ELSEVIER

Biochimica et Biophysica Acta 1312 (1996) 55-62

BBA Biochimica et Biophysica Acta

Evidence for coordinated regulation of osteoblast function by 1,25-dihydroxyvitamin D_3 and parathyroid hormone

Johannes P.T.M. van Leeuwen *, Jan C. Birkenhäger, Gertjan C.M. van den Bemd, Huibert A.P. Pols

Department of Internal Medicine III, Erasmus University Medical School, Dr. Molewaterplein 40, 3015 GD Rotterdam, The Netherlands

Received 18 December 1995; accepted 19 January 1996

Abstract

From several animal studies and clinical observations it became evident that at target tissue level 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) and parathyroid hormone (PTH) must act in an interrelated manner. In the present study we examined the interaction between 1,25-$(OH)_2D_3$ 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-$(OH)_2D_3$ 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-$(OH)_2D_3$-induced up-regulation of VDR. 1,25-$(OH)_2D_3$ 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-$(OH)_2D_3$ 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-$(OH)_2D_3$. Finally, we examined the interaction with respect to osteocalcin synthesis. In ROS 17/2.8 and MG-63 cells 1,25-$(OH)_2D_3$ stimulated osteocalcin production. In ROS 17/2.8 cells PTH as well as stimulation of cAMP production by forskolin enhanced 1,25-$(OH)_2D_3$-induced osteocalcin production whereas, as we have shown previously, activation of protein kinase C does not change 1,25-$(OH)_2D_3$-stimulated osteocalcin production. In MG-63 cells neither PTH nor forskolin significantly changed 1,25-$(OH)_2D_3$ induction of osteocalcin synthesis. From the present study it can be concluded that indeed at target cell level 1,25-$(OH)_2D_3$ and PTH act in a coordinated manner. On basis of the potentiation of 1,25-$(OH)_2D_3$ action by PTH in osteoblasts together with the previously reported inhibition of PTH-stimulated cAMP production by 1,25-$(OH)_2D_3$ we postulate a negative feedback-loop at target cell level. The activation of the cAMP pathway results in an enhancement of the 1,25-$(OH)_2D_3$ action whereas the protein kinase C pathway attenuates the 1,25-$(OH)_2D_3$ action. Finally, the present study provides a basis for the indications from in vivo observations about an interrelated action of 1,25-$(OH)_2D_3$ 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 D_3; Parathyroid hormone; Bone; Osteoblast; Interaction; Second messenger

1. Introduction

1,25-Dihydroxyvitamin D_3 (1,25$(OH)_2D_3$) 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 calciotropic 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 1α-hydroxylase activity [1,2] and can also modulate 1,25$(OH)_2D_3$ receptor (VDR) level in kidney [3]. In return, 1,25$(OH)_2D_3$ lowers prepro PTH mRNA levels [4,5] with a subsequent reduction in PTH secretion [6]. Moreover, at osteoblast level several interactions have been observed. 1,25-$(OH)_2D_3$ 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]

* Corresponding author. Fax: +31 10 4633268; e-mail: vanleeuwen@inw3.fgg.eur.nl.

0167-4889/96/$15.00 © 1996 Elsevier Science B.V. All rights reserved
PII S0167-4889(96)00016-X

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

In calcium homeostasis one of the target tissues for $1,25(OH)_2D_3$ and PTH is bone. For both hormones the receptors in bone are located on the osteoblast [12–14]. Although $1,25(OH)_2D_3$ receptors have been demonstrated in osteoclast progenitors [15] it is generally accepted that $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$ work in concert to mobilize bone mineral [18]. In a recent study we have demonstrated that pretreatment with PTH potentiates the $1,25\text{-}(OH)_2D_3$-stimulated bone resorption in vitro [19].

Besides the above mentioned indications for interactions between $1,25(OH)_2D_3$ 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 $25(OH)D_3$-1α-hydroxylase by PTH occurs which eventually results in an increased serum $1,25(OH)_2D_3$ concentration [1]. Under these circumstances also bone will first be exposed to increased PTH levels before increased $1,25(OH)_2D_3$ levels. However, a positive interaction between PTH and $1,25\text{-}(OH)_2D_3$ in osteoblasts might also result in an early enhancement of $1,25\text{-}(OH)_2D_3$ responses independent of an increase in $1,25\text{-}(OH)_2D_3$ concentration. Moreover, the observation that in patients with hypoparathyroidism normally higher doses of $1,25\text{-}(OH)_2D_3$ than produced endogenously under normal circumstances are needed to obtain normocalcemia suggests a relative resistance to $1,25\text{-}(OH)_2D_3$ in the absence of PTH [20,21].

In the present study we focussed on the interaction between PTH and $1,25\text{-}(OH)_2D_3$ 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 $1,25\text{-}(OH)_2D_3$.

2. Materials and methods

2.1. Materials

Bovine fragment 1-34 PTH (bPTH(1-34), dibutyryl 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-^{3}H]-1,25-$(OH)_2D_3$ (105 Ci/mmol), [26,27-^{3}H]25-$(OH)D_3$ (18.71 Ci/mmol), and [^{32}P]dATP were obtained from Amersham (Aylesbury, Buckinghamshire, UK). Non-radioactive $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ 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 (αMEM), penicillin, streptomycin, and glutamine were purchased from Flow Laboratories (Rockville, NY, USA). Chromsphere 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/cm^2 and cultured for 24 h with αMEM supplemented with 2 mM glutamine, 0.1% glucose, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FCS. Next, medium was replaced by αMEM 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 $1,25\text{-}(OH)_2D_3$ binding assay

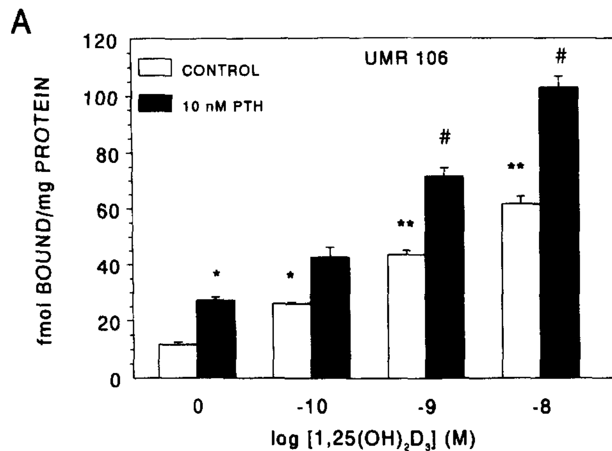
For single point assays, conditions were employed which were previously shown to provide valid estimates of total receptor content in cytosolic extracts [22]. The cells were incubated and cultured as described above. Before preparation of cell extracts, media were aspirated and the cells were incubated 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 [^{3}H]$1,25(OH)_2D_3$ in the absence or presence of a 200-fold molar excess of unlabeled hormone. Receptor bound $1,25(OH)_2D_3$ was separated from unbound sterol by charcoal adsorption [23].

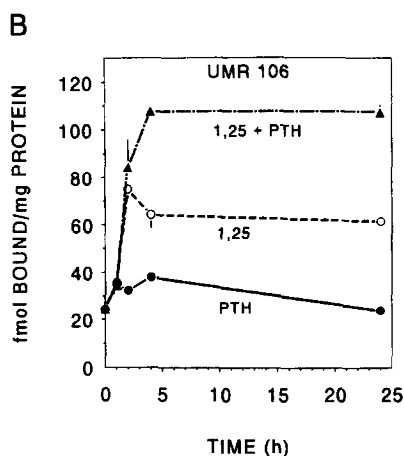
2.4. 24-Hydroxylase acitivity

After incubation with $1,25\text{-}(OH)_2D_3$ 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 [^{3}H]$25(OH)D_3$ in the presence of 0.1% BSA. Subsequently, the formation of $24,25(OH)_2D_3$ 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].





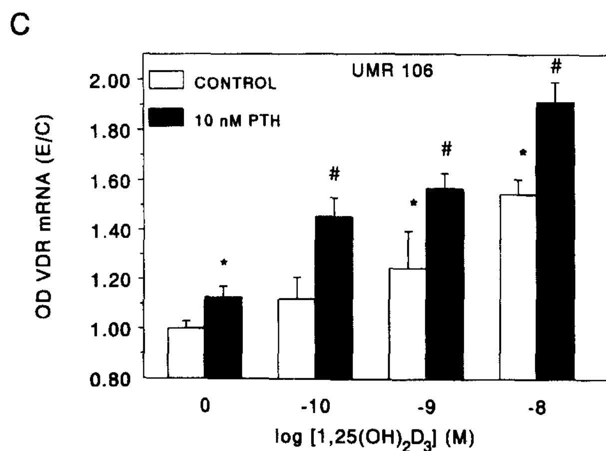


Fig. 1. Effect of PTH on $1,25\text{-}(OH)_2D_3$-induced up-regulation of VDR and VDR mRNA expression in UMR 106 cells. (A) Cells were incubated for 4 h with several concentrations $1,25\text{-}(OH)_2D_3$ and 10 nM PTH. (B) Time-course of coordinated up-regulation of VDR by $1,25\text{-}(OH)_2D_3$ and PTH in UMR 106 cells. Cells were incubated for various periods of time with 10 nM $1,25\text{-}(OH)_2D_3$ and 10 nM PTH. VDR content was assayed in cytosolic extracts as described in Section 2. Data are expressed as fmol of $[^3H]1,25\text{-}(OH)_2D_3$ 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 $1,25\text{-}(OH)_2D_3$ 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 $1,25\text{-}(OH)_2D_3$ and PTH; # $P < 0.005$ calculated as the the significance of interaction between $1,25\text{-}(OH)_2D_3$ and PTH.

2.6. RNA isolation and hybridization

RNA isolation was performed according to the method of Chomszinski [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 NaH_2PO_4, 6% dextran sulfate, 1 μg/ml herring sperm DNA and then hybridized for 16–24 h at 42°C with ^{32}P-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 Na_2EDTA (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 $1,25\text{-}(OH)_2D_3$ in combination with PTH on the VDR

Firstly, we studied the effect of $1,25\text{-}(OH)_2D_3$ in combination with PTH on the VDR in UMR 106 cells. Both

$1,25\text{-}(OH)_2D_3$ 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 $1,25\text{-}(OH)_2D_3$ 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 $1,25\text{-}(OH)_2D_3$ 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 $1,25\text{-}(OH)_2D_3$ and PTH also synergistically induced VDR mRNA expression.

Secondly, the combined actions of $1,25\text{-}(OH)_2D_3$ and PTH on VDR level in ROS 17/2.8 and MG 63 cells were studied. As in UMR 106 cells $1,25\text{-}(OH)_2D_3$ 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 $1,25\text{-}(OH)_2D_3$ 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 $1,25\text{-}(OH)_2D_3$ and PTH had no synergistic effect on VDR (Fig. 2).

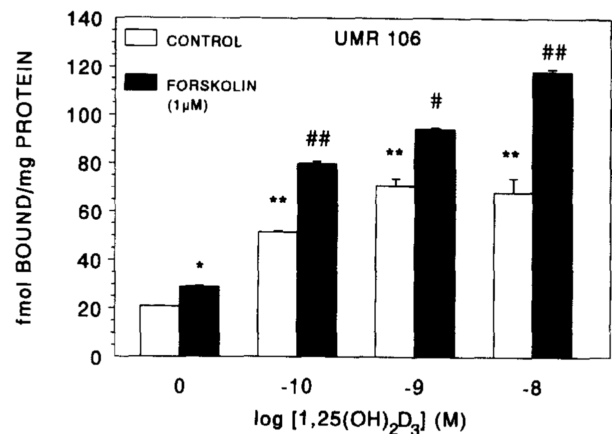


Fig. 3. Effect of forskolin on $1,25\text{-}(OH)_2D_3$-induced up-regulation of VDR in UMR 106 cells. Cells were incubated for 4 h with several concentrations $1,25\text{-}(OH)_2D_3$ and 1 μM forskolin. Next, the VDR content was assayed in cytosolic extracts as described in Section 2. Data are expressed as fmol of $[^3H]1,25\text{-}(OH)_2D_3$ bound per mg protein ± S.D. for at least four different cultures. * $P < 0.01$ and ** $P < 0.001$ versus no addition of $1,25\text{-}(OH)_2D_3$ and forskolin; # $P < 0.05$ and ## $P < 0.005$ calculated as the the significance of interaction between $1,25\text{-}(OH)_2D_3$ and forskolin.

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 $1,25\text{-}(OH)_2D_3$ synergistically up-regulate VDR level in UMR 106 cells. With the cAMP analogue Bt_2cAMP a similar effect was observed (Fig. 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 $1,25\text{-}(OH)_2D_3$-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 $1,25\text{-}(OH)_2D_3$ resulted also in a reduction of homologous up-regulation of VDR (Fig. 4B).

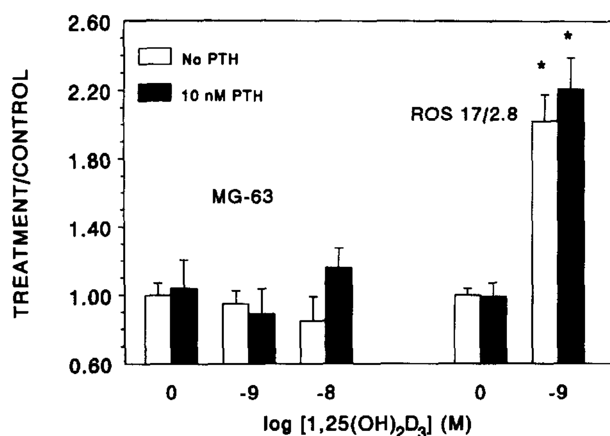


Fig. 2. Effect of PTH and $1,25\text{-}(OH)_2D_3$ on VDR level in ROS 17/2.8 and MG-63 cells. Cells were cultured as described in Section 2 and after incubation with PTH and $1,25\text{-}(OH)_2D_3$ the VDR content was assayed in cytosolic extracts. For reasons of clarity data are expressed as treatment 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 $1,25\text{-}(OH)_2D_3$ and PTH.

3.3. *Regulation of 24-hydroxylase activity by $1,25\text{-}(OH)_2D_3$ and PTH*

In most of the tissues $1,25\text{-}(OH)_2D_3$ induces 24-hydroxylase activity. This enzyme initiates a catabolic cascade (C24-oxidation pathway) for the side-chain oxidation, cleavage and ultimate metabolic elimination of $1,25\text{-}(OH)_2D_3$ and its precursor $25(OH)D_3$ [37]. Also in UMR 106 and MG-63 cells (Fig. 5A and B) but not ROS 17/2.8 cells [38] $1,25\text{-}(OH)_2D_3$ is a potent inducer of 24-hydroxylase activity. After 1 h $1,25\text{-}(OH)_2D_3$ 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 $1,25\text{-}(OH)_2D_3$ 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-

$(OH)_2D_3$ 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-$(OH)_2D_3$ 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-$(OH)_2D_3$-induced 24-hydroxylase activity. In contrast, activation of protein kinase C by PMA caused reduction of 1,25-$(OH)_2D_3$-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.

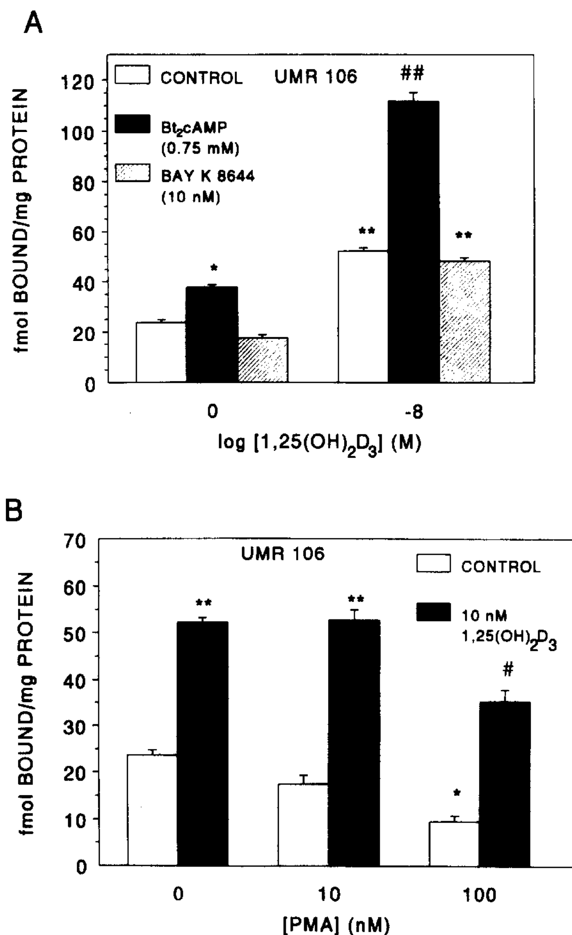


Fig. 4. Effect of (A) the cAMP analogue Bt_2cAMP, the calcium agonist BAY K 8644, and (B) the activator of protein kinase C PMA on 1,25-$(OH)_2D_3$-induced up-regulation of VDR in UMR 106 cells. Cells were incubated for 4 h with 1,25-$(OH)_2D_3$ in the presence or absence of Bt_2cAMP, BAY K 8644 or PMA. Next, VDR content was assayed in cytosolic extracts as described in Section 2. Data are expresed as fmol of $[^3H]$1,25-$(OH)_2D_3$ 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-$(OH)_2D_3$ or any of the other compounds; # $P < 0.05$ and ## $P < 0.001$ calculated as the the significance of interaction between 1,25-$(OH)_2D_3$ and any of the other compounds.

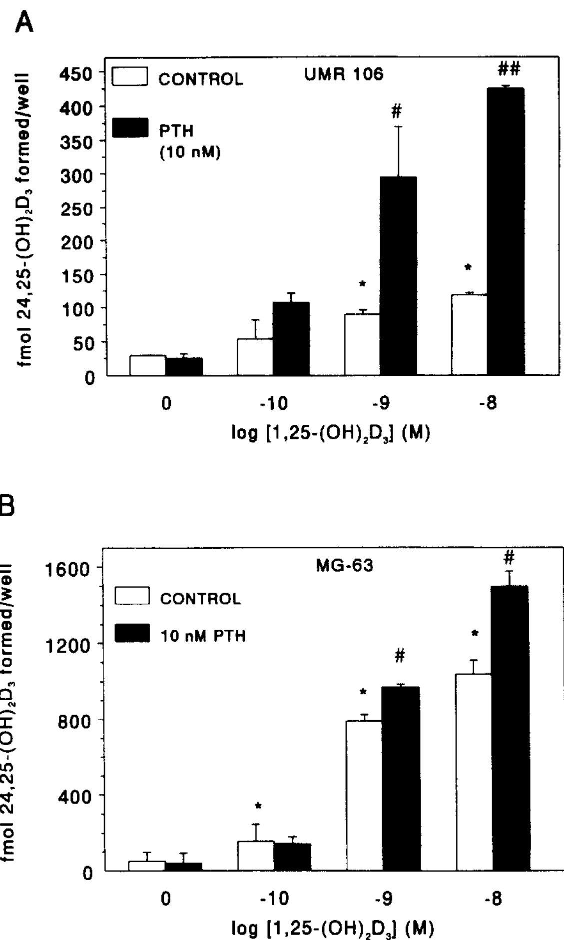


Fig. 5. Effect of PTH on 1,25-$(OH)_2D_3$-induced 24-hydroxylase activity in (A) UMR 106 cells and (B) MG-63 cells. Cells were incubated for 1 h with several concentrations 1,25-$(OH)_2D_3$ and 10 nM PTH followed by an incubation for 1 h with 10 nM $[^3H]$25-$(OH)D_3$ as substrate. Next, after a 2 h wash the 25-$(OH)D_3$ metabolites were extracted and analyzed by HPLC. Data are expressed as fmol 24,25-$(OH)_2D_3$ formed ± S.D. of at least two independent cultures. * $P < 0.001$ versus no addition of 1,25-$(OH)_2D_3$ and PTH; # $P < 0.005$ and ## $P < 0.001$ calculated as significance of interaction between 1,25-$(OH)_2D_3$ and PTH.

3.5. Effect of 1,25-$(OH)_2D_3$ 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-$(OH)_2D_3$. In MG-63 cells PTH did not affect 1,25-$(OH)_2D_3$-stimulated osteocalcin production whereas in ROS 17/2.8 cells cotreatment with PTH enhanced 1,25-$(OH)_2D_3$-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 forkolin did not significantly change 1,25-$(OH)_2D_3$-stimulated osteocalcin production (Fig. 7A). We have previously shown that activation of protein kinase C by phorbol esters did not

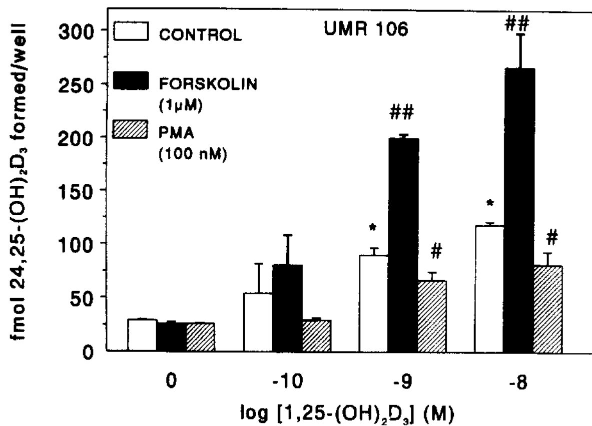


Fig. 6. Effect of forskolin and PMA on $1,25\text{-}(OH)_2D_3$-induced 24-hydroxylase activity in UMR 106 cells. Cells were incubated for 1 h with several concentrations $1,25\text{-}(OH)_2D_3$ and 1 μM forskolin followed by an incubation for 1 h with 10 nM $[^3H]25\text{-}(OH)D_3$ as substrate. Next, after a 2 h wash the $25\text{-}(OH)D_3$ metabolites were extracted and analyzed by HPLC. Data are expressed as fmol $24,25\text{-}(OH)_2D_3$ formed ± S.D. of at least two independent cultures. * $P < 0.001$ versus no addition of $1,25\text{-}(OH)_2D_3$ and forskolin or PMA; # $P < 0.05$ and ## $P < 0.001$ calculated as significance of interaction between $1,25\text{-}(OH)_2D_3$ and forskolin or PMA.

affect basal as well as $1,25\text{-}(OH)_2D_3$-stimulated osteocalcin production [39].

4. Discussion

$1,25\text{-}(OH)_2D_3$ and PTH regulate osteoblast activity via close interactions. On basis of the present observation that PTH potentiates $1,25\text{-}(OH)_2D_3$ action in osteoblasts and the previously reported inhibition of PTH-stimulated cAMP production by $1,25\text{-}(OH)_2D_3$ [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 $1,25\text{-}(OH)_2D_3$ synthesis whereas $1,25\text{-}(OH)_2D_3$ inhibits PTH synthesis by the parathyroid glands.

The currently observed interactions between $1,25\text{-}(OH)_2D_3$ 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 $25\text{-}(OH)\text{-}D_3\text{-}1\alpha$-hydroxylase by PTH occurs which eventually results in an increased serum $1,25\text{-}(OH)_2D_3$ level [1]. Under these circumstances also bone will first be exposed to increased PTH levels before increased $1,25\text{-}(OH)_2D_3$ levels. The present results indicate that despite unchanged serum $1,25\text{-}(OH)_2D_3$ levels already an enhancement of its action on bone cells to restore serum calcium levels could be achieved. The biological significance of $1,25\text{-}(OH)_2D_3$ and PTH interaction is also supported by the observation that some hypoparathyroid patients are resistant to $1,25\text{-}(OH)_2D_3$ 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 $1,25\text{-}(OH)_2D_3$ action in osteoblasts whereas protein kinase C attenuates the $1,25\text{-}(OH)_2D_3$ effects. Our data on interactions between cAMP and $1,25\text{-}(OH)_2D_3$ are in line with recent findings that cAMP enhances the $1,25\text{-}(OH)_2D_3$ action in lymphocytes and fibroblasts [40–43]. However, target tissue/cell-specific interaction between $1,25\text{-}(OH)_2D_3$ 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 $1,25\text{-}(OH)_2D_3$-induced renal 24-hydroxylase activ-

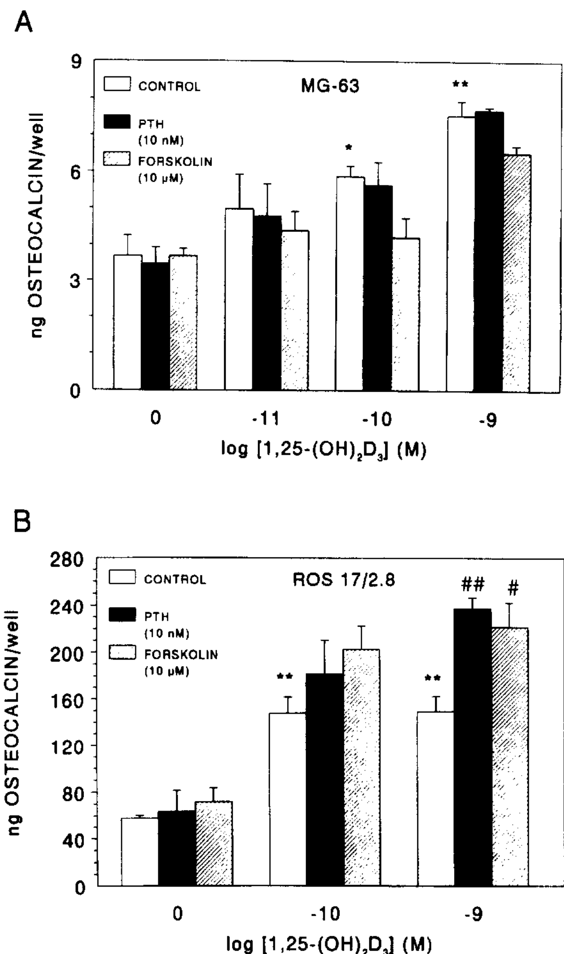


Fig. 7. Effect of PTH and forskolin on $1,25\text{-}(OH)_2D_3$-stimulated osteocalcin production by (A) MG-63 cells and (B) ROS 17/2.8 cells. Cells were incubated for 24 h and next osteocalcin concentration in the medium was analyzed. The changes in cellular osteocalcin content of ROS 17/2.8 cells were 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 $1,25\text{-}(OH)_2D_3$: 52.6 ± 1.8; MG-63 cells: Control: 113.8 ± 4.8 and 10 nM $1,25\text{-}(OH)_2D_3$: 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 $1,25\text{-}(OH)_2D_3$ and PTH or forskolin; # $P < 0.02$ and ## $P < 0.002$ calculated as significance of interaction between $1,25\text{-}(OH)_2D_3$ 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-$(OH)_2D_3$-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-$(OH)_2D_3$-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-$(OH)_2D_3$ 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-$(OH)_2D_3$ 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-$(OH)_2D_3$ 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-$(OH)_2D_3$ 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-$(OH)_2D_3$ 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-$(OH)_2D_3$ 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-$(OH)_2D_3$ 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-$(OH)_2D_3$-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-$(OH)_2D_3$ and PTH are dependent on differentiation status of the osteoblast. Previously it has been shown that the response to 1,25-$(OH)_2D_3$ 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-$(OH)_2D_3$ induction of osteocalcin synthesis may be related to a species difference. An earlier study showed that cAMP enhances 1,25-$(OH)_2D_3$-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-$(OH)_2D_3$ and PTH, two chemically distinct calciotropic 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.

References

[1] Garabedian, M., Holick, M.F., DeLuca, H.F. and Boyle, I.T. (1972) Proc. Natl. Acad. Sci. USA 69, 1673.

[2] Fraser, D.R. and Kodicek, E. (1973) Nature 241, 163.

[3] Reinhardt, T.A. and Horst, R.L. (1990) Endocrinology 127, 942.

[4] Silver, J., Russell, J., Lettieri, D. and Sherwood, L.M. (1985) Natl. Acad. Sci. USA 82, 4270.

[5] Silver, J., Naveh-Many, T., Mayer, H., Schmeizer, H.J. and Popovtzer, M.M. (1986) J. Clin. Invest. 78, 1296.
[6] Cantley, L.K., Russell, J., Lettieri, D. and Sherwood, L.M. (1985) Endocrinology 117, 2114.
[7] Catherwood, B.D. (1985) J. Biol. Chem. 260, 736–743.
[8] Pols, H.A.P., Schilte, J.P., Herrmann-Erlee, M.P.M., Visser, T.J. and Birkenhäger, J.C. (1986) Bone Mineral 1, 397–405.
[9] Pols, H.A.P., Van Leeuwen, J.P.T.M., Schilte, J.P., Visser, T.J. and Birkenhäger, J.C. (1988) Biochem. Biophys. Res. Commun. 156, 588–594.
[10] Van Leeuwen, J.P.T.M., Birkenhäger, J.C., Vink-Van Wijngaarden, T., Van den Bemd, G.J.C.M. and Pols, H.A.P. (1992) Biochem. Biophys. Res. Commun. 185, 881–886.
[11] Reinhardt, T.A. and Horst, R.L. (1990) Endocrinology 127, 942–948.
[12] Kream, B.E., Jose, M., Yamada, S. and DeLuca, H.F. (1977) Science 197, 1086.
[13] Silve, C.M., Kradek, G.T., Jones, A.L. and Arnaud, C.D. (1982) J. Cell. Biol. 94, 379.
[14] Clemens, T.L., Garrett, K.P., Zhou, X-Y., Pike, J.W., Hausler, M.R. and Dempster, D.W. (1988) Endocrinology 122, 1224.
[15] Kumegawa, M., Ishizuka, S., Sumitani, K., Kusuda, M., Hakeda, Y., Okazaki, R., Matsumoto, T. and Ogata, E. (1990) Calcif. Tissue Int. 46(2), A22(Abstract).
[16] Rodan, G.A. and Martin, T.J. (1981) Calcif. Tissue Int. 33, 349.
[17] Raisz, L.G. (1965) J. Clin. Invest. 44, 103.
[18] Garabedian, M., Tanaka, Y., Holick, M.F. and DeLuca, H.F. (1974) Endocrinology 94, 1022.
[19] Van Leeuwen, J.P.T.M., Birkenhäger, J.C., Bos, M.P., Herrmann-Erlee, M.P.M. and Pols, H.A.P. (1992) J. Bone Mineral Res. 7, 303–309.
[20] Ralston, S., Boyle, I.T., Cowan, R.A., Crean, G.P., Jenkins, A. and Thomson, W.S. (1983) Acta Endocrinol. 103, 535–538.
[21] Chesney, R.W. (1984) NY State J. Med. 84, 226–227.
[22] Pols, H.A.P., Birkenhäger, J.C., Schilte, J.P. and Visser, T.J. (1988) Biochim. Biophys. Acta 970, 123–129.
[23] Feldman, D., McCain, T.A., Hirst, M.A., Chen, T.L. and Colston, K.W. (1979) J. Biol. Chem. 200, 10378.
[24] Pols, H.A.P., Schilte, J.P., Nijweide, P.J., Visser, T.J. and Birkenhäger, J.C. (1984) Biochem. Biophys. Res. Commun. 125, 265.
[25] Verhaeghe, J., Van Herck, E., Van Bree, R., Van Assche, F.A. and Bouillon, R. (1988) J. Endocrinol. 120, 143–151.
[26] Chomczinski, P. and Sacchi, N. (1987) Anal. Biochem. 162, 156–159.
[27] Bradford, M.M. (1976) Anal Biochem. 72, 248.
[28] Johnson-Wint, B. and Hollis, S. (1982) Anal. Biochem. 122, 338.
[29] Chase, L.R. and Aurbach, G.D. (1970) J. Biol. Chem. 245, 1520–1526.
[30] Herrmann-Erlee, M.P.M. and Konijn, T.M. (1970) Nature 227, 177–178.
[31] Yamaguchi, D.T., Hahn, T.J., Iida-Klein, E., Kleeman, C.R. and Muallem, S. (1987) J. Biol. Chem. 262, 13168–13173.
[32] Van Leeuwen, J.P.T.M., Bos, M.P., Löwik, C.W.G.M. and Herrmann-Erlee, M.P.M. (1988) Bone Mineral 4, 177-188.
[33] Hruska, K.A., Moskowitz, D., Esbrit, P., Civitelli, R., Westbrook, S. and Huskey, M. (1987) J. Clin. Invest. 79, 230–239.
[34] Abou-Samra, A.S., Jueppner, H., Westerberg, D., Potts, Jr. J.T. and Segre, G.V. (1989) Endocrinology 124, 1107–1113.
[35] Van Leeuwen, J.P.T.M., Birkenhäger, J.C., Schilte, J.P., Buurman, C.J. and Pols, H.A.P. (1990) Cell Calcium 11, 281–289.
[36] Van Leeuwen, J.P.T.M., Birkenhäger, J.C., Buurman, C.J., Van den Bemd, G.J.C.M., Bos, M.P. and Pols, H.A.P. (1992) Endocrinology 130(4), 2259–2266.
[37] Kumar, R. (1986) Kidney Int. 30, 793–803.
[38] Pan, L.C. and Price, P.A. (1987) J. Biol. Chem. 262, 4670–4675.
[39] Van Leeuwen, J.P.T.M., Birkenhäger, J.C., Van den Bemd, G.J.C.M., Buurman, C.J., Staal, A., Bos, M.P. and Pols, H.A.P. (1992) J. Biol. Chem. 267, 12562–12569.
[40] Ravid, A., Koren, R., Narinsky, R., Rotem, C., Novogrodsky, A. and Liberman, U.A. (1990) Endocrinology 70, 1687–1692.
[41] Inaba, M., Okuno, S., Koyama, H., Nishizawa, Y. and Morii, H. (1992) Arch. Biochem. Biophys. 293, 181–186.
[42] Krishnan, A.V. and Feldman, D. (1992) Mol. Endocrinol. 6, 198-206.
[43] Jehan, F., Neveu, I., Naveilhan, P., Brachet, P. and Wion, D. (1993) J. Neurochem. 60, 1843–1853.
[44] Shigematsu, T., Horiuchi, N., Ogura, Y., Miyahara, T. and Suda, T. (1986) Endocrinology 118, 1583–1589.
[45] Shinki, T., He Jin, C., Nishimura, A., Nagai, Y., Ohyama, Y., Noshiro, M., Okuda, K. and Suda, T. (1992) J. Biol. Chem. 267, 13757–13762.
[46] Armbrecht, H.J., Hodam, T.L., Boltz, M.A. and Chen, M.L. (1993) FEBS Lett. 327, 13–16.
[47] Chen, M.L., Boltz, M.A. and Armbrecht, H.J. (1993) Endocrinology 132, 1782–1788.
[48] Krishnan, A.V., Cramer, S.D., Bringhurst, F.R. and Feldman, D. (1994) Endocrinology 136, 705-712.
[49] Lian, J.B., Stewart, C., Puchacz, E., Mackowiak, S., Shalhoub, V., Collart, D., Zambetti, G. and Stein, G. (1989) Proc. Natl. Acad. Sci. USA 86, 1143–1147.
[50] Brown, T.A. and DeLuca, H.F. (1990) J. Biol. Chem. 265, 10025–11029.
[51] Hsieh, J-C., Jurutka, P.W., Galligan, M.A., Terpening, C.M., Haussler, C.A., Samuels, D.S., Shimizu, Y., Shimizu, N. and Haussler, M.R. (1991) Proc. Natl. Acad. Sci. USA 88, 9315–9319.
[52] Darwish, H.M., Burmester, J.K., Moss, V.E. and DeLuca, H.F. (1993) Biochim. Biophys. Acta 1167, 29–36.
[53] Hsieh, J-C., Jurutka, P.W., Nakajima, S., Galligan, M.A., Haussler, C.A., Shimizu, Y., Shimizu, N., Whitfield, K.W. and Haussler, M.R. (1993) J. Biol. Chem. 268, 15118–15126.
[54] Jurutka, P.W., Hsieh, J-C. and Haussler, M.R. (1993) Biochem. Biophys. Res. Commun. 191, 1089–1096.
[55] Jurutka, P.W., Hsieh, J.C., MacDonald, P.N., Terpening, C.M., Haussler, C.A., Haussler, M.R. and Whitfield, G.K. (1993) J. Biol. Chem. 268, 6791–6799.
[56] Noda, M., Yoon, K. and Rodan, G.A. (1988) J. Biol. Chem. 263, 18574–18577.
[57] Skoufos, E. and Sanders, M.M. (1992) Mol. Endocrinol. 6, 1412–1417.
[58] Cho, H. and Katzenellenbogen, B.S. (1993) Mol. Endocrinol. 7, 441–452.
[59] Nordeen, S.K., Bona, B.J. and Moyer, M.L. (1993) Mol. Endocrinol. 7, 731–742.
[60] Sartorius, C.A., Tung, L., Takimoto, G.S. and Horwitz, K.B. (1993) J. Biol. Chem. 268, 9262–9266.
[61] Fujimoto, N. and Katzenellenbogen, B.S. (1994) Mol. Endocrinol. 8, 296–304.
[62] McSheehy, P.M.J. and Chambers, T.J. (1986) Endocrinology 118, 824–828.
[63] Owen, T.A., Aronow, M.S., Barone, L.M., Bettencourt, B., Stein, G.S. and Lian, J.B. (1991) Endocrinology 128, 1496–1504;.
[64] Stein, G.S., Lian, J.B. and Owen, T.A. (1990) FASEB J. 4, 3111–3123.
[65] Theofan, G. and Price, P.A. (1989) Mol. Endocrinol. 3, 36–43.
[66] Beresford, J.N., Gallagher, J.A., Poser, J.W. and Russell, R.G.G. (1984) Metab. Bone Dis. Rel. Res. 5, 229–234.
[67] Evans, D.B., Russell, R.G.G., Brown, B.L. and Dobson, P.R.M. (1989) Biochem. Biophys. Res. Commun. 164, 1076–1085.