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Concomitant Secretion of Calcitonin, β-endorphin and ACTH From Medullary Thyroid Carcinoma *In Vivo* and *In Vitro**

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Abstract—*The present study was performed to investigate the possible synthesis of a common precursor molecule for calcitonin (CT), adrenocorticotropin (ACTH) and β-lipotropin (β-LPH)/β-endorphin (β-EP) by the human medullary thyroid carcinoma (MTC). In a patient with MTC but without Cushing's syndrome, the response of plasma CT, ACTH and cortisol levels to a calcium infusion, lysine vasopressin (LVP) and dexamethasone were measured. A parallel increase of these hormones in response to calcium and LVP was seen, while there was a paradoxical increase of CT during dexamethasone infusion. Incubation of MTC fragments obtained at surgery showed a significant correlation of the secretion of CT, ACTH and β-LPH/β-EP in response to calcium, LVP and dexamethasone. The concomitant release of these hormones* in vivo *and* in vitro *could be compatible with the synthesis of a common precursor molecule for CT, ACTH and β-LPH/β-EP in MTC, although this was not substantiated by gel-chromatography of the tumor extract. Corticotropin releasing factor, a regulator of the normal processing of pro-opiococorticoid precursor molecule in the anterior pituitary gland, is also able to activate ACTH, β-LPH/β-EP and calcitonin secretion from the malignant C-cell of the thyroid.*

INTRODUCTION

IN RECENT years it has become increasingly clear that the main concept in endocrinology, connecting the production of a hormone to a single organ, can no longer be maintained. Especially, developments in the fields of neuroendocrinology and of the gut hormones have triggered this change in view. Many of the hormones originally thought to be confined to the central nervous system, i.e. hypothalamus, have been localised in a number of peripheral tissues such as the gastrointestinal tract. Vice versa, gut hormones have also been recovered from neural tissues [1–3]. This has been explained by the elegant APUD theory of Pearse [4], which points to the common origin of endocrine cells in the alimentary tract and cells in the central nervous system.

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In addition, it has recently been shown that different hormones may be produced and secreted by the same cell. It has been demonstrated that adrenocorticotropin (ACTH) and β-lipotropin (β-LPH) are synthesized as part of a single precursor protein in the pituitary [5, 6]. By cleavage of the precursor in the β-LPH region a family of related peptides are produced: α-endorphin (α-EP), β-EP and γ-EP, representing amino acids 61–76, 61–91 and 61–77 of β-LPH respectively. In many instances ACTH and β-LPH/β-EP are released simultaneously, e.g. during stress [7]. Ectopic production of ACTH and β-LPH may also coincide [8].

It has recently been suggested that calcitonin may be present in ACTH-producing cells in the pituitary [9, 10]. *Vice versa*, ACTH may be produced by medullary thyroid carcinoma (MTC) [11]. In a previous study [12] we found that hypersecretion of ACTH due to Cushing's disease, ectopically ACTH-producing tumors or adrenal insufficiency were not associated

with elevated plasma CT levels. The only exception was a patient with MTC with Cushing's syndrome due to ectopic ACTH production, in whom we observed a simultaneous increase in plasma ACTH and CT after administration of lysine vasopressin (LVP). In the present study we describe the reaction *in vivo* and *in vitro* of ACTH-, CT- and β-LPH/β-EP-like immunoreactivity to various manipulations in a patient with MTC without Cushing's syndrome. The results indicate a concomitant release of these hormones, which could be in accordance with the existence of a common prohormone in MTC which contains CT, ACTH and β-LPH/β-EP.

MATERIALS AND METHODS

Radioimmunoassays

CT was measured in unextracted plasma and media by radioimmunoassay using two antisera (AS). AS G-5 is specific for native CT (mol. wt. 3200 daltons), whereas AS 9654 in addition recognizes big CT (mol. wt. > 30,000 daltons). The latter is usually not present in plasma of patients with MTC and, in contrast to small CT, is not adsorbed onto Florisil (Supelco, Bellefonte, PA) [13]. The concentrations were measured with AS 9654 both before and after extraction with Florisil.

Florisil (200 mg) was shown to absorb 8 ng CT completely from 1 ml serum. Normal values with AS G-5 are less than 0.11 μg/liter, which is in agreement with the difference in immunoreactivity measured with AS 9654 before and after treatment of plasma with Florisil. Normal total CT values with AS 9654 are up to 0.40 μg/liter. The intra-assay variations of determinations with AS G-5 was 5.5% (for 500 duplicate measurements containing 0.02–1.00 μg CT/liter) and the inter-assay variation was 3.2% (mean, 0.10 μg/liter; $n = 20$). For AS 9654 the intra-assay variation amounted to 7.6% (for 1000 duplicates, 0.02–2.00 μg/liter), while the inter-assay variation was 5.4% (mean, 0.12 μg/liter, $n = 15$).

Plasma ACTH was measured by RIA after extraction with micro glass according to Rattcliff and Edwards [14], while media were investigated without the extraction step. Reagents were supplied by the Radiochemical Centre (Amersham, United Kingdom; normal values, <65 ng/liter). Biological activity of ACTH in the media was measured with a bioassay using the steroidogenic response of isolated adrenal cells as described by Lowry *et al.* [15].

β-Endorphin/β-lipotropin (β-EP/β-LPH) immunoreactivity of the media was measured directly as previously described [16], with the standard and antiserum supplied by Dr. R. Guillemin (Salk Institute, La Jolla, USA). As this antiserum recognizes the C-terminal region of β-EP, β-LPH is also measured on an equimolar basis [16]. The antiserum used does not cross-react with ACTH and CT. The detection limit of the assay was 6 pg/tube. Displacement curves for serial dilutions of the media showed lines parallel with the standard displacement curve.

In vitro *studies*

Four grams of the surgically removed tumor in patient 1 were chopped and minced to fragments of about 1–2 mm^3. After rinsing three times with medium, the tumor pieces were divided over 18 vials containing 2 ml Medium 199 (Gibco Biocult, Glasgow, U.K.), 2% human serum albumin and 1.25 mM $CaCl_2$. The vials were incubated for 120 min in a Dubnoff shaker at 37°C in an atmosphere of 95% O_2–5% CO_2 (v/v).

Part of the surgically removed tumor (1 g) was immediately homogenized using a Potter–Elvehjan tube in 4 volumes of 0.1 N HCl at 0°C. The homogenate was kept frozen at −20°C until chromatography [17]. For this, the homogenate was centrifuged for 30 min at 25,000 *g* (0°C) and 0.5 ml of the clear supernatant was applied to a 45 × 1.6 cm Sephadex G-150 (fine) column. The column was equilibrated and eluted with 0.15 M HCl/0.5% bovine serum albumin/0.02% sodium azide, adjusted with HCL to pH 3.3. Flow rate amounted to 4 ml/min and 1.6 ml fractions were collected. The column was calibrated with blue dextran, [^{125}I]-CT, [^{125}I]-β-EP and [^{125}I]-ACTH.

In vivo studies included stimulation tests with lysine vasopressin (LVP, Sandoz, Basel, Switzerland; 10 pressor units intramuscularly), calcium (15 mg Ca/kg infused over 4 hr) and dexamethasone (5 mg infused intravenously over 5 hr).

RESULTS

The patient was a 28-yr-old male with MTC, who did not show signs or symptoms of Cushing's syndrome and who showed a normal suppression of plasma cortisol to 1.7 μg/dl in response to the overnight administration of 1 mg dexamethasone at 11 p.m. Basal plasma CT concentration was found to be elevated in the radioimmunoassays using both antisera, while basal plasma ACTH and cortisol values were normal (Table 1). Calcium infusion (15 mg/kg over 4 hr) resulted in a simultaneous, parallel increase of plasma CT, ACTH and

Table 1. Hormone secretion in response to calcium, lysine vasopressin and dexamethasone in a 28-yr-old patient with medullary carcinoma of the thyroid

		Plasma calcitonin ASG-5 (μg/l)	Plasma ACTH (ng/l)	Plasma cortisol (μg/dl)
Calcium infusion	0 hr	1.24(100%)	62(100%)	11.0(100%)
	2 hr	10.60	201	20.8
	4 hr	17.50(1400%)	278(450%)	22.7(200%)
Lysine vasopressin	0′	2.07(100%)	45(100%)	8.4(100%)
	20′	3.50(170%)	72	12.6
	40′	2.00	71	21.4
	60′	2.20	80(180%)	22.7(270%)
	90′	1.94	47	18.0
Dexamethasone	0 hr	1.60(100%)	124(100%)	10.7(100%)
	3 hr	1.90	53	4.4
	5 hr	3.80(240%)	56	2.3
	6 hr	3.70	50	0.9(8%)
	7 hr	3.00	44(35%)	1.4
Dexamethasone	0 hr	2.25(100%)		
	3 hr	2.20		
	4 hr	3.50		
	5 hr	5.10(225%)		
	7 hr	4.60		
	9 hr	3.60		

Calcium infusion: 15 mg/kg body weight over 4 hr; Lysine vasopressin: 10 pressor units intramuscularly; dexamethasone: 5 mg infused over 5 hr.

cortisol. After LVP administration plasma CT concentration was nearly doubled at 20 min, while the maximal levels of ACTH and cortisol were reached 60 min after injection. The intravenous administration of dexamethasone (5 mg over 5 hr) resulted in a sharp increase of plasma CT concentrations after 5 hr, while both plasma ACTH and cortisol were suppressed. In order to confirm this observation, the intravenous infusion of dexamethasone was repeated, showing essentially the same result with regard to the stimulation of CT secretion.

The reaction of hormone secretion to the same stimuli was studied *in vitro*, using pieces of MTC tissue of this patient (Table 2). CT release followed a linear pattern for 120 min of incubation (after 60 min 1.05 ± 0.28 μg CT/mg protein (AS G-5) had been released into the medium, which is about half of the hormone concentration released after 120 min; see Table 2). Apart from CT, ACTH and β-EP/β-LPH were also released into the medium. LVP significantly increased ACTH release, but the increments in CT and β-EP/β-LPH were not statistically significant. Dexamethasone stimulated both the release of CT and ACTH, but did not affect β-EP/β-LPH release. An increase in the calcium concentration of the medium from 1.25 to 3 mM finally resulted in an increment of the release of CT, ACTH and β-EP/β-LPH. Further investigation of the hormone contents of the media of the 18 vials used in this study revealed a close correlation in CT-concentration as determined with both antisera (Fig. 1a; $P < 0.001$), while there was also a significant correlation between the β-EP/β-LPH and the ACTH content of these media (Fig. 1b; $P < 0.01$). Interestingly, a statistically significant correlation was also observed between the ACTH and CT content of these media (Fig. 1c; for both AS G-5 and AS 9654; $P < 0.01$), and the β-EP/β-LPH and CT content of the media (Fig. 1d; for both AS G-5 and AS 9654; $P < 0.05$).

The immunoreactivity of the ACTH content of the media released from pieces of MTC tissue was compared with its biological activity. Using the dispersed adrenal cell method, control medium did not result in measurable corticosterone production, while the $CaCl_2$ (3 mM)-stimulated medium induced a small increase of corticosterone production. It was calculated from these data that the biological activity of the ACTH secreted by this tumor was 5.9% of its immunoreactive activity.

Gel chromatography of an extract of this tumor showed that virtually all CT-immunoreactivity was contained in one peak

Table 2. Calcitonin, ACTH and β-endorphin/β-lipotropin release by pieces of MTC tissue of patient 1 incubated in vitro *for* 120 min

	Calcitonin AS G-5 (μg/mg protein)	ACTH (pg/mg protein)	β-EP/β-LPH (pg/mg protein)
Control ($n = 4$)	2.25 ± 0.62 (100%)	285 ± 74 (100%)	302 ± 82 (100%)
Lysine vasopressin (1 μM) ($n = 4$)	3.02 ± 0.60 (134%)	1226 ± 241* (430%)	488 ± 119 (162%)
Dexamethasone (5 μg/ml) ($n = 5$)	4.27 ± 0.89* (190%)	1081 ± 94* (380%)	368 ± 96 (122%)
$CaCl_2$ (3 mM) ($n = 5$)	5.18 ± 0.83* (230%)	2229 ± 240* (780%)	708 ± 162* (234%)

Mean ± S.E.M.; 4 or 5 vials per group.
*$P < 0.01$ vs control.

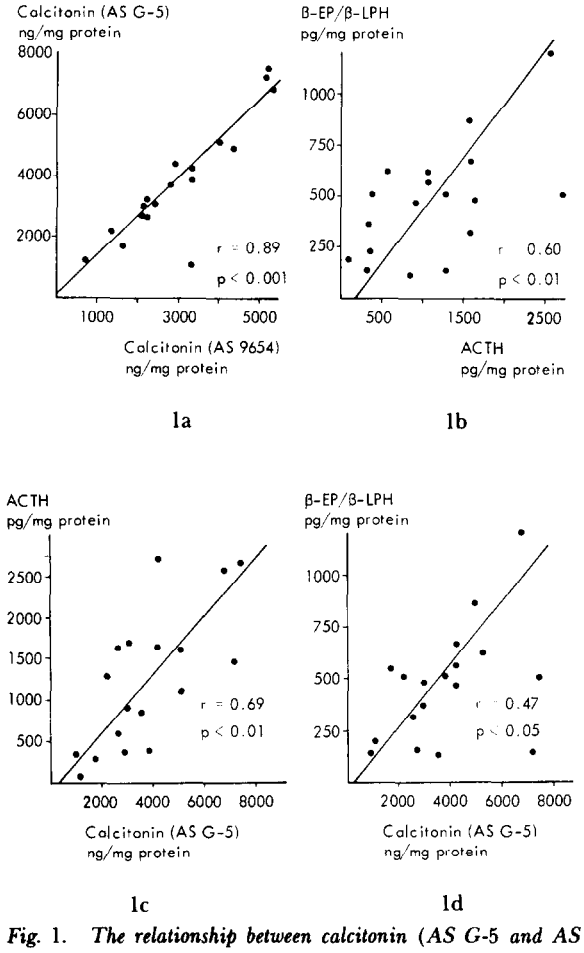

Fig. 1. The relationship between calcitonin (AS G-5 and AS 9654), ACTH and β-EP/β-LPH released into the medium from pieces of MTC tissue incubated in vitro.

representing small, native CT (mol. wt. 3200), while β-EP/β-LPH showed a peak in the high molecular weight region, as well as a broad peak of immunoreactivity around elution peaks of native β-EP. Unfortunately, the ACTH content of all fractions collected was below the detection limit of the assay.

DISCUSSION

It has been well established now that ACTH, β-LPH and β-EP do have a common biosynthetic precursor [5, 6] and that the plasma level of β-EP and β-LPH rises parallel with ACTH following stress in man [7]. The possibility that CT may be part of this precursor molecule has been suggested from different studies. Deftos *et al.* [8] demonstrated, by a specific immunoperoxidase procedure, the presence of CT-containing cells in the rat pituitary gland, while they showed later that these cells were probably the corticotrophs [9]. Organ and cell cultures of pituitary glands of rats, trout and chicken were shown to secrete CT into the medium linearly with time upto 48 hr, which was stimulated by isobutyrylmethylxanthine and TRH [18]. Immunoreactive CT was also shown to be present in extracts of pituitaries from rats, fish, chicken, pigs and sheep, as well as humans [19–21]. Although CT is believed to be the primary polypeptide hormone synthesized and secreted by the C-cell of the thyroid, Cushing's syndrome due to ectopic production of biologically active ACTH by the neoplastic C-cell has frequently been described [22–25]. Apart from the conjoint secretion of CT and ACTH by MTC, ectopic ACTH and CT secretion has also been shown by human tumors from several other locations [26, 27]. The simultaneous presence of ACTH and CT in MTC cells was shown in all 9 tumors studied by Goltzmann *et al.* [11]. Subsequent immuno-

histochemical studies of human MTC tissue and of non-thyroidal CT-producing tumors by Deftos *et al.* [8, 28] suggested that in many instances, CT, ACTH and β-EP were contained within the same cells.

This is in accordance with the earlier suggestion by Lips *et al.* [29] that the primary gene product of different types of carcinoma cells secreting different hormones is contained in a single large hormone precursor or prohormone.

Using an indirect approach by measuring plasma hormone concentrations in patients with disorders of ACTH secretion, we did not find support for the concept of a common precursor molecule for CT and ACTH in the pituitary gland [12]. However, we did find a simultaneous increase in plasma ACTH and CT after LVP administration in a patient with MTC, suggesting that such a common precursor may be synthesised in this tumor [12]. Other arguments against a common precursor molecule containing CT, ACTH and the opioid peptides have been published. The immunocytochemical demonstration of CT into the normal human and rat pituitary gland could not be confirmed by some authors [30, 31]. Nakanishi *et al.* [32, 33] elucidated the primary structure of the larger part of the ACTH/β-LPH precursor of bovine pituitary origin by analysis of its m-RNA sequence and obtained no evidence that it contained the sequence of CT. In studies by Shibasaki *et al.* [17], β-EP-, CT- and ACTH-like immunoreactivity were observed in extracts from both differentiated and anaplastic rat MTC tissue, but the molar ratios of β-EP and ACTH were statistically equal to 1, while no consistent relationship was observed between the concentrations of these two substances and that of immunoreactive CT. Finally, a study of a human oat-cell carcinoma of the lung opposed the presence of a common ACTH/CT-containing precursor molecule [27].

In the present study, involving *in vivo* and *in vitro* studies in a patient with MTC, concomitant secretion of CT, ACTH and β-LPH/β-EP was observed in several conditions. Plasma CT, ACTH and cortisol levels showed a concomitant increase in response to a calcium infusion over 4 hr. No direct stimulation of ACTH secretion by long-term calcium infusion has been reported in normal individuals, and the patient studied did not show stress-related side-effects. The stimulating effects of LVP and dexamethasone on CT secretion were not accompanied by a parallel change in ACTH and cortisol secretion. As in this patient Cushing's syndrome was biochemically and clinically absent, the late rise of ACTH and cortisol after LVP and the normal suppression after dexamethasone probably represents a reaction of the normal anterior pituitary gland. The *in vitro* study with pieces of tumor tissue supports a concomitant release of CT, ACTH and β-LPH/β-EP in reaction to the stimuli mentioned. Monolayer cultures of human CT-secreting cells derived from MTC have also been demonstrated to show a marked increase in hormone secretion if the calcium concentration of the medium is increased [34, 35]. The concomitant response of CT and ACTH secretion by pieces of MTC *in vitro* to dexamethasone and possibly to LVP (the increase of CT was shown here to be statistically significant *in vivo* only), could suggest that at least in the human MTC tumor cell these hormones might originate from a common precursor molecule. This was further underlined by the close correlation between the CT, ACTH and β-EP/β-LPH contents of the media, as shown in Fig. 1. Several arguments, however, can be raised against the existence of such a common precursor molecule: (1) the documented increases in the different hormones did not parallel one another quantitatively either *in vivo* or *in vitro*; (2) the investigation of the tumor extract by gel chromatography was completely inconclusive and did not indicate the presence of such a common precursor molecule in this tumor tissue; and (3) a concomitant reaction of hormone release to these stimuli by itself cannot be used as proof for the origin of these hormones from one single precursor molecule.

The reaction of plasma CT in the *in vivo* tests in this and our previous patient [12] shows that CT secretion by the malignant C-cell is stimulable with LVP, while a concomitant release of CT and ACTH was observed in reaction to calcium infusion. The mechanisms involved in the normal intracellular processing of the ACTH/β-LPH/β-EP precursor molecule in the pituitary gland are currently unknown. It seems likely that different cells are equipped with specific systems for cleavage of the precursor and packaging and storage of the fragments produced. Hormone secretion may then be regulated at several levels, i.e. by effects on the production rate of the precursor, on the activity of the proteolytic enzymes and on the secretory granules. Although the anterior and intermediate lobes of the rat pituitary gland start with a similar molecular weight form of the precursor, differences in processing lead to the release of different hormones [36, 37]. Crude hypothalamic extracts and more purified preparations of CRF [38] have been

shown to stimulate the release of both ACTH-like and β-EP-like components from rat anterior pituitary cells in culture and from AtT-20 mouse pituitary tumor cells. Rat median eminence extracts give a dose-dependent stimulation of ACTH release and intracellular cAMP accumulation in ectopic ACTH-producing dispersed tumor cells from patients with MTC [39, 40]. Our study shows that a naturally occurring hypothalamic CRF-like substance, LVP [41], also stimulated CT secretion *in vivo* in the present patient with MTC tissue, which is in line with our previous report [12]. This observation could be used as an argument for the presence of a common precursor molecule for CT and ACTH in the malignant C-cell of the thyroid, the secretory activity of which is regulated by the naturally occurring CRF.

Exposure of AtT-20 cells to dexamethasone for 24–72 hr results in a reduction in intracellular levels of immunoreactive ACTH and β-EP, as well as a reduced secretion of these hormones, probably due to an inhibition at the level of transcription [42]. The stimulating effect of dexamethasone on CT-secretion *in vivo* and on CT and ACTH release by MTC tissue *in vitro* in our patient seems to point to another, acuter effect of the corticosteroid on this tumor tissue. The nature of the proteolytic enzymes, which play a role in the normal dissection of the precursor molecule prior to release of the hormones from the pituitary cell, is not known. It could very well be that the activity of these enzymes more or less determines which patients with MTC will develop Cushing's syndrome on the basis of ectopic secretion of ACTH. This enzyme activity could determine whether a large enough quantity of ACTH is secreted to stimulate the adrenal cortex, or that the ACTH molecule secreted by the tumor is incompletely liberated and therefore largely biologically inactive, as was the case in our patient.

The present study suggests that CT, ACTH and β-EP/β-LPH in the malignant C-cell of the thyroid are released in a concomitant manner and that CRF, a most important regulator of the processing of this molecule in the anterior pituitary gland, is also able to activate hormone secretion from these cells.

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