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Expression of hypothalamic regulatory peptides in thyroid C cells of different mammals

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ABSTRACT

Besides intervening in calcium homeostasis by means of calcitonin, C cells are also implicated in the synthesis of an increasing number of regulatory peptides that could exert a paracrine regulation on the neighbouring follicular cells. Among the latest peptides reported in C cells, there are several characteristic hypothalamic peptides, such as TRH, CART, and ghrelin, which are mainly involved in the regulation of the metabolism at hypothalamic-pituitary-thyroid axis. The main aim of the present work has been to study the synthesis of the referred hypothalamic peptides by normal and neoplastic C cells of different mammals as well as in C-cell lines of both rat (CA-77, 6-23) and human (TT) origins in order to elucidate whether this is a fact in this kind of vertebrates. With that objective, we have applied the immunoperoxidase technique to analyze the presence of TRH, CART, ghrelin, and somatostatin in thyroid tissues of different species, and immunofluorescence to study those same peptides in C-cell cultures. Furthermore, we have investigated their expression at mRNA level by RT-PCR analysis. Our results demonstrate immunocolocalization of CART, ghrelin, somatostatin and TRH with calcitonin in normal C cells of different mammals, as well as in rat and human neoplastic C cells. We also confirm the expression of those peptides in rat and human C-cell lines by RT-PCR. Consequently, we can conclude that the synthesis of those peptides by C cells is a general event characteristic of the thyroid gland in mammals.

1. Introduction

The thyroid gland is mainly composed of follicular cells but it also contains, although in a smaller proportion, the so-called parafollicular cells or C cells (CC). These neuroendocrine cells are characterized by producing calcitonin (CT) (Bussolati and Pearse, 1967), a hormone which intervenes in the calcium metabolism regulation. In addition, C cells are implicated in the production of numerous regulatory peptides, among which there are several characteristic peptides of certain hypothalamic nuclei. Specifically, somatostatin (SS) has been demonstrated in the thyroid gland of several species of mammals, with a variable extension. Thus, while the frequency for SS immunoreactive (SS-IR) CC varies in rats throughout their lives, as they are scarce in the fetus, abundant at the time of birth and scarce again in adults (Alumets et al., 1980; Kameda et al., 1982; Van Noorden et al., 1977; Zabel et al., 1987), the majority of calcitonin immunoreactive (CT-IR) CC in guinea pigs and rabbits are also SS-IR, besides positivity is seen in the parathyroid gland (Buffa et al., 1979; Kameda et al., 1982). On the contrary, SS-IR CC are observed occasionally in normal pig and human thyroid glands (Fierabracci et al., 1993).

Subsequently, CC implication in the production of other hypothalamic peptides, *in vitro* as well as *in vivo*, was demonstrated. Thus, the presence of prepro-TRH in normal (Gkonos et al., 1989) and neoplastic rat CC (CA-77) (Sevarino et al., 1989; Tavianini et al., 1989) was proved. Ghrelin has also been detected in CC from normal rat and human thyroid glands, as well as in the parathyroid gland (Raghay et al., 2006). Ghrelin is also synthesized in human MTC (Morpurgo et al., 2005; Raghay et al., 2006) and in neoplastic CC lines of human (TT) and rat (6–23) thyroid carcinomas (Kanamoto et al., 2001). Finally, the presence of CART (Cocaine and Amphetamine-Regulated Transcript), an anorexigenic regulatory peptide highly expressed in the brain's appetite control centers (Rogge et al., 2008), has been observed by means of immunofluorescence in CC of pig thyroid glands (Wierup et al., 2007) and

Abbreviations: CC, C cells; CART, cocaine and amphetamine-regulated transcript; GHS-R, growth hormone secretagogue receptor; MTC, medullary thyroid carcinomas; SS, somatostatin; SS-R, somatostatin receptor; TRH, thyrotropin-releasing hormone; TRH-R, TRH receptor.

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human MTC (Landerholm et al., 2011), while its presence in normal thyroids of other species such as rats, mice and humans is not known.

The hypothesis, increasingly found in literature, that C cells, through those regulatory peptides, could exert a paracrine regulation on follicular cells (Martin-Lacave et al., 2009; Morillo-Bernal et al., 2009, 2011; Raghay et al., 2006; Zbucki et al., 2007) is supported by the fact that thyrocytes express the correspondent receptors. In fact, our research group demonstrated the presence of TRH-R in follicular cells (line PC-Cl3) by means of RT-PCR and immunofluorescence (De Miguel et al., 2005). On the other hand, Cassoni et al. analyzed the presence of specific binding-sites for growth hormone secretagogues in normal human thyroid (Cassoni et al., 2000). Recently, we have demonstrated that both C-cells and follicular cells substantially express GHS-r 1α in normal thyroid tissue and C-cell carcinomas in rats (Morillo-Bernal et al., 2011). Likewise, Ain et al. confirmed, by means of RT-PCR, the existence of receptor for SS (SS-R) types 3 and 5 and, to a lesser extent, types 1 and 2, in normal thyroid cell lines (Ain et al., 1997). Moreover, no specific receptor for CART peptides in any tissue, including the thyroid gland, has been identified yet (Keller et al., 2006; Rogge et al., 2008).

If the production of characteristic hypothalamic peptides by CC is confirmed not to be a chance finding but, on the contrary, something common in thyroid glands of different mammals, we would be facing a unique control mechanism of an endocrine gland, since the same peptides that would trigger the thyroid hormone synthesis through the hypothalamic-pituitary-thyroid (HPT) axis, especially TRH, may exert a local regulation of follicular cells. For this reason, the main aim of the present paper has been to study, systematically, the synthesis of the referred hypothalamic regulatory peptides in normal and pathological thyroid CC in different species of mammals as well as in CC lines, in order to check whether this is widespread in this kind of vertebrates.

2. Methods

2.1. Subjects

In this study we have analyzed normal adult thyroid glands from several mammalian species: rat ($n = 7$), human ($n = 5$), guinea pig ($n = 5$), rabbit ($n = 3$) and pig ($n = 2$). Animals were obtained from the Experimental Animal Service at the Medical School of Seville. With the exception of domestic pigs which were killed for other purposes, all animals were anesthetized with i.p. injection of ketamine/Diacepam in a specific dose (mg/kg) depending on the species. All experiments were conducted in accordance with the guidelines proposed in The Declaration of Helsinki (http://www.wma.net) involving the use of laboratory animals. Furthermore, tumoral samples of rat C-cell carcinomas (CCC) ($n = 5$) and human MTC ($n = 5$) were also studied. The human specimens were diagnosed at the Department of Pathology of the Virgen Macarena Hospital in Seville in the eighties. Thyroid glands were formalin-fixed and paraffin-embedded by a standard procedure, sectioned at 4–5 μm thickness, and mounted on silane-coated glass slides for immunohistochemistry. Consecutive tissue sections were stained with hematoxylin and eosin for histological diagnosis and immunostained with a panel of specific antibodies (Table 1).

2.2. Immunohistochemical procedure

Sections were dewaxed, rehydrated and then treated with 3% hydrogen peroxide to block endogenous peroxidase activity for 20 min; nonspecific binding was blocked with 10% normal swine serum. Slides were incubated with the primary antibodies at 4 °C overnight. The specific binding was developed using the commercial LSAB+/HRP kit or the ADVANCE system (Dako, Denmark), according to the manufacturer's instructions, and 3,3′-diaminobenzidine tetrahydrochloride (DAB) solution (Sigma–Aldrich) as chromogen. For immunostaining with anti-CART or anti-ghrelin antibodies, sections were heated for 5 min in a pressure cooker (WMF Perfect Plus) for antigen retrieval before the peroxidase blockade. Slides were counterstained with hematoxylin, dehydrated and coverslipped. Photomicrographs of the samples were performed using an Olympus photomicroscope (Vanox AHBT3) and the Olympus DP10 digital camera.

Controls for specificity of immunohistochemistry were performed by (1) omitting any essential step of the immunoreactions; (2) replacing the primary antibody with an appropriate dilution of normal rabbit serum; (3) preincubating the primary antisera with their corresponding immunogenic peptides (Santa Cruz Biotechnology) 10–20-fold molar excess, for 2 h at RT, followed by the IHC protocol as outlined above.

2.3. Double immunohistochemistry

To analyze the colocalization of CT with SS, TRH, and CART, respectively, a double immunohistochemical labelling with a HIER step in between the two staining sequences to prevent cross-reactions among reagents was performed, according to Lan et al. (1995). Briefly, sections were dewaxed and pretreated in the same way as described above. In the first immunostaining sequence, the specific antibody (anti-SS, anti-TRH or anti-CART) was incubated at 4 °C overnight and followed by the LSAB+/HRP kit or the ADVANCE system. After colour development with DAB as chromogen, sections were heated in a pressure cooker during 1–2 min, in citrate buffer pH 6.0, and then the second immunostaining sequence started. The second specific antibody, anti-CT, was incubated at 4 °C overnight and a LSAB/Alkaline Phosphatase (Dako, Denmark) was used as labelling system. The enzymatic reaction was visualized with Fast-Red as chromogen. Slides were counterstained with hematoxylin and coverslipped in an aqueous permanent medium.

2.4. Cell cultures

The following rat cell lines were used: 6–23 (neoplastic CC, ATCC No. CRL-1607); CA-77 (neoplastic CC, generously provided by Dr. T. Ragot, Institut Gustave Roussy, Paris, France), both grown in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 15% foetal bovine serum (FBS), 2 mM L-Glutamine, 50 U/mL penicillin and 50 μg/mL streptomycin; and PC–Cl3 (follicular cells,

Table 1
Details of primary antibodies used for immunohistochemistry as well as the optimal procedure for each antibody.

Antibody	Source	Supplier	Dilution	Pretreatment	Visualization system
Calcitonin	Rabbit	Dako (A-572)	1:4000	No	LSAB+
Somatostatin	Rabbit	Dako (A0566)	1:1000	No	LSAB+
TRH	Rabbit	Acris (BP5066)	1:5000	No	LSAB+
CART	Rabbit	Phoenix (H-003-62)	1:1000	Yes	ADVANCE
Ghrelin	Rabbit	Santa Cruz (sc-10368)	1:500	Yes	LSAB+
Ghrelin	Rabbit	Phoenix (H-031-30)	1:500	Yes	LSAB+