Upregulation of parathyroid VDR expression by extracellular calcium is mediated by ERK1/2-MAPK signaling pathway

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Submitted 8 September 2009; accepted in final form 18 February 2010

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PARATHYROID HORMONE (PTH) secretion is tightly regulated by extracellular calcium concentration. Parathyroid cells have a specific calcium-sensing receptor (CaSR) (8, 11); the activation of the parathyroid CaSR by calcium not only reduces PTH secretion, but it also decreases the expression of PTH mRNA and inhibits parathyroid cell proliferation (29, 30).

In a previous study, we have shown that an elevation in extracellular calcium concentration upregulates parathyroid vitamin D receptor (VDR) expression (15, 33). Furthermore, the upregulation of the parathyroid VDR expression by its own ligand, calcitriol, was greatly enhanced when the extracellular calcium concentration was high. The signaling pathway involved in the upregulation of parathyroid VDR by high calcium is unknown. Extracellular calcium must act through the CaSR; this receptor belongs to the superfamily of G protein-coupled receptors. Activation of the CaSR results in G_α_{i11} dependent activation of phosphatidylinositol-specific phospholipase C, which in turn results in the accumulation of inositol phosphate-3 (IP_3) and diacylglycerol, a release of calcium from intracellular stores, and the activation of PKC (7, 31).

Previous studies by us and others (3, 6) have shown that CaSR activation stimulates PLA_2 and the production of arachidonic acid (AA), which mediate the inhibition of PTH secretion. Activated PLA_2 is also associated with phosphorylation of MAPK, as ERK 1/2 and p38-MAPK (12, 34).

The present study was designed to characterize the signaling system responsible for the stimulation of parathyroid VDR expression induced by activation of the CaSR.

MATERIALS AND METHODS

In Vitro Studies

Parathyroid tissue. In vitro studies were performed with whole parathyroid glands from male Wistar rats, weighing 170–200 g. The rats were on a 0.6% calcium/0.6% phosphorus diet supplemented with 100 IU/100 g vitamin D. The animals were anesthetized with pentobarbital sodium (50 mg/kg), and blood was drained by aortic puncture; within 2 min, the parathyroid glands were dissected free of the thyroid gland under a dissecting microscope. All animals received humane care in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences.

Incubation conditions. Intact rat parathyroid glands were placed resting inside a nylon basket in individual wells with constant shaking at 37°C in a humid atmosphere. The incubation medium was buffered (7.4) and contained (in mM) 125 NaCl, 5.9 KCl, 0.5 MgCl_2, 1 phosphate (NaH_2PO_4: Na_2HPO_4, 1:2 ratio), 1 Na pyruvate, 4 glucose, and 25 HEPES. Insulin (0.1 IU/ml), 0.1% BSA, penicillin G (100 IU/ml), and streptomycin (100 μg/ml) were added to the medium. CaCl_2 was added to medium to achieve the desired concentration.

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calcium concentration. Ionized calcium concentration was measured using a selective electrode (634 Ca/pH analyzer; Ciba Corning, Essex, UK). After the experimental period, the medium was collected and stored at -20°C until the determination of PTH (Rat Bioactive Intact PTH Elisa Kit, Immunotopics, San Clemente, CA). Assayed agents AA, calcium ionophore (A23187), ERK 1/2 and p38 inhibitors (PD98059 and SB202190, respectively), and the Sp1 inhibitor (mithramycin A) were added to the medium.

Cell viability was assessed in mechanically dispersed cells labeled with two fluorochrome dyes: green fluoriacetate (Sigma, St. Louis, MO) and red propidium iodine (Sigma), which stain live and dead cells, respectively. Stained cells were assessed by flow cytometry (FACScan; Becton-Dickinson, San Jose, CA) using the software LYSIS II. Only tissue samples with >90% of viable cells were used in the experiments.

In Vivo Studies

In vivo studies were performed with normal and uremic rats. Renal failure in rats was induced by two-step 5/6 nephrectomy (5/6 Nx) as follows. On day 1, renal mass ablation of 2/3 of the left kidney and on day 7 nephrectomy of the right kidney were performed. The experimental diet of these rats contained normal calcium (0.6%) and high phosphate (1.2%). Normal, sham-operated rats underwent the same procedures without renal CaCl2 and were fed a normal calcium (0.6%) and normal phosphorus diet (0.6%). Normal (sham-operated) rats received intraperitoneal injections of CaCl2 or a single intramuscular injection of EDTA to obtain 6 h of hypercalcemic or hypocalcemic clamp, respectively, as previously reported (15). Uremic (Nx) rats were also injected CaCl2 to obtain a group of Nx hypercalcemic rats, but not EDTA as they were already hypocalcemic. Another group of rats receiving vehicle was used as a control. For the hypercalcemic clamp, persistently high levels of serum calcium were achieved by intraperitoneal injections of CaCl2 diluted in Ringer lactate. Injections were repeated at 30-min intervals: the first injection of CaCl2 was 58.8 mg; then 44.1 mg at 30 min, 29.4 mg at 60 min, 18.3 mg at 90 min, and 7.0 mg every 30 min until the end of the experiment at 360 min. For the hypocalcemic clamp, rats received one intramuscular injection of EDTA at the beginning of the experiments, which resulted in 6 h of sustained hypocalcemia. Food was removed 12 h before the beginning of the experiments.

VDR mRNA and β-actin mRNA expression. After incubation, intact parathyroid glands were removed and stored at -80°C until RNA extraction. Total RNA was extracted following a modification of Chomczynski and Sacchi's protocol (9). The RNA was dissolved in nuclease-free water (Promega, Madison, WI) and heated for 10 min at 80°C until RNA extraction. Total RNA was quantified by spectrophotometry (ND-1000, NanoDrop, Wilmington, DE). VDR vs. actin was amplified with the kit (RT-PCR Sybr green, Qiagen) using specific primers (sense and antisense, respectively) for actin (5'-TGTCACCAA-CTGGGACGCA- CATGGAG-3'; 5'-GGCGTACAGGGAT AGCACAGCCTGGA-3') and VDR (5'-TGAAGCTGCAAGGCTTCAAGC-3'; 5'-GATG-AACTCTTCTCATCATGGCAGTAC-3'; TIB MOLBIOL, Berlin, Germany). One hundred nanograms of total RNA were used for quantification of VDR and β-actin mRNA. Amplifications were processed by real-time PCR (Lightcycler, Roche Diagnostics, Basel, Switzerland).

Activity of VDR promoter (Luciferase assay). As parathyroid cell line is not available and the common methods used in cell culture may not applied in parathyroid glands, we have used human embryonic kidney cells (HEK293) stably transfected with the human CaR (HEKCaR). We performed a successful transfection in the HEKCaR cells with the plasmid pGL3-PVDR-Luc as a reporter construct (which contains the luciferase gene under the control of the VDR promoter). Forty-eight hours after the transfection, the cells were cultured in calcium concentrations of 0.6 and 1.5 mM. The next day they were washed twice with phosphate-buffered saline and harvested in passive lysis buffer to perform a luciferase assay. The relative luciferase activity was measured by calculating the ratio of firefly luciferase activity to protein concentration. CaSR HEK cells were a generous gift from Jeremy M. Henley (University of Bristol), and pGL3-PVDR-Luc was a generous gift from Madhavi P. Kadakia (Wright State University).

**ERK1/2 phosphorylation.** ERK1/2 phosphorylation was assessed by immunohistochemistry. Parathyroid glands were fixed in 4% formalin. After dehydration through a graded series of ethanol, glands were embedded in paraffin. Three-micrometer sections were deparaffinized and microwave treated in 0.01 mmol/l citrate buffer, pH 7.2, for 15 min. Then, sections were incubated in 0.3% H2O2 in methanol for 30 min. After that, sections were stained (incubated overnight) with primary antibody rabbit anti-phospho-ERK1/2 (monoclonal antibody, 1:60 dilution, Cell Signaling Technology, Beverly, MA). The sections were then incubated for 2 h at room temperature with peroxidase-conjugated anti-rabbit IgG and treated with 3,3′-diaminobenzidine-tetrachloride (1:100 dilution, En-vision System, Dako, Glostrup, Denmark). Every step was followed by three washes with PBS for 10 min. Sections were counterstained with hematoxylin. Immunostaining of ERK1/2 protein was quantified using a Carl Zeiss photomicroscope (Axioskop) coupled to a digital camera and an image-analysis system (Eclipse Net). Images of stained tissue section (×400 magnification) were digitized, converted to grayscale, and analyzed using Image-Pro Plus 4.5 software (Media Cybernetics), which allows discrimination vs. background staining (negative control tissue). The results were expressed as positive cells/area of the entire section of tissue. Approximately nine sections of tissue from each parathyroid gland were immunostained; tissue sections from three glands/experimental group were stained and analyzed together.

Activation of transcription factors Sp1, NF-κB, and activator protein-1. Nuclear protein was extracted from parathyroid tissue using the Qproteome cell compartment kit (Qiagen, Madrid, Spain). Sp1, AP-1, and NF-κB activation (as measured by its specific DNA-binding capacity) was analyzed using respective Trans-AM Transcription Factor Assay Kits (Active Motif North America, Carlsbad, CA) according to the manufacturer's instructions. Briefly, 5 μg of parathyroid tissue nuclear extracts were incubated with an oligonucleotide containing the consensus binding element for each of the transcription factors analyzed bound to a 96-well plate. After extensive washes, the activated transcription factor complexes bound to the oligonucleotide were incubated with an antibody directed against the activated Sp1, activator protein-1 (AP-1), or NF-κB p50 and p65 protein, respectively, at a dilution of 1:1,000. After washes, the plate was subsequently incubated with a secondary antibody conjugated to peroxidase (1:1,000), and the peroxidase reaction was quantified at 450 nm with a reference wavelength of 655 nm using a spectrophotometer (TECAN, Salzburg, Austria).

Chemicals. All chemical products of culture medium, AA, and the calcium ionophore (A23187) and mithramycin were obtained from Sigma. ERK1/2 MAPK and p38 MAPK inhibitors (PD98059 and SB202190, respectively) were purchased from Calbiochem (San Diego, CA).

**Statistical Analyses**

Comparisons between more than two means were performed by ANOVA, followed by Duncan’s post hoc analysis. An unpaired t-test was used for comparison of two means. Tests were performed using SPSS 11.0 for Windows.

**RESULTS**

**Effect of Extracellular Calcium Concentration on Parathyroid VDR mRNA Expression**

The expression of VDR mRNA in parathyroid glands incubated for 6 h in a medium with high calcium (1.5 mM)
Intracellular Calcium-PLA2-AA Pathway and Upregulation of Parathyroid VDR mRNA Expression by Calcium

The increase in intracellular calcium (Ca\textsubscript{i}) by the addition of the calcium ionophore A23187 (10 \mu M) to the low-calcium (0.6 mM) medium for 6 h produced an increase in VDR mRNA expression (Fig. 1). The presence of the calcium ionophore also reduced the PTH secretion to a level similar to that observed in high calcium concentration (1.5 mM) (37 ± 3% vs. 0.6 mM calcium, P < 0.01). AA, which is produced by a Ca\textsubscript{i}-dependent PLA2, plays a crucial role in the parathyroid cell intracellular signaling system. The addition of AA (20 \mu M) to the low-calcium medium (0.6 mM) also produced an increase in VDR mRNA to the same level as that observed with the high-calcium medium (Fig. 1); AA also inhibited PTH secretion despite the low calcium in the medium (to 39 ± 3% vs. 0.6 mM calcium, P < 0.01).

MAPK Activity and Upregulation of Parathyroid VDR mRNA Expression by Calcium

To assess whether MAPK (ERK1/2 or p38) activity was increased twofold with low calcium (0.6 mM) (Fig. 1). The expected inhibition of PTH secretion by calcium (to a 40 ± 5% vs. 0.6 mM calcium, P < 0.01) was also observed. To demonstrate that high calcium increases VDR promoter activity, we produced a reporter construct for VDR. Promoter activity could not be tested in isolated parathyroid cells in culture because dispersion of parathyroid cells is associated to a rapid decrease in the expression of CaSR, and there is no parathyroid cell line available. Thus VDR promoter activity had to be evaluated in HEKCaR cells transfected with the plasmid pGL3-PVDR-Luc. Luciferase activity was consistently increased in 1.5 mM compared with 0.6 mM calcium (1.37 ± 0.03 vs. 1.0 ± 0.04, P < 0.05).

Fig. 1. Intracellular calcium (Ca\textsubscript{i})-PLA2-arachidonic acid (AA) pathway and the upregulation of parathyroid vitamin D receptor (VDR) mRNA expression by calcium. Intact rat parathyroid glands were incubated for 6 h in low calcium (0.6 mM), high calcium (1.5 mM), low calcium plus the ionophore A23187 (10 \mu M; Ca 0.6 mM+I), or low calcium plus AA (20 \mu M; Ca 0.6 mM+AA). VDR mRNA data are expressed as the % of ratio units (the VDR/\beta-actin mRNA ratio) vs. those in low (0.6 mM) calcium. Values are means ± SE. *P < 0.05 vs. 0.6 mM Ca.

Fig. 2. Effect of MAPK inhibitors on VDR mRNA expression. Intact rat parathyroid glands were incubated for 6 h with a high calcium concentration and PD98059 (10 \mu M), a specific ERK1/2 inhibitor, or SB202190 (10 \mu M), a specific p38 inhibitor. The results are shown in Fig. 2. High calcium increased VDR expression. The inhibition of ERK1/2 by PD98059 prevented the increase in VDR expression by calcium. However, the inhibition of p38 by SB202190 did not reduce the high calcium-induced VDR expression. These results suggest that calcium regulates VDR expression via ERK1/2 activation. Inhibition of ERK1/2 also had an effect on PTH secretion. In a high calcium-concentration medium, PTH secretion was increased when ERK1/2 was inhibited by the addition of PD (75 ± 6% vs. 0.6 mM calcium in 1.5 mM calcium+PD compared with 52 ± 3% in 1.5 mM calcium, P < 0.05). In contrast, inhibition of p38 did not interfere with the reduction of PTH by high calcium (57 ± 4% in 1.5 mM calcium+PD compared with 52 ± 3% in 1.5 mM calcium, not significant).

Effect of ERK1/2 on VDR Expression is Regulated Upstream by AA

Both AA and ERK1/2 seem to be involved in the upregulation of VDR mRNA expression. Additional experiments were performed to determine whether AA regulates VDR expression via activation of ERK1/2.

The addition of AA to the 0.6 mM calcium medium increased VDR mRNA expression. This effect of AA was no longer present when PD98059, the ERK1/2 inhibitor, was added to the culture medium. This was not the case when SB202190, the p38 inhibitor, was added to the medium; SB202190 did not have an effect on the stimulation of VDR mRNA expression by AA (Fig. 3). Thus AA needs ERK1/2 activation to stimulate VDR expression. The addition of PD98059 also prevented the inhibition of PTH secretion by AA [98 ± 5 and 40 ± 4% vs. 0.6 mM calcium in 0.6 calcium+AA+PD, (not significant) and 0.6 calcium+AA (P < 0.01), respectively], but the addition of SB202190 did not (40 ± 3% vs. 0.6 mM calcium, P < 0.01).
AA Induces Activation of ERK1/2

The effect of AA on parathyroid ERK1/2 phosphorylation was assessed by immunohistochemistry (Fig. 4). Parathyroid glands incubated with AA for 6 h showed an increase in the number of parathyroid cells stained for phosphorylated ERK1/2 [88 ± 2 and 24 ± 6 positive cells/area of the entire section of tissue in 0.6 mM calcium + AA and 0.6 mM calcium (P < 0.01), respectively]. In parathyroid glands incubated with a high calcium concentration (1.5 mM), the number of cells stained for phosphorylated ERK1/2 was 115 ± 5 (P < 0.01 compared with 0.6 mM calcium).

Sp1 But not AP-1 or NF-κB Is Involved in Upregulation of VDR Expression by Calcium

The VDR promoter possesses sites for interaction with several transcription factors such as the AP-1, Sp1, and NF-κB. Thus we assessed the activation of these through the PLA2- AA-ERK1/2 pathway which mediates the upregulation of the VDR expression by calcium. DNA binding of activated Sp1 was evaluated in glands exposed to high calcium. As shown in Table 1, a high calcium concentration (1.5 mM) in the medium produced an increase in Sp1 activation compared with 0.6 mM calcium. The addition of AA to the low-calcium medium increased Sp1 activation to almost the same level as 1.5 mM calcium. Furthermore, inhibition of ERK1/2 by PD98059 prevented the activation of Sp1 induced by AA in low calcium.

Activation of p50 and p65, the two main components of the NF-κB family, and of AP-1 were also evaluated by measuring the DNA-binding capacity. Experiments using high calcium, AA, or PD98059 did not produce activation of either NF-κB p50 or p65 or AP-1 (Table 1).

To demonstrate that the upregulation of VDR expression by calcium is directly dependent on Sp1 activation, parathyroid glands were incubated in low (0.6 mM) and high (1.5 mM) calcium in the presence of mithramycin A (a Sp1 inhibitor). As shown in the Fig. 5, mithramycin A, which prevents Sp1 binding to GC-rich regions in chromatin, at a 1 μM concentration prevented the upregulation of VDR mRNA by high calcium.

Upregulation of VDR Expression by Calcium in Uremia

To ascertain whether the upregulation of VDR expression by calcium is maintained in hyperplasic parathyroid glands under uremic conditions, we performed in vivo experiments with a rat model of secondary hyperparathyroidism (5/6 nephrectomy...
and a high-phosphate diet). Normal (sham-operated) rats received intraperitoneal injections of CaCl₂ or a single intramuscular injection of EDTA to obtain a 6-h hypercalcemic or hypocalcemic clamp, respectively. This experimental setup has been performed previously in our laboratory (14). Uremic (Nx) rats were also injected with CaCl₂ to obtain a group of Nx hypercalcemic rats; EDTA was not given to Nx as they were already hypocalcemic. Another group of rats receiving vehicle was used as a control. In hypercalcemic Nx rats, serum calcium was higher than in hypocalcemic Nx rats (1.19 ± 0.05 vs. 0.75 ± 0.04, P < 0.01); however, the serum calcium in hypercalcemic Nx rats was not as high as in hypercalcemic sham rats (1.64 ± 0.08, P < 0.01). As shown in the Fig. 6, both hypercalcemic sham and Nx rats, had similar increased levels of VDR mRNA compared with the groups of hypocalcemic rats.

**DISCUSSION**

We have previously demonstrated that CaSR activation by increased extracellular calcium or calcimimetics upregulates rat parathyroid VDR expression in vivo (15, 33). The present study was designed to elucidate the signaling pathway that mediates the stimulation of the parathyroid VDR gene expression induced by extracellular calcium.

Our study shows that a high calcium concentration increased VDR mRNA expression in rat parathyroid glands in vitro. High extracellular calcium also increased the vdr promoter activity in HEKCaR cells transfected with the plasmid pGL3-PVDR-Luc. Thus the increase in the VDR mRNA levels observed in the parathyroid glands is likely due to an increase in the transcriptional activity.

AA plays a crucial role in the parathyroid cell intracellular signaling system. We and others have shown that in parathyroid cells, the PLA₂-AA pathway is stimulated by high extracellular calcium and mediates the inhibition of PTH secretion (3, 6, 18). In the presence of a low extracellular calcium concentration, the addition of AA inhibits the PTH secretion (3, 6). Furthermore, the activation of the CaSR by extracellular calcium results in a PKC-mediated activation of PLA₂ to release AA and subsequent ERK1/2 activation (13, 19). Bordeau et al. (5, 6) showed that the downstream-oxygenated products of AA produced through the 12-and 15-lipoxygenase pathway, 12-, and 15-HETES, are potent inhibitors of PTH secretion.

In this work we have evaluated whether this pathway is also involved in the stimulation of VDR expression by calcium. The results indicate that this effect seems to be mediated by AA since the addition of AA to the low-calcium medium produced an increase in VDR mRNA of the same magnitude as that observed with high calcium in the medium. An effect of AA, directly or through the products of its metabolism, on the regulation of gene expression has been previously observed in other cell types (4, 20). In parathyroid cells, the Ca₉ level increases in response to the CaSR-dependent PLC activation (8), and previous studies by us demonstrated the activation of the PLA₂ and AA production via a Ca₉ increase (3). In this study, the incubation of parathyroid glands with low calcium and the calcium ionophore (which mimics the increase in Ca₉ after activation of the CaSR) resulted in an increase in the expression of VDR mRNA. Therefore, our results show that the stimulation of VDR gene expression by extracellular calcium is mediated by the Ca₉-PLA₂-AA pathway, although the role of other intracellular pathways cannot be discarded.

Most of G protein-coupled receptors cause activation of the ERK1/2-MAPK pathway (29, 36), which in turn regulates PLAS activity (15, 20, 21). The CaSR is a G protein-coupled receptor. Kifor et al. (19) suggested that in CaSR-transfected HEK cells, the activation of PLAS is dependent on ERK1/2 MAPK signaling. In the present study, we have shown that the
specific inhibition of ERK1/2 activity prevented stimulation of VDR expression by high calcium concentration. The inhibition of other MAPK pathways, such as p38 MAPK, had no effect on VDR expression. Furthermore, the effect of AA on PTH secretion and VDR expression was also blocked by the inhibition of ERK1/2, which suggests that the stimulation of VDR expression by AA is mediated through the ERK1/2-MAPK signaling. Thus we evaluated the effect of AA on ERK1/2 activation. Parathyroid glands incubated with high (1.5 mM) calcium showed higher levels of phosphorylated (activated) ERK1/2 compared with those incubated in low (0.6 mM) calcium. The addition of AA to the low-calcium medium resulted in the upregulation of VDR mRNA and the stimulation of ERK1/2 activation, which confirms that AA regulates parathyroid VDR gene expression through the activation of the MAPKs. These results strongly suggest a specific role of ERK1/2 in the regulation of VDR expression in parathyroid glands.

Different studies have shown that activation of the CaSR induces phosphorylation of ERK1/2 in a variety of cell types, such as Rat-1 fibroblasts (24), NIH/3T3 fibroblasts (17), HEK-RCa transfected cells, bovine parathyroid cells (19), and human parathyroid gland slices (13). Nevertheless, there are not reports of a genomic effect of MAPK (ERK1/2) activation in parathyroid cells. This is not the case for other cell types in which activation of MAPKs leads to changes in gene expression (14, 28). An involvement of MAPKs in VDR expression in parathyroid cells has not been considered before. However, there is one study in the osteosarcoma cell line MC3T3-E-1 showing that the activation of the ERK1/2-MAPK was associated with an increase in VDR protein expression (28). A number of studies have demonstrated that AA and/or its metabolites are able to activate the MAPK pathway (1, 23, 32, 35). Our results show that in parathyroid glands, ERK1/2-MAPK is activated when AA is added to the medium and this is followed by an increase in VDR gene expression.

The VDR promoter has response elements to several transcription factors as the AP-1, Sp1, and NF-κB (26). In addition, there is a report on the activation of AP-1 by AA-MAPK-dependent signals in the hepatic cell line HepG2 (4). Alimov et al. (2) have suggested that the Sp family had an important role in the transcription of the PTH gene by interacting with a highly conserved Sp1 DNA element in the gene’s promoter. In our experiments, we did not find an involvement of AP-1 and NF-κB in the regulation of VDR mRNA expression by extracellular calcium. However, activation of a CaSR-dependent signaling pathway, which upregulated VDR mRNA expression, caused the activation of Sp1. The addition of AA to the low-calcium medium also increased Sp1 activation to almost the same level as high calcium concentration. Furthermore, activation of Sp1 induced by AA was prevented by the inhibition of ERK1/2. Thus our results demonstrate that activation of CaSR stimulates Sp1 activation through the ERK 1/2 pathway. We confirmed that the upregulation of VDR expression by calcium is directly dependent on Sp1 activation by the use of the Sp1 inhibitor mithramycin A, a GC box inhibitor that specifically blocks interaction between Sp1 and the GC motif by masking GC-rich sequences (10). Incubation of parathyroid glands with mithramycin A prevented the upregulation of VDR mRNA by high calcium. This suggests that following the activation of the CaSR, Sp1 interacts with its response element at the VDR promoter to modulate VDR expression by extracellular calcium.

Figure 7 summarizes the signaling pathway by which the activation of CaR likely regulates the expression of the VDR to facilitate the inhibitory action of calcitriol on parathyroid cell function.

Finally, in vivo studies revealed that uremic hyperplasic parathyroid glands retain the ability to upregulate VDR expression by calcium. Hypocalcemic uremic rats showed a reduced expression of VDR mRNA. After 6 h of hypercalcemia, the VDR mRNA levels increased to levels similar to those observed in normal rats. It suggests that the signaling pathway involved in the regulation of VDR expression by calcium remains functional in our uremic rats with (diffuse) parathyroid hyperplasia. This is in agreement with previous results reporting that the treatment with the calcimimetic R-568 increased VDR mRNA and protein in human parathyroid glands with diffuse hyperplasia in vitro (33).

![Diagram](http://ajprenal.physiology.org/)
The results of the present study add new knowledge of the mechanisms by which serum calcium regulates the inhibitory effect of calcitriol on the parathyroid glands by modulating parathyroid VDR expression. Downregulation of parathyroid VDR caused by low calcium is an important concept in the pathogenesis of secondary hyperparathyroidism because hypocalcemia may not allow a normal inhibition of parathyroid cells by calcitriol. Thus any effort to avoid an important decrease in serum calcium in patients with secondary hyperparathyroidism may facilitate the inhibitory effect of vitamin D on the parathyroids. Furthermore, it would allow for improved efficacy of vitamin D therapy if calcimimetics are also given to the patients (33). Elucidation of the signaling pathways involved in this effect may lead to a comprehensive understanding of the mechanisms of action of CaR activators as the calcimimetics. The OPTIMA study demonstrated that calcinac-tet allowed the attainment of key metabolic targets for patients with secondary hyperparathyroidism with a lower dose of vitamin D sterols (25). Thus it seems reasonable to assume that a key component of its therapeutic effect is related to the upregulation of VDR expression by this pathway.

In conclusion, our results demonstrate that extracellular calcium stimulates VDR expression in parathyroid glands through the elevation of the cytosolic calcium level and the subsequent stimulation of the PLA2-AA-dependent ERK1/2-pathway. Furthermore, our results suggest that the transcription factor Sp1 mediates the regulation of parathyroid VDR expression by extracellular calcium.

GRANTS

Financial support was obtained from Instituto Carlos III (FIS 07-0287, FIS 07/0315) and Junta de Andalucía (0025/07, 0127/08). Y. Almadén is a senior researcher supported by the Fundación Progreso y Salud, Consejería de Salud (Junta de Andalucía).

DISCLOSURES

No conflicts of interest are declared by the authors.

REFERENCES


AJP-Renal Physiol • VOL 298 • MAY 2010 • www.ajprenal.org


