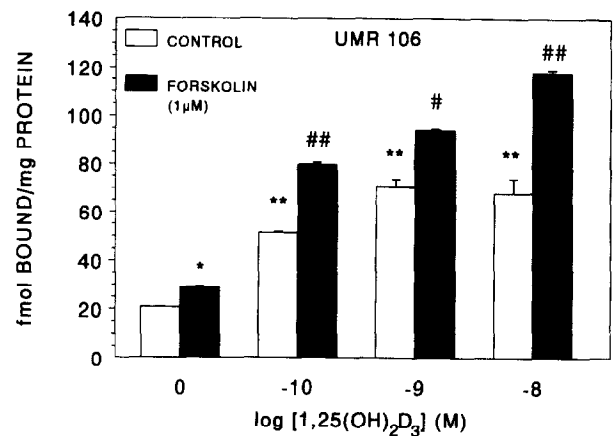


Fig. 3. Effect of forskolin on 1,25-(OH)₂D₃-induced up-regulation of VDR in UMR 106 cells. Cells were incubated for 4 h with several concentrations 1,25-(OH)₂D₃ and 1 μM forskolin. Next, the VDR content was assayed in cytosolic extracts as described in Section 2. Data are expressed as fmol of [³H]1,25-(OH)₂D₃ bound per mg protein ± S.D. for at least four different cultures. * *P* < 0.01 and ** *P* < 0.001 versus no addition of 1,25-(OH)₂D₃ and forskolin; # *P* < 0.05 and ## *P* < 0.005 calculated as the significance of interaction between 1,25-(OH)₂D₃ and forskolin.

4A). Calcium has been shown to be involved in the heterologous up-regulation of VDR by PTH [35]. However, increasing intracellular calcium concentration with the calcium agonist BAY K 8644 did not result in potentiation of 1,25-(OH)₂D₃-induced up-regulation of VDR (Fig. 4A). Recently, we have shown that activation of protein kinase C by phorbol ester treatment for 4 h caused down-regulation of VDR [36]. Addition of the phorbol ester PMA together with 1,25-(OH)₂D₃ resulted also in a reduction of homologous up-regulation of VDR (Fig. 4B).

3.3. Regulation of 24-hydroxylase activity by 1,25-(OH)₂D₃ and PTH

In most of the tissues 1,25-(OH)₂D₃ induces 24-hydroxylase activity. This enzyme initiates a catabolic cascade (C24-oxidation pathway) for the side-chain oxidation, cleavage and ultimate metabolic elimination of 1,25-(OH)₂D₃ and its precursor 25(OH)D₃ [37]. Also in UMR 106 and MG-63 cells (Fig. 5A and B) but not ROS 17/2.8 cells [38] 1,25-(OH)₂D₃ is a potent inducer of 24-hydroxylase activity. After 1 h 1,25-(OH)₂D₃ caused a strong induction of 24-hydroxylase activity whereas 10 nM PTH alone was not effective (Fig. 5A and B). However, when both UMR 106 and MG-63 cells were treated by 1,25-(OH)₂D₃ together with 10 nM PTH a synergistic induction of 24-hydroxylase activity was observed (Fig. 5A and B).

3.4. Effect of cAMP and protein kinase C on induction of 24-hydroxylase activity

As in both UMR 106 and MG-63 cells a synergistic induction of 24-hydroxylase activity by PTH and 1,25-

(OH)₂D₃ was observed the role of cAMP and protein kinase C was further examined in UMR 106 cells. Cells were incubated for 1 h with 1,25-(OH)₂D₃ in the absence and presence of 1 μM forskolin or 100 nM PMA. Activation of the cAMP pathway by forskolin resulted in potentiation of 1,25-(OH)₂D₃-induced 24-hydroxylase activity. In contrast, activation of protein kinase C by PMA caused reduction of 1,25-(OH)₂D₃-induced 24-hydroxylase activity (Fig. 6). Comparison of Fig. 5A and Fig. 6 demonstrates that 10 nM PTH was more potent than 1 μM forskolin. This is in accordance with their potencies to stimulate cAMP production: Control: 0.77 ± 0.08, 10 nM PTH: 71.95 ± 4.37, and 1 μM forskolin: 4.83 ± 0.28 pmol cAMP.

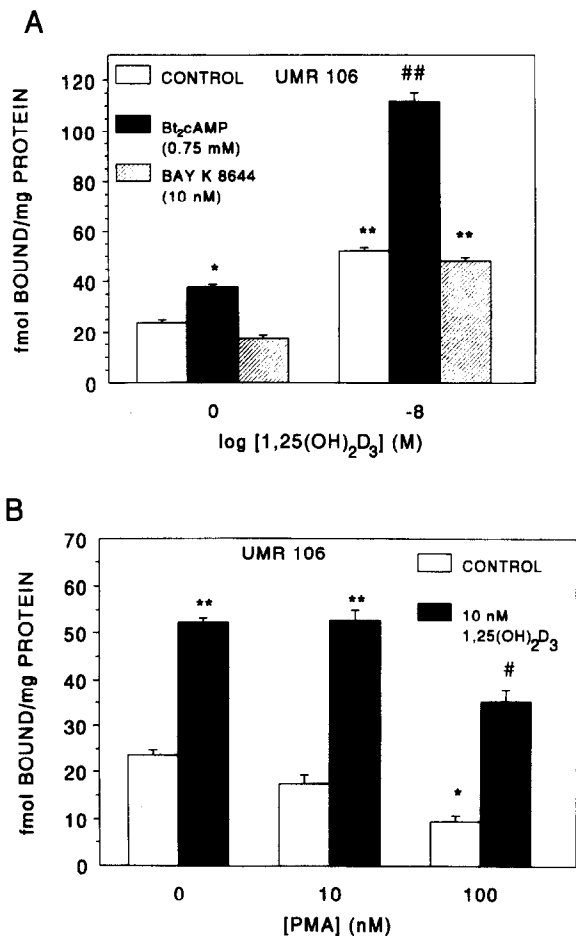


Fig. 4. Effect of (A) the cAMP analogue Bt₂cAMP, the calcium agonist BAY K 8644, and (B) the activator of protein kinase C PMA on 1,25-(OH)₂D₃-induced up-regulation of VDR in UMR 106 cells. Cells were incubated for 4 h with 1,25-(OH)₂D₃ in the presence or absence of Bt₂cAMP, BAY K 8644 or PMA. Next, VDR content was assayed in cytosolic extracts as described in Section 2. Data are expressed as fmol of [³H]1,25-(OH)₂D₃ bound per mg protein ± S.D. for at least four different cultures. * *P* < 0.025 and ** *P* < 0.005 versus no addition of 1,25-(OH)₂D₃ or any of the other compounds; # *P* < 0.05 and ## *P* < 0.001 calculated as the the significance of interaction between 1,25-(OH)₂D₃ and any of the other compounds.

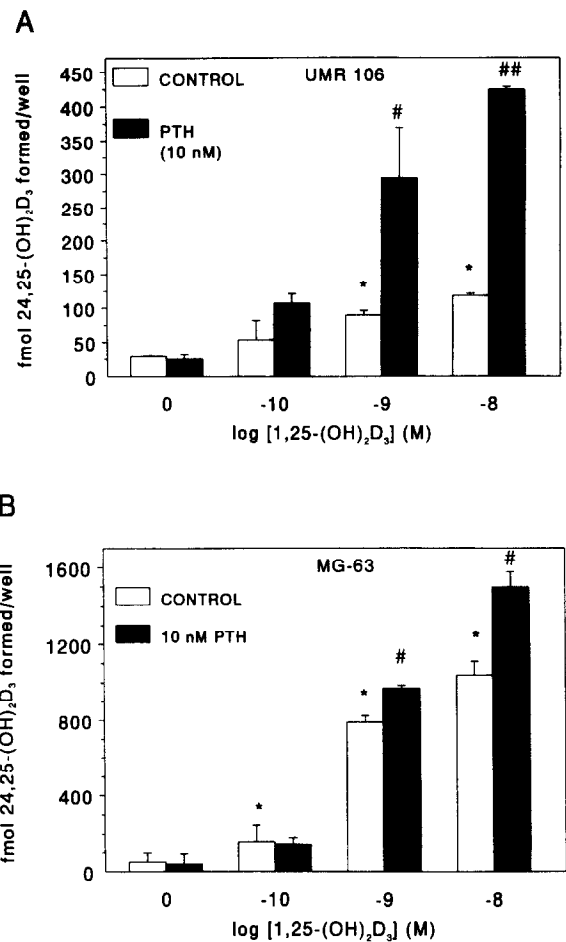


Fig. 5. Effect of PTH on 1,25-(OH)₂D₃-induced 24-hydroxylase activity in (A) UMR 106 cells and (B) MG-63 cells. Cells were incubated for 1 h with several concentrations 1,25-(OH)₂D₃ and 10 nM PTH followed by an incubation for 1 h with 10 nM [³H]25-(OH)D₃ as substrate. Next, after a 2 h wash the 25-(OH)D₃ metabolites were extracted and analyzed by HPLC. Data are expressed as fmol 24,25-(OH)₂D₃ formed ± S.D. of at least two independent cultures. * *P* < 0.001 versus no addition of 1,25-(OH)₂D₃ and PTH; # *P* < 0.005 and ## *P* < 0.001 calculated as significance of interaction between 1,25-(OH)₂D₃ and PTH.

3.5. Effect of 1,25-(OH)₂D₃ and PTH on osteocalcin synthesis

In contrast to UMR 106 cells, both MG-63 and ROS 17/2.8 cells synthesize osteocalcin which is stimulated by 1,25-(OH)₂D₃. In MG-63 cells PTH did not affect 1,25-(OH)₂D₃-stimulated osteocalcin production whereas in ROS 17/2.8 cells cotreatment with PTH enhanced 1,25-(OH)₂D₃-stimulated osteocalcin production (Fig. 7A and B). PTH by itself did not induce osteocalcin production in both cell lines. Activation of the cAMP pathway with forskolin mimicked the potentiating effect of PTH in ROS 17/2.8 cells (Fig. 7B) and in MG-63 cells forskolin did not significantly change 1,25-(OH)₂D₃-stimulated osteocalcin production (Fig. 7A). We have previously shown that activation of protein kinase C by phorbol esters did not

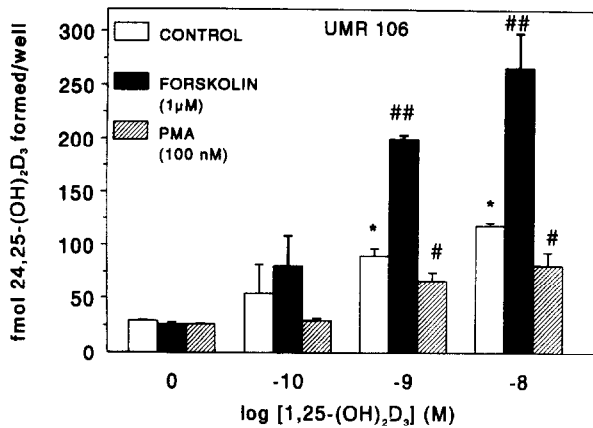


Fig. 6. Effect of forskolin and PMA on 1,25-(OH)₂D₃-induced 24-hydroxylase activity in UMR 106 cells. Cells were incubated for 1 h with several concentrations 1,25-(OH)₂D₃ and 1 μM forskolin followed by an incubation for 1 h with 10 nM [³H]25-(OH)₂D₃ as substrate. Next, after a 2 h wash the 25-(OH)₂D₃ metabolites were extracted and analyzed by HPLC. Data are expressed as fmol 24,25-(OH)₂D₃ formed ± S.D. of at least two independent cultures. * *P* < 0.001 versus no addition of 1,25-(OH)₂D₃ and forskolin or PMA; # *P* < 0.05 and ## *P* < 0.001 calculated as significance of interaction between 1,25-(OH)₂D₃ and forskolin or PMA.

affect basal as well as 1,25-(OH)₂D₃-stimulated osteocalcin production [39].

4. Discussion

1,25-(OH)₂D₃ and PTH regulate osteoblast activity via close interactions. On basis of the present observation that PTH potentiates 1,25-(OH)₂D₃ action in osteoblasts and the previously reported inhibition of PTH-stimulated cAMP production by 1,25-(OH)₂D₃ [7,8] a negative feedback-loop at target cell level can be postulated. This mimics the well-known systemic feedback-loop in which PTH stimulates renal 1,25-(OH)₂D₃ synthesis whereas 1,25-(OH)₂D₃ inhibits PTH synthesis by the parathyroid glands.

The currently observed interactions between 1,25-(OH)₂D₃ and PTH are logical from a physiological point of view. Within minutes a reduction in serum calcium is followed by a rise in serum PTH level. Subsequently, within hours, stimulation of renal 25-(OH)-D₃-1 α-hydroxylase by PTH occurs which eventually results in an increased serum 1,25-(OH)₂D₃ level [1]. Under these circumstances also bone will first be exposed to increased PTH levels before increased 1,25-(OH)₂D₃ levels. The present results indicate that despite unchanged serum 1,25-(OH)₂D₃ levels already an enhancement of its action on bone cells to restore serum calcium levels could be achieved. The biological significance of 1,25-(OH)₂D₃ and PTH interaction is also supported by the observation that some hypoparathyroid patients are resistant to 1,25-(OH)₂D₃ for the treatment of hypocalcemia [20,21].

The analysis of the role of intracellular signal pathways

demonstrated that in general activation of protein kinase A via increased cAMP production enhances the 1,25-(OH)₂D₃ action in osteoblasts whereas protein kinase C attenuates the 1,25-(OH)₂D₃ effects. Our data on interactions between cAMP and 1,25-(OH)₂D₃ are in line with recent findings that cAMP enhances the 1,25-(OH)₂D₃ action in lymphocytes and fibroblasts [40–43]. However, target tissue/cell-specific interaction between 1,25-(OH)₂D₃ and PTH (cAMP, protein kinase C) is evident from comparison of the data obtained on renal 24-hydroxylase and our results. In the kidney PTH, via cAMP, reduces 1,25-(OH)₂D₃-induced renal 24-hydroxylase activ-

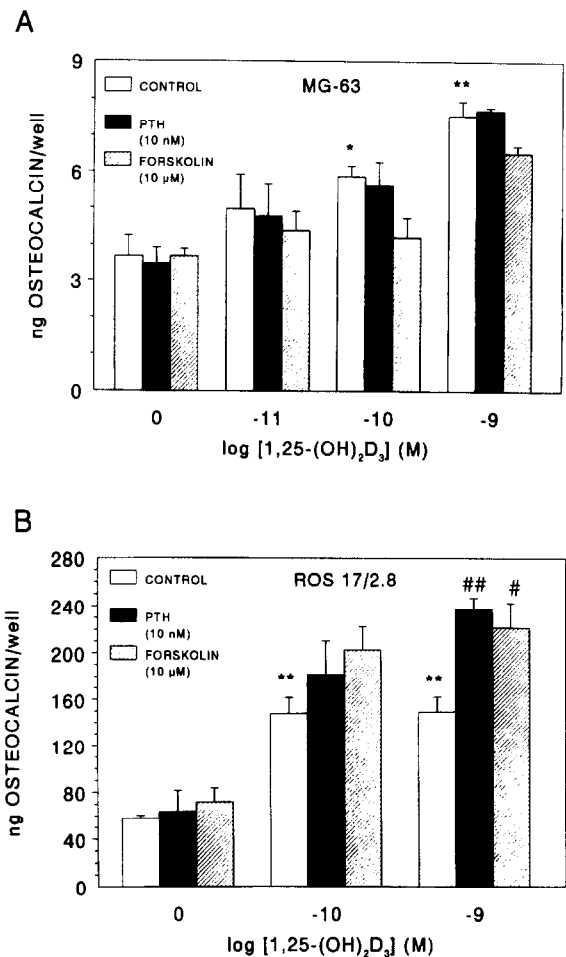


Fig. 7. Effect of PTH and forskolin on 1,25-(OH)₂D₃-stimulated osteocalcin production by (A) MG-63 cells and (B) ROS 17/2.8 cells. Cells were incubated for 24 h and next osteocalcin concentration in the medium was analyzed. The changes in cellular osteocalcin content of ROS 17/2.8 cells were similar as the changes observed in the medium. The cellular osteocalcin content of MG-63 cells was below detection level. Within the 24 h incubation period vitamin D did not change the proliferation. Expressed as μg DNA/well the following DNA values were obtained after 24 h incubation, ROS 17/2.8 cells: Control: 49.3 ± 1.7 and 10 nM 1,25-(OH)₂D₃: 52.6 ± 1.8; MG-63 cells: Control: 113.8 ± 4.8 and 10 nM 1,25-(OH)₂D₃: 117.7 ± 0.1 (expressed as μg DNA/well). Data are presented as ng osteocalcin ± S.E.M. of 2–6 independent cultures. * *P* < 0.02 and ** *P* < 0.002 versus no addition of 1,25-(OH)₂D₃ and PTH or forskolin; # *P* < 0.02 and ## *P* < 0.002 calculated as significance of interaction between 1,25-(OH)₂D₃ and PTH or forskolin.

ity as well as 24-hydroxylase mRNA expression [44,45] whereas activation of protein kinase C by phorbol esters potentiates 1,25-(OH)₂D₃-induced renal 24-hydroxylase mRNA expression [46,47]. From the present study and previously reported data on the absence of effect of protein kinase C activation on 1,25-(OH)₂D₃-stimulated responses in both renal and bone tissue [39,43–46] it is clear that cAMP and protein kinase C have opposite effects on 1,25-(OH)₂D₃ action. The fact that PTH stimulates both cAMP production and protein kinase C activity [33,34] suggests that the PTH-activated cAMP pathway overrules the protein kinase C pathway.

The present study demonstrates interactions in osteoblasts between 1,25-(OH)₂D₃ and PTH when the two hormones are tested simultaneously. This is in contrast to a recent study on pretreatment of the cells with PTH. Pretreatment with PTH resulted in up-regulation of the VDR and subsequently an enhanced 1,25-(OH)₂D₃ induction of 24-hydroxylase mRNA expression [48]. It is, however, for the following reasons unlikely that the synergistic up-regulation of VDR is involved in the potentiation of 24-hydroxylase activity in UMR 106 and MG-63 cells and osteocalcin production in ROS 17/2.8 cells. In UMR 106 cells both up-regulation of VDR and induction of 24-hydroxylase activity are initiated simultaneously upon addition of both hormones. Moreover, in ROS 17/2.8 cells there is no synergistic up-regulation of VDR whereas 1,25-(OH)₂D₃ induction of osteocalcin synthesis is augmented by PTH, and finally, in MG-63 cells no up-regulation of VDR whereas stimulation of 24-hydroxylase activity is potentiated by PTH. Several mechanisms to account for the interaction between 1,25-(OH)₂D₃ and PTH can be put forward. Firstly, direct interaction at gene level: In the osteocalcin promoter both vitamin D response elements and consensus cAMP-regulatory elements have been identified [49]. Secondly, phosphorylation of VDR: Both homologous- and heterologous-induction of phosphorylation of the VDR have been shown to be important for transcriptional activation [50–52]. The VDR can be phosphorylated at specific sites by cAMP-dependent protein kinase A, protein kinase C and casein kinase II [52–55]. Thirdly, stabilization of mRNA: For osteocalcin mRNA it has been reported that parathyroid hormone increases its stability [56]. Although it is conceivable that one or more of these mechanisms are involved in the interactions between 1,25-(OH)₂D₃ and PTH their exact role or the involvement of a yet unidentified process remains to be established.

It is becoming evident that steroid and peptide regulators of cell function do not act independently. The analysis of the cross-talk between the signal transduction pathways of these two chemically distinct compounds is crucial for the understanding of their eventual biological response. Although limited, interactions between the cAMP pathway and the steroid hormone mechanism of action have been described in other systems [57–60]. Even a complete change of function has been observed. In the presence of

cAMP both the progesterone antagonist RU486 and the estrogen antagonist tamoxifen display agonist-like activities [59–61]. Most of these studies concern experiments with reporter constructs transfected into cells. The present study demonstrates coordinated regulation of endogenous cellular responses (i.e. not transfected reporter constructs) by hormones acting via an intracellular receptor or via a membrane bound receptor.

Although PTH is active in all three cell lines tested (Figs. 1 and 5B, Fig. 7B), some of the observed interactions with 1,25-(OH)₂D₃ appeared to be partially cell line- and response-specific. Considering the osteoblast-differentiation scheme defined by Stein et al. [62] the three osteoblastic cell lines used may represent different stages of osteoblast differentiation. UMR 106 resemble poorly differentiated osteoblasts as no basal and 1,25-(OH)₂D₃-stimulated osteocalcin production is present. MG-63 cells may be representative for the intermediate-early differentiation phenotype: high collagen type I and low osteocalcin production levels, while on the basis of the high osteocalcin levels the ROS 17/2.8 cells are fully differentiated osteoblasts. Therefore, the present observations suggest that interaction(s) between 1,25-(OH)₂D₃ and PTH are dependent on differentiation status of the osteoblast. Previously it has been shown that the response to 1,25-(OH)₂D₃ is related to the proliferative and differentiated state of osteoblasts [63]. The observation by Chambers and McSheehy [64] that conditioned medium of UMR 106 cells is a potent stimulator of bone resorption whereas conditioned medium of ROS 17/2.8 cells hardly stimulate bone resorption is indicative for the fact that these cells indeed represent osteoblasts with different functional roles, i.e. involved in regulation of bone resorption or in bone formation. However, to address this aspect more specifically studies with a well defined osteoblast differentiation model are needed [62]. The different effects of PTH on 1,25-(OH)₂D₃ induction of osteocalcin synthesis may be related to a species difference. An earlier study showed that cAMP enhances 1,25-(OH)₂D₃-induction of osteocalcin mRNA expression in ROS 17/2.8 cells [65] but not in human bone cells derived from surgical specimens [66,67].

Together, the present study shows that 1,25-(OH)₂D₃ and PTH, two chemically distinct calcitropic hormones, act at cellular level in a coordinated manner to regulate osteoblast activity. This adds up to systemic interaction with respect to the mutual regulation of each others production and secretion.

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