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Additive effects of dexamethasone and calcium on the calcitonin mRNA level in adrenalectomized rats

Philippe Besnard*, Valérie Jousset and Jean-Michel Garel

Endocrinologie Moléculaire et Métabolique, Université Pierre et Marie Curie, 4 place Jussieu, 75252 Paris-Cédex 05, France

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Northern hybridizations were used to evaluate the effects of dexamathasone and calcium on calcitonin mRNA levels in adrenalectomized female rats Two weeks after adrenalectomy, a 3 6-fold decrease in the calcitonin mRNA level was observed (28% vs 100% in sham-operated controls) After 5 days of dexamethasone treatment (1 5 mg/kg b wt), a 2 6-fold rise in calcitonin mRNA occurred in adrenalectomized rats (73% vs 28%) This increment was higher when dexamethasone treated animals were injected with calcium (100% vs 73%) The effect of calcium on the calcitonin mRNA level of adrenalectomized rats treated or not with dexamethasone was similar, and additive in the former case Our data suggest that calcium and dexamethasone elevate calcitonin mRNA by two different mechanisms

Messenger RNA, Calcitonin, Dexamethasone, Calcium, Adrenalectomy, (Rat)

1. INTRODUCTION

Glucocorticoids are a major class of steroid hormones produced by the adrenal cortex and are important physiological regulators of gene expression. In several target tissues including pituitary gland, liver, intestine and adrenal medulla, they control the expression of a small number of genes by increasing or decreasing mRNA levels (for review, see [1]). In the thyroid, recent in vitro studies showed that DEX, a synthetic glucocorticoid, may change the mRNA concentration of CT, a Ca-lowering peptide hormone elaborated by the C-cells. In TT cells, a continuous human C-cell line derived from a medullary thyroid carcinoma, and in CA-77 cells, a rat medullary thyroid carcinoma cell line, it has been reported that DEX enhanced the specific CT mRNA levels [2,3], but a decrease was observed in 44-2 cells, another murine medullary thyroid carcinoma cell line [4]. The basis of such differences in response to glucocorticoids remains unclear, although the properties of the different transformed clonal cell strains used in these studies might explain these conflicting data. Curiously, as yet, in vivo data are lacking. To determine if such a modulation of CT mRNA levels is present in the normal thyroid glands, ADX adult rats were injected for 5 days with DEX, and thyroid CT mRNAs were measured by Northern hybridization using a specific CT cDNA probe. Ca being the main CT secretagogue, an acute Ca injection was also performed in ADX and ADX + DEX rats to ascertain whether a glucocorticoid pretreatment potentiated the short-term response of C-cells to a Ca load. We report here that the marked drop in CT mRNA level occurring after ADX is partially compensated by the DEX treatment or a Ca load. These 2 stimuli have an additive effect on the CT mRNA level. No change in plasma CT level was observed 24 h after the last DEX injection, but the acute Ca load triggered a greater CT release in DEX-treated rats than in untreated ADX animals. This in vivo study suggests that the control of the CT gene expression by glucocorticoids and Ca implies different mechanisms and that endogenous glucocorticoids may specifically enhance the thyroidal release of CT in response to hypercalcemic stress in rats.

Correspondence address J.-M. Garel, Endocrinologie Moléculaire et Métabolique, Université Pierre et Marie Curie, 4 Place Jussieu, 75252 Paris-Cédex 05, France

Abbreviations CT, calcitonin, ADX, adrenalectomized, DEX, dexamethasone, Ca, calcium; SO, sham-operated, NEN, Nensorb column

* *Present address* Laboratoire de Physiologie de la Nutrition, ENSBANA, Campus Montmuzard, 21100 Dijon, France

2. MATERIALS AND METHODS

2 1 *Animals and treatments*

Female Wistar rats weighing 242 ± 3 g were purchased from CERJ (Le Genest, France). They were fed a commercial diet (UAA A03, Usine d'Alimentation Rationnelle, Villemoisson/Orge) containing 0 84% calcium, 0.78% phosphorus, 0.17% magnesium and 3000 IU vitamin D/kg. All surgery was performed under light ether anesthesia Adrenals were removed with fine-curved forceps. ADX females were maintained on 0 15 M NaCl in their drinking water Control rats were SO. One week after surgery, animals were s c injected daily for 5 days with either 1.5 mg DEX (Serva, Heidelberg,

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FRG)/kg b.wt (ADX + DEX) or 15 μg DEX/kg b.wt (ADX + dex) and were killed 24 h after the last injection. Similarly, SO rats and a group of ADX females received the vehicle alone (100 μl absolute ethanol). One hour before sacrifice, half of ADX and ADX + DEX groups were i.p. injected with 35 mg Ca as calcium glucono-galactogluconate (Calcium Sandoz)/kg b.wt giving the ADX + Ca and ADX + DEX + Ca groups, respectively. Animals were killed by aortic puncture under light ether anesthesia. Thyroid glands were quickly removed and stored in liquid nitrogen until RNA extractions.

2.2. *Preparation of ^{32}P-labeled probe*

The CT specific probe was the *Bgl*II-*Nsi*I fragment of the human CT cDNA. The plasmid containing near full-length human CT cDNA was kindly provided by M.G. Rosenfeld (University of California San Diego, La Jolla, CA 92093). Component HL1-Blue cells (Stratagene, La Jolla, CA 92037) were transformed by the pUC 8 plasmid. Recombinant plasmids were selected on ampicillin plates. Plasmid DNA was extracted by the alcaline lysis method [5]. After enzymatic hydrolysis, the fragment *Bgl*II-*Nsi*I was electroeluted from the agarose gel, and labeled with [^{32}P]dCTP to a specific activity of 5×10^7 cpm/μg by Nick-translation (Nick-translation kit, Amersham, England).

A control probe was used to verify that equivalent amounts of RNAs were loaded. A 24-mer oligonucleotide specific of the rat 18S ribosomal RNA was 5′ end-labeled using T_4 polynucleotide kinase and (γ^{32}P)ATP and then purified on Nensorb columns (NEN). Specific activity of $4 \cdot 10^8$ cpm/μg were achieved routinely.

2.3. *RNA extractions and Northern blot hybridizations*

RNAs were extracted from batches of 5 thyroid glands with phenol-chloroform and purified with LiCl precipitation [6] according to previously published procedures [7,8]. Aliquots of 30 μg total RNAs were denatured by formaldehyde [9], and electrophoresed on 1% agarose gel overnight with 1× MOPS buffer (1× MOPS: 0.02 M morpholinopropane sulfonic acid, 0.005 M sodium acetate, 0.001 M EDTA, pH 7.0). RNAs were transferred to gene-screen membranes (NEN) using 0.025 M sodium phosphate buffer pH 6.5. Membranes were dried and then baked for 2 h at 80°C. For the CT probe membranes were prehybridized 4 h at 42°C in 50% formamide, 5× SSC, 5× Denhardt, 0.05 M sodium phosphate buffer pH 6.5, and 100 μg/ml of denaturated herring sperm DNA. Hybridization was carried out in a modified prehybridization buffer (i.e. 1× Denhardt, 0.02 M sodium phosphate buffer, and 10% dextran sulfate) containing 10 ng/ml of labeled human CT cDNA denatured by heating (100°C for 10 min). Membranes were washed by 2 changes of 2× SSC at room temperature for 5 min, and then 2 changes of 2× SSC, 1% SDS at 55°C for 30 min, and finally 2 changes of 0.1× SSC at room temperature for 30 min. For the ribosomal probe prehybridization was carried out at 42°C during 4 h in 5× SSC, 0.1% Denhardt, 0.05 M sodium phosphate buffer pH 6.5, 0.1% SDS, and 250 μg/ml salmon sperm DNA. Hybridization was done overnight in the same solution in the presence of 10^4 cpm of labeled oligonucleotide. Membranes were washed 20 min in 2× SSC at room temperature. Dried membranes were exposed at −80°C to Kodak Royal X-Omat AA5 films. Autoradiographs were quantified in an automatic densitometric scanner (Shimadzu Scientific Instruments).

2.4. *Other methods*

Blood samples were collected in polyethylene tubes cooled on ice water. After centrifugation at 4°C, aliquots of plasma were used immediately for calcium determination by flame photometry (Eppendorf, FCM 6341) and phosphorus analysis [10], the remainder being frozen (−35°C) until CT was assayed. The CT in plasma was measured by a RIA which has been described in detail [11]. The G 813 antibody (goat antiserum raised against synthetic human CT) was a gift of Dr H. Heath (Mayo Clinic and Mayo Foundation, Rochester, MN, USA). The detection limit of the assay was 40 pg/ml. All plasma samples were run in the same assay and intra-assay variations were 5%.

Results were expressed as means ± SE. Significance of differences between groups was determined using Student's-*t* test except for Northern results were all probabilities were calculated using the Mann–Whitney *U*-test for ranked non-parametric data.

3. RESULTS

According to previous data [12], DEX-treated rats exhibited a 10% weight loss consecutively to the known catabolic effect of glucocorticoids on muscles, and a small decrease in food intake. The body weight loss was more important with high than low concentrations of DEX (220 ± 2 g and 233 ± 3 g, respectively vs 244 ± 2 g in ADX controls on day 5 of treatment; $P < 0.001$). By contrast, no difference was noted between ADX and SO groups (244 ± 2 g vs 245 ± 2 g).

As shown in fig.1 and in agreement with previous studies [12,13], plasma Ca levels in ADX- and in DEX-treated rats were not different from values found in SO animals. Because a glucocorticoid excess inhibits the Ca fixation in bones [13], the same Ca load yielded an increment in plasma Ca stronger in ADX + DEX than in ADX rats (18.02 ± 0.25 vs 16.55 ± 0.38 mg/dl; $P < 0.001$). The plasma phosphorus concentrations were significantly lower in SO controls than in all other groups (fig.1).

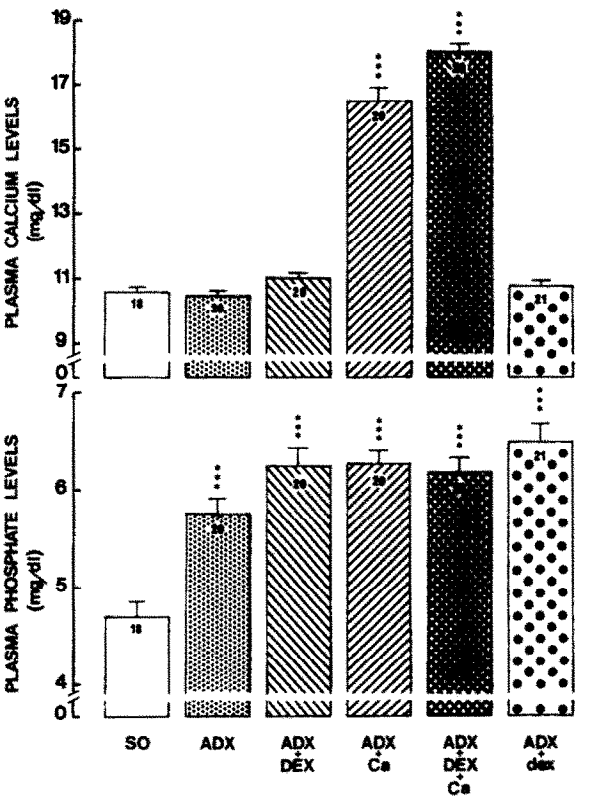

Fig.1. Effects of DEX and/or Ca treatments on plasma Ca and phosphate levels of ADX female rats. Mean ± SE and the number of estimations. ***$P < 0.001$ compared to SO controls. ADX + DEX: 1.5 mg DEX/kg b.wt/day for 5 days; ADX + dex: 15 μg DEX/kg b.wt/day during 5 days; + Ca: 35 mg Ca/kg b.wt 1 h before sacrifice.

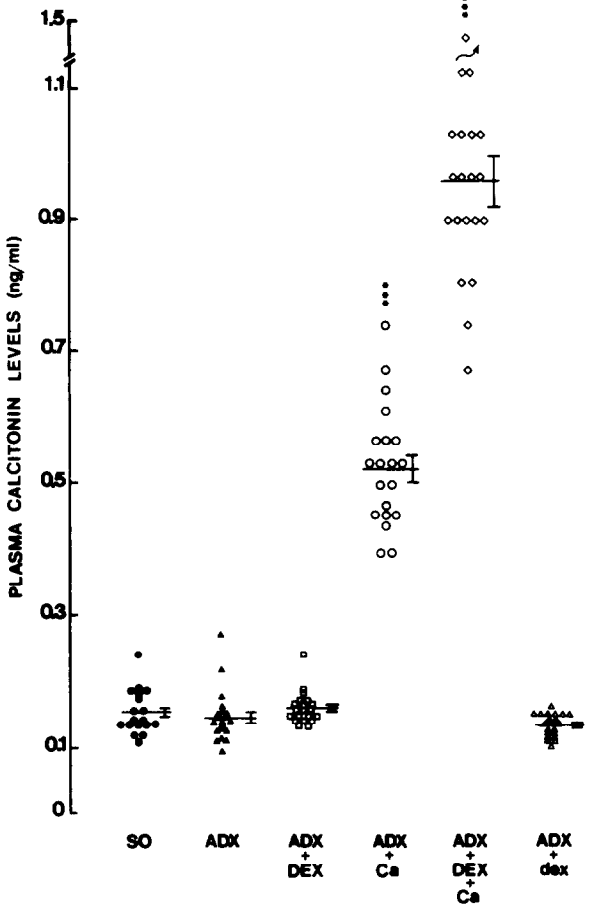

Fig.2 Effects of DEX and/or Ca treatments on plasma CT levels of ADX female rats Mean ± SE, individual values were plotted ***$P<0.001$ from SO controls For details, see legend of fig 1

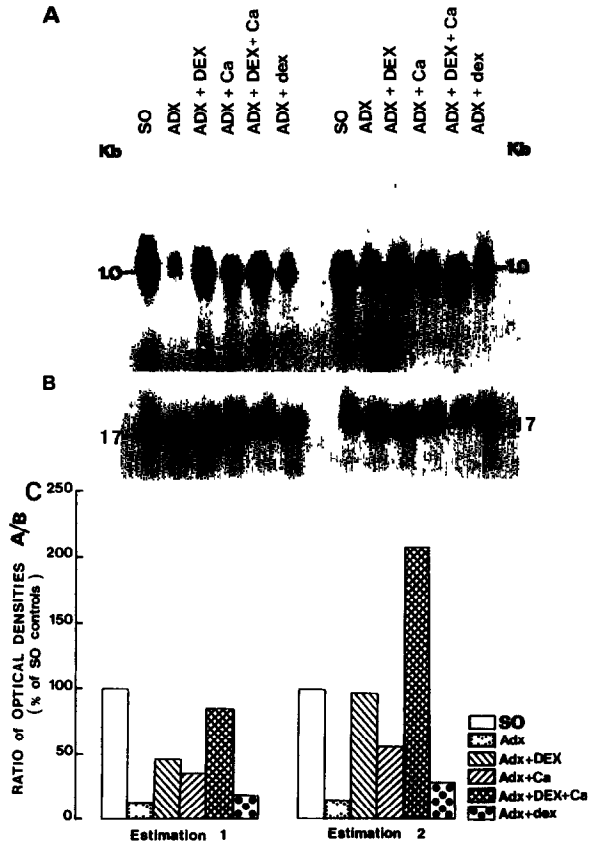

Fig 3 Effects of DEX and/or Ca treatments on CT mRNA levels of thyroid glands from ADX female rats Two typical Northern blots are shown; each Northern blot represents RNAs extracted from groups of 5 animals In each case 30 μg of total RNAs were loaded The values indicate the RNA size in kb relative to λ *Hin*dIII markers (A) Hybridization with the CT specific probe (B) Hybridization with a 18S rRNA probe to verify that equivalent amounts of RNAs were sampled (C) Bar graphs derived from densitometric scans of A/B ratios The results are given as percent of SO controls For details see legend of fig 1

No difference in plasma CT concentrations were found in SO, ADX-, and DEX-treated rats (fig.2). The intraperitoneal injection of Ca induced respectively a 3.5-fold and a 6.4-fold increase in plasma CT levels in ADX + Ca and ADX + DEX + Ca groups, respectively, as compared to SO controls (0.52 ± 0.02 and 0.96 ± 0.04 ng/ml vs 0.15 ± 0.01 ng/ml in SO; $P<0.001$). After DEX treatment, the Ca load induced a rise in plasma CT levels 1.8-fold higher than in untreated ADX rats.

In order to determine whether ADX, DEX and Ca induced changes in CT gene expression, levels of thyroidal mRNA encoding for CT were determined by Northern hybridization. After densitometric scanning of autoradiograms, the CT mRNA level was expressed as the ratio of hybridizing signals between the CT probe and the 18S rRNA control probe (fig.3). A marked drop in CT mRNA occurred 2 weeks after ADX (fig.3). More than a 3.6-fold decrease was observed in ADX females as compared to SO controls (28% vs 100% in SO; mean values of 3 independent estimations; $P<0.001$). Five days of exposure to a high DEX concentration yielded a 2.6-fold rise in thyroidal CT mRNA concentrations (73% vs 28% in ADX). In comparison, a 100-fold lower dose of DEX had no significant effect on CT mRNA levels. An acute Ca load induced a greater increment of the CT specific mRNA in DEX-treated ADX than in untreated ADX rats (100% and 49% respectively vs 28% in ADX controls).

4. DISCUSSION

These experiments were carried out to determine the effects of glucocorticoids on the thyroidal CT mRNA level of the rat. We have used DEX rather than corticosterone, the main natural glucocorticoid in rats, because this synthetic compound has an increased affinity for glucocorticoid receptors and a delayed plasma clearance which enhances tissue exposure. In addition, DEX has a negligible mineralocorticoid effect and thus does not produce sodium retention, hypokaliemia, and hypertension.

The dramatic fall in CT mRNA levels observed 2 weeks after ADX was partially compensated by the high DEX supply. This result might be explained in 2 ways. Firstly, the glucocorticoid induces a selective growth of C-cells so that the observed effect is strongly related to a shift in glandular cell population. The fact that DEX markedly inhibits the C-cell growth in vitro [3,4] argues against this hypothesis. Furthermore, a great modification in the number of C-cells might be associated with detectable changes in plasma CT levels, an effect which does not occur here. Secondly, glucocorticoids are required for the maintenance of CT mRNA to the high level which is known in the rat. Indeed, in this species, the CT-secreting cells are very active since the mRNA coding for CT accounts for 3.8% of total mRNAs from the thyroids although the C-cells constitute less than 10% of total glandular cells [14]. This last hypothesis, which is consistent with earlier in vitro studies [2,3], suggests a direct regulation of CT gene expression by endogenous glucocorticoid hormones since DEX does not alter the stability of CT mRNA in the TT cell line [3].

Independently of the DEX effect, an increment in the specific CT mRNA concentration occurs 1 h after Ca injection. This is the first time that a clear-cut effect of Ca on the hybridizable CT mRNA level is observed [15,16]. The additive influence of DEX and Ca suggests a control of CT gene expression by two different ways for these 2 stimuli. Further studies will be required to determine whether glucocorticoids and Ca increase the rate of transcription and/or have an effect on the half-life of the messenger transcript.

In vitro, DEX was reported to decrease [4] or to increase [17] the CT release by C-cell lines. In our experimental conditions, no variation in plasma CT levels was observed after DEX treatment. According to Muszynski et al. [17], the acute secretory response to Ca was enhanced in DEX-treated females. For the same Ca load, plasma CT levels were 1.8-fold higher in DEX than in untreated ADX rats. The hypercalcemia triggered by the Ca injection does not alone explain such a CT release. These findings suggest that DEX potentiates the Ca secretagogue effect. Thus, the regulation of CT mRNA by DEX leads to an increase in the amount of CT in the plasma only following a hypercalcemic stress. It is still unclear why, under basal conditions, there is no increase in plasma CT after DEX treatment. It is possible that the extremely potent secretagogue effect of Ca is necessary to reveal a concomitant increase in plasma CT and cellular CT mRNA.

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