Increases in the size, number and proliferation rate of VIP-immunoreactive pituitary cells induced in vitro by TRH are associated with increases in VIP release

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SUMMARY

Pituitary VIP levels and pituitary VIP-expressing cell numbers are increased in hypothyroidism, but the regulating role of TRH as regards morphological changes remains obscure. In order to determine whether TRH is involved in regulating the maintenance of pituitary VIP-expressing cells and its cellular proliferation a double immunocytochemical study for VIP and proliferating cell nuclear antigen (PCNA) was carried out in pituitary monolayer cultures treated with TRH. TRH induced a significant *in vitro* increase in the numerical density ($p<0.01$) and percentage ($p<0.001$) of VIP-expressing cells. These changes were accompanied by increases in VIP release ($p<0.01$) and in the size ($p<0.01$) of VIP-expressing cells. Our results suggest that TRH could regulate the maintenance of the percentage of pituitary VIP-expressing cells, their activity and their proliferation in a similar way to the way in which it regulates the release of VIP.

Key words: Pituitary gland – VIP – TRH – Immunohistochemistry – Cellular proliferation

INTRODUCTION

The existence of pituitary VIP-expressing cells is well documented (Morel et al., 1982; Nagy et al., 1988; Carrillo and Phelps, 1992; Carretero et al., 1992, 1994) and several factors have been implicated in the regulation of the synthesis and release of VIP, such as estrogens (Prysor-Jones et al., 1988; Köves et al., 1990; Lam et al., 1990; O'Halloran et al., 1990; Lam, 1991; Carrillo et al., 1991; Lasaga et al., 1991; Kasper et al., 1992; Watanobe and Takebe, 1992; Carretero et al., 1997), prolactin (Sarkar, 1989) or glucocorticoids (Jones et al., 1990; Lam et al., 1992). Additionally, thyroid hormones inhibit the synthesis of pituitary VIP (Segerson et al., 1989; Lam and Reichlin, 1989; Lam et al., 1989; Lam et al., 1990; Jones et al., 1994; Buhl et al., 1995); this inhibitory effect could be modulated by hypothalamic factors because it is abolished following anterolateral hypothalamic deafferentiation (Michalkiewicz and Suzuki, 1994).

The hypothalamic regulation of pituitary VIP may be mediated by inhibitory effects of dopamine, as previously demonstrated in our laboratory (Carretero et al., 1992, 1994, 1996), or by stimulatory signals induced by TRH, because TRH increases the *in vitro* pituitary secretion of VIP in glands from hypothyroid rats (Lam y Reichlin, 1989).

However, it is not known whether, like dopamine (Carretero et al., 1996), TRH is involved in regulating the proliferation and maintenance of pituitary VIP-expressing cell numbers.

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In order to determine whether TRH is involved in the regulation of VIP-expressing cell proliferation, a double immunocytochemical study for VIP and PCNA (proliferating cell nuclear antigen) was carried out on pituitary monolayer cultures obtained from the glands of euthyroid male rats. VIP release was determined by RIA and the numerical density, percentage, and cellular area of VIP-expressing cells and proliferating VIP-expressing cells were calculated.

Material and methods

Pituitary cultures. Following anaesthesia with isoflurane, male Wistar rats (175-200 g) were killed by decapitation and the anterior pituitary glands were removed and washed in Earle's balanced salt solution. Enzymatic dispersion was carried out by incubation for 15 minutes at 37ºC in Hank's solution to which 0.15% $MgCl_2$, 0.1% papain, 0.01% DNase and 0.1% of neutral protease had been added. Mechanical dispersion was achieved by passing the pituitaries through Pasteur pipettes and 20 to 22 gauge needles. After centrifugation, the supernatant was removed and the cells were resuspended in an appropriate volume of Dulbecco's modified Eagle's medium, supplemented with 10% calf serum, 2.5% foetal calf serum, 2% L-glutamine, 1000 IU/ml of penicillin and 1000 IU/ml of streptomycin. Cells were seeded on 40 culture chamber slides (Nunc, 1ml) at a final concentration of 2.5 x 10^5 cells/ml and incubated at 37ºC in a 5% CO_2/95% air atmosphere for 7 days. On the 4th day of incubation, the medium was replaced by fresh medium. On the 7th day of culture, the medium was removed and was replaced by medium to which 10^{-7}M TRH had been added with and the cultures were incubated for 3 hours. At the end of the incubation periods, the media were collected in ice-cold glass tubes containing 100ml 1N HCl, boiled for 5 min, and frozen until assayed. The chambers were carefully washed with Dulbecco's sterile PBS and the cells were then fixed in Somogyi solution for 1 hour, followed by careful rinsing in PBS. In order to verify the results obtained, two pituitary cultures were made under identical experimental conditions.

Radioimmunoassay (RIA) of VIP. VIP levels in the culture media were determined from duplicate aliquots of media by RIA, as described previously (Carretero et al., 1996). VIP was quantified by radioimmunoassay using an antiserum raised in rabbits against porcine VIP. VIP was conjugated to BSA with carbodiimide hydrochloride (Lorenzo et al., 1989). The dilution of the antiserum was 1: 250,000. Assay sensitivity was 7 pg/ml, the within- and between-assay variations being 4-6 and 10-15% respectively. Antiserum specificity was tested against pentagastrin, secretin, pancreatic polypeptide, insulin, glucagon, somatostatin, Arg-vasopressin, GnRH, b-endorphin, gastric intestinal peptide, GHRH and PHI, but none of these was observed to decrease the binding of ^{125}I-VIP.

Immunohistochemistry. A double-labelling immunohistochemical method for PCNA and VIP was developed. Endogenous peroxidase was blocked with H_2O_2 in methanol and non-specific reactions of the secondary antibody by incubation in normal goat serum (Dako, diluted 1:30). Cultures were incubated overnight at 4ºC with the mouse PC10 mAb (Dako, lot. 121 diluted 1:2000 in TBS). Biotinylated goat anti-mouse IgG (Dako, diluted 1:100) and Avidin-Biotinylated horseradish peroxidase complex (Dako, ABC kit, diluted 1:100) were successively applied at room temperature for 40 min and 30 min, respectively. The reaction was developed in freshly prepared 3-3' DAB (Sigma, 25% in TRIS buffer containing 0.03% of H_2O_2). Following the PCNA immunolabelling, the peroxidase-antiperoxidase (PAP) reaction was performed for the detection of VIP, using rabbit antiserum against VIP (Dako, diluted 1:1200). Following saturation of the specimens with normal mouse serum and normal swine serum, incubations with the primary antibody were performed overnight at 4ºC. Swine anti-rabbit IgG (Dako, diluted 1:100) was applied at room temperature for 30 min, followed by rabbit PAP complex (Dako, diluted at 1:100) for 30 min at room temperature. The second immunoreaction was developed in 4-chloro-1-naphthol (Sigma, 1.7x10^{-3}M in 3% absolute ethanol and TRIS-buffer containing 0.03% H_2O_2).

Quantification of VIP and PCNA-VIP immunoreactive cells. Four thousand cells per chamber were evaluated using an Axioplan Zeiss microscope equipped with an ocular grid at a final magnification of 400x. The cells were randomly selected from different areas of the chambers. Chambers with a strong agglomeration of overlapping cells were rejected and only non-overlapping cells were considered. Because the PCNA protein is known to have a 20-hour half-life in *in vitro* systems (Bravo et al., 1987) and is detected in the cytoplasm of cells that have recently completed mitosis (Hall et al., 1990; Coltrera and Gown, 1991) only nuclear PCNA expression was considered, except in metaphasic-mitotic cells. VIP-positive and VIP- and PCNA-positive cells were accounted and the percentage of VIP- and PCNA-positive cells from the total number of VIP-positive cells was calculated.

Numerical density of VIP cells. Numerical density was calculated as the number of immunoreactive cells per mm^2, from 100 micrographs (x500) of the chambers per treatment group chosen according to a randomised sys-

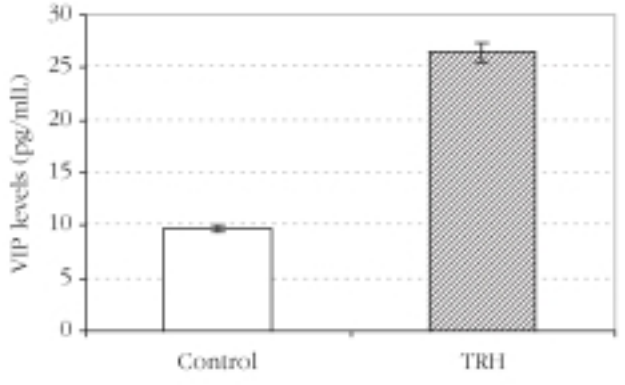

Figure 1.- Plot showing mean values of VIP levels in the culture media (pg/ml) obtained in control and TRH-treated cultures.

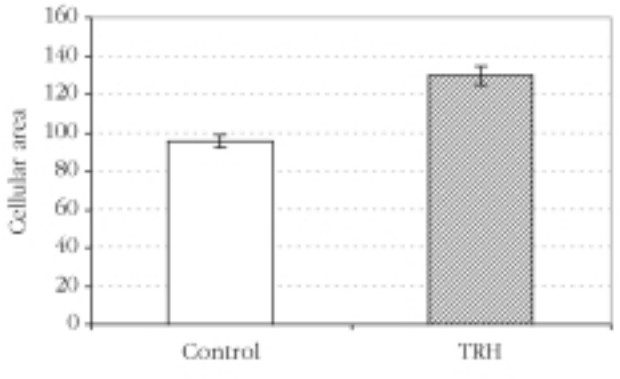

Figure 2.- Plot showing mean values of the cellular areas of VIP-positive cells (μm^2) obtained in control and TRH-treated cultures.

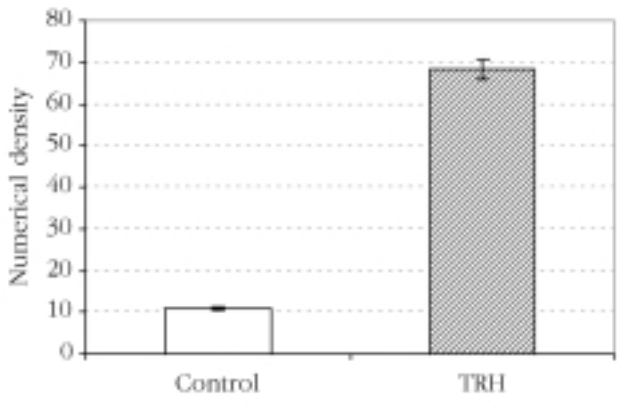

Figure 3.- Plot showing mean values of the numerical density of VIP-positive cells (number of cells/mm^2) obtained in control and TRH-treated cultures.

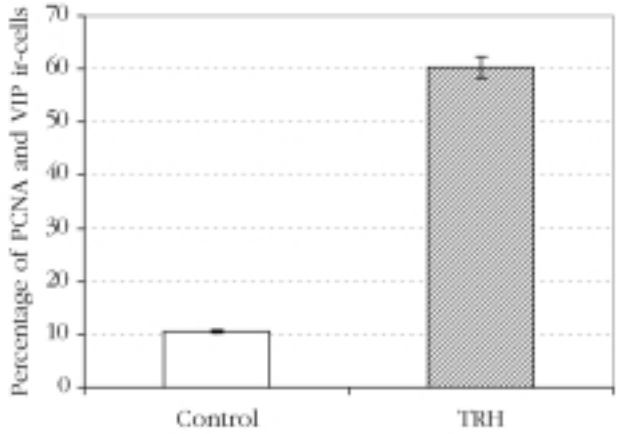

Figure 4.- Plot showing mean values of the percentage of proliferating (PCNA-positive) VIP-positive cells obtained in control and TRH-treated cultures.

tematic method (Weibel, 1969) from a pool of 500 micrographs (25 per chamber and group).

Morphometry. Using a MIP-2 automatic image analyser (IMCO-10, Kontron), the cellular area of 50 VIP-immunopositive cells per chamber was calculated in an interactive fashion. The cells were randomly selected from different zones of the chambers. For this purpose, the surface of the chamber was subdivided into 10 similar areas and 5 immunoreactive cells per area were measured.

Statistical analysis. Two pituitary cultures were carried out under similar experimental conditions in order to verify the reproducibility of the results (within-assay error was <1.5%). For each parameter evaluated (n=20), the values obtained were processed statistically. Data from individual experiments were subjected to ANOVA, followed by the Scheffé F test for multiple comparisons, accepting $p<0.05$ as significant. Results are expressed as arithmetical means ± SEM.

RESULTS

VIP levels in the culture media (Figure 1) were 2.5 fold higher after treatment with TRH than in control chambers (26.50±3.92 vs. 9.77±0.31 pg/ml, $p<0.01$).

After 3 hours of treatment, TRH had induced a significant increase in the cellular area (Figure 2) of the VIP-positive cells present in the cultures (129.77±6.49 vs. 95.62±4.78, $p<0.01$). Moreover, the numerical density of VIP-positive cells (Figure 3) was 6-fold higher after treatment with TRH than in control cultures (68.33±3.42 vs. 10.82±0.54, $p<0.001$).

The increase of numerical density observed following treatment with TRH was associated with an increase of the percentage of VIP-positive cells expressing PCNA. The percentages of proliferating VIP-positive cells were 5-fold higher in TRH-treated than in control cultures (60.38±3.02 vs. 10.52±0.53, $p<0.001$).

Discussion

PCNA is an auxiliary protein of DNA polymerase δ, necessary for DNA replication. It is expressed in the G1 phase, reaches a maximum in the S phase, and thereafter declines during the G2 and M phases of the cell cycle (Mathews et al., 1984; Tan et al., 1986; Prelich and Stillman, 1988). Although some discrepancies have been reported between PCNA immunocytochemical expression and data obtained with other cell proliferation-assessing methods, several studies have shown that the PCNA labelling index represents a valuable approach for the evaluation of cellular proliferation in the anterior pituitary gland (Oishi et al., 1993; Carretero et al., 1995a, 1995b, 1996, 1997). Moreover, the kinetics of anterior pituitary cells have been studied using the PCNA labelling index; this has been reported to be more sensitive than other methods, such as the BrdU labelling index (Oishi et al., 1983).

The presence of VIP-positive cells in the pituitary gland is well documented (Carrillo and Phelps, 1992) and the percentage of these cells is modified by changes in the endocrine environment or following hormonal treatment (Segerson et al., 1989; Lam et al., 1989; Köves et al., 1990; Lam 1991; Carretero et al., 1994).

We have previously demonstrated *in vitro* that sex hormones modify the percentage and proliferation of pituitary VIP-positive cells (Carretero et al., 1995, 1997, 1998). The findings of the present study, using PCNA as a marker of cellular proliferation, demonstrate that the proliferation rate of VIP pituitary cells is low in the control cultures and is significantly increased after treatment with TRH.

Also in previous studies, we have reported the *in vivo* and *in vitro* hypothalamic regulation of pituitary VIP (Carretero et al., 1992) and have described an important inhibitory role for dopamine (Carretero et al., 1994, 1996).

TRH could be another hypothalamic factor involved in the regulation of pituitary VIP, because incremental doses of TRH stimulate the release of VIP from hypothyroid pituitary cell cultures (Lam and Reichlin, 1989). However, no previous studies addressing the proliferation of VIP pituitary cells after *in vitro* treatment with TRH have been carried out.

This is the first *in vitro* study demonstrating that at doses that increase the release of pituitary VIP TRH increases the number, size and proliferation rate of pituitary VIP-positive cells.

Our findings suggest that increases in TRH could be involved in regulating the maintenance of the VIP-cell population in the pituitary gland and in the proliferative response of VIP pituitary cells to hypothyroidism.

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