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Melatonin-synthesizing enzymes and melatonin receptor in rat thyroid cells

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Summary. Melatonin is an indoleamine with a wide spectrum of biological activities other than transmitting photoperiod information, including antioxidant, oncostatic, anti-aging and immunomodulatory properties. Although melatonin is synthesized mainly in the pineal gland, other tissues have the same capacity. In the present study, we examined whether two key enzymes in melatonin biosynthesis, arylalkylamine Nacetyltransferase (AANAT) and hydroxyindole-O-methyltransferase (HIOMT) and its receptor MT_1 are expressed in the two endocrine thyroid cells of the rat, follicular cells and C cells. Reverse transcriptase polymerase chain reaction analyses demonstrated that both AANAT and HIOMT mRNAs are expressed in the rat thyroid C-cells, and MT_1 expression has been detected in C cells and follicular cells. Immunofluorescence revealed that AANAT protein is localized in C-cell cytoplasm, and MT_1 protein in both cell populations. These findings demonstrate that the rat thyroid expresses AANAT, HIOMT, and its receptor MT_1, showing that C cells are the main melatonin-synthesizing sites in the thyroid. This local C-cell-secreted melatonin may protect follicular cells from the oxidative stress inherent to the thyroid gland, and could also have paracrine and autocrine functions.

Key words: Melatonin, MT_1, Thyroid, C cells, Follicular cells

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Introduction

Melatonin, an important indoleamine secreted by the pineal gland during the night, is mainly implicated in circadian rhythm control of mammalian and other vertebrates. Besides playing an important role as a transmitter of photoperiodic information, this indoleamine has antioxidant (Martinez-Cruz et al., 2002; Reiter et al., 2005, 2009; Mogulkoc et al., 2006), anti-aging (Reiter et al., 2002; Tajes et al., 2009) antiproliferative, and, potentially, anticancerogenic activities, including suppressing effects on secretory and growth processes of the thyroid gland (Lewinski and Karbownik, 2002). Melatonin production is catalyzed by two well-characterized enzymatic reactions from tryptophan. First, serotonin is converted to N-acetylserotonin (NAS) by the enzyme arylalkylamine N-acetyltransferase (AANAT) (Voisin et al., 1984). NAS is subsequently methylated by hydroxyindole-O-methyltransferase (HIOMT) to form melatonin (Axelrod and Weissbach, 1960). Although the pineal gland is considered the main site of melatonin synthesis, many extrapineal tissues have been identified as melatonin synthesizers, such as retina (Mennenga et al., 1991; Iuvone et al., 2002; Tosini et al., 2007), Harderian gland (Djeridane et al., 1998), gut (Raikhlin et al., 1975; Konturek et al., 2007), ovary (Itoh et al., 1997, 1999; Nakamura et al., 2003), immune system (Guerrero and Reiter, 2002; Carrillo-Vico et al., 2004, 2005; Naranjo et al., 2007), skin (Slominski et al., 2002; Fischer et al., 2008), and testes (Tijmes et al., 1996). Moreover, melatonin has been found in the rat thyroid gland (Kvetnoy, 1999).

Considering the great production of melatonin in many organs and its wide spectrum of biological

activities, one can hypothesise that extrapineal melatonin may play key autocrine and paracrine roles for the local coordination of intercellular relationships. In fact, many neighbouring cells in different organs have melatonin membrane receptors (Barrett et al., 1994; Sallinen et al., 2005).

It is well known that melatonin has inhibitory effects on the pituitary gland. This substance inhibits TSH expression and accumulation in *rat pars tuberalis*-TSH cells (Aizawa et al., 2007), regulating diurnal changes in TSH concentration. Many effects of melatonin on the thyroid gland have also been described. In rodents, high doses of melatonin inhibit basal and TSH-stimulated mitotic activity of thyroid follicular cells *in vivo* and in primary culture (Lewinski and Sewerynek, 1986). Besides, melatonin has a direct inhibitory effect on T_4 secretion and, also, depresses the response of the thyroid to TSH (Wright et al., 1997, 2000). Furthermore, melatonin has a protective role against oxidative stress in the rat thyroid gland (Karbownik and Lewinski, 2003; Makay et al., 2009; Rao and Chhunchha, 2010).

The thyroid gland has two different endocrine cell populations, namely, follicular cells, the most abundant cells in the gland and responsible for secreting T_3 and T_4, and C cells or parafollicular cells, which produce calcitonin. Apart from their role in calcium homeostasis, C cells are probably also involved in the intrathyroidal regulation of follicular cells. This hypothesis is supported by different features, such as their characteristic 'parafollicular' position, their predominance in the central region of the thyroid lobe - the so-called *C-cell region* (McMillan et al., 1985) - the expression of thyrotropin receptors by C cells (Morillo-Bernal et al. 2009), the parallel evolution of C cells and follicular cells in different thyroid status (Martín-Lacave et al., 2009) and their implication in the secretion of many different regulatory peptides (Scopsi et al., 1990; Ahrén, 1991; Sawicki, 1995), some of them with an inhibiting action on thyroid hormone secretion, whereas others act as local stimulators of thyroid hormone synthesis. In fact, the presence of certain receptors for some of these regulatory peptides has been demonstrated in follicular cells, receptors that could be implicated in local fine-tuning of follicular-cell activity (De Miguel et al., 2005; Morillo-Bernal et al. 2011). Although melatonin has been detected previously in thyroid C-cells by immunohistochemistry (Kvetnoy, 1999), nothing is known about melatonin-synthesizing-enzyme expression in rat thyroid gland.

In order to demonstrate the biosynthesis of melatonin by C cells in the rat thyroid gland, in the present work, we have analyzed the mRNA expression pattern of the key enzymes implicated in melatonin synthesis: AANAT and HIOMT (Wurtman and Axelrod, 1968). Moreover, we have studied mRNA expression of melatonin receptor (MT_1), and AANAT and melatonin receptor (MT_1) protein localization by immunofluorescence, in both follicular and C-cell lines and rat thyroid tissue.

Materials and methods

Cells and culture requirements

The following cell lines were used: PC-C13 (rat follicular cells, generously provided by Dr. Massimo Santoro, Centro di Endocrinologia e Oncologia Sperimentale di C.N.R Naples) and CA77 (rat C-cells, generously provided by Dr. T. Ragot, Institut Gustave Roussy, Paris, France). PC-C13 were grown in Coon's modified Ham's F12 medium suplemented with 5% FBS and a mixture of six growth factors (1 nM TSH, 10 μg/ml insulin, 10 ng/ml somatostatin, 5 μg/ml transferrin, 10 nM hydrocortisone, and 10 ng/ml glycyl-L-histidyl-L-lysine acetate). CA77 were grown in Dulbecco's Modified Eagle's medium (DMEM) with 15% fetal bovine serum. Cell cultures were maintained at 37°C in a humidified atmosphere with 5% CO_2. Cell cultures were always used at 70-80% confluence.

Total RNA extraction and reverse transcription

Total RNA from cultured cells was extracted by using TriPure Isolation Reagent (Roche, Mannheim, Germany) according to the manufacturer's instructions. After cell lysis and RNA extraction, RNA was precipitated with isopropanol, and the pellet was washed in 75% ethanol. RNA samples were recovered by centrifuging at 14,000 g for 5 min and then dried. Each RNA pellet was dissolved in 50 μl RNase-free water and quantified spectrophotometrically. In order to discard DNA contamination before cDNA synthesis, RNA samples were incubated in gDNA wipeout buffer (Quantitect Reverse Transcription Kit, Qiagen. Hilden, Germany) at 42°C for 2 min and then used directly for reverse transcription. cDNA synthesis was carried out using the Quantitech Reverse Transcription Kit (Qiagen. Hilden, Germany) in a reaction containing 14 μl of 1 μg RNA in 6 μl of a mixture formed by 1X reverse transcription master mix containing 1 μl reverse transcriptase, 4 μl RT buffer and 1 μl RT primer mix. The mix was incubated for 15 min at 42°C and then inactivated at 95°C for 3 min.

PCR

For the detection of transcripts, non quantitative PCRs were carried out in a reaction containing 5 μL of RT product as template DNA, 1X PCR buffer, $MgCl_2$ in an appropriate concentration, 0.4 mM each deoxynucleotide, 2.5 U ECOTAQ DNA polymerase (Ecogen, Barcelona, Spain), 0.2 μM sense and antisense primers of housekeeping gene (b-actin) and 0.2 μM sense and antisense primers of the gene under study, in a final volume of 25 μl. The PCR reaction was started by a 10 min activation of hotstart DNA polymerase at 94°C followed by a 40 cycles of target cDNA amplification. The template was initially denatured at 94°C for 1min followed by 40-cycle program with 1 min annealing and

1 min elongation at 72°C. Beta-actin gene was used as internal control. Reaction mixture, without the cDNA, was used as negative control in each run. cDNA from rat pineal and brain were used as positive controls. The primers sequences and annealing temperatures are shown in Table 1.

Immunofluorescent AANAT and MT_1 detection in cell cultures

Immunofluorescent analyses were performed to localize MT_1 and AANAT, in cultured CA77 cells, and MT_1 in PC-C13 cells. Before the experiments, cells were cultivated in serum and factor free medium for 24 h and then cells were fixed in 4% paraformaldehyde at room temperature for 20 min. For AANAT immunodetection, cells were permeabilized with 0.3% triton X-100 for 5 min. After this, slides were washed with PBS and blocked by incubation with donkey normal serum for 1h at room temperature. Anti-MT_1 (1/50, goat, Santa Cruz sc-13186, CA, USA), anti-AANAT (1/100, goat, Santa Cruz sc-55612, CA, USA), anti-calcitonin (1/1000, rabbit, DAKO, Glostrup, Denmark) and anti-thyroglobulin (1/1000, rabbit, DAKO, Glostrup, Denmark) antibodies were applied overnight at 4°C in a humidity chamber. DAPI was added to the primary antibody solution for nuclei counterstaining. Afterwards, slides were washed in PBS and incubated for 30 min at RT with secondary antibodies raised in different species, labelled either with Cy-2 or Cy-3 (1/100, Jackson Immunoresearch Laboratories, Suffolk, UK) for single or simultaneous double immunofluorescence. Controls for immunoreaction specificity were performed by omitting the primary antibody step.

Finally, after PBS washing, slides were mounted in 90% glycerol, 2% n-propylgallate (Sigma, St. Louis, MO, USA) and observed under a confocal laser scanning microscope (TCS SP2, Leica Microsystems, Heidelberg GmbH, Germany) or a fluorescence microscope (BX50, Olympus, Japan).

Immunolocalization of AANAT and MT_1 in thyroid samples

Immunofluorescent analyses were performed in sections of formalin-fixed paraffin-embedded thyroid glands obtained from normal rats (n=3). The same antibodies previously used were applied overnight at 4°C to the 5 μm thick-thyroid sections at the following dilutions: anti-calcitonin, 1/2000; anti-thyroglobulin, 1/4000; anti-MT_1, 1/100; anti-AANAT, 1/200. An antigen-retrieval method (10 mM citric acid buffer pH 6) was employed. DAPI was added to the primary antibody solution for nuclei counterstaining. Antigen-antibody binding was detected by using Cy-2 or Cy-3 labelled secondary antibodies (Jackson Immunoresearch Laboratories. Suffolk, UK). Controls for specificity of immunoreactions were performed by omitting the primary antibody or any essential step of the technique.

Melatonin determination

Supernatants obtained from 80%-confluence cultures of CA77 and PC-C13 cells were collected for melatonin determination. Culture media with and without FBS were used as controls to eliminate any contribution to the melatonin content from the culture medium itself. Melatonin content in the culture medium was assayed by ELISA kit (DRG Diagnostics, Marburg, Germany) as already reported (Naranjo et al., 2007). Melatonin from 500 μl of the samples, standards and controls was extracted (90-100% yield recovery) using C18 reversed-phase columns (IBL-Hamburg, Germany) and methanol elution. The dried extracts (after evaporating methanol) were stored at -20°C for up to 48 h. Melatonin levels were measured in duplicate using 96-well microtiter plates coated with captured goat antirabbit antibodies. Each microtiter plate was filled either with 50 μl blank reagent, extracted calibrators, extracted samples or extracted standard solutions (containing 0, 3, 10, 30, 100 or 300 pg/ml melatonin). Then, 50 μl melatonin biotin

Table 1. Sequences of MT_1, MT2, HIOMT, AANAT and ß-actin primers used in RT-PCR study.

Primer	5´-sequence-3´	Annealing Temperature	PCR fragment
MT_1 Forward MT_1 Reverse	CAGTACGACCCCCGGATCTA GGCAATCGTGTACGCCG	58°C	65 bp
HIOMT Forward HIOMT Reverse	AGTGACATCATGGGTGGGAATTTATGACTT CCCTACCCCACCATTACTGTGACATC	60°C	105 bp
AANAT Forward AANAT Reverse	GAGATCCGGCACTTCCTCACCCTGTGTCCAGA CCCAAAGTGAACCGATGATGAAGGCCACAAGA	68°C	94 bp
ß-actin Forward ß-actin Reverse	CAGATGTGGATCAGCAAGCAGGAGTACGAT GCGCAAGTTAGGTTTTGTCAAAGAA	62°C	126 bp

MT_1: melatonin receptors type 1; HIOMT: hydroxiindol-O-methyltransferase; AANAT: arylalkyl-N-acetyltransferase.

and 50 μl rabbit antiserum were added into each well, shaken carefully, sealed with adhesive foil and incubated overnight at 2-8°C.

After washing three times with 250 μl diluted assay buffer, 150 μl anti-biotin conjugate to alkaline phosphatase was added into each well and incubated for 2 h at room temperature. The reaction was developed using p-nitrophenyl phosphate and optical densities were determined at 450 nm in an automatic microplate reader. The sensitivity of the melatonin assay was 3.0 pg/ml.

Statistics

All experiments for melatonin determination were performed in triplicate. Melatonin data were represented as mean ± SD. Data were compared using Student's test, p values of less than 0.05 were accepted as significant.

Results

Non-quantitative PCR

PCR analyses demonstrated that both AANAT and HIOMT mRNAs are expressed in CA77 cells, but not in PC-C13 cell line. Moreover, both cell lines were also positive for the expression of MT_1. Expression for these three genes was also positive in rat thyroid-gland tissue (Fig. 1). All these fragments were parallely amplified from rat pineal gland and brain mRNAs, which were used as positive controls for AANAT/HIOMT and MT_1, respectively.

Melatonin production by thyroid cells

Melatonin content in CA77 and PC-C13 culture media and controls without cells was measured by ELISA and expressed as pg melatonin/ml. Our results showed a significant amount of melatonin in CA77 culture, although some melatonin was derived from the medium's FBS (Fig. 2). Melatonin found in PC-C13 culture was not significant because it represented similar values as those detected in control medium with FBS. Melatonin was not detected in PC-C13 or CA77 culture media without FBS. Three experiments were carried out to confirm these results.

Immunofluorescence microscopy

We have studied the cellular localization of AANAT in rat C-cell cultures (CA77) and thyroid tissue by immunofluorescence. AANAT immunoreactivity was found in the cytoplasm of CA77 cells - colocalized with

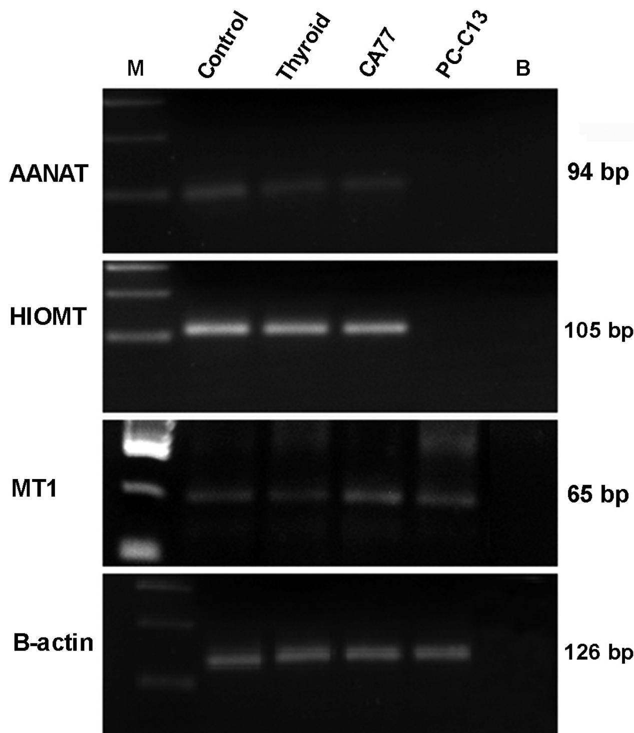

Fig. 1. mRNA expression of AANAT, HIOMT and MT_1 by RT-PCR from rat thyroid gland, CA77 and PC-C13 cell lines. Control refers to pineal gland for AANAT and HIOMT mRNA expression and brain for MT_1 mRNA expression.The right lane (B) represents the negative control in which cDNA is replaced with distilled water.

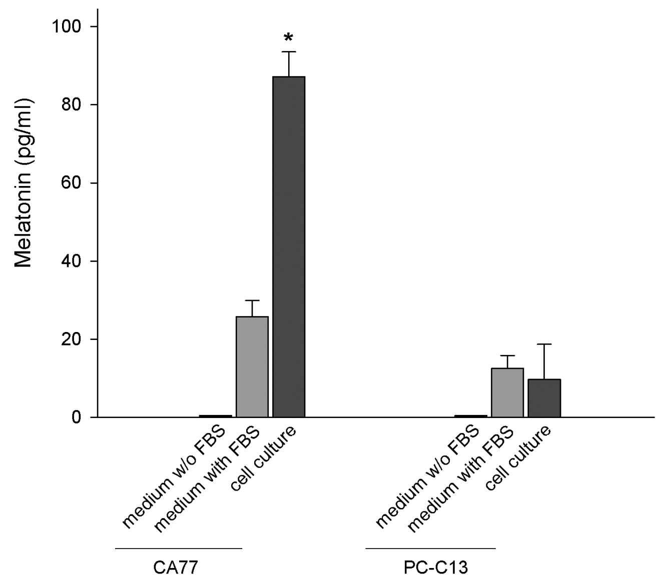

Fig. 2. Melatonin determination in culture medium. Melatonin was measured by ELISA in supernatants of cell cultures that reached 80% confluence. In order to eliminate any contribution to the melatonin content from the culture medium itself, melatonin was also measured in culture medium (without cells) with and without FBS. Graph shows significant amounts of melatonin in the CA77 cell line. The melatonin content observed in the PC-C13 cultures came from melatonin present in the FBS of culture medium (Coon's). *p<0.05 vs. CA77-culture medium (DMEM) without cells.

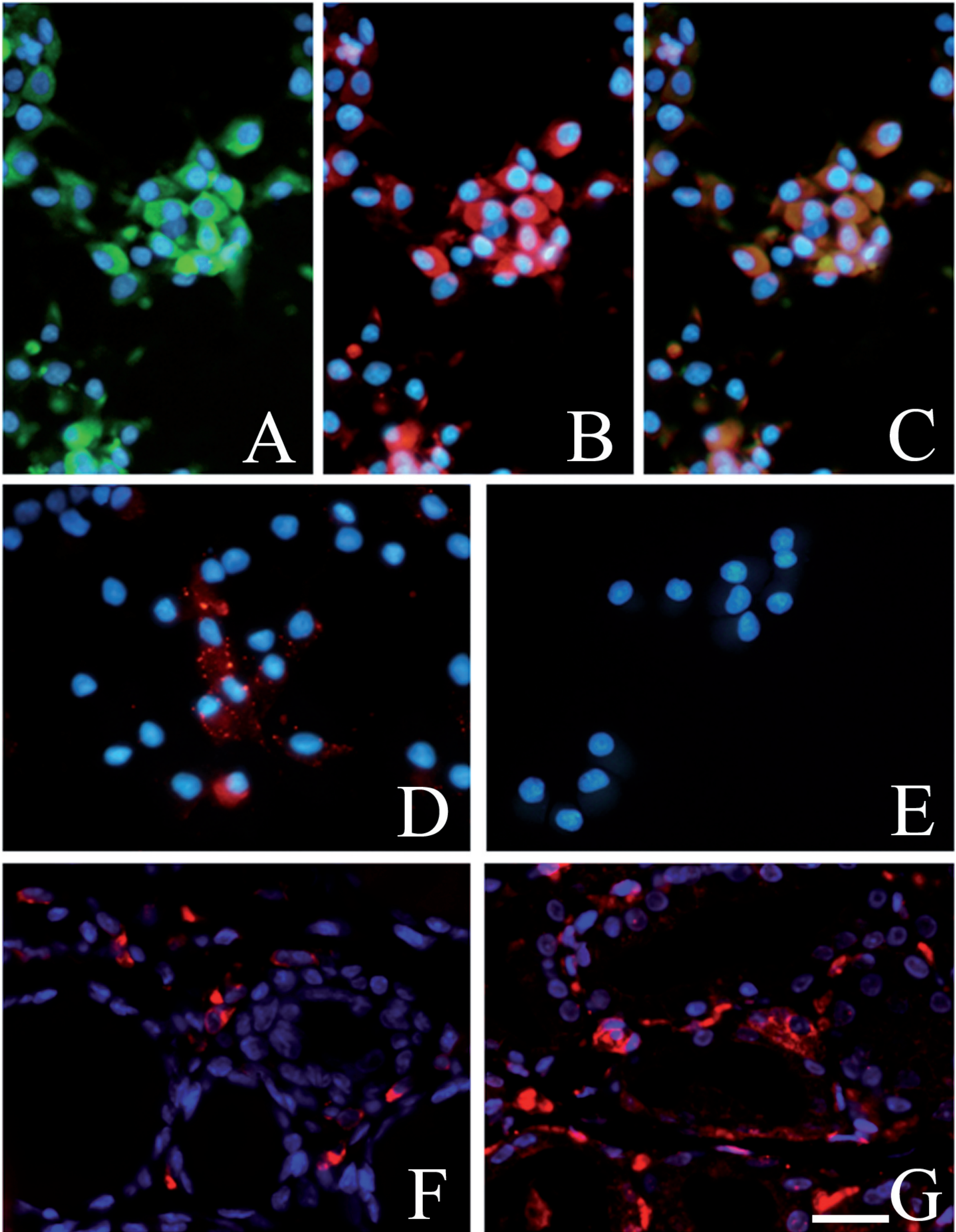

Fig. 3. Immunolocalization of AANAT and MT_1 in CA77 C-cell line **(A-E)** and rat thyroid tissue **(F, G)** by immunofluorescence. Immunopositivities for AANAT and MT_1 were detected in C cells of both origins according to a cytoplasmatic, and heterogeneous immunostaining patterns, respectively. Calcitonin **(A)**, AANAT **(B, F)**, merge **(C)**, MT_1 **(D, G)** and negative control **(E).** Bar: 25 μm.

calcitonin - and in thyroid C cells (Fig. 3A,F). Additionally, the cellular localization of MT_1 was examined in the same samples. A heterogeneous immunostaining pattern was observed in both C-cell line and thyroid sections, detecting MT_1 immunopositivity according to a cytoplasmic and membrane pattern (Fig. 3D,G).

Immunocolocalization for MT_1 and TGB in follicular cell cultures (PC-C13) and thyroid samples was also examined, detecting coexpression of both markers in the cytoplasm of some follicular cells (Fig. 4).

Discussion

C cells are mainly known for secreting calcitonin, a serum calcium decreasing hormone. Nevertheless, besides their theoretical role in calcium homeostasis, C cells may be involved in the intrathyroidal regulation of follicular cells, suggesting a possible interrelationship between the two endocrine populations in the thyroid gland. In this sense, C cells also synthesize and release a number of different regulatory peptides. Specifically, calcitonin-producing C-cells coexpress neuroendocrine

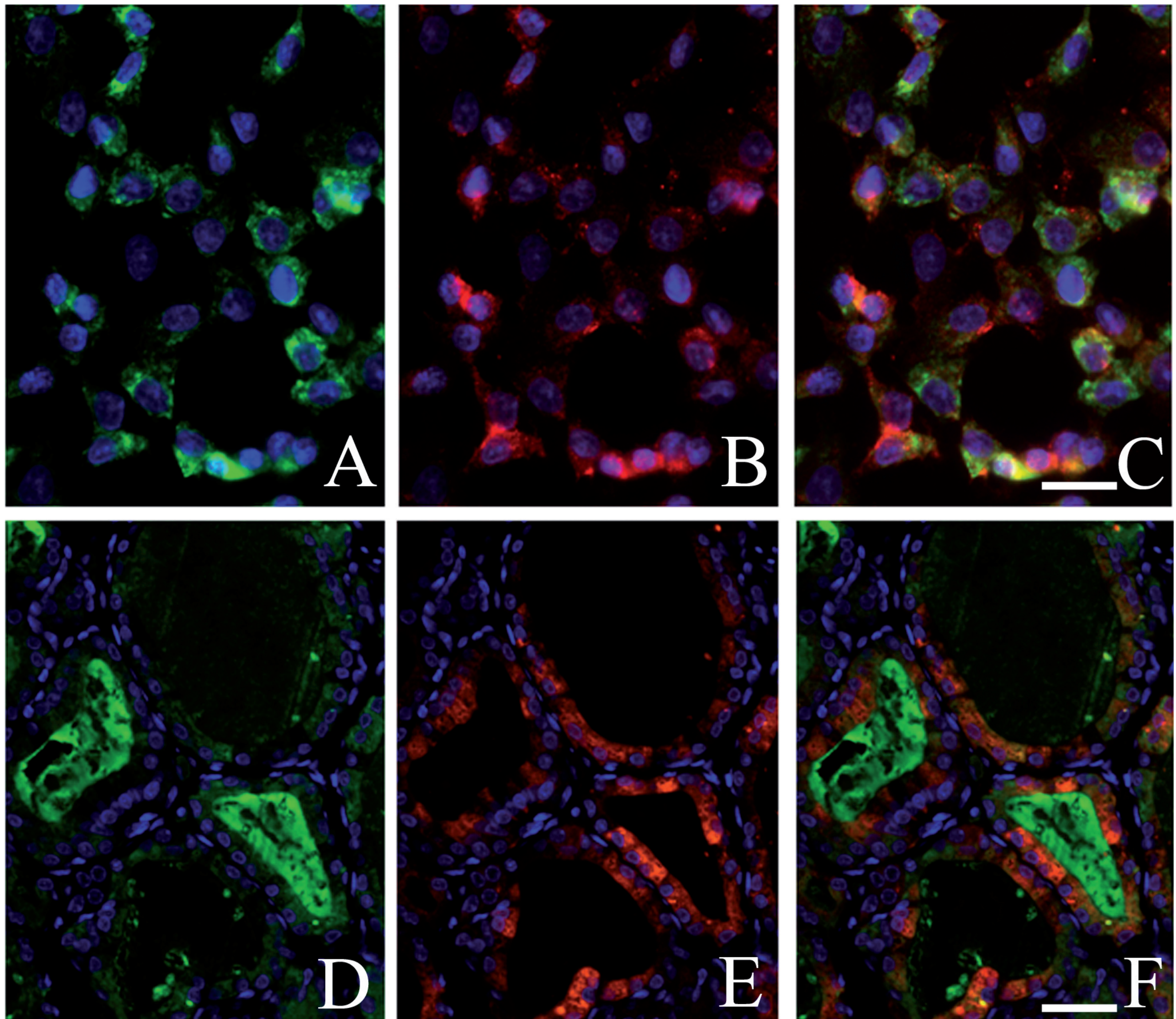

Fig. 4. Immunocolocalization of MT_1 and TGB in PC-C13 follicular cells **(A-C)** and rat thyroid tissue **(D-E)** by immunofluorescence. MT_1 was coexpressed with thyroglobulin in some follicular cells at cytoplasmatic level in cell cultures as well as in the follicular epithelium of thyroid sections. Thyroglobulin **(A, D)**, MT_1 **(B, E)**, merge **(C, F)**. Bars: A-C, 20 μm; D-F, 25 μm

peptides, such as somatostatin, serotonin, calcitonin gene-related peptide (CGRP), helodermin, pancreatic polypeptide and C-terminal gastrin/CCK (Van Noorden et al., 1977; Kameda et al., 1982; Cohn et al., 1984; Zabel, 1984; Nitta et al., 1986; Kameda, 1987; Zabel et al., 1987; Arias et al., 1989; Grunditz et al., 1989). Despite the fact that receptors for some of them, like somatostatin or serotonin, are expressed by follicular cells (Tamir et al., 1992, 1996; Ain et al., 1997), there is not a clear role assigned for these C-cell-secreted regulatory peptides yet. Recently, a few new regulatory peptides have been added to the list of markers expressed by C cells; however, in contrast to those listed above, these ones have well known roles on the hypothalamus–pituitary thyroid axis. Thus, Wierup et al. (2007) demonstrated that C cells express the cocaine and amphetamine regulated transcript (CART), a peptide expressed by neurons in the arcuate nucleus and involved in the inhibition of food intake, stimulation of energy expenditure and regulation of the hypothalamic-pituitary axis (Fekete et al., 2006; Fekete and Lechan, 2006). Furthermore, ghrelin and TRH, the two additional regulatory peptides which orchestrate the hypothalamic control of the thyroid function through thyrotropin, are also expressed in C cells (Gkonos et al., 1989; Howard et al., 1996; Skinner et al., 1998; Korbonits et al., 2001; De Miguel et al., 2005; Fekete and Lechan, 2006; Raghay et al., 2006). Although, the role of most of these molecules secreted by C cells within the thyroid gland remains unclear, our group has recently demonstrated a potentiating effect of ghrelin, on the TSH-induced hormone synthesis and proliferative activities, in rat thyrocytes (Morillo-Bernal et al., 2011).

It is well known that melatonin is not a pineal exclusive hormone. In the thyroid gland, immuno-positive C-cells using antibody to MT have been detected (Kvetnoy, 1999). However, this research does not implicate an endogenous synthesis due to the fact that melatonin can cross the biological membranes. In the present study, an endogenous melatonin biosynthesis in rat thyroid C-cell has been demonstrated. First, we have detected significant levels of melatonin in the CA77 culture medium. Second, the mRNA expression of two key melatonin biosynthetic enzymes, AANAT and HIOMT, has been confirmed in a rat C-cell line. Moreover, AANAT protein has been detected by immunofluorescense in rat C-cells. On the other hand, RT-PCR studies have shown that AANAT and HIOMT expression was either negative in thyroid follicular cells or too low to be detected by PCR. These results are in accordance with those of melatonin determination in culture media, which were also negative for PC-C13 cell line.

The presence of melatonin receptor in CA77, PC-C13 cell lines and thyroid tissue was also analyzed. If the hypothesis of melatonin having an additional role as local regulator synthesized by C cells is true, thyroid follicular cells must contain receptors for this hormone. The present study shows, for the first time, the presence of MT_1 at both mRNA and protein levels in the rat thyroid follicular cells in both cell line and thyroid tissue. Furthermore, immunopositive staining for MT_1 seemed to be higher in PC-C13 than in CA77, which could be related to differences in expression levels. Additional gene expression quantitative analyses are necessary to confirm this finding. Moreover, *in vivo* and *in vitro* experiments have also demonstrated a direct inhibitory effect of melatonin on intrathyroid hormone production (Pevet, 2000; Wright et al., 2000). On the other hand, melatonin synthesis by C cells may play a role in antioxidant defense to protect thyroid cells from oxidative stress. In this respect, many researchers have suggested the role of melatonin in the protection against oxidative damage during both physiological and pathological processes in the thyroid (Karbownik and Lewinski, 2003b; Mogulkoc et al., 2006; Rao and Chhunchha, 2010). Reactive oxygen species (ROS) are involved in cellular processes of the thyroid gland, as occurs in other organs. For example, hydrogen peroxide participates in different steps of hormone synthesis, as well as in the Wolff-Chaikoff's effect and in hypothyroidism caused by iodine excess in the thyroid (Karbownik and Lewinski, 2003a).

Finally, we have also described the presence of MT_1 in CA77 cells and rat thyroid tissue C-cells. It is interesting to consider the possibility of an autocrine regulation, common in endocrine cells, in the secretion of melatonin by thyroid C-cells. It is interesting the fact that a similar pattern was also exhibited by rat thyroid tissue, which contributes to clear up the possibility of unexpected AANAT, HIOMT or MT_1 expression as a consequence of cell transformation usually described in cell lines.

In conclusion, we have demonstrated that rat thyroid C-cells express the two key enzymes in melatonin biosynthesis, AANAT and HIOMT, suggesting that this hormone could be another local regulator synthesized by C-cells. This endogenous melatonin, together with pineal melatonin and other hormonal and non-hormonal agents, could modulate and regulate thyroid function and homeostasis (Sainz et al., 2003; Mocchegiani et al., 2006). On the other hand, rat thyroid C-cells and follicular cells express melatonin receptor MT_1. Although further studies are needed to evaluate the significance of MT_1 expression in thyroid cells and the paracrine/autocrine effects of melatonin in the thyroid, our data provide new evidence for a putative novel intrathyroidal regulatory pathway of thyroid regulation via paracrine/autocrine melatonin signalling that may be involved in thyroid-hormone synthesis or redox homeostasis.

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