Increased level of calcitonin mRNA after 1,25-dihydroxyvitamin D_3 injection in the rat

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Vitamin D metabolites are able to change plasma calcitonin (CT) levels, but nothing is known about a possible effect at the CT gene level. Here we have investigated the acute effects of 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) on the CT biosynthetic activity of thyroid glands from adult rats. Plasma CT levels were significantly increased ($\times 2$) 1 and 2 h after 1,25-$(OH)_2D_3$ injection in the face of unchanged plasma calcium values. The thyroidal CT content also was unchanged. A 2-fold increase in CT mRNA level measured by dot-blot hybridization occurred 1 and 2 h after 1,25-$(OH)_2D_3$ administration. Expression of CT gene products was examined in the rabbit reticulocyte lysate cell-free translation assay. After polyacrylamide gel electrophoresis, specific immunoprecipitates were autoradiographed and quantified by integration. A single precursor of $M_r \cong 15000$ could be specifically immunoprecipitated with CT antisera. A 3–4-fold rise in translatable CT mRNA activity was observed 1 and 2 h after 1,25-$(OH)_2D_3$ injection. Thus, parallel changes in CT mRNA level, CT mRNA activity and plasma CT levels were observed in adult female rats after administration of 1,25-$(OH)_2D_3$. These findings demonstrate for the first time that 1,25-$(OH)_2D_3$ enhanced CT gene expression in the face of unchanged plasma calcium levels.

mRNA level *mRNA activity* *Calcitonin* *1,25-$(OH)_2D_3$* *Adult rat*

1. INTRODUCTION

The biologically active metabolite of vitamin D, 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$), is considered to act on target cells by mechanisms similar to those of the classical steroid hormones [1,2]. Recently, it has been shown that 1,25-$(OH)_2D_3$ increases the relative amount of prolactin mRNA in GH_4C_1 cells and that the full expression of this effect is dependent on extracellular calcium [3]. Vitamin D_3 metabolites are known to increase the calcitonin (CT) secretion rate in pigs [4], and to decrease the thyroidal CT content before and after weaning in rats [5]. The structure of the biosynthetic precursor of CT produced in C cells has been determined [6–12], but as yet, there is no report on a possible effect of 1,25-$(OH)_2D_3$ at the CT gene level. This prompted us to investigate the effects of 1,25-$(OH)_2D_3$ on the CT mRNA level measured by dot-blot hybridization with ^{32}P-labeled CT cDNA, and on the CT mRNA activity evaluated by translation assay [13].

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2. MATERIALS AND METHODS

2.1. *Animals*

Female Wistar rats weighing 211 ± 2 g were purchased from CERJ (Le Genest, France). They were fed a commercial diet (UAR 103, Usine d'Alimentation Rationnelle, Villemoisson/Orge, France) containing 0.92% calcium, 0.92% phosphorus, 0.15% magnesium and 4000 IU vitamin D/kg. After an overnight fast of 16 h, the females were subcutaneously injected with 1 μg 1,25-$(OH)_2D_3$/kg

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body wt (Hoffmann-La Roche, Basel). The rats were killed by aortic puncture under light ether anesthesia 1, 2, 4, 8 and 16 h after 1,25-$(OH)_2D_3$ injection. Thyroid glands were removed and stored in liquid nitrogen until extracted.

2.2. *mRNA extraction and translation studies*

RNA were extracted from batches of 10 hemithyroid glands with phenol-chloroform, purified with LiCl precipitation [14], and poly(A)-rich RNA separated by oligo(dT) cellulose [15] according to published procedures [10,16].

1 μg mRNA (in 1 μl water) was added to 10 μl rabbit reticulocyte lysate obtained from Amersham (England) containing 50 μCi [^{35}S]methionine and incubated for 60 min at 37°C. Radioactivity incorporated into synthesized proteins was estimated by precipitation with trichloroacetic acid [17]: 1-μl aliquots were spotted on 3 MM Whatman paper wetted with 20% trichloroacetic acid, boiled for 10 min in 1 l of 10% trichloroacetic acid, dried with diethyl ether, and counted. For immunoprecipitation, 5- or 6-μl aliquots were incubated for 24 h at 4°C in 300 μl of 0.1 M phosphate buffer (pH 7.4) containing 0.5% Triton X-100, 0.2% human albumin, 0.1% methionine in the presence of 0.7 μl sheep antiserum (M 732) against human synthetic CT saturated with 10 μg human CT or without. Immunoglobulins were then precipitated by the addition of 25 μl anti-sheep (IgG) for 24 h at 4°C. Precipitates were collected by centrifugation over 200 μl of a 1 M sucrose cushion in a 1.5 ml polypropylene tube (Eppendorf), and washed 5 times with the immunoprecipitation buffer. Precipitates were dissolved and heat-denatured in 25 μl of 60 mM Tris (pH 6.8) containing 2% SDS, 5% 2-mercaptoethanol and 0.4% bromophenol blue. Aliquots were counted in scintillation mixture (Biofluor, NEN), specificity of the immunoprecipitates was checked by 0.1% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (20% acrylamide) [18] and autoradiography performed using Kodak X-Omat AR5 film (8-day exposure at −80°C). Autoradiographies were quantified in an automatic densitometric scanner.

2.3. *mRNA-cDNA hybridization assays*

*Pst*I-cleaved insert of pBR 3271 containing the entire coding sequence of human preprocalcitonin [12] was labeled to an activity of 10^8 cpm/μg by nick translation using polymerase I (Kornberg) [19].

Aliquots of poly(A)-rich RNA (1 μg) were denatured by glyoxal [20] and were spotted on 'Gene Screen' membranes (NEN) previously washed and equilibrated with 20 × standard saline citrate (NaCl/Cit, 1 × NaCl/Cit = 0.1 M NaCl, 0.015 M sodium citrate). Membranes were dried under a lamp and baked for 2 h at 80°C. They were prehybridized in 50% formamide, 5 × NaCl/Cit, 5 × Denhart (1 × Denhart = 0.2 mg/ml bovine serum albumin, Ficoll and polyvinyl pyrrolidone), 50 mM sodium phosphate buffer pH 6.5, 100 μg/ml of denatured herring sperm DNA and 1 μg/ml of poly(A) (PL Biochemicals). Membranes were incubated 4 h at 42°C and hybridized in a modified prehybridization buffer (i.e., 1 × Denhart, 20 mM sodium phosphate buffer and 10% dextran sulfate) containing 0.3 μCi of hCT cDNA denatured by heating (100°C for 10 min). Hybridization was carried out for 18 h at 42°C. Membranes were washed by 3 changes of 2 × NaCl/Cit, 0.1% SDS for 5 min at room temperature and then 2 changes of 0.1 × NaCl/Cit, 0.1% SDS for 30 min at 55°C. Dried membranes were exposed at −80°C to Kodak Royal X-Omat AR5 film for 2 days.

2.4. *Other methods*

Blood samples were collected in polyethylene tubes cooled in ice water. After centrifugation at 4°C, aliquots of plasma were used immediately for calcium and phosphorus determinations, the remainder being frozen (−35°C) until CT was assayed. The thyroid glands were dissected under a microscope at 4°C and sonicated (MSE 150 W Ultrasonic Desintegrator Mk2, Crawley, England) at 4°C for 30 s in barbital buffer (0.025 M, pH 8.6) containing peptidase inhibitors [5]. The CT in plasma and sonicates was measured by a radioimmunoassay (RIA) which has been described [21]. The plasma calcium concentration was measured by flame photometry (Eppendorf, FCM 6341), and the plasma phosphate level was evaluated as in [21].

Results were expressed as means ± SE. Significance of difference between groups was determined using Student's *t*-test. For plasma CT, some values were below the limits of detectability so that

a mean value could not be calculated. In this case significance of difference between groups was determined using the non-parametric U-test of Mann and Whitney.

3. RESULTS AND DISCUSSION

After s.c. injection of 1,25-$(OH)_2D_3$, plasma calcium was unchanged until 16 h, where a slight increase was observed (10.86 ± 0.16 vs 10.42 ± 0.09 mg/dl, $P < 0.05$). A trend towards lower values occurred 2 h after injection (fig.1). Plasma phosphate concentrations were significantly increased at all times (except at 2 h) following administration of 1,25-$(OH)_2D_3$ (fig.1). A peak in plasma CT levels was apparent 1 and 2 h after in-

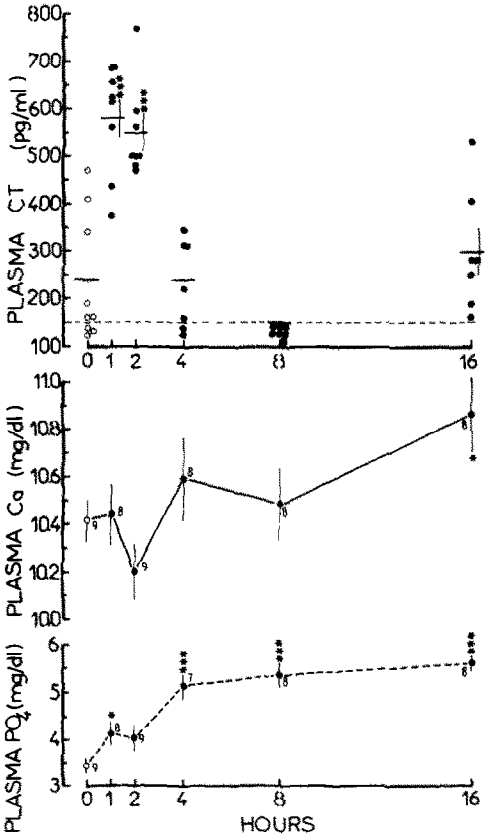

Fig.1. Plasma calcitonin (CT), calcium and phosphate concentrations after s.c. injection of 1 μg 1,25-$(OH)_2D_3$/kg in adult female rats. Means ± SE, and the number of animals. Individual values were given for plasma CT. * $P < 0.05$, *** $P < 0.001$ from time 0.

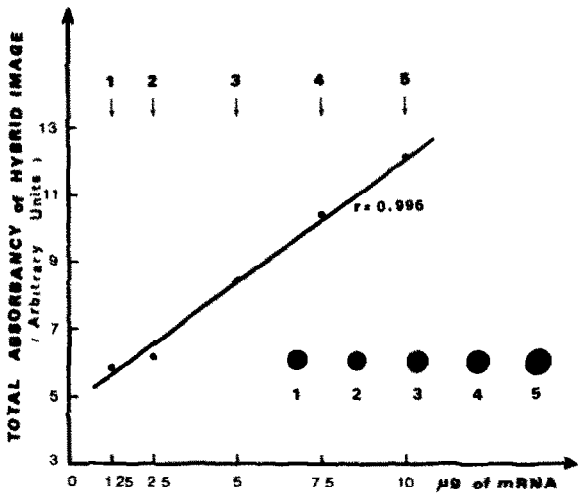

Fig.2. Dot hybridization assay. Aliquots of rat poly(A)-rich RNA (1.25–10 μg) were spotted on Gene Screen and hybridized with 0.3 μCi of a ^{32}P-labeled cDNA complementary to human CT mRNA. Autoradiograms were scanned on a gel densitometer set at maximum gain and results expressed as arbitrary units. The numbered arrows denote the position of the plot of each similarly numbered hybrid image of the autoradiography shown in the insert. Regression curve and correlation coefficients were calculated by standard statistical techniques.

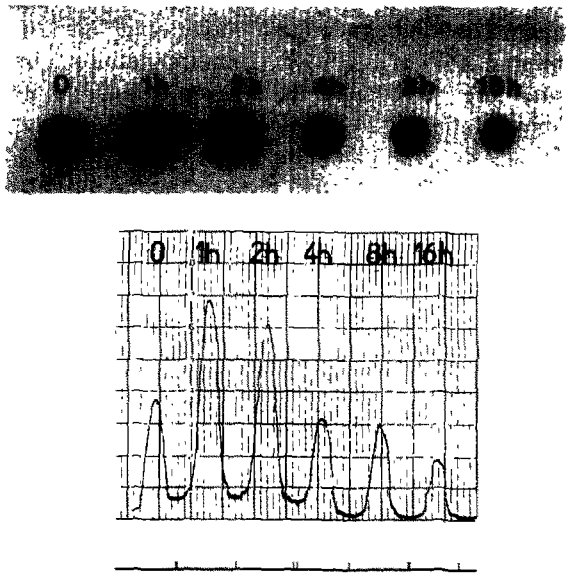

Fig.3. Autoradiographies and densitometric scans of dot-blot hybridization of mRNA with ^{32}P-labeled CT cDNA. Rat mRNA (1 μg) was immobilized on Gene Screen (NEN) and hybridized to ^{32}P-labeled cDNA excised by the restriction enzyme *Pst*I, from pBR 3271.

jection, these plasma values returned to basal levels by 4 h, and decreased subsequently (becoming undetectable) by 8 h. A further increase was noted at 16 h (fig.1). The thyroidal CT stores remained unchanged throughout the course of the experiment (not shown). Similar patterns for CT secretion and plasma calcium have been reported following 1,25-$(OH)_2D_3$ injection [22]. To ascertain the quantities of CT related mRNAs produced in our experiments, we performed RNA/DNA hybridization assays with cloned radiolabeled human CT cDNA [12]. It was found previously that our cDNA probe to human CT mRNA cross-reacts with rat CT mRNA [10]. The amount of ^{32}P-labeled specific cDNA probe hybridized to rat poly(A)-rich RNA increased linearly with increasing amounts of mRNA (1.25–10 μg mRNA) (fig.2). As shown in fig.3, a 2-fold increase in the CT mRNA level could be detected by the dot-blot assay 1 and 2 h after 1,25-$(OH)_2D_3$ injection. Control values were reached by 4 h. A small and slow decline was observed afterwards (fig.3). When 1 μg poly(A)-rich RNA was translated in the rabbit reticulocyte cell-free translation assay, SDS-polyacrylamide gel electrophoresis and autoradiography of the specific immunoprecipitates showed the presence of a major band (M_r ≅15000)

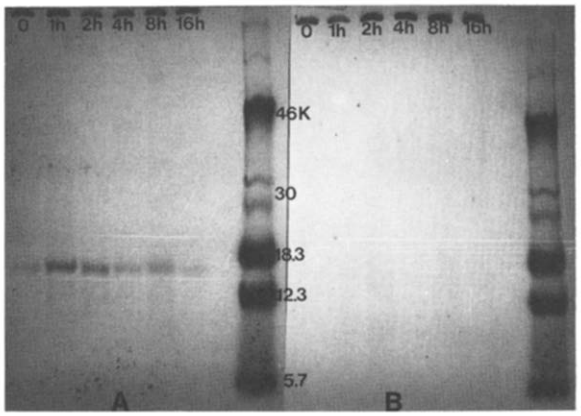

Fig.4. Autoradiographies of specific immunoprecipitates obtained by translation of 1 μg thyroid mRNA. The proteins synthesized and immunoprecipitated were analysed by 0.1% SDS-20% PAGE. (A) Without unlabeled human CT; (B) with an excess of unlabeled human CT (10 μg). Numbers on the middle lane represent $M_r \times 10^{-3}$ of labeled markers: insulin, 5700; cytochrome *c*, 12300; lactoglobulin A, 18300; carbonic anhydrase, 30000; ovalbumin, 46000.

(fig.4A). This band was absent using specific antiserum saturated with unlabeled CT (fig.4B). Translatable CT mRNA activity was clearly increased 1 and 2 h after injection (× 3–4) (figs 4,5), and then decreased to control at 4 h. A slight increase occurred 8 h after 1,25-$(OH)_2D_3$ administration followed by a decline to pre-injectional levels at 16 h (figs 4,5). Radioactivity incorporated in specific immunoprecipitates (table 1) showed a 3–4-fold increase 1 and 2 h after 1,25-$(OH)_2D_3$ administration, and declined slowly thereafter.

The present results show that the increased plasma CT level induced by 1,25-$(OH)_2D_3$ is closely related to the rise in CT mRNA. Of particular interest was the finding that the response occurred in the face of unchanged plasma calcium level since it has been shown that an acute calcium stimulation elicits a rapid increase in CT mRNA [10]. A

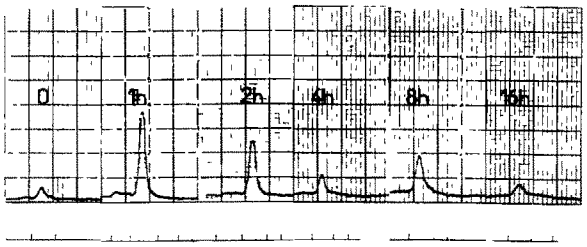

Fig.5. Densitometric scans of autoradiographies shown in fig.4A.

Table 1

Radioactivity incorporated in translation products specifically immunoprecipitated by CT antiserum

Time after treatment with 1,25-$(OH)_2D_3$ (h)	cpm in specific immunoprecipitates
0	431 ± 41
1	1669 ± 81
2	828 ± 57
4	547 ± 46
8	573 ± 47
16	401 ± 40

Radioactivities were estimated as the difference between two measurements (in the absence of human CT minus in the presence of an excess of human CT: 10 μg). Data represent mean ± 2 SD of radioactivity incorporated in preprocalcitonin

good parallelism was found between the CT mRNA level measured by dot-blot hybridization and the CT mRNA activity evaluated in the translation assay.

As the present effects of 1,25-$(OH)_2D_3$ have been demonstrated at supra-physiological hormone concentrations, it would be premature to attempt to relate these findings to any possible physiological action of vitamin D on CT synthesis and secretion. Further studies using vitamin D-deficient rats injected with physiological doses of 1,25-$(OH)_2D_3$ will be required to determine the physiological relevance of these observations. Nevertheless, our data demonstrate for the first time a 1,25-$(OH)_2D_3$ mediated enhancement of CT gene expression independently of plasma calcium.

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