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Glucocorticoids Modulate the Biosynthesis and Processing of proThyrotropin Releasing-Hormone (proTRH)

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The thyrotropin- (TRH) releasing hormone precursor (26 kDa) undergoes proteolytic cleavage at either of two sites, generating N-terminal 15 kDa/9.5 kDa or C-terminal 16.5/10 kDa intermediate forms that are processed further to yield five copies of TRH-Gly and seven non-TRH peptides. Glucocorticoids (Gcc) have been shown to enhance TRH gene expression in three different cell systems in vitro, an effect that occurs, at least in part, through transcriptional activation. Although this implies that an increase of TRH prohormone biosynthesis would take place, this had not been demonstrated as yet. We report here that the synthetic glucocorticoid dexamethasone (Dex) substantially elevated the *de novo* biosynthesis of the intact 26-kDa TRH prohormone and its intermediate products of processing in cultured anterior pituitary cells, an observation that is consistent with an overall upregulation of both the biosynthesis and degradation of the TRH precursor. We reasoned that Gcc may act not only at the transcriptional, but also at the translational/posttranslational level. To address this question we chose a different cell system, AtT_{20} cells transfected with a cDNA encoding preproTRH. Since TRH gene expression in these cells is driven by the CMV-IE promoter and not by an endogenous "physiological" promoter, these cells provide an ideal model to study selectively the effects of Gcc on the translation and posttranslational processing of proTRH without interference from a direct transcriptional activation of the TRH gene. Dex caused a significant 75.7% increase in newly synthesized 26-kDa TRH prohormone, suggesting that the glucocorticoid raised the translation rate. We then demonstrated that Dex treatment accelerated TRH precursor processing. Of interest, processing of the N- vs the C-terminal intermediate was influenced differentially by the glucocorticoid. Although the N-terminal intermediate product of processing accumulated, the C-terminal intermediate was degraded more rapidly. Consistent with these observations was the finding that the intracellular accumulation of the N-terminally derived peptide $preproTRH_{25\text{-}50}$ was enhanced, but levels of the C-terminally derived peptide $preproTRH_{208\text{-}255}$ were reduced. Accumulation of TRH itself, whose five copies are N- and C-terminally derived, was also enhanced.

We conclude that Gcc induce changes in the biosynthesis and processing of proTRH by increasing the translation rate and by differentially influencing the processing of N- vs C-terminal intermediates of the precursor molecule. These effects of Gcc at the translational and posttranslational levels result in an increase in TRH production accompanied by differential effects on the accumulation of N- and C-terminal non-TRH peptides.

Key Words: AtT_{20} cells; glucocorticoids; peptide biosynthesis; proTRH; TRH.

Introduction

Rat preproTRH is a 29-kDa polypeptide composed of 255 amino acids; it contains an N-terminal 25 amino acid leader sequence, five copies of the TRH progenitor sequence Gln-His-Pro-Gly flanked by paired basic amino acids (Lys or Arg), and seven non-TRH peptides *(1)*. The primary function of hypothalamic TRH (pGlu-His-ProNH_2) is to stimulate the secretion and biosynthesis of both thyroid-stimulating hormone (TSH), which in turn regulates thyroid function, and prolactin *(2–5)*. TRH is widely distributed within the central nervous system (CNS) where it acts as both a neurotransmitter and a neuromodulator *(6–8)*. Much of the knowledge about the posttranslational processing of proTRH has come from studies that were carried out in our laboratory *(9,10)*. Using a cell line (AtT_{20} cells) transfected

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with a cDNA-encoding preproTRH, we demonstrated that proTRH is present as a 26-kDa protein and that this precursor is cleaved at two mutually-exclusive sites to generate the first intermediate forms *(11,12)*.

Glucocorticoids (Gcc) evoke a broad spectrum of responses in many eukaryotic cells by stimulating or repressing the transcription of Gcc-regulated genes, including those of peptide hormones within the CNS or anterior pituitary *(13–21)*. The primary effect of Gcc on gene transcription can occur either by specific binding of the steroid receptor complex to DNA at the site of glucocorticoid response elements *(22)* or by interfering with the action of other transcription factors through protein–protein interactions *(22–24)*. In addition to direct effects on gene transcription, Gcc have been shown to elicit secondary modulatory effects at the posttranscriptional, translational, and posttranslational level *(25–30)*. For example, Gcc have been shown to stimulate the processing of the precursors of atrial natriuretic factor as well as neurotensin *(25,30)*. Gcc also regulate the posttranslational maturation, the intracellular trafficking, and the extracellular release of the mouse mammary tumor virus *(28,31–33)*.

We previously reported that Gcc stimulate TRH gene expression in primary cultures of diencephalic neurons as well as anterior pituitary (AP) cells, an effect that was found to be the result of transcriptional activation *(34,35)*. Similarly, Gcc induce TRH gene transcription in medullary carcinoma CA 77 tumor cells *(36)*. To characterize further the effect of Gcc on the biosynthesis and processing of the TRH precursor, we initially undertook radiolabeling experiments in anterior pituitary (AP) cells. To differentiate between transcriptional and posttranscriptional/posttranslational effects of Gcc, we employed another cell system, AtT_{20} cells transfected with a cDNA encoding preproTRH. TRH gene expression in this system is driven by the cytomegalovirus immediate-early promotor (CMV-IE) *(9)*. Thus, the effects of Gcc on the translation and posttranslational processing of proTRH can be investigated without interference from a direct transcriptional activation of the TRH gene owing to the absence of a physiological promoter.

Results

Effect of Dexamethasone (Dex) on proTRH Biosynthesis in AP Cells

Dex treatment (10^{-8} *M* for 72 h) caused a pronounced 10.7-fold rise in the intact, *de novo* synthesized 26-kDa TRH prohormone in AP cells that were radiolabelled for 4 h (Fig. 1). This was accompanied by an increase in radiolabeled 15-, 9.5-, and 6-kDa moieties, all of which are intermediate products of processing that are derived from the N-terminal portion of proTRH *(12,44)*. Anti-pCC_{10} recognizes the intact TRH precursor as well as these processing products (Table 1).

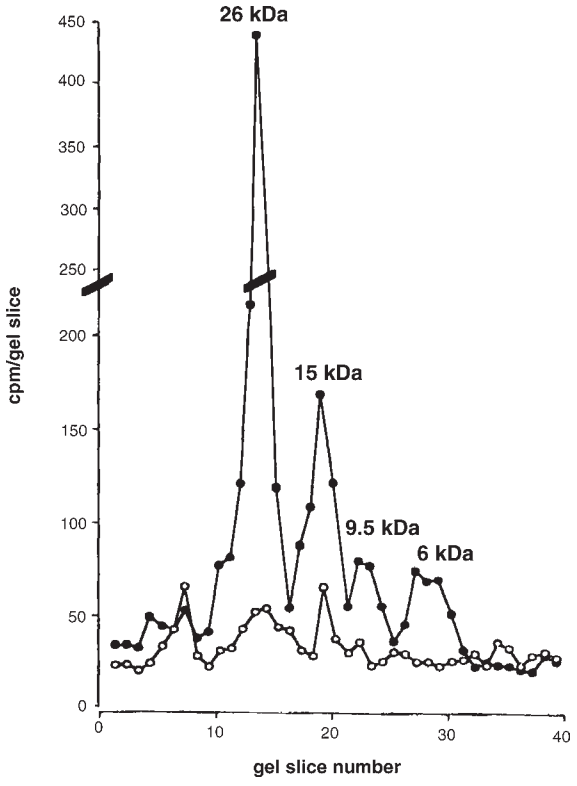

Fig. 1. Effect of Dex on proTRH biosynthesis in anterior pituitary cells. Cultured AP cells were treated with Dex (10^{-8} *M*) for 72 h and then radiolabeled for 4 h with ^{3}H-leucine. Anti-pCC_{10}, an antibody (*see* Table 1 for characteristics) that recognizes the intact TRH prohormone, the N-terminal 15-kDa intermediate, and its products of processing, was used for immunoprecipitation prior to electrophoretic separation on SDS-polyacrylamide gel. (●), dexamethasone (10 m*M*); (○), control.

Effect of Dex on proTRH Biosynthesis in AtT_{20} Cells Transfected with a preproTRH cDNA

Dex treatment (10^{-7} *M* for 72 h) caused a 75.7% increase ($p < 0.02$) in radiolabeled 26-kDa TRH prohormone in AtT_{20} cells that were radiolabeled for 10 min (Fig. 2). Since the preproTRH cDNA is under control of the cytomegalovirus immediate-early promotor in these cells, this increase in TRH biosynthesis is likely the result of a change in the translation rate of preproTRH mRNA. To confirm that Gcc did not affect preproTRH mRNA levels in this system, we performed Northern blot analysis of total RNA isolated from wild-type and transfected AtT_{20} cells in the presence or absence of Dex (10^{-7} *M* for 72 h) and found no significant difference in GAPDH-corrected preproTRH mRNA levels following Dex exposure (not shown).

Effect of Dex on proTRH Processing in AtT_{20} Cells Transfected with a preproTRH cDNA

To evaluate further whether Gcc affect the stoichiometric ratio of the N-terminal 15-kDa vs the C-terminal 16.5-kDa intermediate products of processing *(11,12)*, AtT_{20} cells were subjected to a 30-min pulse followed by a short

Table 1
Polyclonal Antibodies Used in This Study[a]

	Antibodies made against	Moieties recognized	
Anti-pCC_{10}	Synthetic decapeptide Cys-Lys-Arg-Gln-His-Pro-Gly-Lys-Arg-Cys	prepro-TRH_{25-255}	(26 kDa)
		prepro-TRH_{25-151}	(15 kDa)
		prepro-TRH_{25-112}	(9.5 kDa)
		prepro-TRH_{25-74}	(6 kDa)
Anti-pYE_{17}	prepro-$TRH_{240-255}$	prepro-TRH_{25-255}	(26 kDa)
		prepro-$TRH_{115-255}$	(16.5 kDa)
		prepro-$TRH_{160-255}$	(10 kDa)
		prepro-$TRH_{208-255}$	(5.4 kDa)
Anti-pAC_{12}	prepro-$TRH_{208-219}$	prepro-$TRH_{208-255}$	(5.4 kDa)
Anti-pYE_{27}	prepro-TRH_{25-50}	prepro-TRH_{25-50}	(4 kDa)

[a]Listed are the different epitopes of the proTRH sequence used to generate these antibodies and the peptides they recognize.

15-min chase, and equally divided samples obtained from these experiments were immunoprecipitated with anti-pCC_{10} and anti-pYE_{17}, respectively. Figure 3 depicts data from a representative experiment. After 30 min of labeling, the 26-kDa TRH precursor and its 15-, 9.5-, and 6-kDa intermediate peptides from the N-terminal region (pCC_{10}; *see* Table 1) were formed. Moieties corresponding to the 16.5- and 10-kDa intermediate products from the C-terminal region (pYE_{17}; *see* Table 1) were also detectable (Fig. 3A). Dex treatment increased the relative amount of radiolabeled 15- and 16.5-kDa intermediate products compared to the intact 26-kDa TRH prohormone, but did not affect the ratio of 15-/16.5-kDa proteins (Fig. 3B). After 15 min of chase, the 15-/16.5-kDa ratio (1.3) in untreated cells increased slightly (Fig. 3C), and in Dex-treated cells, this ratio (0.9) was slightly lower (Fig. 3D), suggesting that Dex did not significantly affect TRH precursor cleavage at the first two mutually exclusive cleavage sites that generate the 15- and 16.5-kDa forms.

Differential Effect of Dex on the Processing of N- and C-terminal Intermediate Forms of proTRH

To investigate the long-term effect of Gcc under steady-state labeling conditions, AtT_{20} cells were incubated with two concentrations of Dex (10^{-8} and 10^{-7} *M*) for 72 h and subsequently radiolabeled with ^{3}H-leucine for 7 h. Figure 4A depicts a typical electrophoretic profile of N-terminal-derived peptides that were immunoprecipitated with anti-pCC_{10}. Dex treatment induced a dose-dependent decrease in the intact 26-kDa TRH prohormone (1×10^{-8} *M*: 13% decrease; 1×10^{-7} *M*: 20% decrease) accompanied by a 60 and 130% increase, respectively of the 15-kDa intermediate form of processing. To evaluate further the processing of the 15-kDa moiety to its smaller forms, we monitored the intracellular accumulation of preproTRH_{25-50} (pYE_{27}), a non-TRH peptide derived from the full processing of this N-terminal intermediate *(10)*. Figure 4B shows that Dex increased the accumulation of radiolabeled

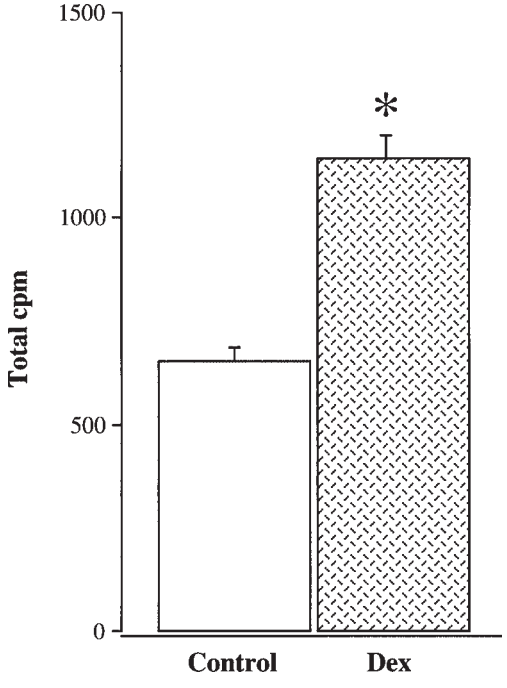

Fig. 2. Effect of Dex on proTRH biosynthesis in AtT_{20} cells transfected with a preproTRH cDNA. AtT_{20} cells that had been treated with 1×10^{-7} *M* Dex for 72 h were radiolabeled for 10 min with ^{3}H-leucine. The harvested material was immunoprecipitated with anti-pCC_{10} followed by electrophoretic separation on SDS-polyacrylamide gel. Data represent mean cpm ± SEM ($n = 3$ independent experiments) of the integrated peak area (26 kDa). *$p < 0.02$ compared to control.

pYE_{27} in a dose-dependent manner. A similar profile of accumulation was also observed for the N-terminal non-TRH peptide, preproTRH_{83-106} (data not shown).

We then monitored the intracellular processing of C-terminal intermediates. Electrophoretic fractionation of immunoprecipitated peptides shows that Dex caused a dose-dependent decrease in the intact 26-kDa TRH prohormone (Dex 1×10^{-8} *M*: 23%; 1×10^{-7} *M*: 33%) (Fig. 5A).

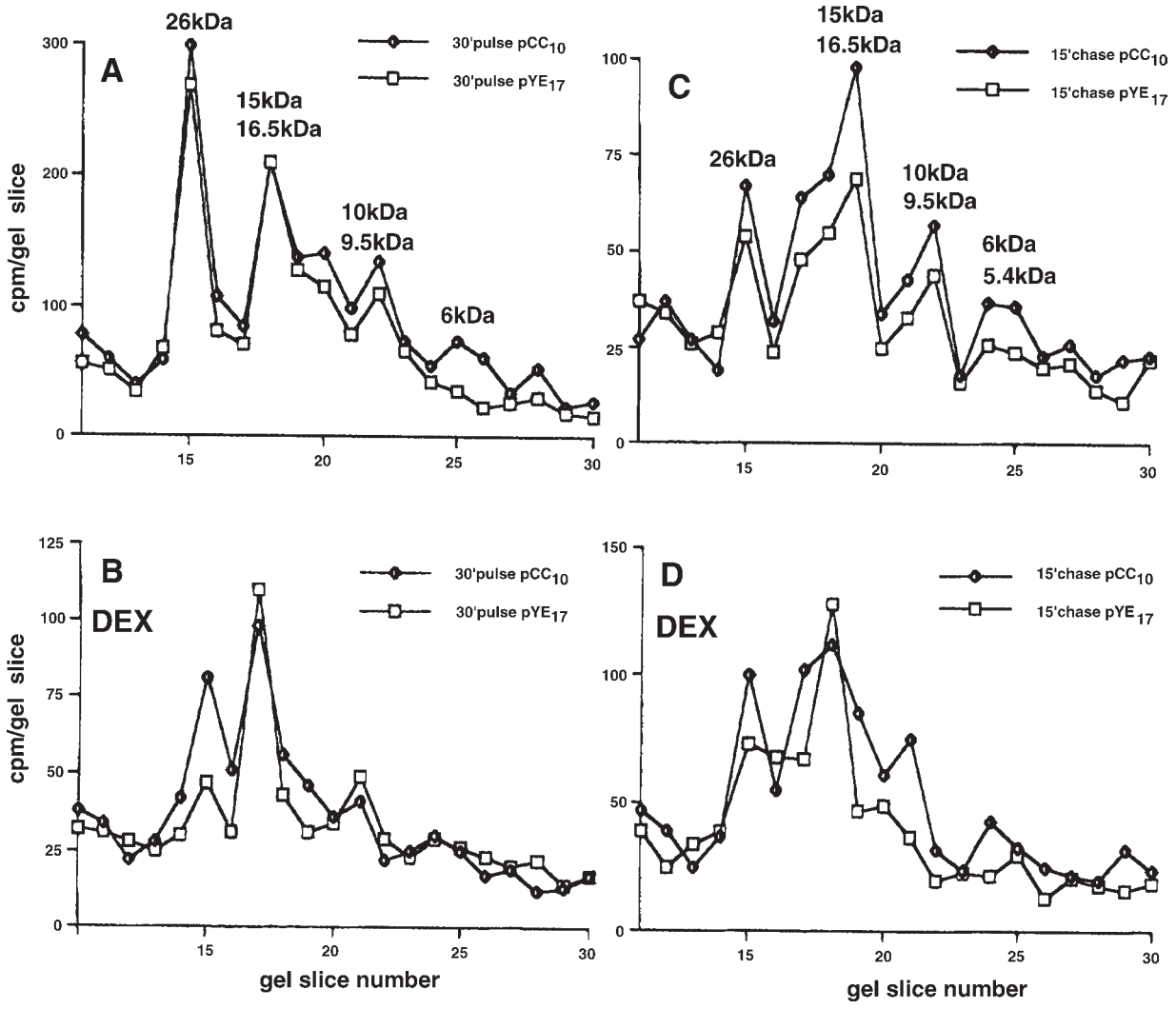

Fig. 3. Effect of Dex on proTRH processing in AtT_{20} cells transfected with a preproTRH cDNA. AtT_{20} cells were treated with Dex ($1 \times 10^{-7} M$, 72 h) and either labeled with ^{3}H-leucine for 30 min **(A,B)** or pulsed for 30 min followed by a 15-min chase with regular culture media containing 0.5 mg/mL of cold leucine **(C,D)**. The harvested material was divided and immunoprecipitated with anti-pCC_{10} or anti-pYE_{17} (*see* Table 1 for characteristics) followed by electrophoretic separation on SDS-polyacrylamide gel. A representative experiment is depicted.

Furthermore, a dose-dependent decrease in the 16.5-kDa C-terminal intermediate form (Dex $1 \times 10^{-8} M$: 22.2%; $1 \times 10^{-7} M$: 35.7%) was observed. This suggests that cleavage of both the prohormone and the 16.5-kDa form was simultaneously accelerated. When we monitored the formation of the 5.4-kDa ($preproTRH_{208\text{-}255}$) peptide, a peptide derived from the processing of the N-terminal 16.5-kDa intermediate, we found that Dex dramatically increased the degradation of this peptide such that it already disappeared at a dose of $1 \times 10^{-8} M$ (Fig. 5B).

Differential Effect of Dex on the Accumulation and Release of proTRH-Derived Peptides

To investigate how differential effects of Dex on the processing of N- and C-terminal intermediates affect the final products of processing in AtT_{20} cells, we chose to determine the intracellular accumulation and basal release of the N-terminal peptide $preproTRH_{25\text{-}50}$, the C-terminal peptide $preproTRH_{208\text{-}255}$, and TRH itself whose five copies are derived from both N- and C-terminal intermediates. Increasing concentrations of Dex caused an intracellular accumulation of $preproTRH_{25\text{-}50}$ and TRH ($p < 0.05$ for Dex $10^{-7} M$ compared to control). Although high doses of Dex reduced the basal secretion of $preproTRH_{25\text{-}50}$, TRH release remained unchanged (Fig. 6A,C). In contrast, Dex ($10^{-7} M$) caused a marked reduction in the cellular content of $preproTRH_{208\text{-}255}$ ($p < 0.05$) that was accompanied by a reduced secretion of this C-terminal-derived peptide (Fig. 6B).

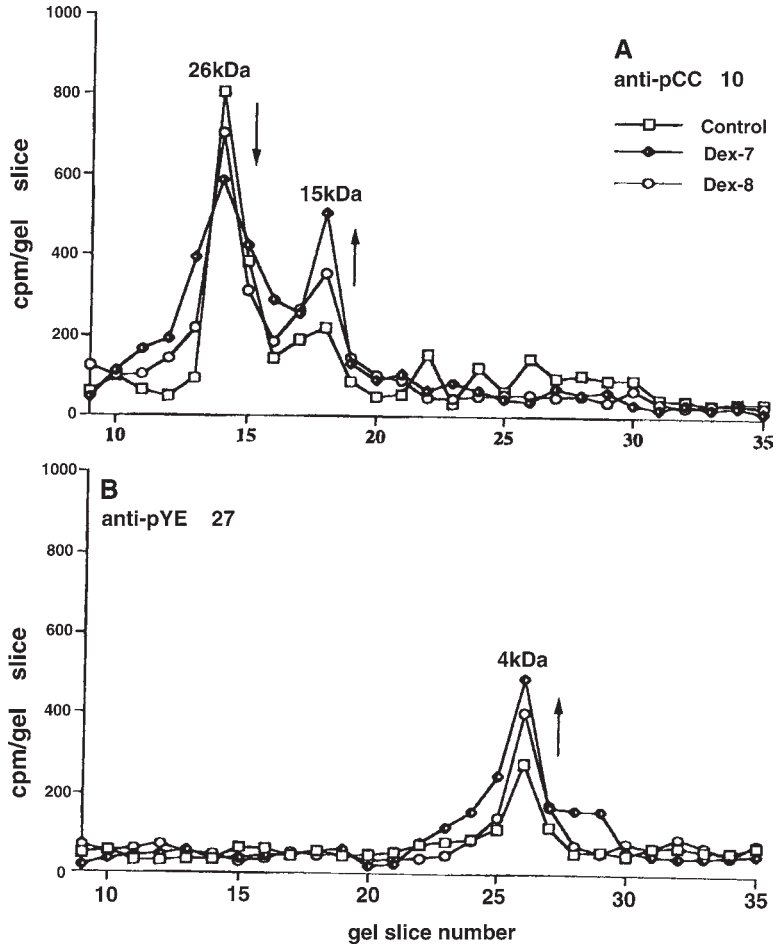

Fig. 4. Effect of Dex on the processing of proTRH and its N-terminal intermediate in AtT_{20} cells transfected with a preproTRH cDNA. AtT_{20} cells were treated with Dex (1×10^{-8} or 1×10^{-7} *M*) for 72 h and radiolabeled for 7 h with ^{3}H-leucine. The harvested material was immunoprecipitated with anti-pCC_{10} **(A)** or anti-pYE_{27} **(B)** (*see* Table 1 for characteristics) followed by electrophoretic separation on SDS-polyacrylamide gel. A representative experiment is depicted.

Discussion

In this study, we provide evidence for the first time that Gcc regulate the biosynthesis and processing of the TRH prohormone at the translational/posttranslational level. This, in addition to the already documented effect of Gcc on TRH gene transcription *(34–36)*, adds a new level of complexity to how Gcc influence TRH expression. In all in vitro systems tested so far, including hypothalamic neurons, anterior pituitary cells or a thyroidal C-cell line, Gcc stimulate TRH gene expression regardless of the cell type or whether the cells were in primary culture or a tumor cell line *(34–36)*. In vivo, more diverse, tissue-specific effects of Gcc on TRH gene expression have been reported. Neurons outside the hypothalamic paraventricular nucleus (PVN) do not appear to be affected by changes in adrenal status, whereas Gcc suppress TRH mRNA levels in the PVN *(45)*. The action of Gcc on paraventricular TRH neurons likely involves other brain areas, including hippocampus and amygdala, that have been found to convey the negative feedback action of Gcc on corticotropin-releasing hormone expressing neurons in the PVN *(20)*. Thus, the direct positive regulation by Gcc may be overridden by an indirect negative regulation causing a reduction of gene expression in paraventricular TRH neurons, whereas positive and negative regulation may amount to no change of gene expression in TRH neurons outside the PVN *(45)*.

A prerequisite for a direct effect of Gcc on TRH gene expression in isolated cells is the expression of Gcc receptors as well as the presence of glucocorticoid-responsive element(s) in the promoter region of the TRH gene, both of which have been documented *(46–48)*. Having previously reported that Gcc increase the expression of TRH mRNA in cultured AP cells, we wanted to establish that the synthetic glucocorticoid dexamethasone indeed stimulates the *de novo* biosynthesis of the TRH precursor in this system. We report here that Dex substantially elevated the biosynthesis of the intact 26-kDa TRH prohormone and its intermediate products of processing consistent with an overall upregulation of both the biosynthesis and degradation of the TRH pre-

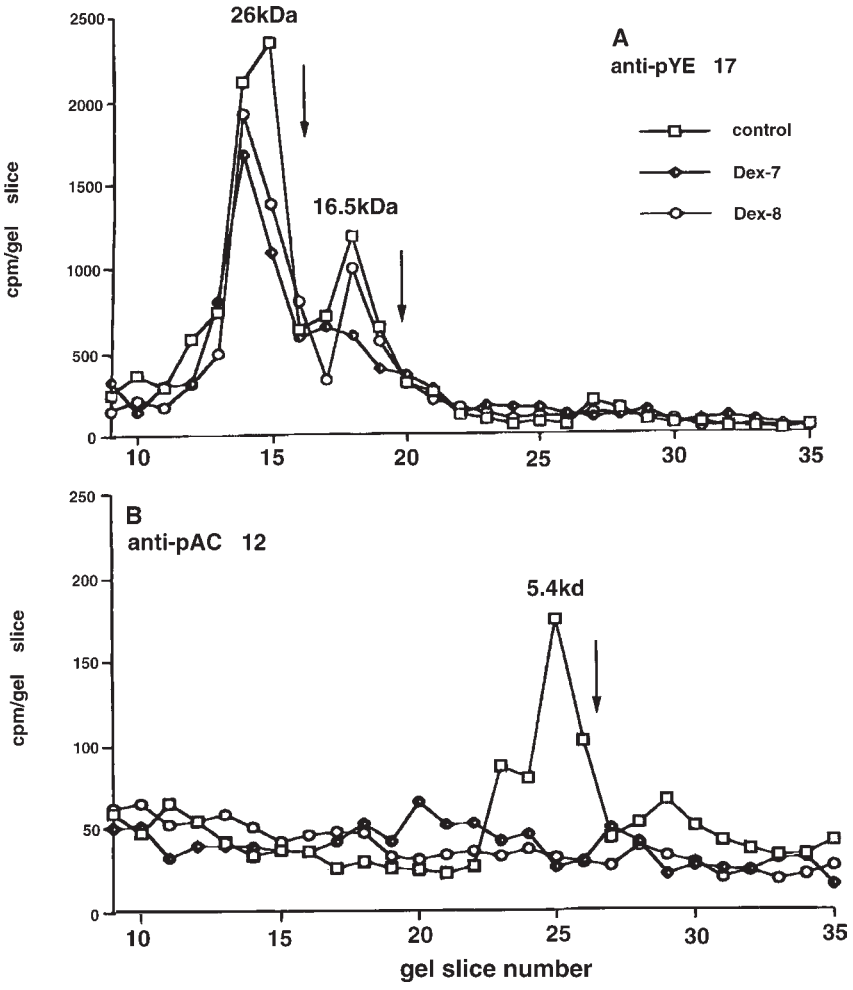

Fig. 5. Effect of Dex on the processing of proTRH and its C-terminal intermediate in AtT_{20} cells transfected with a preproTRH cDNA. AtT_{20} cells were treated with Dex (1×10^{-8} or 1×10^{-7} *M*) for 72 h and radiolabeled for 7 h with ^{3}H-leucine. The harvested material was immunoprecipitated with anti-pYE_{17} **(A)** or anti-pAC_{12} **(B)** (*see* Table 1 for characteristics) followed by electrophoretic separation on SDS-polyacrylamide gel. A representative experiment is depicted.

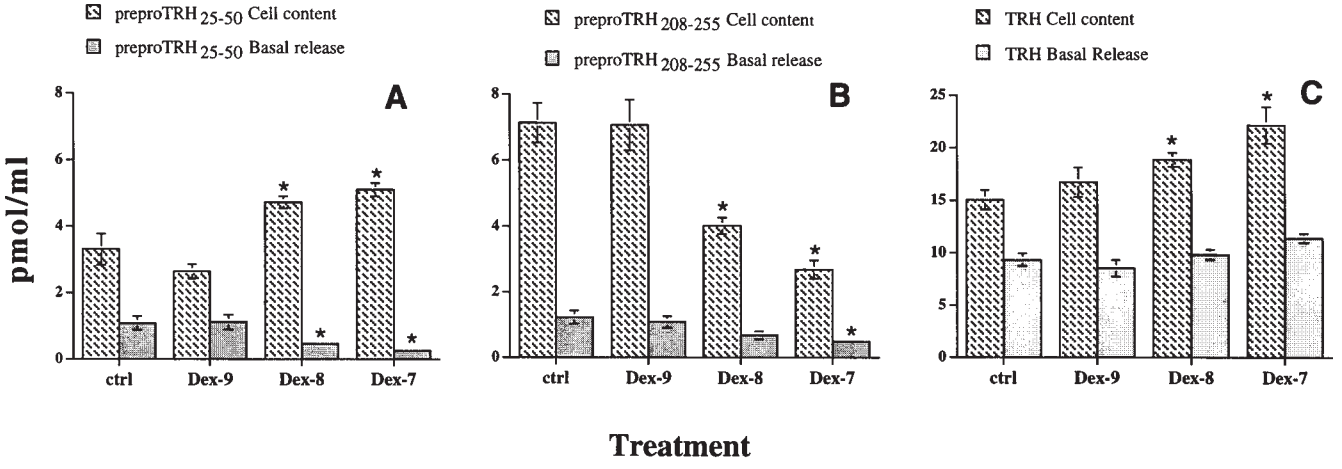

Fig. 6. Effect of Dex on the accumulation and release of proTRH-derived peptides in AtT_{20} cells transfected with a preproTRH cDNA. AtT_{20} cells were cultured in six-well plates; cellular extracts and release media (2 h basal release) were processed further for the determination by RIA of the intracellular accumulation and basal release of the N-terminal peptide $preproTRH_{25–50}$ **(A)**, the C-terminal peptide $preproTRH_{208–255}$ **(B)**, and TRH itself **(C)** whose five copies are derived from both N- and C-terminal intermediates. A representative experiment ($n = 6$ wells for each group) is depicted. Data are presented as mean values ± SEM. $^{*}p < 0.05$ compared to control.

cursor. This observation raised the question regarding whether Gcc stimulate TRH biosynthesis not only by elevating TRH gene transcription, but also by affecting translational and posttranslational mechanisms. To address this question, we chose a different cell system, AtT_{20} cells transfected with a cDNA encoding preproTRH. Since TRH gene expression in these cells is driven by the CMV-IE promoter and not by an endogenous "physiological" promoter, this system would allow investigating the effects of Gcc on the translation and processing of the TRH prohormone without interference from transcriptional events *(9)*.

Using a short labeling pulse of 10 min we noted that Dex treatment significantly increased the *de novo* biosynthesis of intact, 26-kDa TRH prohormone in transfected AtT_{20} cells, suggesting that the Gcc treatment raised the translation rate. A longer-term labeling (30 min) revealed that Dex treatment raised the relative amount of the 15-kDa N-terminal as well as the 16.5-kDa C-terminal intermediate product of processing, suggesting that changes in the activity of enzymes, including PC1 and PC2, that have been implicated in TRH precursor processing might have occurred *(42,44)*. Alternatively, an increase in precursor biosynthesis following Dex treatment could simply provide more substrate for cleavage enzymes and, thus, result in an increase in the levels of intermediates without an enhancement in posttranslational processing. To address this issue more appropriately, we chose long-term labeling conditions (7 h) to reach steady-state labeling conditions. These experiments revealed that Gcc differentially regulate TRH precursor processing. Increasing doses of Dex caused a reduction of intact TRH prohormone labeling accompanied by an accumulation of the N-terminal 15-kDa intermediate whose final products of processing were also markedly increased as assessed by determining the formation of $preproTRH_{25\text{-}50}$. These data suggest that Dex accelerated cleavage of the 26-kDa precursor at the site responsible for the formation of the 15-kDa intermediate. In contrast, Dex reduced the formation of the C-terminal 16.5-kDa intermediate, suggesting accelerated processing of this intermediate. The rapid degradation of the C-terminal intermediate resulted in a dramatic reduction of labeled final products of processing as determined by measuring $preproTRH_{208\text{-}255}$. These data suggest that Dex not only accelerated the cleavage of the intact TRH prohormone at the site that has been implicated for the formation of the 16.5-kDa intermediate, but also caused rapid degradation of this intermediate. Steady-state measurements of the cellular content of the N-terminal proTRH-derived peptide $preproTRH_{25\text{-}50}$ and the C-terminal peptide $preproTRH_{208\text{-}255}$ corroborated these findings. Of interest, the overall result of the differential effects of Dex on TRH prohormone processing was that the glucocorticoid caused an increase in the intracellular accumulation of TRH itself. Complete processing of the TRH precursor results in five copies of TRH, two of which are derived from the N-terminal intermediate and three of which come from the C-terminal intermediate.

How could Dex differentially affect the processing of the N- vs the C-terminal intermediate? We suggest that glucocorticoid-induced changes in the expression of processing enzymes as well as alterations in the morphology of AtT_{20} cells may bring about the differential effects of Dex on proTRH processing.

The first evidence of the ability of PC1 to cleave proTRH to its predicted products came from studies with partially purified recombinant PC1 derived from transfected fibroblast cells *(42,49)*. Recently, we were able to demonstrate that TRH is produced from proTRH by a number of proconverting enzymes using a vaccinia virus system to coinfect proTRH mRNA with different prohormone convertase mRNAs *(12)*. Multiple coinfection experiments suggest that PC1 is primarily responsible for all cleavage events, and that the secondary role of PC2 may be related to the final removal of TRH from TRH-extended forms *(12)*. We proposed that the 87-kDa form of PC1 present in the Golgi complex may be responsible for the first cleavage of proTRH *(42,50)*. Thus, the results presented here suggest that Dex may directly or indirectly stimulate expression of the 87-kDa form of the prohormone convertase PC1, which is responsible for the cleavage of proTRH at the basic amino acid pairs Lys-$Arg^{152\text{-}153}$ or Arg-$Arg^{158\text{-}159}$ and Lys-$Arg^{107\text{-}108}$ or Arg-$Arg^{113\text{-}114}$, resulting in the formation of the N-terminal 15-kDa and C-terminal 16.5-kDa intermediate, respectively, from an accelerated degradation of the 26-kDa TRH prohormone. In support of this hypothesis, it has been previously shown that Gcc affect PC1 gene expression in AtT_{20} cells *(51,52)*. In view of the accumulation of the 15-kDa intermediate, it appears that the activity of PC1 and possibly PC2, whose expression is generally low in AtT_{20} cells and that is responsible for cleaving the 15-kDa moiety into the 6- and 3.8-kDa fragments, is either reduced or not as enhanced by Dex compared to the activity that causes the cleavage of the 26-kDa to produce the 15-kDa peptide. It is possible that Dex reduces the maturation of smaller forms of PC1, but not of the 87-kDa form since the initial cleavage of proTRH was accelerated. Although our data on the overall importance of PC1 for TRH prohormone processing are conclusive, we can not exclude the possibility that other enzymes whose expression is induced by Gcc may play a role in the processing of proTRH intermediates and, thus, may contribute to the differential effects of Dex on the their degradation.

Changes in proTRH processing following Dex treatment may also occur as a result of morphological alterations within several organelles of AtT_{20} cells. We have observed an enlargement of the Golgi apparatus during glucocorticoid treatment *(53)*. Although speculative, these changes may slow down the normal transport of the 15-kDa intermediate peptide from the *trans*-Golgi net-

work to immature secretory granules where further processing takes place and, thus, contribute to the accumulation of this intermediate *(11)*.

In conclusion, we have demonstrated that Gcc induce changes in the biosynthesis and processing of proTRH by affecting the translation rate and by differentially influencing the processing of N- vs C-terminal intermediates of the precursor molecule. These effects of Gcc at the translational and posttranslational level result in an increase in TRH production accompanied by differential effects on the accumulation of N- and C-terminal non-TRH peptides.

Materials and Methods

Tissue Culture

AP cells were cultured as previously described *(37,38)*. Briefly, AP tissue was separated from posterior/intermediate lobes, collected into sterile Hank's balanced salt solution, enzymatically dispersed with neutral protease (1.5 U/AP) (Sigma, St. Louis, MO), and plated in a monolayer on 35-mm wells at a density of 1000 cells/mm^2. The cells were cultured for up to 10 d in a modified L-15/DMEM (Gibco-BRL, Grand Island, NY) medium containing 10% fetal calf serum (Gibco-BRL, Grand Island, NY) *(37,38)*.

AtT_{20} cells transfected with a cDNA encoding preproTRH were grown in 75-cm^2 flasks at 37°C in an atmosphere of 5% CO_2, 95% air, and 90% humidity. Each flask was plated with 2 million cells and cultures were maintained for 7 d in a Dulbecco's Modified Eagle's Essential Medium (DMEM) (Gibco-BRL, Gaithersburg, MD) containing 10% fetal calf serum (FCS) as previously described *(39)*. Culture medium was replaced every 2 d with fresh medium. Experiments were performed at confluency using flasks containing between 25 and 30 × 10^6 cells (~80% confluency) with a total protein content of 10.5 ± 0.3 mg (n = 6). The protein content (Bio-Rad Protein Assay Bio-Rad Laboratories, Hercules, CA) of each flask was determined to correct for small differences in cell number between flasks.

RNA Extraction and Northern Blot Analysis

Total RNA was isolated from cultured cells at 80% confluence by acid guanidinium thiocyanate-phenol-chloroform extraction *(40)*. Equal amounts of total RNA (5 μg) were separated by electrophoresis in 1.1% agarose/formaldehyde gel, blotted onto a nitrocellulose filter, and hybridized for 18 h at 42°C with either a random primed ^{32}P-labeled (10^9 cpm/μg) preproTRH cDNA or GAPDH cDNA probe *(41)*. Filters were washed twice for 20 min at room temperature in 2X standard saline citrate/0.1% SDS followed by two 30-min washes in 0.1X standard saline citrate/0.1% SDS at 55°C, and then exposed to Kodak XAR-5 film. Filters were stripped twice for 3 min in 1% glycerol at 80°C between hybridizations.

Glucocorticoid Treatment

AP cells were cultured for 4 d under standard conditions; then Dex was added to the medium at a dose of 10^{-8} *M* for 72 h. This dose had been previously shown to increase TRH gene expression approx 10-fold *(35)*.

After 2 d in culture, AtT_{20} cells were treated with graded doses of Dex ranging from 10^{-10} to 10^{-7} *M* for 72 h. Within the various Dex concentrations tested, we found that 10^{-7} *M* Dex was the most effective Dex concentration in producing changes in proTRH processing, as judged by radiolabeling protocols followed by SDS-PAGE analysis (not shown) and RIA *(see below)*. Analysis of the time-dependent (12, 24, 48, 72, and 96 h) effect of Dex revealed that changes in proTRH processing were more pronounced after 72 h of exposure to Gcc (not shown). Therefore we chose to expose cells to 10^{-7} *M* Dex for 72 h.

Antibodies and RIAs

The following antibodies were utilized in these studies (Table 1):

1. Anti-pCC_{10}, which recognizes $preproTRH_{25\text{-}255}$ (26 kDa), $preproTRH_{25\text{-}151}$ (15 kDa), $preproTRH_{25\text{-}112}$ (9.5 kDa), and $preproTRH_{25\text{-}74}$ (6 kDa);
2. The C-terminal antibody, anti-pYE_{17}, which recognizes $preproTRH_{25\text{-}255}$ (26 kDa), $preproTRH_{115\text{-}255}$ (16.5 kDa), $preproTRH_{160\text{-}255}$ (10 kDa), and $preproTRH_{208\text{-}255}$ (5.4 kDa);
3. Anti-pAC_{12}, which recognizes $preproTRH_{208\text{-}255}$ (5.4 kDa); and
4. Anti-pYE_{27}, which recognizes $preproTRH_{25\text{-}50}$ (4 kDa).

The RIAs for TRH, pCC_{10}, pYE_{27} ($preproTRH_{25\text{-}50}$), pYE_{17} ($preproTRH_{240\text{-}255}$), and pAC_{12} ($preproTRH_{208\text{-}219}$) have been described in detail elsewhere *(11,42)*.

Radiolabeling Experiments

Following the Dex treatment, AP cells were incubated with leucine-free DMEM containing 2.5% dialyzed FCS for 30 min and then labeled with 200 μCi of (3,4,5, ^{3}H)-leucine (156 Ci/mmol) for 4 h prior to harvesting.

Experiments were conducted at 80% confluency (30 × 10^6 cells) on d 5 of culture; before radiolabeling, cells were incubated for 30 min with 6 mL of leucine-free DMEM containing 2.5% dialyzed FCS. Then cells were pulsed with 200 μCi of (3,4,5, ^{3}H)-leucine (156 Ci/mmol) for 7 h prior to harvesting, pulsed for 30 min and chased for 15 min prior to harvesting, or pulsed for 10 min with 400 μCi of (3,4,5, ^{3}H)-leucine (156 Ci/mmol), and chased for 30, 60 and 90 min prior to harvesting. Following incubation, the media were removed and radiolabeled peptides were extracted as previously described *(10)*.

Immunoprecipitation

An immunoprecipitation protocol was carried out as described previously *(10)*. Briefly, lyophilized cell extracts were resuspended in 10 μL of 0.2% bovine serum albumin (BSA) and 200 μL of hypotonic buffer A (10 m*M* $NaPO_4$,

pH 7.2/1 m*M* EDTA/0.1% Triton X-100). Following resuspension, cell extracts were incubated for 24 h at 4°C with 20 µL of protein G-purified antibodies *(46)* directed against specific cryptic peptides. Twenty-five microliters of goat-antirabbit IgG were then added along with 75 µL of buffer B (500 m*M* KCl/50 m*M* NaH_2PO_4, pH 7.4/5 m*M* NaEDTA/0.25% Triton X-100). Samples were further incubated for 4 h at 4°C. Immunoprecipitates of cell extracts were washed once with buffer B and once with buffer C (10 m*M* NaH_2PO_4, pH 7.2/15 m*M* NaCl), which removes EDTA and Triton X-100. The immunoprecipitates were then resuspended in sample buffer (0.0625 *M* Tris, pH 6.8/1% SDS/15% glycerol/15 m*M* dithiothreitol), and boiled for 4 min prior to SDS-PAGE. Immunoprecipitation using nonimmune serum and immune serum directed against pCC_{10} and pYE_{17} in the presence of an excess of synthetic pCC_{10} and pYE_{17} peptides did not result in visible peaks (not shown).

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Equal amount of total proteins from untreated cells and those that had been treated with Dex were loaded onto a discontinuous tricine-polyacrylamide gel electrophoresis system *(43)*. A stacking gel was made to 3% crosslinking (acrylamide/*bis*-solution), and the separating gel was made to 6% crosslinking (acrylamide/*bis*-solution). Gels were run in the Protean 16-cm cell system (Bio-Rad). Following electrophoresis, gels were cut into 1-mm slices in a gel slicer (Hoefer Scientific Instruments, San Francisco, CA), and prepared for either counting or radioimmunoassay. For tritium analysis, immunoprecipitated peptides were extracted from gel slices by incubation in 1 mL of 1 *N* acetic acid for 24 h at 4°C. Scintillation fluid (Bio Safe II, RPI, IL) was added, and samples were counted in a scintillation counter. Preparation for RIA included the same acetic acid extraction as described above, but following incubation, gel slices were removed. Samples were then lyophilized and resuspended in the appropriate RIA buffer. Recovery of peptides from gel slices has been shown to be approx 90% as determined by RIA prior to and following the electrophoresis. For SDS-PAGE, the following mol-wt markers were used: prestained bovine serum albumin (BSA), 80.0 kd; ovalbumin, 49.5 kd; carbonic anhydrase, 32.5 kd; soybean trypsin inhibitor, 27.5 kd; lysozyme, 18.5 kd (Bio-Rad); trypsin inhibitor, 20.4 kd; myoglobin, 16.95 kd; myoglobin fragment IV, 14.4 kd; myoglobin fragment III, 8.16 kd; myoglobin fragment II, 6.2 kd; myoglobin fragment I, 2.5 kd (Diversified Biotech, Newton, MA).

Analysis of Content and Release of TRH-Derived Peptides

Content and basal release of TRH and selected cryptic peptides were monitored by incubating cells, grown on 35-mm 6-well plates, for 2 h in release media (MEM containing 0.003% bacitracin and 0.1% BSA). Total peptide content (cellular content) as well as levels of TRH and proTRH-derived peptides in the release media were evaluated by RIAs specifically recognizing different sequences of the TRH prohormone. Six wells were used for each experimental condition. Following incubation, the media were removed, boiled in acetic acid, and lyophilized.

Statistics

Graphs were generated by plotting cpm or RIA values against the gel slice number, which corresponded to a particular mol-wt peptide. Protein assay results were used to correct for minor variations in total cell number. Data were displayed as pmol/mg of protein or cpm/gel slice. Analysis of variance (ANOVA) followed by a multiple comparison (Tukey-Kramer test) was employed when appropriate.

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