Expression and processing of procholecystokinin in a rat medullary thyroid carcinoma cell line

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A rat medullary thyroid carcinoma cell line, CA-77, was shown to express the cholecystokinin (CCK) gene. Measurements using a library of sequence-specific radioimmunoassays before and after enzymic treatment of extracts and chromatographic fractions showed that the cells contained 1.0 pmol of α -carboxyamidated cholecystokinins/10⁶ cells, 0.4 pmol of glycine-extended intermediates/10⁶ cells and 1.0 pmol of further C-terminal-extended pro-CCK/10⁶ cells. Gel chromatography and reverse-phase h.p.l.c. revealed both sulphated and nonsulphated CCK-8 in the cells. The growth medium contained in addition α -amidated CCK-33, glycine-extended CCK-8 and pro-CCK. Exposure to 0.1 μ M-dexamethasone for 6 days increased the cellular content and secretion of all of the described CCK peptides by 2–3-fold. The increase was first noted after 3 days of treatment. Monensin inhibited the synthesis of α -carboxyamidated CCK and the secretion of all of the CCK forms measured. Colchicine at a low concentration (0.2 μ mol/l) apparently increased the synthesis and secretion of α -carboxyamidated CCK, whereas higher concentrations inhibited CCK synthesis. Finally, chloroquine inhibited the α -carboxyamidation of CCK. We conclude that the CA-77 cell line is a useful tool for studies of the expression and post-translational processing of pro-CCK.

INTRODUCTION

Cholecystokinin (CCK) is one of the classical gut hormones. It stimulates pancreatic exocrine secretion and gall bladder contraction (Mutt & Jorpes, 1971). CCK is also synthesized in cerebral neurons, where it acts as a neurotransmitter (for review, see Vanderhaeghen & Crawley, 1985).

The CCK gene encodes a 114 (pig) or 115 (man and rat) amino acid precursor (Deschenes *et al.*, 1984; Gubler *et al.*, 1984; Takahashi *et al.*, 1985), which is processed by proteolysis, amidation and sulphation (Rehfeld *et al.*, 1988). The active site at the C-terminus, Trp-Met-Asp-Phe-NH₂, is identical with that of gastrin. Several active (i.e. α -amidated) forms of CCK have been described, i.e. CCK-5, CCK-8, CCK-33, CCK-39 and CCK-58 (Rehfeld & Hansen, 1986). Of these, CCK-8 is predominant in the brain, whereas the larger forms are more abundant in the gut (Dockray, 1978; Rehfeld, 1978b).

Expression of CCK has been detected in medullary carcinomas of the thyroid gland from rats (Deschenes *et al.*, 1984), and also from man (Rehfeld *et al.*, 1990). In addition, trace amounts have been found in normal thyroid tissue from dogs (Laurberg & Rehfeld, 1987). In view of the elaborate biogenesis of CCK peptides, cell lines producing CCK would be a useful tool in further studies of CCK biosynthesis. The rat medullary thyroid carcinoma cell line, CA-77, seems to comply with this requirement. In the present study we have characterized and modulated the synthesis of CCK peptides in the CA-77 cell line.

MATERIALS AND METHODS

Cell culture

CA-77 cells derived from a rat medullary thyroid carcinoma (Muszynski *et al.*, 1983) were obtained through T. W. Schwartz, State University Hospital, Copenhagen, Denmark. The cells were grown in nutrient mixture F-10 (Ham) containing Dulbecco's modified Eagle medium 1885 (1:1, v/v) with 4.8 g of glucose/litre, 5 μ g of transferrin/ml, 5 μ g of insulin/ml and 5 ng of selenous acid/ml (Muszynski *et al.*, 1983) Penicillin

(100 units/ml), streptomycin (100 g/ml) and 2 mM-L-glutamine were added to the culture medium. Cells were grown in 4 ml of growth medium in Nunclon culture flasks (50 ml), and maintained in a humidified incubator at 37 °C in air/CO₂ (9:1).

Extraction of cells

After removal of the medium, each culture flask was rinsed twice with phosphate-buffered saline (PBS; 15 mM-NaH₂PO₄/140 mM-NaCl, pH 7.4) before scraping the attached cells (approx. 3 × 10⁶ cells) into PBS. The cells were counted and centrifuged before extraction. The cell pellet was boiled for 3 min in 2 ml of 3 M-acetic acid, followed by centrifugation at 10000 g for 15 min. The supernatant was collected and adjusted to pH 8 by NaOH. Cell extracts and growth media were frozen at –18 °C until use.

Radioimmunoassays

The following antisera were used.

(1) Ab 2609, which is directed against the common α -amidated active site of gastrin and CCK, i.e. -Trp-Met-Asp-Phe-NH₂. It measures all biologically active forms of CCK (i.e. α -carboxyamidated cholecystokinins) and gastrin, irrespective of their degree of tyrosine O-sulphation (Rehfeld, 1978a).

(2) Ab 2604, which is directed against the C-terminal part of amidated bioactive gastrins. The cross-reactivity with CCK peptides is negligible (Rehfeld *et al.*, 1972).

(3) Ab G160, which is specific for CCK. It is directed against amino acids 25–30 of CCK-33 and requires O-sulphation of the tyrosine residue. Hence it measures amidated sulphated CCK, but not non-sulphated CCK peptides (Cantor, 1986).

(4) Ab 3208, which is directed against the common glycine-extended C-terminal sequence of CCK (designated glycine-extended intermediates) and gastrin (Hilsted & Rehfeld, 1986). After sequential cleavage with trypsin (1 mg/ml, in 0.05 M-sodium phosphate, pH 7.5) and carboxypeptidase B (100 μ l/ml of sample), as previously detailed (Hilsted & Rehfeld, 1986), precursors of both CCK (pro-CCK) and gastrin are bound to Ab 3208.

Abbreviations used: CCK, cholecystokinin; PBS, phosphate-buffered saline; TRH, thyrotropin-releasing hormone.

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Chromatography

Sephadex G-50 (superfine) columns (10 cm × 10 mm) were eluted with 0.125 M-ammonium bicarbonate buffer, pH 8.2, at 4 °C. Void and total volumes of the columns were determined using ^{125}I -albumin and $^{22}\text{NaCl}$. The columns were calibrated with sulphated CCK-33, CCK-22, glycine-extended CCK-8 (CCK-8-Gly) and sulphated CCK-8. Selected gel chromatographic fractions were pooled, lyophilized, reconstituted in the starting buffer and applied to reverse-phase h.p.l.c. C_8 column (220 mm × 4.6 mm) eluted with a 10–40% CH_3CN gradient in 10 mM-ammonium acetate.

Statistical method

Student's *t* test was used for statistical analysis.

RESULTS

Characterization of CCK by chromatography

On gel chromatography of cell extracts, all of the carboxy-amidated immunoreactivity was eluted as sulphated CCK-8 (Fig. 1). The medium contained mainly CCK-8, but also α -amidated immunoreactivity corresponding to CCK-33 and CCK-22 (Fig. 2).

CCK precursor peptides were eluted mainly as glycine-extended CCK-8, but larger precursor peptides were also present (Fig. 3). Reverse-phase h.p.l.c. of the CCK-8 peak from the gel chromatography of the cell extract showed that approx. 60% of the CCK-8 was sulphated and 40% was non-sulphated (Fig. 4). CCK in the medium was eluted similarly (results not shown). By h.p.l.c. the salt peak (fractions 4–7) interfered with the radioimmunoassay. Apparent immunoreactivity was also detected in the first fractions after the salt peak. This might represent degradation products of CCK or interference of other low-molecular-mass substances.

Effects of dexamethasone

Exposure to 0.1–1 μM -dexamethasone for 6 days increased the

Fig. 1. Gel chromatography of an extract from CA-77 cells

Extract (1 ml) was applied to a calibrated Sephadex G-50 superfine column (10 cm × 10 mm) and eluted at 4 °C with 0.125 M-ammonium bicarbonate buffer, pH 8.2, at a flow rate of 3.6 ml/h; fraction vol. 1.2 ml. The elution was monitored radioimmunochemically using Ab 2609 (●) and Ab 2604 (○). The fractions indicated by the bar were pooled, lyophilized and after reconstitution applied to reverse-phase h.p.l.c. (see Fig. 4).

Fig. 2. Gel chromatography of the growth-medium from CA-77 cells

The conditions were as in Fig. 1. The elution was monitored radioimmunochemically using Ab 2609 (●) and Ab 2604 (○).

Fig. 3. Gel chromatography of the growth medium from CA-77 cells: use of proteolytic cleavage for demonstration of pro-CCK

The conditions were as described in the legend to Fig. 1. The elution was monitored radioimmunochemically using Ab 3208 before (○) and after (●) the fractions had been cleaved sequentially by trypsin and carboxypeptidase B.

content of α -carboxyamidated CCK and of pro-forms of CCK in the cells by 2-fold (Table 1a). The concentrations of CCK in the growth medium are shown in Table 1(b). These concentrations reflected the accumulated synthesis of CCK for 3 days, e.g. 3–6 days after initiation of the treatment. The concentrations of the different CCK forms increased by 3-fold. The increase appeared to be most pronounced for the more post-translationally processed CCK forms. The time course of the effect of dexamethasone is shown in Figs. 5 and 6.

Fig. 4. Reverse-phase h.p.l.c. of pooled gel chromatographic fractions (Fig. 1)

A 150 μ l sample was applied to a calibrated C_8 Aquapore RP-300 column (220 mm $\times$ 4.6 mm) which was eluted at 40 $^{\circ}$ C with a CH_3CN gradient in 10 mM-ammonium acetate, indicated by the broken line. The flow rate was 1 ml/min. Fractions of 1 ml were collected, dried and subjected to radioimmunoassay using Ab 2609 ($\bullet$) or Ab G160 ($\circ$). Peaks: 1, sulphated CCK-8; 2, non-sulphated CCK-8.

Effect of monensin

Incubation with monensin affected the morphology of the cells (results not shown), and cells incubated with a concentration of 50 μ mol/l died. The concentrations of all CCK forms decreased in the growth medium, whereas in the cells only the concentration of α -carboxyamidated CCK decreased (Table 2).

Effect of colchicine

After treatment with colchicine many detached cells were seen in the growth medium. A colchicine concentration of 10 μ mol/l inhibited synthesis and secretion of all CCK forms (Table 3), and

Fig. 5. Time course of the effect of dexamethasone on the synthesis of CCK and its precursors in the CA-77 cell line

Cells were grown to subconfluency. Treatment with 100 nM-dexamethasone was then started, with intervals of 3 days. Finally, cells were harvested and contents (pmol/ 10^6 cells; means $\pm$ s.d., $n = 4$) of CCK ($\bullet$), glycine-extended CCK ($\circ$) and pro-CCK ($\blacksquare$) were determined. Significantly higher values than controls are indicated by * $P < 0.001$ and ** $P < 0.05$.

1 μ mol/l also inhibited total synthesis of CCK, as judged from the total concentration of CCK in the growth medium. However, the synthesis and secretion of α -carboxyamidated CCK was increased in cells incubated with 0.2–1 μ mol of colchicine/l (Table 3).

Effect of chloroquine

The CA-77 cell line was only affected by chloroquine at a concentration of 250 μ mol/l, which caused the content of α -carboxyamidated CCK in the cells to decrease. At the same concentration the levels of precursor forms in the growth media increased (Table 4).

Table 1. Effect of dexamethasone on the synthesis of CCK (a) and secretion of CCK from the CA-77 cell line into the growth medium (b)

Subconfluent cultures were treated with dexamethasone at various concentrations for 6 days. Cells were then harvested and growth medium from the last 3 days of treatment was collected. The cell contents (a) and the concentrations in the media (b) of CCK, glycine-extended CCK and pro-CCK were measured. Means $\pm$ s.d. of four experiments are given. Compared with controls, the changes were significant at the following levels: * $P < 0.05$, $\dagger P < 0.02$, $\ddagger P < 0.01$, $\S P < 0.001$.

(a)	Dexamethasone (M)	α -Carboxyamidated CCK (pmol/ 10^6 cells)	Glycine-extended intermediates (pmol/ 10^6 cells)	Pro-CCK (pmol/ 10^6 cells)
	0	0.19 $\pm$ 0.03	0.39 $\pm$ 0.11	0.46 $\pm$ 0.11
	10^{-9}	0.15 $\pm$ 0.03	0.38 $\pm$ 0.03	0.43 $\pm$ 0.10
	10^{-8}	0.18 $\pm$ 0.04	0.39 $\pm$ 0.08	0.38 $\pm$ 0.09
	10^{-7}	0.29 $\pm$ 0.03 $\dagger$	0.67 $\pm$ 0.12*	0.75 $\pm$ 0.28
	10^{-6}	0.41 $\pm$ 0.06 $\S$	0.63 $\pm$ 0.15*	0.75 $\pm$ 0.26
(b)	Dexamethasone (M)	α -Carboxyamidated CCK (pmol/l)	Glycine-extended intermediates (pmol/l)	Pro-CCK (pmol/l)
	0	37 $\pm$ 4	140 $\pm$ 17	1049 $\pm$ 94
	10^{-9}	45 $\pm$ 3	156 $\pm$ 41	1143 $\pm$ 196
	10^{-8}	43 $\pm$ 8	198 $\pm$ 30 $\dagger$	1630 $\pm$ 117 $\S$
	10^{-7}	107 $\pm$ 16 $\S$	441 $\pm$ 49 $\S$	2551 $\pm$ 280 $\S$
	10^{-6}	139 $\pm$ 34 $\S$	475 $\pm$ 74 $\S$	1818 $\pm$ 372 $\dagger$

Table 2. Effect of monensin on the processing (a) and secretion (b) of CCK

Subconfluent cultures were treated with monensin for 2 days. Means $\pm$ s.d. of three experiments are given. Significant differences compared with controls are indicated by * $P < 0.05$, † $P < 0.01$, ‡ $P < 0.001$.

(a)	Monensin (μ mol/l)	α -Carboxyamidated CCK (pmol/ 10^6 cells)	Glycine-extended intermediates (pmol/ 10^6 cells)	Pro-CCK (pmol/ 10^6 cells)
	0	0.82 $\pm$ 0.06	0.65 $\pm$ 0.3	1.10 $\pm$ 0.05
	1	0.23 $\pm$ 0.08‡	1.40 $\pm$ 0.5	1.45 $\pm$ 0.50
	10	0.19 $\pm$ 0.11†	2.00 $\pm$ 0.95	2.00 $\pm$ 1.50
(b)	Monensin (μ mol/l)	α -Carboxyamidated CCK (pmol/l)	Glycine-extended intermediates (pmol/l)	Pro-CCK (pmol/l)
	0	205 $\pm$ 17	133 $\pm$ 11	1073 $\pm$ 115
	1	54 $\pm$ 28‡	50 $\pm$ 25†	355 $\pm$ 46‡
	10	16 $\pm$ 13‡	71 $\pm$ 38*	346 $\pm$ 42‡

Table 3. Effect of colchicine on the processing (a) and secretion (b) of CCK

Subconfluent cultures were treated with colchicine for 2 days. Means $\pm$ s.d. of four experiments are given. Values differ significantly from controls at * $P < 0.01$ or † $P < 0.001$.

(a)	Colchicine (μ mol/l)	α -Carboxyamidated CCK (pmol/ 10^6 cells)	Glycine-extended intermediates (pmol/ 10^6 cells)	Pro-CCK (pmol/ 10^6 cells)
	0	0.47 $\pm$ 0.09	0.26 $\pm$ 0.05	0.35 $\pm$ 0.10
	0.2	0.51 $\pm$ 0.19	0.42 $\pm$ 0.02	0.50 $\pm$ 0.13
	1	0.31 $\pm$ 0.02*	0.95 $\pm$ 0.17†	1.50 $\pm$ 0.45*
	10	0.07 $\pm$ 0.01†	0.31 $\pm$ 0.04	0.53 $\pm$ 0.17
(b)	Colchicine (μ mol/l)	α -Carboxyamidated CCK (pmol/l)	Glycine-extended intermediates (pmol/l)	Pro-CCK (pmol/l)
	0	67 $\pm$ 4	83 $\pm$ 12	1118 $\pm$ 229
	0.2	365 $\pm$ 49†	78 $\pm$ 6	983 $\pm$ 6
	1	137 $\pm$ 26*	35 $\pm$ 9*	499 $\pm$ 73†
	10	19 $\pm$ 5†	10 $\pm$ 1†	296 $\pm$ 47†

Table 4. Effect of chloroquine on the processing (a) and secretion (b) of CCK

Subconfluent cultures were treated with chloroquine for 2 days. Means $\pm$ s.d. of four experiments are given. Values differ significantly from control at * $P < 0.01$.

(a)	Chloroquine (μ mol/l)	α -Carboxyamidated CCK (pmol/ 10^6 cells)	Glycine-extended intermediates (pmol/ 10^6 cells)	Pro-CCK (pmol/ 10^6 cells)
	0	0.96 $\pm$ 0.22	0.21 $\pm$ 0.33	0.90 $\pm$ 0.20
	10	1.19 $\pm$ 0.66	0.31 $\pm$ 0.15	0.95 $\pm$ 0.30
	25	0.97 $\pm$ 0.27	0.30 $\pm$ 0.05	1.15 $\pm$ 0.35
	100	0.79 $\pm$ 0.17	0.25 $\pm$ 0.05	0.85 $\pm$ 0.20
	250	0.48 $\pm$ 0.07*	0.45 $\pm$ 0.25	0.90 $\pm$ 0.40
(b)	Chloroquine (μ mol/l)	α -Carboxyamidated CCK (pmol/l)	Glycine-extended intermediates (pmol/l)	Pro-CCK (pmol/l)
	0	184 $\pm$ 40	175 $\pm$ 35	1082 $\pm$ 145
	10	240 $\pm$ 70	150 $\pm$ 30	1031 $\pm$ 200
	25	237 $\pm$ 70	134 $\pm$ 10	1021 $\pm$ 340
	100	210 $\pm$ 26	257 $\pm$ 115	1495 $\pm$ 475
	250	184 $\pm$ 12	257 $\pm$ 10*	1642 $\pm$ 257*

Fig. 6. Time course of the effect of dexamethasone on the synthesis and secretion of CCK and its precursors from the CA-77 cell line into the growth medium

Cells were grown to subconfluency. Growth medium was changed every third day and treatment with 100 nM-dexamethasone was begun with intervals of 3 days. Growth medium from the last 3 days of treatment was collected and the concentrations (pmol/l; means $\pm$ S.D., $n = 4$) of CCK (●), glycine-extended CCK (○) and pro-CCK (■) were measured. Significantly higher values than controls are indicated by * $P < 0.001$ and ** $P < 0.05$.

DISCUSSION

The present study shows that the CA-77 cells synthesize sulphated α -carboxyamidated CCK-8. Thus the CA-77 cells are able to process pro-CCK in a manner similar to that in normal CCK-producing neuroendocrine cells. The processing includes cleavage at dibasic and monobasic cleavage sites, α -amidation and tyrosine *O*-sulphation (Rehfeld *et al.*, 1988). The amidation process is crucial, as only α -amidated peptides have biological activity, and the activity is further potentiated by sulphation (Rehfeld *et al.*, 1988). Procalcitonin is also processed to amidated calcitonin in the CA-77 cells as occurs in normal tissue (Birnbbaum *et al.*, 1986). Antiserum Ab 2609 cross-reacts with gastrin; however, the CA-77 cell line synthesizes no gastrin, as judged from measurements with the gastrin-specific antiserum Ab 2604.

The concentration of α -carboxyamidated CCK in the CA-77 cell line was approx. 1 pmol/ 10^6 cells (Tables 2 and 4). However, as the generation number of the cell line exceeded 25, the concentration decreased in three passages to about 0.2 pmol/ 10^6 cells (Table 1). This appeared to be due to decreased α -carboxyamidation of CCK, but possibly also to decreased synthesis of CCK, as the concentration of pro-CCK in the cells decreased from 1 to 0.5 pmol/ 10^6 cells.

The first cell line shown to synthesize CCK was a rat islet tumour cell line (Madsen *et al.*, 1986). Later, Haun *et al.* (1989) established a cell line (WE 4/2, also from a rat medullary thyroid carcinoma) that produced CCK and also processed pro-CCK to sulphated CCK-8. Finally, Schneider *et al.* (1989) recently demonstrated CCK synthesis in two human neuroepithelioma cell lines. One of these (SK-PN-AU) was able to process CCK to α -carboxyamidated CCK-8, whereas the other (SK-N-MC) pro-

cessed pro-CCK poorly. O'Hare & Schwartz (1989) found that several neuroendocrine cell lines amidated neuropeptide Y efficiently, whereas processing at the dibasic cleavage sites was partly impaired compared with normal tissues. Lapps *et al.* (1988) transfected cholecystokinin cDNA into a murine anterior pituitary cell line (AtT-20) and a monkey kidney epithelial cell line (COS). The AtT-20 cells were able to synthesize sulphated CCK-8, whereas the COS cells predominantly released non-sulphated pro-CCK into the medium.

In the present investigation, larger forms of CCK were found mainly in the growth medium. Similar results have been detected with the islet tumour cell line (Madsen *et al.*, 1986). The accumulation of CCK precursors in the medium may be due to constitutive secretion of CCK without the final maturation, which takes place in the secretory granules (Kelly, 1985). This assumption is supported by the results obtained when the cells were incubated with chloroquine (Table 4). Chloroquine inhibits the sorting of proteins to the secretory granules, apparently by preventing the intravesicular decrease of pH that occurs in the clathrin-coated immature secretory granules of the trans-Golgi network. Instead, the proteins are secreted unprocessed through the constitutive export route (Moore *et al.*, 1983; Orci *et al.*, 1987). Accordingly, the content of α -carboxyamidated CCK decreased in the cells and the concentration of unprocessed CCK increased in the medium.

Monensin inhibits intracellular transport within the Golgi apparatus. This is accompanied by decreased proteolytic processing and secretion of proteins (Tartakoff, 1983; Orci *et al.*, 1984). Accordingly, we found significantly lower concentrations of all CCK products in the growth medium of cells treated with monensin. In the cells, only the concentration of α -carboxyamidated CCK was decreased. Tyrosine sulphation of secretory proteins takes place in the trans-Golgi apparatus (Lee & Huttner, 1985), and the degree of sulphation of α -carboxyamidated CCK, as measured by antiserum G160, appeared unchanged (results not shown). In contrast, the content of glycine-extended CCK as well as that of pro-CCK was elevated. Therefore the C-terminal proteolytic cleavage in CCK seemed to be unaffected by monensin. This is in contrast with the results obtained with proinsulin in pancreatic β -cells (Orci *et al.*, 1984), but is similar to the processing of the egg-laying hormone, the initial cleavage of which is not inhibited by monensin (Sossin *et al.*, 1990).

Colchicine inhibits the secretion of intracellular proteins by interference with vesicular transport along microtubules, e.g. colchicine inhibits the assembly of tubulin into microtubules (Lambeir & Engelborghs, 1980). The transport of vesicles from the endoplasmic reticulum to the Golgi apparatus also appears to be inhibited (Alonso, 1988). In the present investigation, synthesis, processing and secretion of CCK were inhibited by 10 μ M-colchicine. In contrast, after treatment with 0.2 μ M-colchicine, an increased concentration of α -carboxyamidated CCK was detected in the growth medium, whereas the content was unchanged in the cells (Fig. 3). This might be due to increased synthesis and secretion of α -carboxyamidated CCK or decreased degradation in the growth medium, e.g. by a decreased secretion of various enzymes. The fact that the growth medium of untreated cells contained many CCK precursor peptides, and that only one-fifth of a CCK tracer (10 fmol) added to the medium was degraded (results not shown), argues against the latter possibility. Therefore 0.2 μ M-colchicine appeared to increase the α -carboxyamidation and secretion of CCK. Enhanced secretion of intracellular material caused by colchicine has also been detected in other cells (Temple & Wolff, 1973; Gordon & Werb, 1976). We observed a decreased adhesion of the cells treated with colchicine. A similar observation was made by Thomas *et al.* (1987) with cultured guinea-pig endothelial cells.

As is the case for calcitonin, α -calcitonin gene-related peptide and thyrotropin-releasing hormone (TRH) (Muszynski *et al.*, 1983; Russo *et al.*, 1987; Tavianini *et al.*, 1989), the production of CCK in the CA-77 cells was stimulated by dexamethasone. The increased content of CCK in the cells was not due to an increase in cell size, since the amount of protein extracted from the cells was unaffected by dexamethasone treatment (results not shown). The secretion of CCK into the growth medium increased even more than did the content of CCK in the cells (Table 1). The amounts of α -carboxyamidated CCK and precursor peptides increased. This is consistent with the assumption that corticosteroids increase the frequency of transcription (Schütz, 1988; Russo *et al.*, 1988). The rise in CCK synthesis occurred rather slowly after the treatment with dexamethasone was begun. Similar results have been obtained with calcitonin (Muszynski *et al.*, 1983) and TRH (Tavianini *et al.*, 1989).

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