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Decrease in calcitonin and parathyroid hormone mRNA levels and hormone secretion under long-term hypervitaminosis D_3 in rats

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Summary. In calcium homeostasis, vitamin D_3 is a potent serum calcium-raising agent which *in vivo* regulates both calcitonin (CT) and parathyroid hormone (PTH) gene expression. Serum calcium is the major secretagogue for CT, a hormone product whose biosynthesis is the main biological activity of thyroid C-cells. Taking advantage of this regulatory mechanism, long-term vitamin D_3-induced hypercalcemia has been extensively used as a model to produce hyperactivation, hyperplasia and even proliferative lesions of C-cells, supposedly to reduce the sustained high calcium serum concentrations. We have recently demonstrated that CT serum levels did not rise after long-term hypervitaminosis D_3. Moreover, C-cells did not have a proliferative response, rather a decrease in CT-producing C-cell number was observed. In order to confirm the inhibitory effect of vitamin D_3 on C-cells, Wistar rats were administered vitamin D_3 chronically (25,000 IU/d) with or without calcium chloride ($CaCl_2$). Under these long-term vitaminD_3-hypercalcemic conditions, calcium, active metabolites of vitamin D_3, CT and PTH serum concentrations were determined by RIA; CT and PTH mRNA levels were analysed by Northern blot and *in situ* hybridization; and, finally, the ultrastructure of calciotrophic hormone-producing cells was analysed by electron microscopy. Our results show, that, in rats, long term administration of vitamin D_3 results in a decrease in hormone biosynthetic activities of both PTH and CT-producing cells, albeit at different magnitudes. Based upon these results, we conclude that hypervitaminosis D_3-based methods do not stimulate C-cell activity and can not be used to induce proliferative lesions of calcitonin-producing cells.

Key words: Vitamin D_3, Calcitonin, PTH, C cells, Parathyroid cells, Hypercalcemia

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Introduction

Calcitonin (CT), parathyroid hormone (PTH) and 1,25-dihydroxycholecalciferol (1,25-$(OH)_2D_3$), the active metabolite of vitamin D_3, are the main hormones involved in regulating calcium homeostasis. CT is produced mainly in the C cells, which represent about 5% of all the endocrine cells in the rat thyroid gland (Martín-Lacave et al., 1992), while PTH is synthesized in the chief cells of the parathyroid gland. A high calcium stimulates CT secretion whereas a low calcium stimulates PTH secretion (Naveh-Many et al., 1992).

Expression of the PTH gene is regulated by 1,25-$(OH)_2D_3$ and estrogens (Silver et al., 1985; Naveh-Many and Silver, 1990; Naveh-Many et al., 1992). PTH mRNA levels are reduced over 90% 24 hours after 1,25-$(OH)_2D_3$ administration (Silver et al., 1985, 1986; Naveh-Many and Silver, 1990). Moreover, 1,25-$(OH)_2D_3$ is a powerful inhibitor of CT gene transcription that reduces CT expression to 4% of basal within 24 hours (Silver and Naveh-Many, 1993). In addition, estrogens, as in the case of PTH, induce CT gene expression. It has also been reported that chronic hypercalcemia induced by large doses of vitamin D_3 produces hypertrophic and hyperplastic changes of C-cells and has even been used as a model to induce proliferative lesions derived from them (Thurston and Williams, 1982; Capen and Martin, 1989; Okada et al., 1991). However, in those studies, plasma calcitonin levels were not measured to verify the supposed increasing hormonal synthesis by C-cells.

We have recently demonstrated that during a long-term hypercalcemia induced by vitamin D_3, calcitonin serum levels, and, also, the number of calcitonin immunopositive cells of the thyroid gland, slightly decreased (Martín-Lacave et al., 1998). Those last results are in sharp contrast to previous papers reporting a hypertrophic and hyperplastic effect on C-cells after vitamin-D treatment (Zabel, 1976; Okada et al., 1991).

Nevertheless, the effect of prolonged treatment with vitamin D_3 metabolites on CT and PTH transcription

and secretion by specialized endocrine cells has not been examined yet. In this study, we present the different behaviour of C-cells and chief cells in the synthesis and release of calciotrophic hormones under a long-term treatment with pharmacological doses of oral vitamin D_3. We demonstrate that vitamin D_3-induced hypercalcemia does not stimulate biosynthetic CT activity. Moreover, vitamin D_3 long-term administration leads to a decrease in CT mRNA and CT serum levels and, also, in PTH mRNA and seric PTH; results which are not compatible with hyperactivation-mediated proliferative changes.

Materials and methods

Experimental model

Male Wistar rats (n=90), aged 2 months, were divided into groups. Group 0 (n=10) was the no treatment control. Group I (n=20) was the control utilized at each timepoint. Group II consisted of 20 animals receiving 50,000 IU of vitamin D_3 (previously diluted in an alcoholic vehicle) per 100 ml of drinking water for either 1 or 2 months. On average, each subject drank over 50 ml of this solution every day. Group III consisted of 20 animals receiving the same treatment as group II plus 0.1% of $CaCl_2$. Finally, group IV: 20 animals which received only 0.1% $CaCl_2$. In the course of the experiment, the rats were housed and handled according to approved guidelines, maintained under a standard laboratory diet and allowed water *ad libitum*. Animals were sacrificed under ether anaesthesia. Blood samples were taken by aortic puncture and thyro-parathyroid complexes were extracted and immediately either fixed or frozen in liquid nitrogen.

Serum analysis

Total serum calcium was determined using a calcium analyzer (Merck Vitalab Selectra). The level of serum CT was determined using commercially available kits (Nichols Institute Diagnostics, CA, USA). The levels of serum PTH were determined using the Rat PTH (IRMA) Kit (Nichols Institute Diagnostics, CA, USA). Serum concentrations of 25-OHD_3 were measured by RIA using a 25-hydroxyvitamin D, ^{125}I RIA Kit (INCSTAR Corporation-Stillwater, Minnesota, USA). The measurement of 1,25-$(OH)_2D_3$ levels was carried out using a RIA for the determination of 1,25-dihydroxyvitamin D (Nichols Institute Diagnostics CA, USA). Results were expressed as pg 1,25-$(OH)_2D_3$/ml rat serum.

Probes

For CT mRNA, a 45 base antisense oligodeoxynucleotide probe, complementary to 3' end of exon 4 (Jacobs et al., 1981) was used. For PTH mRNA, a cocktail of two 45-base oligodeoxynucleotide antisense probes, corresponding to 3' and 5' end coding regions, respectively, was used (Heinrich et al., 1984). For GAPDH mRNA, a cocktail of two 45-base oligodeoxynucleotide antisense probes corresponding to NH_2 and COOH ends of mature protein, respectively, was used. Sequences were chosen using the *gcg* Wisconsin Package and showed no homology with any other known sequence of Genebank Database. Antisense probes and complementary sense chains, used as negative controls, were 3'-OH end-tailed with Digoxigenin-11-dUTP/dATP, according to the manufacturer's instruction (Oligonucleotide Tailing Kit, Roche).

Northern blot analysis

Total thyro-parathyroid RNA was isolated as previously described (Chomczynski and Sacchi, 1987). Twenty μg of total RNA was size fractionated by electrophoresis through 1% agarose/2% formaldehyde gel and transferred to nylon membrane (Bionylon Z^+, Bioprobe). For hybridization, 30 ng/ml of specific probe was diluted in 5xSSC, 0.1% N-Laurylsarcosyne, 0.02% SDS, 1% Blocking Reagent (Roche) and incubated overnight at 60 °C. After stringency washes, digoxigenin-labelled nucleotides were detected with alkaline phosphatase-linked anti-digoxigenin antibodies which were finally revealed by quimioluminescence. CT and PTH hybrid signals were normalized by subsequent hybridization of membranes with the GAPDH mRNA specific probe. Analysis of autoradiographs was performed using an image analysis program (NIH Image 6.01). mRNA levels (the ratio of integrated intensities CT/GAPDH and PTH/GAPDH) were expressed in arbitrary units. CT and PTH mRNA levels in treated groups were expressed as % of control values.

In situ hybridization

After the isolation, the thyro-parathyroid complex was fixed in 4% paraformaldehyde and paraffin-embedded. Paraffin-embedded 5 μm sections were deparaffinized, rehydrated and permeabilised with Proteinase K (Roche). For hybridization, 2 ng/μl of specific antisense, or control sense oligonucleotide probe, was diluted in 50% deionized formamide, 10% dextran sulphate, 2xSSC, 0.1xTris-EDTA, 1 μg/μl sonicated sperm salmon DNA, 5xDenhardt's solution. Sections were incubated overnight with 40 μl of this solution, under coverslip, at 42 °C in humidity chamber. After extensive stringency washes, digoxigenin was detected using an anti-digoxigenin alkaline phosphatase-conjugated antibody which was subsequently detected by a standard histoenzymatic color reaction. Finally, sections were methyl green-counterstained and mounted with glycerol-gelatin.

Electron microscopy

Small fragments of thyroid-parathyroid complexes

Table 1. Body weight (g) and total serum calcium (mg/dl) in control and experimental animals.

TREATMENT	BODY WEIGHT (g) Months			SERUM CALCIUM (mg/dl) Months		
	0	1	2	0	1	2
Control	339±33	480±56	538±71	11.07±0.30	10.54±0.17	10.16±0.10
Vit D_3	371±41	360±49*	353±46**		14.81±0.18**	13.33±0.40***
Vit D_3 + $CaCl_2$	347±45	442±42	401±43*		14.31±0.39***	13.06±0.38***
$CaCl_2$	369±57	468±60	398±67		10.40±0.10	10.54±0.11

Mean±SD. *: P<0.05; ** : P<0.01 ***: P<0.001. Statistical differences versus control rats of same age.

were fixed in 2.5% glutaradehyde and 2% paraformaldehyde in 0.1M phosphate buffer, pH 7.4, for 4 h, postfixed in OsO_4 in the same buffer, dehydrated through graded alcohols, and embedded via propylene oxide in Spur. Ultrathin sections were counterstained with uranyl acetate and lead citrate.

Statistical analysis

Data were subjected to ANOVA. The mean values in each experiment were compared with the control group using Student's test. Results were given as mean ±SEM. P values of less than 0.05 were accepted as significant.

Results

Effects on serum calcium and rat body weight

The administration of vitamin D_3 produced a marked increase in serum calcium concentration as compared with controls. Calcium concentration one month after treatment with vitamin D_3 raised from 10.54 mg/ml (normocalcemia) to 14.81 mg/ml (Table 1), a state that can be characterized as severe sustained hypercalcemia. There was no significant change in calcium levels after two months of treatment. There was a significant decrease in the weight of the treated rats in comparison with the control rats. This decrease was more pronounced in rats without calcium chloride supplement in the drinking water. These rats also showed generalized weakness and increasing signs of cachexia following the treatment. In contrast, animals treated with calcium chloride alone showed stable serum calcium levels and body weights. Therefore, further studies were limited to animals treated with vitamin D_3.

Effects on serum concentration of vitamin D_3 metabolites

The hypervitaminosis D_3 state was mainly due to the second main active metabolite of vitamin D_3, the 25-OHD_3. The administration of vitamin D_3, combined or not with $CaCl_2$, produced an approximately 10-fold increase of 25-OHD_3 serum levels in all treated rats, as compared with control rats, whose basal 25-OHD_3 serum levels did not vary at the different time-points of the experiment (Fig. 1B). In contrast with 25-OHD_3 data, the administration of vitamin D_3 did not raise the serum levels of the most active metabolite of vitamin D_3

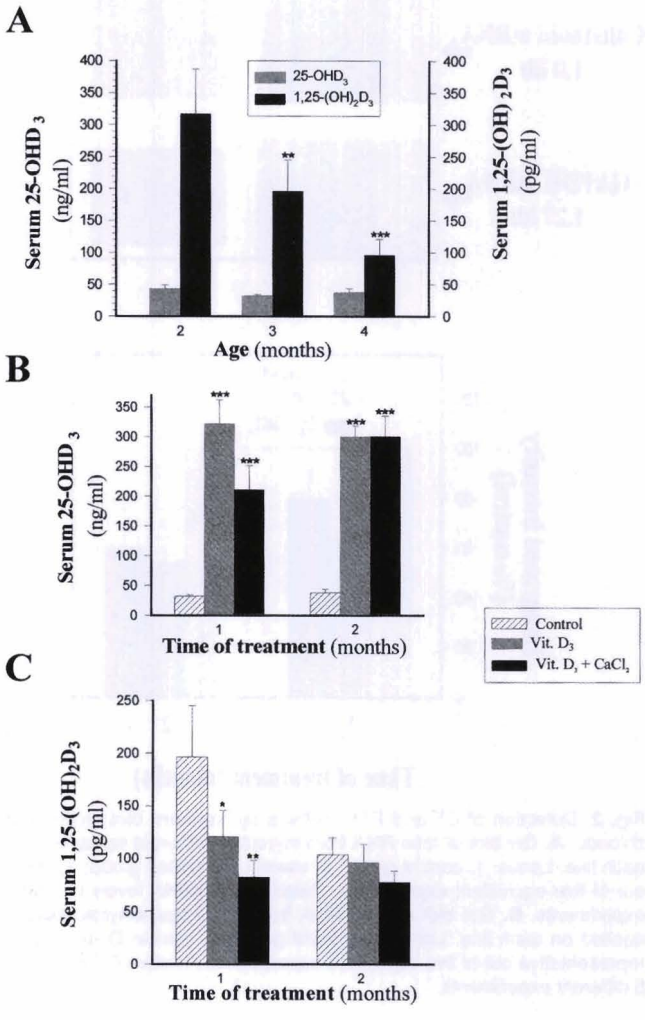

Fig. 1. Serum levels of vitamin D_3 metabolites in control rats and in rats treated either with vitamin D_3 or vitamin D_3 plus calcium chloride. **A.** Serum 25-OH-vitamin D_3 and 1,25-$(OH)_2D_3$ levels in control rats. **B.** Serum 25 OHD_3 levels in control and experimental animals. **C.** Serum 1,25-$(OH)_2D_3$ levels in control and experimental animals. Each bar represents the mean±SEM of 10 rats. *: P<0.05;**: P<0.01; ***: P<0.001 versus untreated rats.

$1,25\text{-}(OH)_2D_3$ (Fig. 1C). As a result of the treatment, decreased serum $1,25\text{-}(OH)_2D_3$ values were found in all experimental animals. This effect was more evident after the first month of treatment. In relation to this, a significant decrease in basal levels of $1,25\text{-}(OH)_2D_3$ was seen in 2-, 3- and 4-month-old control rats, which diminished more than 60% during this period of their lives (Fig. 1A).

Table 2. Changes of serum calcitonin and PTH levels under long-term hypervitaminosis D_3.

TREATMENT	SERUM CALCITONIN (pg/ml) months		SERUM PTH (pg/ml) months	
	1	2	1	2
Control	21.94±2.95	49.30±10.05	17.22±5.14	50.58±11.68
Vit D_3	25.16±9.19	26.79±6.09***	1.32±0.96*	1.42±1.44***
Vit D_3 + $CaCl_2$	29.60±7.49	30.28±3.67***	0.88±0.43*	0.63±0.74***

Mean±SD. *: P<0.05; ***: P<0.001. Statistical differences versus control rats of same age.

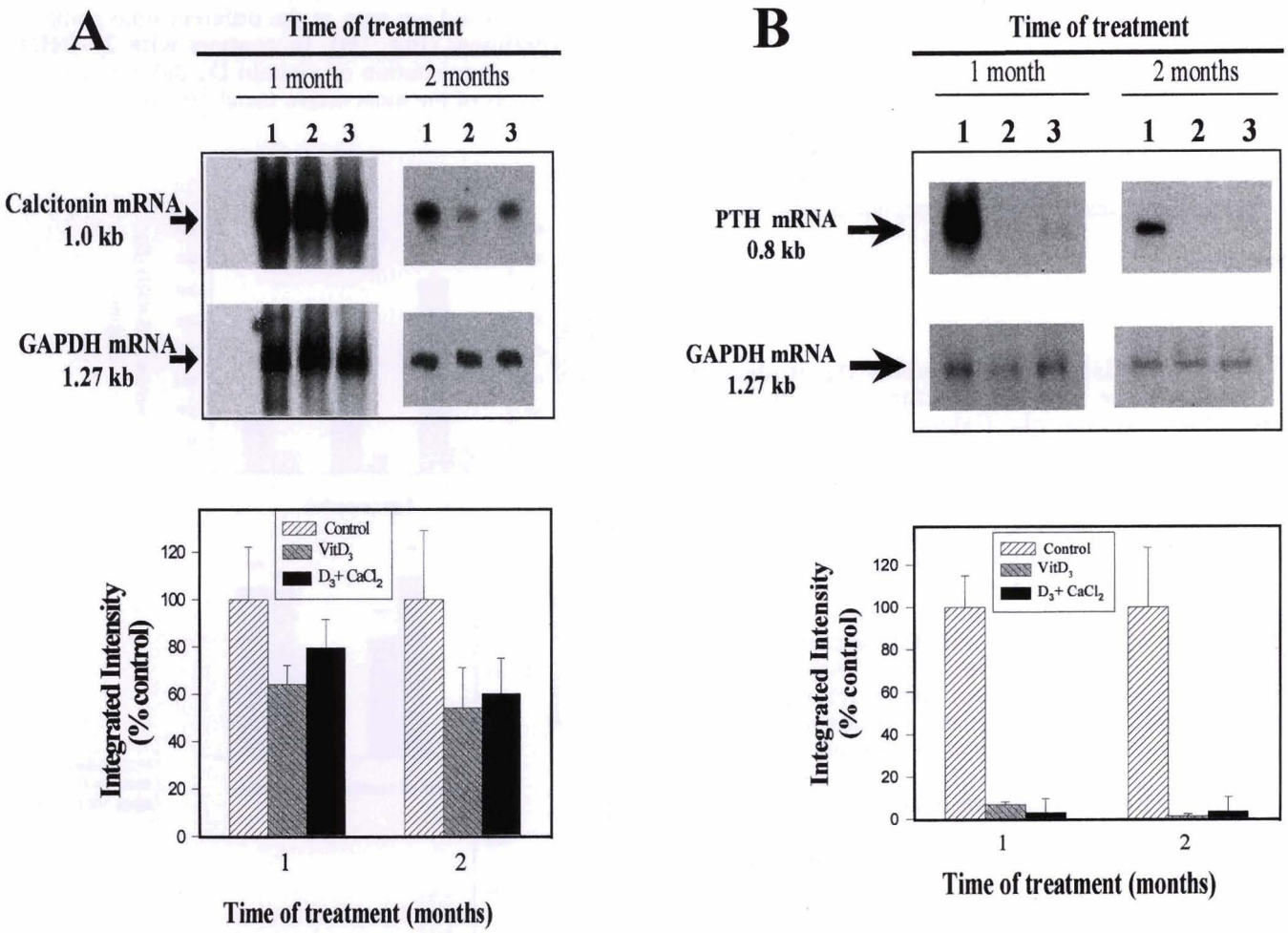

Fig. 2. Detection of CT and PTH mRNAs by Northern blot analysis after 1 and 2 months of treatment with vitamin D_3 and vitamin D_3 with calcium chloride. **A.** Gel blot of total RNA from thyroid-parathyroid tissue hybridized sequentially for CT mRNA and GAPDH mRNA. Twenty μg were applied on each line. Lanes: 1, control group; 2, vitamin D_3-treated group; 3, vitamin D_3+$CaCl_2$-treated group. Figure represents the results of one representative out of five equivalent experiments. Relative CT mRNA levels of control and experimental rats. Results are shown as the mean±SEM for 5 different experiments. **B.** Gel blot of total RNA from thyroid-parathyroid tissue hybridized sequentially for PTH mRNA and GAPDH mRNA. Twenty μg were applied on each line. Lanes: 1, control group; 2, vitamin D_3-treated group; 3, vitamin D_3+$CaCl_2$-treated group. Figure represents the results of one representative out of five equivalent experiments. Relative CT mRNA levels of control and experimental rats. Results are shown as the mean±SEM for 5 different experiments.

Effects on CT expression and CT serum concentration

A moderate decrease in the steady-state CT mRNA levels was found by Northern blot analysis after one month of vitamin D_3 treatment (Fig. 2). This decrease was fairly small in the group treated with vitamin D_3 and calcium chloride. CT gene expression decreased more significantly in both groups after two months of

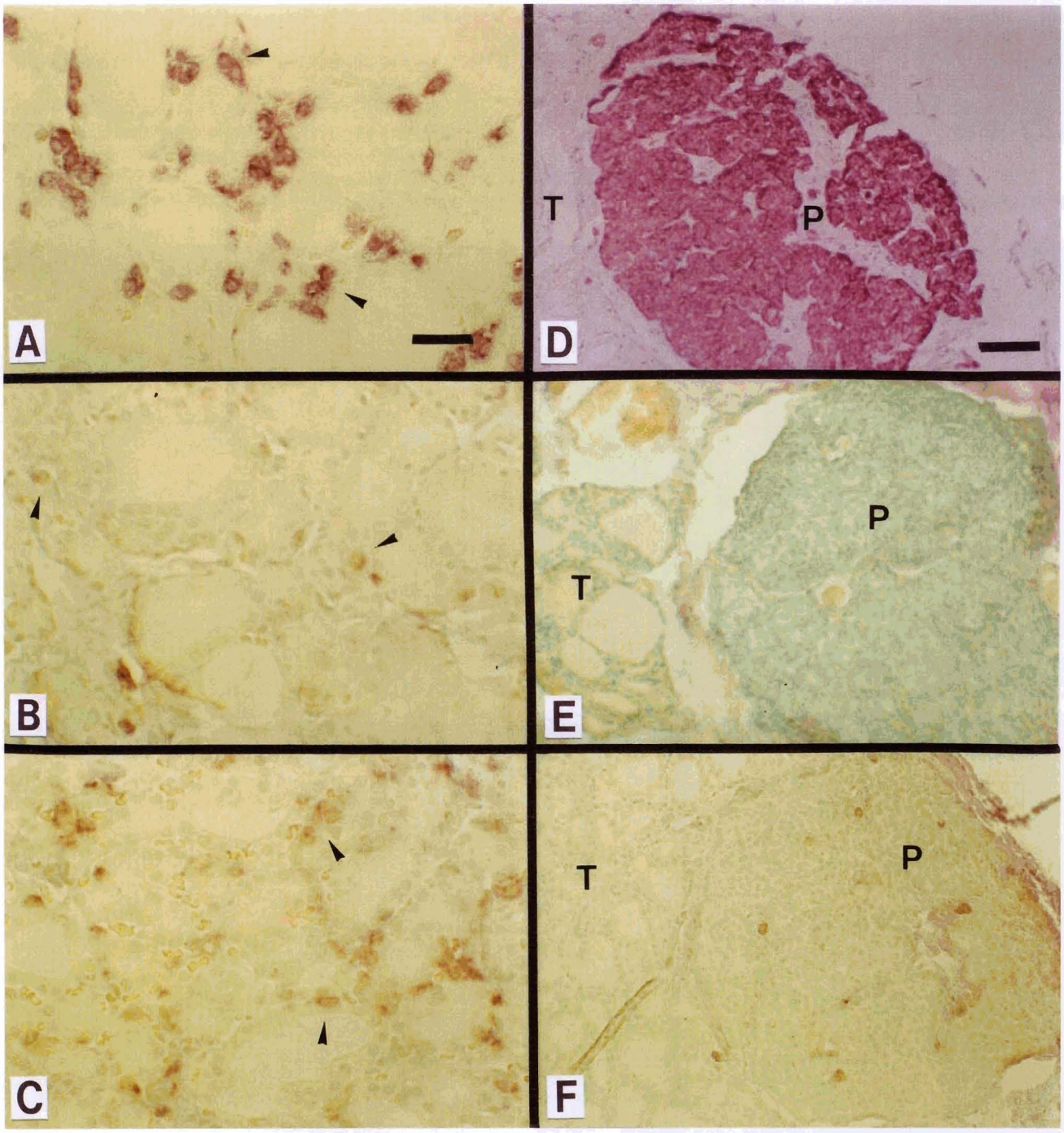

Fig. 3. Detection of CT **(A-C)** and PTH mRNAs **(D-F)** by *in situ* hybridization on rat thyro-parathyroid gland after 2 months of treatment. **A.** Control rat: There is a clear localization of CT mRNA in all C cells. **B.** Vitamin D_3-treated rats: There is a reduction in the amount of CT mRNA which makes the identification of the C cells difficult in comparison with control glands. **C.** Vitamin D_3+$CaCl_2$-treated rats: the staining of C cells with the CT probe is more reduced than in control glands but slightly higher in comparison with vitamin D_3-treated rats. x 400. Bar: 25 μm. **D.** Control rat: There is a clear localization of PTH mRNA in all chief cells. **E.** Vitamin D_3-treated rats: There is a complete reduction in the amount of PTH mRNA which makes the parathyroid gland negatively stained. **F.** Vitamin D_3+$CaCl_2$-treated rats. P: parathyroid gland; T: thyroid gland. x 200. Bar :50 μm.

treatment. Thyroid sections stained by *in situ* hybridization corroborate the decrease in the amount of CT mRNA in C cells after vitamin-D treatment, however the proportion of CT mRNA positive cells seemed not to vary in comparison to controls (Fig. 3). Similarly, the decrease in serum CT concentration was more pronounced in the group treated with vitamin D_3, and, also, more evident after the second month of treatment (Table 2).

Effects on PTH expression and PTH serum concentration

The administration of vitamin D_3 strongly inhibited the expression of the PTH gene. As can be seen in Figs. 2, 3, the amount of PTH mRNA became undetectable by Northern blot analysis of 10 μg of total thyroparathyroid RNA and by *in situ* hybridization. No differences were observed with the addition of calcium chloride. As a result, PTH levels in serum were markedly decreased (Table 2), becaming undetectable after one month of treatment.

Effects on the ultrastructure of C cells and chief cells

In comparison with control rats, secretory granules of C-cells in all treated animals were markedly reduced in number but they did not disappear completely. The Golgi apparatus was moderately developed and the endoplasmic reticulum was mostly vesicular in shape

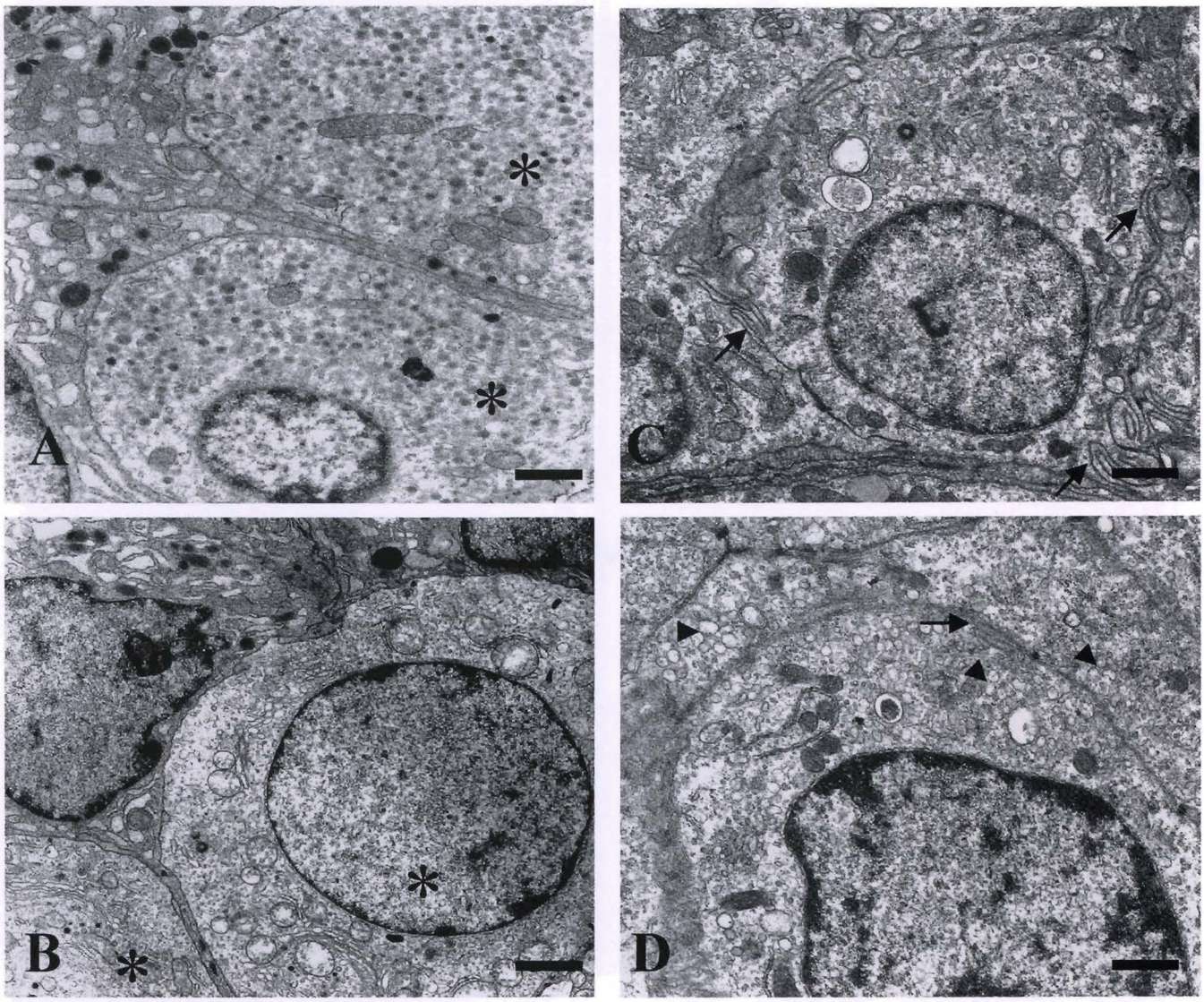

Fig. 4. Ultrastructural images of C-cells **(A, B)** and chief cells **(C, D)** belonging to control rats **(A, C)** and vitamin D_3-treated rats **(B, D)**. As can be observed, in the thyroid gland (A, B) there is a marked decrease in the number of secretory granules contained in the cytoplasm of the C cells (*) under hypervitaminosis D_3. In the parathyroid gland (C, D), under hypervitaminosis D_3 conditions, there is a reduction in the complexity of membrane infoldings (arrows) in the chief cells, and an increase in the number of empty vesicles (arrow heads). x 4,400. Bar: 2,3 μm.

and somewhat decreased in volume. Therefore, the cytoplasm of the degranulated cells was much clearer than that of the normally granulated cells (Fig. 4A,B). In the parathyroid glands of treated rats, the majority of chief cells were characterized by the presence of numerous empty vesicles of different sizes in the cytoplasm (Fig. 4C,D). Furthermore, the experimental rats presented chief cells with less complex infoldings of the cellular membrane than in control animals. Different organelles such as rough endoplasmic reticulum and free ribosomes, appeared to be similar to those of non-treated rats.

Discussion

The results of our investigations, together with some literature data, support our first hypothesis that C-cells, in a hypercalcemic state due to an intoxication with vitamin D_3, do not respond with hypertrophic and hyperplastic changes, but rather in these conditions, the main biosynthetic activity of C-cells is inhibited (Martín-Lacave et al., 1998). The hypervitaminosis D_3 state obtained in our experimental model was mainly due to the rising of 25-OHD_3 serum levels. 25-OHD_3 is the first active and most abundant metabolite in the pathway of vitamin D_3 activation (Parfitt, 1999). Conversion of vitamin D_3 in 25-OHD_3 is not subjected to strict regulation and basically depends on vitamin D_3 availability (Reichel et al., 1989). In our experiment, non-treated experimental rats, aged 2, 3 and 4 months, showed similar serum 25-OHD_3 values, whereas the oral administration of vitamin D_3 led to a ten-fold increased in serum levels of 25-OHD_3. In contrast, synthesis and degradation of 1,25-$(OH)_2D_3$ (the most active vitamin D_3 metabolite) are strictly down regulated by serum calcium, and up regulated by calcitonin and PTH (Kawashima et al., 1981; Shigematsu et al., 1986; Sinki et al., 1992). Based on this, as a result of the hypercalcemia and decreased levels of serum calcitonin and PTH, lower levels of 1,25-$(OH)_2D_3$ were observed in both treatment groups. In our study we have also observed a decrease in 1,25-$(OH)_2D_3$ serum levels with age in control animals. A similar decrease has also been described in humans with aging (Fujisawa et al., 1984; Quesada et al., 1994). This observation, in our experimental model, might be related to developmental calcium requirements rather than changes caused by the prolongation of the treatment.

In chronic hypervitaminosis D_3, such as in the experimental model we have presented, parathyroid cells must respond to both Ca^{++} and vitamin D_3, which act synergistically on PTH expression and secretion. Steady-state levels of PTH mRNA dramaticaly decreased (as assessed by either Northern blotting or *in situ* hybridization). This, in turn, results in undetectable levels of serum PTH. These patterns of PTH expression and secretion are similar to those elicited by short-term treatments with 1,25-$(OH)_2D_3$ (Karmali et al., 1989; Naveh-Many et al., 1989, 1992; Naveh-Many and Silver 1990; Moallem et al., 1998). Ultrastructural appearance of the parathyroid gland in long-term vitamin-treated rats also coincides with that seen in chief cells under short-term hypercalcemia or hypervitaminosis D (Setoguti et al., 1981, 1995; Wild et al., 1985). The apparent degradation of storage granules by hydrolysis, and the decrease of the cell surface area, seems to be closely associated with an intracellular regulatory mechanism for PTH secretion.

Hypervitaminosis D_3 affects CT-producing cells in a distinctly different manner. C-cell population may respond to the increase in extracellular calcium by releasing CT stored in granules through exocytosis. However, vitamin D_3 reduces the transcription of the CT gene. CT secretion gives the cells an almost completely degranulated appearance under electron microscopy. Nonetheless, they still conserve morphological signs of the persistence of some hormonal synthesis. These ultrastructural findings concur with those obtained by *in situ* hybridization and Northern blot analysis. Based upon these, C-cells, still have a moderate rate of CT synthesis which is not observable in short-term treatments, where CT mRNA decreased 4% of baseline level at 48 h (Naveh-Many and Silver, 1988; Silver and Naveh-Many, 1993). Importantly, in these studies, 1,25-$(OH)_2D_3$ did not raise serum calcium. These results indicate that in the case of C-cells, where hypercalcemia and hypervitaminosis D_3 are antagonic stimuli, a second hormone synthesis regulatory mechanism, besides the vitamin D_3-mediated transcriptional inhibition or mRNA stability, would be present.

In the group treated with vitamin D_3 and $CaCl_2$, CT mRNA levels were higher than those elicited by vitamin D_3 alone. In this case, the effect of calcium ingestion on the release of different mediators at the gastrointestinal level, such as gastrin, cholecystokinin and glucagon, might be a decisive factor. These hormones are well-known CT secretagogues and are also involved in regulating calcitonin gene transcription (Naveh-Many and Silver, 1988; Raue et al., 1991, 1993). The administration of $CaCl_2$ with vitamin D_3 in the drinking water may thus act as a "buffer" against the 25-OHD_3 - induced blockade of CT synthesis.

In numerous studies in which vitamin D_3 has been used as a serum-calcium-raising agent, to investigate the reaction of C cells to long-term hypercalcemia, C-cell hypertrophy and hyperplasia has been described (Zabel, 1976; Thurston and Williams, 1982; Kameda et al., 1985; Capen and Martin, 1989; Okada et al., 1991). We have recently described (Martín-Lacave et al., 1998) the effect of long-term administration of vitamin D_3 at hypercalcemic doses on the thyroid gland of the rat. In contrast to previous reports, we have found that C cells did not respond with hypertrophic and hyperplastic changes. In addition, the number of C cells that were immunopositive for CT decreased. This observation concurs with the findings of our current study in which vitamin D_3 does not stimulate the C-cell population, but rather inhibits CT synthesis. Based upon these results it is difficult to accept that hypervitaminosis D_3, which inhibits the main biosynthetic activity of C-cells, could

induce proliferative changes in this endocrine population. We conclude that the oral administration of vitamin D_3 at serum-calcium-raising doses for prolonged periods reduces the rate of biosynthesis and secretion of both PTH and CT. Therefore, supporting our previous data (Martín-Lacave et al., 1998), hypervitaminosis D_3-based methods are not reliable models to induce proliferative lesions of calcitonin-producing cells, as has been extensively described in the literature.

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