

Regulation of Parathyroid Vitamin D Receptor Expression by Extracellular Calcium

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Abstract. Low extracellular calcium (Ca) stimulates parathyroid hormone (PTH) secretion and also increases the renal synthesis of calcitriol (CTR), which is known to decrease PTH production. This study began with the hypothesis that the parathyroid cell response to CTR may be modulated by extracellular Ca concentration through an effect on parathyroid cell vitamin D receptor (VDR). In the present study, rat parathyroid glands were incubated in low (0.6 mM) and high (1.5 mM) Ca concentration. The parathyroid VDRmRNA was higher in 1.5 than 0.6 mM Ca. Furthermore, this effect was not observed in incubated slices of kidney cortex and medulla, tissues which also possess both Ca and vitamin D receptors. Experiments were also performed to evaluate the effect of Ca on VDR expression *in vivo*. Male Wistar rats received intraperitoneal injections of CaCl₂ or a single intramuscular injection of

EDTA to obtain 6 h of hypercalcemic (ionized Ca, 1.4 to 1.6 mM) or hypocalcemic (ionized Ca, 0.85 to 0.95 mM) clamp; a third group of rats was used as control. A small dose of CTR was administered to hypercalcemic rats to match the serum CTR levels of hypocalcemic rats. Parathyroid gland VDRmRNA and VDR protein were increased in hypercalcemic rats as compared with hypocalcemic rats. Increasing doses of CTR upregulated VDRmRNA and VDR only in hypercalcemic rats. Additional experiments showed that the decrease in VDR in hypocalcemic rats prevented the inhibitory effect of CTR on PTHmRNA. In conclusion, our study shows that extracellular Ca regulates VDR expression by parathyroid cells independently of CTR and that by this mechanism hypocalcemia may prevent the feedback of CTR on the parathyroids.

The parathyroid cell increases parathyroid hormone (PTH) secretion in response to a decrease in the extracellular calcium (Ca) concentration. A cell membrane Ca sensor receptor enables these cells to respond to small changes in serum Ca concentration (1). PTH acts on its target organs, mainly kidney and bone, to increase the serum Ca to its normal level. Calcitriol (CTR), the production of which is stimulated by hypocalcemia and also by PTH, is another hormone that contributes to restoration of normocalcemia by stimulating intestinal absorption of Ca (2). Thus there are two different hormones, PTH and CTR, with a calcemic effect, and both share the commitment of preserving normocalcemia. The elevation of serum Ca feeds back on the parathyroid cell, inhibiting PTH secretion and synthesis (2); in addition, high CTR also inhibits PTH synthesis by acting on specific vitamin D receptors (VDR) (3). The inhibitory effect of CTR on parathyroid cells has been widely

demonstrated to the point that CTR is used to reduce PTH levels in uremic patients with advanced hyperparathyroidism (4). However, it would not seem appropriate that CTR should inhibit PTH synthesis if hypocalcemia persists; such inhibition would counteract the function of the parathyroid cell, which is to restore normocalcemia. Furthermore, it would seem paradoxical that the parathyroid cells are exposed to two contradictory effects; low Ca is stimulating parathyroid cell function at the same time as CTR is inhibiting parathyroid cell function.

A study by Naveh-Many *et al.* (5) showed that the ability of exogenous CTR to inhibit PTHmRNA in vitamin D–deficient rats was markedly impaired when serum Ca was low, suggesting that the inhibition of PTHmRNA by CTR was not possible if hypocalcemia was present. In a more recent publication, Sela-Brown *et al.* (6) have shown that a decrease in Ca produces an increase in the expression of calreticulin, a cytoplasmic protein that prevents the binding of vitamin D receptor–retinoid X receptor- β to the PTH vitamin D response element (VDRE). Whether the serum Ca concentration has an effect on parathyroid VDR expression is not clear. Russell *et al.* (7) found that 6 d of dietary Ca restriction decreased VDRmRNA in chicken parathyroid glands and that CTR administration only upregulated VDR expression when chickens were fed a normal Ca diet. In studies of shorter duration, Naveh-Many *et al.* (8) showed that an acute (1 to 6 h) change in serum Ca did

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not affect parathyroid VDRmRNA and that CTR administration produced upregulation of the VDRmRNA. In another study, Brown *et al.* (9) evaluated the effect of CTR administration on parathyroid VDR expression in rats fed for 6 wk with diets containing different vitamin D and Ca levels. Analysis of covariance suggested that upregulation of VDR by CTR in the parathyroid glands may be mediated primarily by an increased serum Ca concentration. In this study, an independent effect of CTR on parathyroid gland VDRmRNA was not observed.

It is important to know whether Ca exerts an effect on VDR expression, because CTR administration is a standard treatment for uremic hyperparathyroidism, a condition in which hypocalcemia is common. Another important consideration is that hypocalcemia may exacerbate the development of secondary hyperparathyroidism in renal failure, perhaps by negating the effect of any endogenous CTR on the parathyroids.

The present study was designed to determine whether extracellular Ca has an independent effect on parathyroid VDR expression and how this effect may modify the regulation of PTHmRNA by CTR. The experiments have been performed *in vivo* using rats and *in vitro* by incubating intact rat parathyroid glands.

Materials and Methods

In Vitro Studies

In vitro studies were performed using parathyroid glands obtained from male Wistar rats weighing 170 to 200 g. The rats were on a 0.6% Ca, 0.6% P diet supplemented with 100 IU of vitamin D/100 g. The animals were anesthetized with thiopental (50 mg/kg), blood was drained by aortic puncture; within 2 min, the parathyroid glands were dissected free of the thyroid under a dissecting microscope and removed.

Intact rat parathyroid glands were placed resting inside a nylon basket in individual wells containing 2 ml of incubation medium; the glands were maintained in a constant rocking and shaking motion (AOS-0; SBS Instruments SA, Badalona, Spain) at 37°C. Cell viability was >80% (10). The incubation medium was buffered (pH 7.4) and contained 125 mM NaCl, 5.9 mM KCl, 0.5 mM MgCl₂, 1 mM Na-pyruvate, 4 mM glutamine, 12 mM glucose, 25 mM Hepes with 0.1 IU/ml human insulin, 0.1% BSA, 100 IU/ml penicillin G, and 100 mg/ml streptomycin. A phosphate concentration of 1 mM was obtained by adding NaH₂PO₄ and Na₂HPO₄ in 1:2 proportion. Calcium chloride was added to obtain Ca concentrations of 0.6 and 1.5 mM (as measured by a selective electrode, model 634; Ciba Corning, Essex, England). All chemical products were obtained from Sigma, St. Louis, MO. After 6 h of incubation, the parathyroid tissue was processed for VDRmRNA and VDR protein analysis. This time period was chosen because longer incubation periods (9 h) did not further increase parathyroid VDRmRNA levels. To determine if the effect of extracellular Ca on VDR expression was specific for parathyroid tissue, additional experiments were performed using small slices of kidney cortex and medulla under incubation conditions that were identical to the experiments with parathyroid glands. Renal cortex and medulla samples were obtained from control rats. The rat kidney was quickly removed and placed at 4°C in a 10-cm-well plate containing saline. The kidney was cut longitudinally, and samples of cortex and medulla were dissected, avoiding the area of transition from cortex-medulla. Then, the tissues were cut in small (1 mm³) pieces with the help of small scissors and a Dumont #5 tweezers. Experiments were per-

formed in 12-well plates. Each well contained ten of these small tissue slices in 2 ml of the same medium used for parathyroid glands incubation. These slices of kidney tissue were shown to increase cAMP production in response to 1 to 34 rat PTH (10⁻⁶ M) added to the incubation medium.

In Vivo Studies

The Effect of Extracellular Ca and CTR on Parathyroid VDRmRNA and VDR Protein *In Vivo*. *In vivo* studies were performed with rats weighing 200 g and fed as described in *in vitro* experiments. Rats were divided into three groups, which underwent 6 h of hypercalcemia, normocalcemia, or hypocalcemia. In the hypercalcemic group, persistently high levels of serum Ca were achieved by intraperitoneal injections of CaCl₂ diluted in Ringer lactate. Injections were repeated at 30-min intervals: 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, 14.7 mg at 120 min, 11 mg at 150 min, and 7.5 mg each 30 min until the end of the experiment at 360 min. A hypocalcemic clamp was performed following a modified protocol described by Thomas *et al.* (11); rats received one intramuscular injection of EDTA (300 mg/kg) at the beginning of the experiments, which resulted in 6 h of sustained hypocalcemia. Normocalcemic rats received intraperitoneal injections of Ringer lactate or an intramuscular injection of water. Food was removed 12 h before the beginning of the experiments. To evaluate the effect of CTR on VDR expression during hypocalcemia and hypercalcemia, some rats from each group received increasing doses of CTR. Normocalcemic and hypocalcemic rats received vehicle or 10 or 20 pmol of CTR intraperitoneally at 30 min intervals until the end of the 6 h experiment. Hypercalcemic rats received vehicle or 5 or 10 pmol of CTR intraperitoneally every 30 min; in this group, administration of higher dose of CTR (20 pmol) was considered unnecessary.

The Effect of Hypocalcemia on the Regulation of PTHmRNA by CTR. Sustained hypocalcemia of 30 h duration was achieved by intramuscular injections of 300 mg/kg EDTA every 6 h. All the animals were fed the standard diet, as described in *in vitro* experiments, during the 30 h. These rats were divided into three groups: a first group received a bolus of CTR (200 pmol intraperitoneally) 10 min before the initiation of hypocalcemia, a second group received the same bolus of CTR 6 h after the initiation of hypocalcemia (this period of time is enough for hypocalcemia to induce a decrease in VDR); a third group received vehicle 10 min before or 6 h after initiation of hypocalcemia. Two additional groups of normocalcemic rats were also studied, one of these groups received vehicle, and the other received a bolus of CTR (200 pmol intraperitoneally) 10 min before the initiation of the experiments. All rats were sacrificed after 30 h by aortic puncture, blood and parathyroid glands were stored until processing. 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 Science and published by the National Institutes of Health (NIH publication No 86–23, revised 1985).

Blood and Tissue

Fresh parathyroid glands from each rat were dry-frozen in liquid nitrogen and stored at –80°C until RNA isolation. To measure VDR protein, parathyroid glands from ten rats were placed in KTED pH 7.4 buffer containing 300 mM KCl, 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 5 mM DTT, and 5 μl of Protease Inhibitor Cocktail (Sigma, St. Louis, MO) and stored in liquid nitrogen until processing. In preliminary experiments, renal cortex gave consistent measurement of

VDR while brain did not, so renal cortex and brain were extracted as positive and negative controls for VDR, respectively.

After measurement of ionized Ca (model 634; Ciba Corning, Essex, England), the serum was stored at -20°C . Serum phosphate was measured by a spectrophotometric method (Sigma Diagnostics, St Louis, MO), PTH was quantified by a rat-specific IRMA assay (Nichols Institute, San Juan de Capistrano, CA), and CTR by RIA (IDS Kit, Boldon, UK).

RNA Isolation and RT-PCR

One milliliter of phenol-guanidine isothiocyanate solution (Tri-Reagent; Sigma, St. Louis MO) was added to the glands. The glands were ultrasonicated for 5 min at 4°C to allow for complete cell rupture. Thereafter, total RNA was extracted following a modification of Chomczynski and Sacchi's protocol (12). Extracted total RNA was dissolved in nuclease-free water (Promega, Madison WI) and heated for 10 min at 60°C . Total RNA was quantified by spectrophotometry (13). VDR *versus* actin and PTH *versus* actin were amplified separately with the kit Access RT-PCR System (Promega, Madison WI) using specific primers and 100 ng of total RNA per sample. DNA amplifications were processed by a Genetic Analyser Abi Prism 310 (Perkin Elmer, Foster City, CA). Data were analyzed using a specific software Gene Scan v 3.1/1998 (Perkin Elmer, Foster City, CA). The amount of VDR/Actin-mRNA and PTH/Actin-mRNA were expressed as percent of the control group.

Parathyroid VDR Protein Extraction and Western Blot Analysis

Samples were sonicated on ice in KTED pH 7.4 buffer for 1 min using a Vibra Cell Sonifier (Sonic Materials Inc, Danbury, CT). The homogenate was centrifuged at $18,000 \times g$ for 40 min at 4°C . The supernatant was collected and stored at -20°C for Western blot analysis. Protein concentration was measured by the method of Bradford using BSA as standard (14). VDR extracts from parathyroid glands, renal cortex, and brain (80 μg of protein each one) were electrophoresed on a 10% SDS-polyacrylamide gel (Western blotting system; BioRad, Hercules, CA) and electrophoretically transferred (Transfer systems; BioRad, Hercules, CA) from the gels onto nitrocellulose membranes (BioTrace^{NT}, Ann Arbor, MI). The following steps were performed with gentle shaking. Membranes were incubated in TTBS-L blocking solution containing Tris-HCl pH 7.6 20 mM, Tween 20 0.2%, NaCl 150 mM, and nonfat dry milk 5% at room temperature for 2 h to block the non specific binding. Membranes were then washed with TTBS buffer (same composition as TTBS-L without nonfat dry milk) at room temperature and then incubated overnight at 4°C with 1:500 dilution of VDR monoclonal antibody raised in mice. The membranes were washed with TTBS buffer again and then re-incubated with anti-mouse IgG conjugated with alkaline phosphatase at room temperature for 2 h. The membranes were then washed with PBA buffer containing 100 mM Tris-HCl (pH 9.0), 100 mM NaCl, and 5 mM MgCl_2 at room temperature and incubated with a 10 ml of PBA solution also containing 66 μl of Nitroblue tetrazolium (5% in N-N dimethylformamide at 70% in water) and 33 μl of Toluidin salt (5% in N-N dimethylformamide). The signals were quantified using a densitometric scanner (Gelprinter Plus, Frederick, MD) and expressed as areas under the peaks. The amount of VDR protein was expressed as percent of the control group.

Other Chemicals

CTR was obtained from Abbot Laboratories S.A. (Madrid, Spain). Primers for RT-PCR were obtained from Life Technologies (Paisley,

UK), DTT, TEMED, 2-mercaptoethanol, and Blotting grade blocker nonfat dry milk from BioRad (Hercules, CA), 2-propranolol and chloroform from Panreac (Barcelona, Spain), and absolute ethanol from Merck (Darmstadt, Germany). All the other chemicals were purchased from Sigma (St. Louis, MO). Monoclonal IVG8C11 primary antibody for VDR was raised in mice.

Statistical Analyses

Values were expressed as mean \pm SE. Difference between means of two different groups was determined by *t* test; difference between means of three or more groups was assessed by ANOVA. $P < 0.05$ was considered statistically significant.

Results

The Effect of Extracellular Ca on Parathyroid VDRmRNA *In Vitro*

To evaluate a direct effect of extracellular Ca concentration on parathyroid VDRmRNA, intact rat parathyroid glands were incubated for 6 h in Ca concentrations of 0.6 and 1.5 mM. The results are presented in Figure 1. The VDRmRNA was significantly greater in high-Ca than in a low-Ca concentration measured as percent of 0.6 mM Ca values ($270 \pm 20\%$ *versus* $100 \pm 40\%$, respectively; $P < 0.05$). Similar experiments were performed in renal slices of cortex and medulla; however, in these tissues the VDRmRNA content was unaffected by the Ca concentration in the medium after 6 h and even 12 h of incubation. Parathyroid VDR protein was quantified by Western blot after 6-h incubation in 0.6 and 1.5 mM Ca. Densitometric values showed that at 1.5 mM Ca the VDR protein was significantly increased to $220 \pm 30\%$ ($P < 0.05$; $n = 3$ experiments) measured as percent of 0.6 mM Ca values. Thus, the *in vitro* increase in VDRmRNA induced by Ca was accompanied by a concomitant increase in VDR protein.

The Effect of Extracellular Ca on Parathyroid VDRmRNA and VDR Protein *In Vivo*

Rats were maintained during a 6 h period with high serum Ca (mean serum ionized Ca ranging from 1.5 to 2.2 mM), normal serum Ca (1.12 to 1.22 mM) or low serum Ca (0.8 to

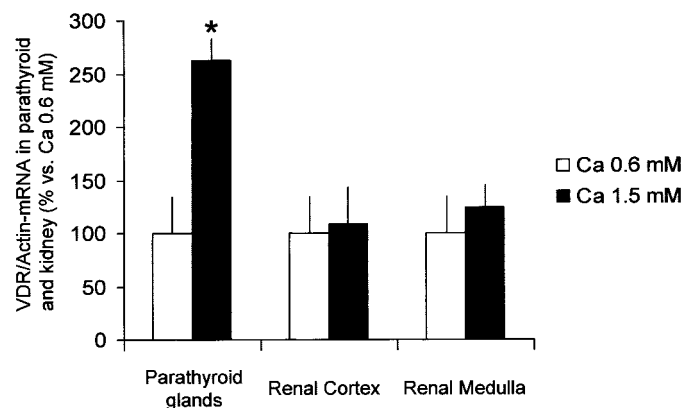


Figure 1. Vitamin D receptor (VDR)/Actin-mRNA (% *versus* Ca 0.6 mM) in parathyroid and kidney incubated *in vitro* in 0.6 and 1.5 mM Ca for 6 h. * $P < 0.05$ *versus* control. $n = 20$ rats per group.

0.95 mM) (Figure 2A). In hypocalcemic rats, the serum CTR concentration increased progressively so that serum CTR at the end of the experiment was 140% of the baseline value. In normocalcemic and hypercalcemic rats CTR levels did not change. CTR may regulate VDRmRNA content; therefore, an additional group of hypercalcemic rats received CTR intraperitoneally, 5 pmol every 30 min throughout the 6 h experimental period. With this dose of CTR, hypercalcemic rats reached serum CTR levels similar to that observed in hypocalcemic rats (Figure 2B), but the administration of CTR did not produce a further increase in serum Ca concentration (Figure 2A). At the end of the 6-h experiment, PTH values were increased in hypocalcemic rats (66 ± 12 pg/ml) and decreased in hypercalcemic rats with and without the addition of CTR (1 ± 0.8 and

2 ± 0.7 pg/ml, respectively). In normocalcemic rats PTH concentration was 12 ± 2 pg/ml (Table 1).

Vitamin D receptor mRNA was measured at the end of the 6-h experimental period, and the results are shown in Figure 2C. In hypocalcemic rats, the VDRmRNA was lower and the CTR level was higher than normocalcemic rats. Hypercalcemic rats had similar VDRmRNA and CTR levels as normocalcemic rats. In CTR-supplemented hypercalcemic rats the VDRmRNA was fourfold greater than in hypocalcemic rats, despite similar CTR level, and VDRmRNA was twofold greater than normocalcemic and hypercalcemic rats without CTR supplementation. The VDR level was also decreased in hypocalcemic rats and increased in hypercalcemic rats with CTR supplementation (twofold increase as compared with hypocalcemic rats),

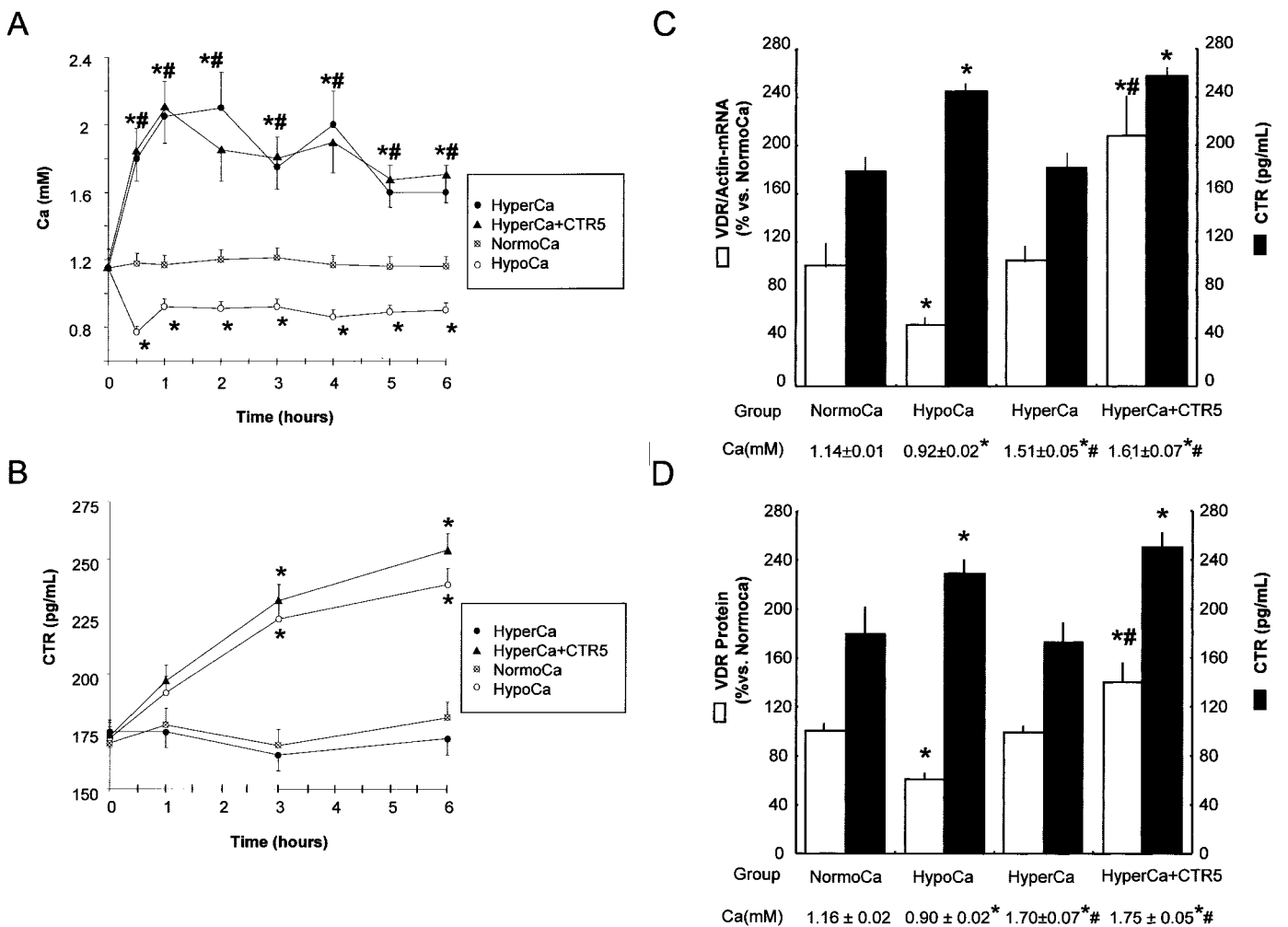


Figure 2. (A) Blood ionized Ca ($n = 10$ rats per group) and (B) serum calcitriol (CTR) concentration ($n = 8$ rats per group) in rats with normocalcemia (NormoCa), hypocalcemia (HypoCa), hypercalcemia (HyperCa), and hypercalcemia plus CTR injections (5 pmol/30 min intraperitoneally) (HyperCa+CTR5). * $P < 0.05$ versus NormoCa and # $P < 0.05$ versus HypoCa. (C) Parathyroid VDR/Actin-mRNA (% versus NormoCa) and the serum CTR concentration in rats after 6 h of normocalcemia (NormoCa), hypocalcemia (HypoCa), hypercalcemia (HyperCa), and hypercalcemia plus CTR injections (5 pmol/30 min intraperitoneally) (HyperCa+CTR5). * $P < 0.05$ versus NormoCa and # $P < 0.05$ versus HypoCa. $n = 12$ rats per group. (D) Parathyroid VDR (% versus NormoCa) and serum CTR concentration in rats after 6 h of normocalcemia (NormoCa), hypocalcemia (HypoCa), hypercalcemia (HyperCa), and hypercalcemia plus CTR injections (5 pmol/30 min intraperitoneally) (HyperCa+CTR5). * $P < 0.05$ versus NormoCa and # $P < 0.05$ versus HypoCa. $n = 4$ experiments with 10 rats each one per group.

Table 1. CTR and PTH levels at each time in normo-, hypo-, and hypercalcemic clamps with two additional doses of exogenous calcitriol^a

Groups	CTR (pg/ml)			PTH (pg/ml) 6 h
	1 h	3 h	6 h	
1. NormoCa	178 ± 9	169 ± 6	181 ± 8	12 ± 2
2. NormoCa+CTR10	204 ± 8 ^b	316 ± 6 ^b	309 ± 3 ^b	8 ± 3
3. NormoCa+CTR20	336 ± 15 ^{bc}	451 ± 25 ^{bc}	334 ± 2 ^{bc}	13 ± 3
4. HypoCa	192 ± 9	224 ± 4 ^b	239 ± 7 ^b	66 ± 12 ^b
5. HypoCa+CTR10	227 ± 5 ^{bd}	309 ± 7 ^{bd}	310 ± 7 ^{bd}	78 ± 11 ^b
6. HypoCa+CTR20	278 ± 19 ^{bde}	531 ± 21 ^{bde}	330 ± 9 ^{bde}	74 ± 14 ^b
7. HyperCa	175 ± 8	165 ± 7	172 ± 9	2 ± 0.7 ^b
8. HyperCa+CTR5	197 ± 8	232 ± 9 ^{bf}	254 ± 5 ^{bf}	1 ± 0.8 ^b
9. HyperCa+CTR10	218 ± 8 ^{bg}	300 ± 9 ^{bg}	281 ± 3 ^{bg}	1 ± 0.9 ^b

^a n = 6 rats per group. CTR5, 10 and 20 means the respective number of pmols administered each 30 min intraperitoneally.
^b P < 0.05 versus group 1.
^c P < 0.05 versus group 2.
^d P < 0.05 versus group 4.
^e P < 0.05 versus group 5.
^f P < 0.05 versus group 7.
^g P < 0.05 versus group 8.

whereas the VDR content in hypercalcemic rats without CTR administration was similar to controls (Figures 2D and 3). *In vitro* experiments also showed that a Ca concentration of 0.9 mM (same values as *in vivo* hypocalcemic experiments) decreased parathyroid VDRmRNA levels as compared with 1.5 mM Ca (100 ± 28% versus 222 ± 33%, respectively; P < 0.05); data are shown as percent of 0.9 mM Ca values.

The Effect of CTR on VDRmRNA and VDR Level

The effect of CTR on VDRmRNA and VDR protein was assessed in rats with low, normal, and high Ca concentrations. Different levels of CTR were achieved by intraperitoneal CTR injections at 30-min intervals throughout the 6-h experimental

period. Rats with low and normal serum Ca concentrations received injections of vehicle or 10 or 20 pmol of CTR and rats with hypercalcemia received vehicle or 5 or 10 pmol of CTR. During the 6-h experiment, the administration of CTR did not increase the serum Ca concentration and the serum CTR levels increased according to the dose of CTR administered (Table 1). VDRmRNA after 6 h of experiment is shown in Figure 4. In hypercalcemic rats, the administration of 5 and 10 pmol of CTR produced a dose-dependent increase in VDRmRNA to levels so high that the administration of higher doses of CTR

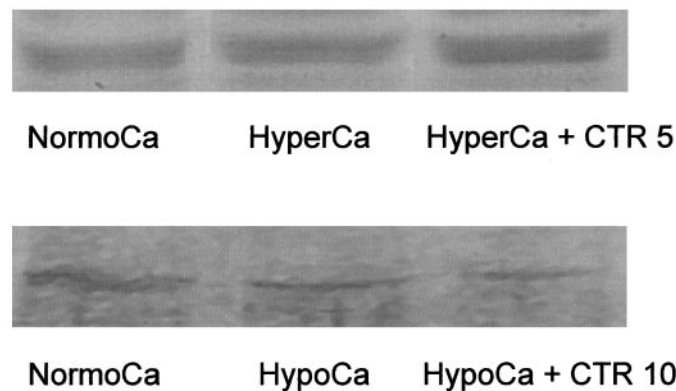


Figure 3. Parathyroid VDR Western blot from rats (ten glands per group) after 6 h of normocalcemia (NormoCa), hypercalcemia (HyperCa), and hypercalcemia plus calcitriol injections (5 pmol/30 min intraperitoneally) (HyperCa+CTR5) (upper panel); and normocalcemia (NormoCa), hypocalcemia (HypoCa), and hypocalcemia plus calcitriol injections (10 pmol/30 min intraperitoneally) (HypoCa+CTR10) (lower panel).

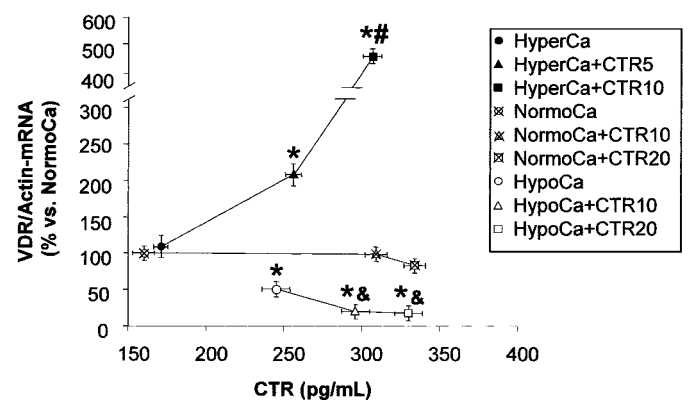


Figure 4. Parathyroid VDR/Actin-mRNA versus serum calcitriol (CTR) concentration in rats after 6 h of normocalcemia (NormoCa), hypocalcemia (HypoCa), and hypercalcemia (HyperCa) without and with CTR supplementation (5, 10, or 20 pmol) given intraperitoneally every 30 min during the 6-h experiment. Parathyroid VDR/Actin-mRNA is expressed as % versus normocalcemic rats (NormoCa) without calcitriol administration. *P < 0.05 versus NormoCa, #P < 0.05 versus HyperCa with CTR (5 pmol/30 min intraperitoneally) and &P < 0.05 versus HypoCa. n = 12 rats per group.

(20 pmol/30 min) was considered unnecessary. In normocalcemic rats, the increase in CTR concentration did not produce a significant change in VDRmRNA. Finally, the increase in CTR in hypocalcemic rats resulted in a significant decrease in VDRmRNA. However, Western blot analysis revealed that in these hypocalcemic rats, the decrease in VDRmRNA induced by 10 pmol of CTR was not accompanied by a decrease in VDR protein (Figure 3). In normocalcemic and hypercalcemic rats the administration of CTR produced changes in VDR similar to those observed in VDRmRNA (data not shown).

The Effect of Hypocalcemia on the Regulation of PTHmRNA by CTR

Thirty hours of sustained hypocalcemia produced a more than twofold increase in PTHmRNA and also stimulated the production of CTR. The administration of a bolus of CTR (200 pmol) before the induction of the 30-h period of hypocalcemia reduced the PTHmRNA to levels that were below control. By contrast, PTHmRNA was not significantly reduced in hypocalcemic rats receiving the CTR bolus 6 h after hypocalcemia was started when VDR levels were decreased (Figure 5). At the end of the 30 h of hypocalcemia, the serum CTR levels were similar in the three hypocalcemic groups, but the serum CTR level measured 8 h after administration of the CTR bolus was 1148 ± 301 pg/ml.

Discussion

The present study was designed to determine whether the extracellular Ca concentration regulates VDR expression. The

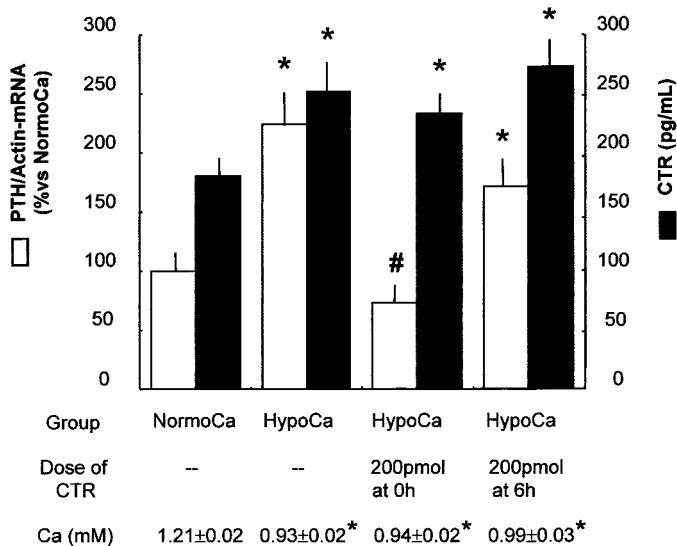


Figure 5. PTH/Actin-mRNA expressed as % versus normocalcemic rats without calcitriol administration (% versus NormoCa) and serum calcitriol concentration (CTR) in rats after 30 h of normocalcemia (NormoCa), hypocalcemia (HypoCa), hypocalcemia (HypoCa) plus one bolus of calcitriol (200 pmol) given 10 min before the initiation of hypocalcemia (0h) and hypocalcemia (HypoCa) plus one bolus of calcitriol (200 pmol) given 6 h after the initiation of hypocalcemia (6h). * $P < 0.05$ versus NormoCa, # $P < 0.05$ versus HypoCa. $n = 10$ rats per group.

results show that (1) extracellular Ca directly regulates VDRmRNA and VDR, (2) CTR upregulates the VDRmRNA only when the serum Ca concentration is elevated, and (3) the decrease in VDR induced by hypocalcemia may prevent the expected CTR induced reduction of PTHmRNA.

In vitro studies performed in intact parathyroid glands showed that the extracellular Ca concentrations directly affect VDRmRNA. Furthermore, this effect was not observed in the slices of kidney cortex and medulla, tissues which also possess both Ca and vitamin D receptors. Thus, the effect of Ca on VDR appears to be specific for the parathyroid tissue. This *in vitro* model has been useful to evaluate PTH secretion in our previous work (10). After a 6-h incubation, intact rat parathyroid glands demonstrate an adequate PTH response to Ca. However, some experiments performed in isolated parathyroid cells have shown a stable PTH response to Ca within the first 24 h (15). Nevertheless, we are not aware of a previous *in vitro* study using either dispersed cells or parathyroid slices, which has addressed a potential role of Ca on VDRmRNA. Therefore, the present study shows for the first time that Ca regulates parathyroid VDRmRNA *in vitro*.

To show a direct effect of Ca on parathyroid VDR expression *in vivo* we used a rat model in which modifications in serum Ca lasted long enough to detect changes in VDRmRNA and VDR protein and at the same time the experiments were not too prolonged so that parathyroid hyperplasia induced by low Ca could be avoided. A 6-h period was sufficient to detect significant changes in VDRmRNA and VDR protein. This period of time was chosen on the basis of results obtained *in vitro* using a 6-h incubation period.

Hypocalcemia stimulates CTR production directly and through an increase in PTH, which can also stimulate CTR production (16). Calcitriol is a factor that has been shown to regulate the VDR expression (8). Thus, to evaluate the effect of hypocalcemia and hypercalcemia on VDR *in vivo*, serum CTR must be similar in both conditions. In our hypocalcemic rats, serum CTR levels increased, thus an additional group of hypercalcemic rats received a small dose of CTR intraperitoneally repeated at short intervals (30 min) to reproduce the increase in CTR observed in hypocalcemic rats.

The results of the *in vivo* experiments showed that both VDRmRNA and VDR protein were decreased in hypocalcemic rats despite an increase in CTR levels. Moreover, for equal CTR concentration the hypercalcemic rats exhibited a marked increase in VDR protein and VDRmRNA as compared with hypocalcemic rats. Although other authors have evaluated the effect of Ca on parathyroid VDR, the results have not been uniform. This difference in results can be attributed to a disparity in the models used. Russell *et al.* showed that the administration of CTR in vitamin D-deficient chickens for 3 to 6 d increased parathyroid VDRmRNA, and this effect was enhanced when serum Ca levels were normal; furthermore, the authors found that in these vitamin D-depleted birds a dietary-induced elevation in serum Ca alone was capable of increasing the VDRmRNA, but the VDR content was not measured in that study. Our results are in agreement with those obtained by Russell *et al.* (7), although our experimental model was dif-

ferent. Russell *et al.* (7) used vitamin D–depleted chickens, thus serum CTR and Ca levels were low and this would result in parathyroid hyperplasia, which may affect both Ca-sensing receptor (17) and VDR content (18). In our study, rats were not vitamin D–depleted. In addition, we did not observe an increase in the percent of parathyroid cells in the S phase of the cell cycle as assessed by flow cytometry (data not shown).

In a different study, Brown *et al.* (9) maintained rats for 6 wk in a vitamin D–deficient status and showed that an elevation of serum Ca induced by high-Ca diet produced upregulation of parathyroid VDRmRNA and increased vitamin D binding; but they did not find an independent effect of CTR on VDRmRNA. In that study, rats were fed a low-Ca diet and may have had parathyroid gland hyperplasia that could affect both Ca receptor and VDR content (17,18). Our results showing an effect of Ca on VDR are in agreement with Brown *et al.* (9). In our 6-h study, we observed that CTR produced an increase in VDR levels when Ca concentration was elevated; however, CTR did not increase VDR expression in rats maintained during 6 h with hypocalcemia (and no evidence of parathyroid hyperplasia). Not all authors have shown a direct effect of Ca on parathyroid VDR. Naveh-Many *et al.* (8) showed that parathyroid VDR was upregulated by CTR, and they did not observe a change in rat parathyroid VDRmRNA after acute (1 to 6 h) changes in serum Ca levels. However, in the same study, rats with hypocalcemia after 3 wk of low Ca with normal vitamin D diet did not have high parathyroid VDRmRNA despite increased serum CTR levels, suggesting that a normal Ca is necessary for CTR to upregulate the VDR gene expression.

Our results show that CTR rapidly upregulates VDRmRNA and VDR (within 6 h) when serum Ca is above normal. These results are similar to the Russell *et al.* study (7) in birds. Our study also shows that when serum Ca is low, an increase in CTR levels not only did not increase VDRmRNA but even resulted in a decrease. Russell *et al.* (7) and Brown *et al.* (9) did not observe a negative effect of CTR administration on VDRmRNA during hypocalcemia. However, it is important to note that in these studies the animals were on a vitamin D–deficient diet combined with a low-Ca diet, which may have produced a marked reduction in parathyroid VDR expression with little margin for any further decrease. By contrast, the serum levels of CTR in our hypocalcemic rats were not decreased and the parathyroid VDRmRNA was not markedly suppressed before CTR administration.

Although the present study was focused on VDR regulation in parathyroids, we have observed that *in vitro* changes in Ca concentration did not affect kidney VDRmRNA levels. These results are not in the same line as the reported by Brown *et al.* (9) and Sandgren and DeLuca (19), which showed that *in vivo* an increase in extracellular calcium was associated with upregulation of kidney VDRmRNA. The difference may be due to the fact that our results are from *in vitro* experiments and the results of Brown *et al.* and Sandgren and DeLuca were obtained from *in vivo* experiments. Nevertheless, reports on the effect of calcium on kidney VDRmRNA *in vivo* are not uniform, because Rougi-Zineb *et al.* (20) have observed that in

vitamin D–deficient rats, Ca supplementation with the corresponding increase in serum Ca did not produce an increase in kidney VDRmRNA.

Because CTR decreases parathyroid gene transcription of PTHmRNA, additional experiments were performed to evaluate whether the decrease in VDR expression induced by hypocalcemia prevented the normal downregulation of PTHmRNA by CTR (5). Thus a bolus of CTR was administered to rats before induction of hypocalcemia while other rats received the bolus after 6 h of hypocalcemia (when VDR was already downregulated by hypocalcemia). Administration of CTR after 6 h of hypocalcemia did not significantly reduce PTHmRNA, whereas the PTHmRNA was downregulated in rats receiving CTR before the initiation of hypocalcemia. It is likely that the low VDR after 6 h of hypocalcemia made the CTR ineffective, whereas there was VDR available in rats that received the CTR before hypocalcemia, and it was stabilized by the binding to CTR (21). These results suggest that the downregulation of VDR by hypocalcemia resulted in impairment of the inhibitory action of CTR on parathyroid cell function. Other authors (5,7) have shown that the inhibition of PTHmRNA by CTR is relatively ineffective when serum Ca concentration is low. This finding could be related to the low VDR expression induced by low Ca. Low Ca is known to directly increase PTHmRNA by stabilizing the molecule through the increased production of AUF-1 protein (22); however, in our study serum Ca levels were equally decreased in the two groups of hypocalcemic rats receiving the bolus of CTR. The results of the present study suggest that a low serum Ca may increase PTHmRNA directly by a post-transcriptional effect and also indirectly by lowering the parathyroid VDR, which makes CTR less effective. Recent work by Sela-Brown *et al.* (6) demonstrated that low serum Ca stimulates the production of calreticulin, which interferes with the binding of the VDR-CTR complex to VDRE. This is a mechanism by which low Ca may prevent the actions of CTR on the parathyroid cell (reduction of PTHmRNA and the upregulation of the VDR). The lack of upregulation of VDR by CTR in hypocalcemic rats could be explained by an increase in calreticulin, but the *in vitro* studies performed in the absence of CTR suggest that the downregulation of VDR by low Ca is not mediated by a lack of upregulation of VDR by CTR. Nonetheless, our *in vivo* studies cannot rule out that the effect of Ca on VDR expression could be mediated by the changes in the synthesis of calreticulin. However, we have observed that the VDR was reduced after 6 h of hypocalcemia, even though PTHmRNA was not yet increased (data not shown). If calreticulin had played a role in decreasing VDR through the inhibition of VDR-VDRE binding, an increase in PTHmRNA would have been expected.

Downregulation of parathyroid VDR caused by low Ca is an important concept in the pathogenesis of secondary hyperparathyroidism because hypocalcemia may not allow a normal inhibition of parathyroid cells by CTR, even when CTR levels are normal, which is the usual case in early renal failure because the tendency to hypocalcemia stimulates CTR production of the remnant kidney (23).

The inhibitory effect of CTR on parathyroid cells has been

widely demonstrated by different authors (24). However, the main purpose of the parathyroid cell is to maintain serum Ca through the secretion of PTH; therefore, it seems reasonable that the inhibitory action of CTR on parathyroid cell function be regulated by the extracellular Ca. The results of the present study and by other authors (7,9) suggest that the feedback mechanism of CTR on the parathyroids should not occur if hypocalcemia has not been corrected, otherwise the function of the parathyroid cell would be defeated.

In conclusion, this study shows that extracellular Ca regulates VDR expression by parathyroid cells independently of CTR and that this is a mechanism by which serum Ca may modulate the feedback of CTR on the parathyroids.

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