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Regulation by vitamin D metabolites of messenger ribonucleic acid for preproparathyroid hormone in isolated bovine parathyroid cells

(calcium/sterol/control/culture)

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ABSTRACT **We have recently determined that high calcium concentrations, in parallel with their suppressive effects on parathyroid hormone (PTH) secretion, reversibly and specifically decrease preproPTH mRNA in cultured bovine parathyroid cells. In order to determine whether vitamin D metabolites also regulate the content of preproPTH mRNA, we tested their effects on bovine parathyroid cells in the same culture system. Levels of preproPTH mRNA were determined by dot-blot hybridization or blot hybridization with a labeled cloned cDNA probe. Incubation with 1,25-dihydroxycholecalciferol at doses varying from 10 pM to 0.1 μM caused a direct decrease in mRNA down to 50% of control values at 48 hr. There was no evidence that 1,25-dihydroxycholecalciferol, even at the highest concentrations, had any toxic effects on cell number or viability or on total RNA or RNA synthesis. Levels of α-actin mRNA did not change in the same experiments, and the suppression of preproPTH mRNA was reversible. When the relative potency of various vitamin D metabolites in suppressing preproPTH mRNA was evaluated, 1,25-dihydroxycholecalciferol > 24,25-dihydroxycholecalciferol > 25-hydroxycholecalciferol > vitamin D_3 (cholecalciferol). These effects were highly specific and suggest that vitamin D metabolites play an important role in regulating the production of PTH.**

Parathyroid hormone (PTH) and 1,25-dihydroxycholecalciferol (1,25-$(OH)_2D_3$) are the principal hormones regulating calcium homeostasis (1–3). Both hormones mobilize calcium from bone, with PTH also acting on the kidney to increase calcium reabsorption as well as the synthesis of 1,25-$(OH)_2D_3$. The resultant increase in serum calcium decreases the secretion of PTH and subsequently the production of 1,25-$(OH)_2D_3$ (1). What has not been well established is whether 1,25-$(OH)_2D_3$ itself has any direct effects on the synthesis and secretion of PTH, analogous to the feedback of steroid hormones on pituitary peptide hormone release. While some authors have suggested that vitamin D metabolites acutely suppress PTH release, other studies show either an increase or no effect (4–8).

Receptors for 1,25-$(OH)_2D_3$ have been well documented in the intestine and bone (9, 10), both recognized as target tissues for the hormone. Similar cytoplasmic receptors, which bind 1,25-$(OH)_2D_3$ at a high affinity ($K_d = 5 \times 10^{-10}$ M), have been demonstrated in parathyroid cells from the chicken and other species (11, 12). Moreover, after administration of 1,25-$(OH)_2[^3H]D_3$ to chicks and rats, there is marked accumulation of the metabolite in the parathyroid glands, particularly in the nuclei (13, 14). Vitamin D, being a sterol and analogous to steroid hormones, might be expected to have biological actions that take place over hours rather than minutes. These findings suggest a possible role for the vitamin D metabolites in regulating the synthesis of PTH, and the current studies were designed to test that hypothesis. The results showed that 1,25-$(OH)_2D_3$ produced a specific and significant fall in steady-state levels of preproPTH mRNA over 24–48 hr and that it was much more effective than other vitamin D metabolites in doing so.

MATERIALS AND METHODS

Preparation of Primary Cultures. Adult bovine parathyroid glands were obtained from a local slaughterhouse and transported in sterile medium on ice to the laboratory. Glands were washed briefly in 70% ethanol, rinsed in sterile medium and trimmed and minced into pieces approximately 1 mm^3 in size prior to digestion. Digestion was carried out for 90 min under sterile conditions in Dulbecco's modified Eagle's medium containing collagenase at 2 mg/ml (CLS Grade, Worthington). Cells were filtered through sterile 200-μm gauze, rinsed three times with sterile medium, and plated on 16-mm plates (1×10^6 cells) in 1 ml of Dulbecco's modified Eagle's medium containing 1.25 mM Ca^{2+}, 10% fetal calf serum, and 1% penicillin and streptomycin. Prior to incubation, cell number and viability were determined by direct cell count with a hemocytometer and by Trypan blue dye exclusion, respectively.

Bovine parathyroid cells were maintained in primary monolayer culture for 24–72 hr, at which time old medium was removed and replaced with fresh medium containing either one of the vitamin D metabolites to be tested or vehicle (10 μl of ethanol) alone. Each set of conditions was carried out in quadruplicate. At the end of each experiment, cell number and viability were determined as described above after removing the cells with trypsin/EDTA (GIBCO). In addition, cells cultured for 48 hr were tested for their ability to respond acutely to high and low calcium by incubation with fresh medium containing bovine serum albumin at 2 mg/ml; 1.0 mM magnesium; 0.5, 1.25, or 2.5 mM calcium; and no 1,25-$(OH)_2D_3$ at 37°C for 30 min. Medium was then removed for radioimmunoassay of PTH (1).

Extraction of Total RNA. At different time intervals, the medium was aspirated, cells were removed and washed, and total cellular RNA was extracted with guanidine thiocyanate by the method of Ulrich *et al.* (15). Total RNA was redissolved in sterile water and quantitated by reading the absorbancy at 260 nm. The absorbancy at 280 nm also was determined, and the ratio of A_{260}/A_{280} in all cases was between 1.9–2.0.

In some cases the cells were pulsed with [^{3}H]uridine (10 μCi/ml of medium; 1 Ci = 37 GBq) for 6 hr prior to extraction of RNA as described above. Unincorporated label was removed by two successive precipitations with 2.5 vol of ethanol in the presence of 0.3 M sodium acetate (pH 5.2).

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Abbreviations: PTH, parathyroid hormone; D_3, cholecalciferol; 1,25-$(OH)_2D_3$, 1,25-dihydroxycholecalciferol; 24,25-$(OH)_2D_3$, 24,25-dihydroxycholecalciferol; 25-OH-D_3, 25-hydroxycholecalciferol.

Preparation of RNA for Hybridization Analysis. PreproPTH mRNA was determined by the dot-blot procedure described by White and Bancroft (16), and, in some cases, total RNA was subjected to gel electrophoresis (17) and transferred to a nitrocellulose filter for blot hybridization as described by Maniatis *et al.* (18). RNA to be dot-blotted was denatured by incubating for 15 min at 55°C in a solution containing 50 μl of sterile water, 30 μl of 20× NaCl/Cit (1× NaCl/Cit is 0.15 M NaCl/0.01 M Na citrate, pH 7.0) and 20μl of formaldehyde. Aliquots containing either 0.25 or 0.5 μg of total RNA were applied to a nitrocellulose filter by using a multiple filtration manifold supplied by Schleicher & Schuell. Nonspecific background hybridization was determined by blotting total bovine liver RNA at concentrations of 1, 5, and 10 μg. Filters were baked in a vacuum oven at 90°C for 2 hr.

Hybridization with p-cDNA-10. Preparation and characterization of a cDNA fragment specific for preproPTH mRNA (p-cDNA-10) was as described (19). The cDNA fragment used covered the entire coding sequence for preproPTH. This cDNA fragment was labeled with [α-^{32}P]dCTP (7000 Ci/mmol, carrier-free; Amersham) by nick-translation to a specific activity of 10^8 cpm/μg (20). Filters were hybridized with labeled cDNA at 2×10^6 cpm/ml overnight at 43°C in sealed plastic bags containing 10 ml of a solution composed of 50% formamide, 5× NaCl/Cit, 1× Denhardt's solution (0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin) and denatured salmon sperm DNA at 100 μg/ml. Filters were washed three times in 2× NaCl/Cit containing 0.1% $NaDodSO_4$ at room temperature and three times in 0.2× NaCl/Cit containing 0.1% $NaDodSO_4$ at 55°C. The dried filters were exposed to x-ray film (SB-5, Eastman Kodak) for 6 hr, and the intensity of the spots was determined by densitometry scanning with a Beckman DU-8 spectrophotometer (19).

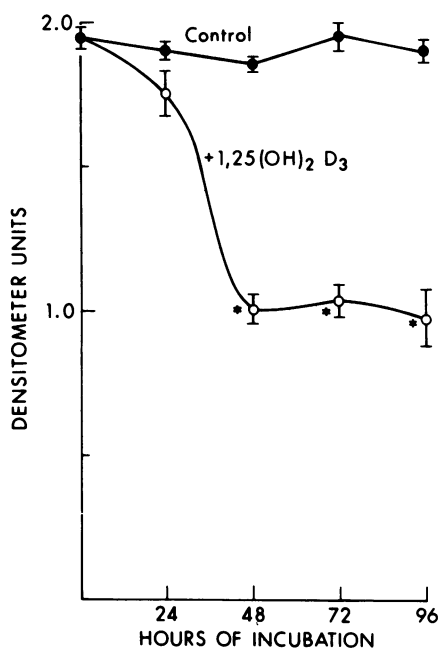

FIG. 1. Time course of 1,25-$(OH)_2D_3$ effects on preproPTH mRNA content of parathyroid cells in primary culture. Cells were maintained in quadruplicate cultures in Dulbecco's modified Eagle's medium with 10% fetal calf serum and 1.25 mM calcium. The medium was then replaced with fresh medium, and either vehicle (10 μl of ethanol) or 0.1 μM 1,25-$(OH)_2D_3$ in ethanol was added. Cells were extracted at various time intervals after the addition of 1,25-$(OH)_2D_3$ or vehicle. The experimental results are plotted in densitometer units, and the mean ± SEM for quadruplicate plates is shown. *, $P < 0.01$ compared with control.

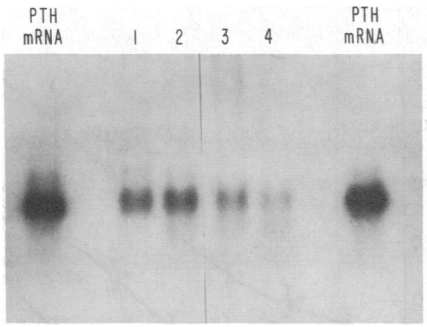

FIG. 2. Blot hybridization of total RNA from parathyroid cells. Total RNA (3 μg) was subjected to electrophoresis on 1.5% agarose blotted on a nitrocellulose filter and hybridized with p-cDNA-10 labeled with ^{32}P by nick translation (19). This was followed by autoradiography. Lanes: 1 and 2, RNA from cells incubated with vehicle only for 24 and 48 hr, respectively; 3 and 4, RNA from cells treated with 0.1 μM 1,25-$(OH)_2D_3$ for 24 and 48 hr. PreproPTH mRNA standard is biologically active poly(A)$^+$ RNA from intact bovine glands purified by sucrose density gradient.

RESULTS

Fig. 1 shows the time course of mRNA suppression when cells were incubated with 0.1 μM 1,25-$(OH)_2D_3$ in quadruplicate at various time intervals. An initial decrease of 15–20% was noted at 24 hr, with a decline to almost 50% at 48 hr ($P < 0.01$) and a plateau of 50% suppression in the subsequent time period. Although there was always a decrease in mRNA at 24 hr, it was only statistically significant in some experiments. Analysis of total RNA by agarose gel electrophoresis also showed a progressive decrease in preproPTH mRNA over 48 hr of incubation (Fig. 2). A single band on the blot hybridizations was noted at the same position as purified preproPTH mRNA.

When the effects of 1,25-$(OH)_2D_3$ were tested at various concentrations, a significant effect was apparent at 10 pM that increased progressively at higher concentrations up to a maximum at 0.1 μM (Table 1 and Fig. 3). The effect of a second metabolite, 24,25-dihydroxycholecalciferol [24,25-$(OH)_2D_3$], was approximately 1/100th that of 1,25-$(OH)_2D_3$, although it caused significant suppression at 1 and 100 nM. The metabolite 25-hydroxycholecalciferol (25-OH-D_3) only showed a significant effect at 1 μM, and cholecalciferol (vitamin D_3) itself had no effect, even at concentrations as high as 1 μM. A representative densitometry scan of a dot blot containing RNA from cells incubated with different concentrations of the various vitamin D metabolites is shown in Fig. 4. Suppressive dose–response effects were noted with 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ at two different concentrations of total RNA. Dot blots of total bovine liver RNA produced negligible background, even when 10 μg was applied to the filter.

In order to be certain of the specificity of the effects, the cytoplasmic concentrations of mRNA from an unrelated protein, α-actin, were also studied with a ^{32}P-labeled probe of

Table 1. Effects of 1,25-$(OH)_2D_3$ on mRNA for preproPTH and α-actin at 48 hr

1,25-$(OH)_2D_3$, nM	PreproPTH mRNA, densitometer units	α-Actin mRNA, densitometer units
None	2.38 ± 0.14	1.40 ± 0.02
0.01	1.91 ± 0.02 ($P < 0.05$)	1.42 ± 0.03 (NS)
1.0	1.52 ± 0.02 ($P < 0.05$)	1.48 ± 0.03 (NS)
100.	1.31 ± 0.04 ($P < 0.05$)	1.32 ± 0.08 (NS)

Cells were plated in quadruplicate. *P* values are from a comparison with the control. NS, not significant compared with control.

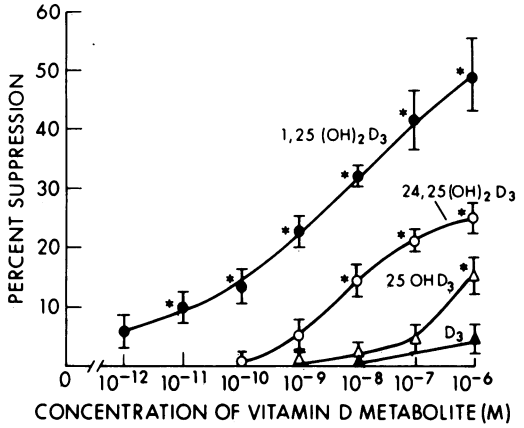

FIG. 3. Dose–response of vitamin D_3 and its metabolites on the percentage suppression of cellular preproPTH mRNA content is shown at 48 hr. ●, 1,25-$(OH)_2D_3$; ○, 24,25-$(OH)_2D_3$; △, 25-OH-D_3; and ▲, vitamin D_3. The results represent mean ± SEM in quadruplicate plates. *, $P < 0.01$ compared with control.

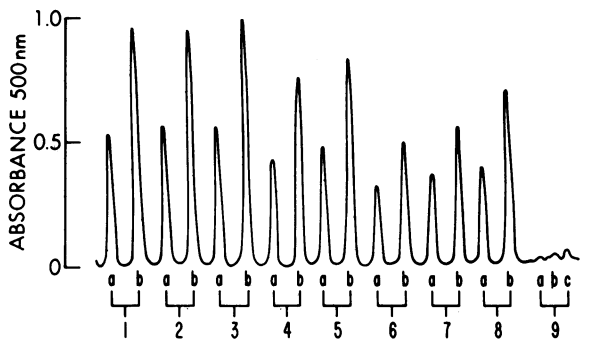

FIG. 4. Densitometer scan of a dot-blot hybridization using total RNA from parathyroid cells incubated with various vitamin D metabolites for 48 hr. Peaks a and b of plots 1–8 represent 0.25 and 0.5 μg of total RNA, respectively. The experimental conditions in the plots were as follows: 1, vehicle alone (control); 2, 0.1 μM vitamin D_3 (no effect); 3, 0.1 μM 25-OH-D_3 (no effect); 4, 0.1 μM 24,25-$(OH)_2D_3$; 5, 1μM 24,25-$(OH)_2D_3$; 6, 0.1 μM 1,25-$(OH)_2D_3$; 7, 1 nM 1,25-$(OH)_2D_3$; and 8, 10 pM 1,25-$(OH)_2D_3$. Peaks 9a, 9b, and 9c correspond to 1, 5, and 10 μg, respectively, of total bovine liver RNA and do not show significant nonspecific hybridization.

chicken muscle α-actin (21). Cells that had been in culture with 1,25-$(OH)_2D_3$ in the medium at varying concentrations for 48 hr showed no change in α-actin mRNA, despite a striking decrease in preproPTH mRNA (Table 1). There was also no effect of 1,25-$(OH)_2D_3$ on total RNA (with and without 0.1 μM 1,25-$(OH)_2D_3$) or on [^{3}H]uridine incorporation into RNA at both 24 and 48 hr by methods reported previously (19) (see Table 2).

At the end of the experiment, cell number and viability were determined as described in *Methods*. Cell number was generally 85–90% of that plated originally, and cell viability was typically >95%. The response of parathyroid cells that had been in culture for 48 hr to acute changes in calcium concentration was as expected. Low calcium (0.5 mM) increased the amount of PTH secreted into the medium from 2.1 ng per 10^5 cells per hr (at 1.25 mM calcium) to 3.8 ng per 10^5 cells per hr, while high calcium (2.5 mM) suppressed PTH secretion to 1.6 μg per 10^5 cells per hr (22). In addition, electron micrographs of cells cultured with 0.1 μM 1,25-$(OH)_2D_3$ showed intact subcellular organelles and no evidence of toxicity compared with cells incubated under similar conditions in the absence of the vitamin D_3 metabolite (courtesy of Steven Baum, Departments of Cell Biology and Medicine, Albert Einstein College of Medicine).

In order to determine whether the effects of 1,25-$(OH)_2D_3$ on the suppression of mRNA were reversible, 0.1 μM 1,25-$(OH)_2D_3$ was added to cells that were in culture for periods of 48 and 96 hr, respectively, and compared with cells in culture without 1,25-$(OH)_2D_3$ (Fig. 5). In one set of quadruplicate plates after 48 hr of 1,25-$(OH)_2D_3$ exposure, the medium was replaced without added 1,25-$(OH)_2D_3$, and the cells were incubated for another 48 hr. There was significant suppression of mRNA at 48 hr and at 96 hr compared with control plates for those cells exposed to 1,25-$(OH)_2D_3$ over that time period. In the cells that were switched to medium containing no 1,25-$(OH)_2D_3$, there was a significant increase in the concentration of mRNA compared with those that had been exposed continuously to 1,25-$(OH)_2D_3$, although it had not returned completely back to control levels at that point (up to 85% of control). Cultures were not continued beyond 96 hr.

DISCUSSION

The results of this study showed that 1,25-$(OH)_2D_3$ decreased steady-state levels of preproPTH mRNA in a dose-dependent manner, with greater potency than the less active vitamin D metabolites 24,25-$(OH)_2D_3$ and 25-OH-D_3. In other target tissues, 1,25-$(OH)_2D_3$ has also been the most potent metabolite, with 24,25-$(OH)_2D_3$ generally having <1% of its activity (3). The effects in our system were first noted at 24 hr or less, were maximal at 48 hr, and then appeared to reach a plateau. There was no evidence of cellular toxicity from 1,25-$(OH)_2D_3$, with cell number and viability being the same as that of the control. Levels of total RNA, RNA synthesis, and α-actin mRNA were also the same in vitamin D-treated and control cells. The electron microscopic appearance of vitamin D-treated cells was healthy, with intact subcellular organelles and no evidence of toxicity.

The effects on mRNA were statistically significant at physiologic concentrations and maximal at pharmacologic concentrations of 1,25-$(OH)_2D_3$. It is possible that the methods used for cell dispersion rendered the cells less sensitive than otherwise to vitamin D metabolites, but, by all the criteria mentioned above, the cells appear to be functioning

Table 2. Effect of 1,25-$(OH)_2D_3$ on total RNA and RNA synthesis in cultured parathyroid cells

Addition	Total RNA, μg		Urd incorp., cpm × 10^{-4} per μg of RNA	
	24 hr	48 hr	24 hr	48 hr
Control	6.65 ± 0.4	6.40 ± 0.6	3.13 ± 0.127	3.75 ± 0.162
1,25-$(OH)_2D_3$	6.45 ± 0.35*	6.40 ± 0.45*	2.85 ± 0.149*	3.94 ± 0.154*

Total RNA represents average values from quadruplicate plates containing 10^6 cells each. RNA was extracted as described. Uridine incorporation (Urd incorp.) was achieved by the addition of 10 μCi of [^{3}H]uridine per ml of medium 6 hr prior to extraction.
*Not significant compared with control.

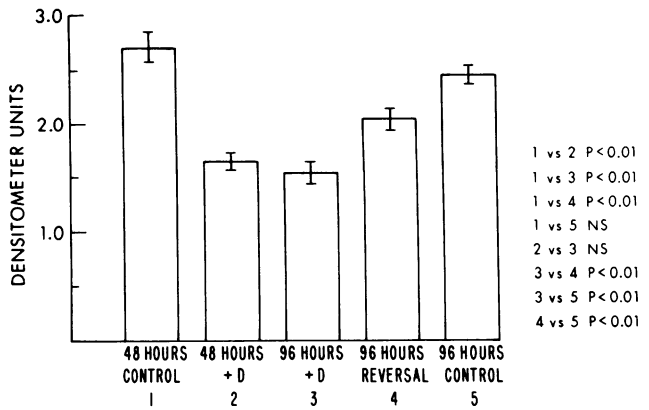

FIG. 5. Response of preproPTH mRNA content of parathyroid cells cultured sequentially in medium with and without 0.1 μM 1,25-$(OH)_2D_3$. PreproPTH mRNA content was measured at 48 hr in cells cultured without added 1,25-$(OH)_2D_3$ (control, column 1), with 1,25-$(OH)_2D_3$ (0.1 μM) added for 48 hr (+D, column 2) or 96 hr (+D, column 3), with 1,25-$(OH)_2D_3$ (0.1 μM) added for 48 hr and then replaced by medium without 1,25-$(OH)_2D_3$ (96 hr, reversal, column 4), and cells at 96 hr with no added 1,25-$(OH)_2D_3$ (control, column 5). The results represent the mean ± SEM of quadruplicate plates, and the statistics are shown.

normally. The presence of 10% fetal calf serum also results in the binding of vitamin D metabolites to serum proteins, so that the effects we observed would probably have been even more sensitive in the absence of serum. Currently, we are working with chemically defined serum-free medium to determine whether there are any differences.

In two other vitamin D target organs, the intestine and bone, 1,25-$(OH)_2D_3$ also affects mRNA concentrations (23, 24). In the intestine, it stimulates the synthesis of calcium-binding protein mRNA and in rat calvarial bone cells, it inhibits the synthesis of procollagen mRNA. In pituitary cells in culture, it stimulates prolactin synthesis and prolactin mRNA levels (25). Our findings *in vitro* showed that levels of preproPTH mRNA were responsive to vitamin D metabolites, supporting the concept that the parathyroid gland is a target organ for vitamin D. Whether the effects we noted were due to changes in rates of transcription and/or changes in the half-life of mRNA is currently being determined. The presence of documented receptors in the parathyroid cell for this sterol and the localization of 1,25-$(OH)_2D_3$ to the nucleus support the physiologic observations made in this study. As is the case for all *in vitro* systems, however, these results will need to be tested *in vivo*.

More complex questions surround the effects of vitamin D metabolites on acute and chronic PTH secretion (4–8); in our recent studies, it has been shown that 1,25-$(OH)_2D_3$ over 24–48 hr leads to decreased release of PTH in response to low calcium. This is in striking contrast to the lack of any acute effect of 1,25-$(OH)_2D_3$ on PTH secretion (22). These observations are complementary to and supported by the decreases in mRNA caused by vitamin D metabolites in this study.

This work was performed while J.S. was on sabbatical leave from the Department of Nephrology, Hebrew University, Hadassah Medical School, Jerusalem, Israel. We are grateful to Dr. Leslie Leinwand (Albert Einstein College of Medicine) for supplying the α-actin probe and to Dr. M. Uskakovic (Hoffman–LaRoche) for supplying the vitamin D metabolites. The studies were supported in part by U.S. Public Health Service Grants AM 28556 and HD 15891.

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