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Inverse effects of estradiol and testosterone on the in vitro proliferation rate of rat VIP-immunoreactive pituitary cells

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Summary

Using double immunocytochemical labelling for Proliferating Cell Nuclear Antigen (PCNA) and Vasoactive Intestinal Peptide (VIP), a study was conducted to elucidate the repercussions of various doses (ranging from 10^{-8} to 10^{-5}M) of estradiol or testosterone (from 1 to 24 hours) administered to monolayer pituitary cultures on the proliferation rate, expressed as the PCNA-labelling index, of VIP-immunoreactive cells (PCNA-LI). The results are compared with those obtained in control cultures. Estradiol induced significant increases in the percentages of PCNA- and VIP-immunoreactive cells at each dose assayed as early on as one hour postadministration. The most efficient effect was observed for 10^{-5}M estradiol. For all time-points assayed, the percentages of PCNA- and VIP-immunoreactive cells were higher than in control dishes. Similar findings were observed when percentages of VIP-immunoreactive cells were analyzed. Testosterone decreased the percentages of VIP-immunoreactive or PCNA- and VIP-immunoreactive cells with respect to control dishes at all doses and time points analyzed; from 6 to 24 hours of treatment, the effects were less evident for 10^{-7} and 10^{-8}M testosterone than for the other doses assayed. The modifications observed in the proliferation rate and numerical density of VIP-immunoreactive cells were accompanied by increases, in the case of estradiol, and decreases, in the case of testosterone, in the release of VIP to the culture medium. In conclusion, our results suggest that estradiol and testosterone have opposite effects on the release of VIP from pituitary monolayer cultures and on the regulation of the *in vitro* proliferation of pituitary VIP-immunoreactive cells.

Key words: Pituitary - VIP - Gonadal steroids - Cellular proliferation - Immunocytochemistry

Introduction

The presence of VIP in the pituitary has been demonstrated by immunocytochemistry, RIA, HPLC and "in situ" hybridization (Samson et al., 1979; Besson et al., 1979; Rosztejn et al., 1980; Morel et al., 1982; Arnaout et al., 1986; Segerson et al., 1989; Lam et al., 1990; Köves et al., 1990; Lam, 1991) and the presence of pituitary VIP-immunoreactive cells is well documented (Köves et al., 1990; Carretero et al., 1992; Carrillo et al., 1992). However, there are gender-related differences –not well elucidated– between the levels of VIP, which are higher in males than in females, and the numbers of VIP-immunoreactive cells, which are similar for both sexes. These differences have been explained in terms of the notion that changes in the content of VIP could reflect parallel changes in cellular VIP contents rather than in the number of VIP-producing cells (Carrillo et al., 1992). This is because a sexual dimorphism in the regulation of the VIP gene transcription can be inferred from VIP mRNA contents, which are several-fold greater in the anterior pituitary of males than in females (Lam, 1991).

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The findings hitherto reported are conflictive. In this sense, treatment with estrogens modifies the content of VIP in the pituitary gland (Maletti et al., 1982; Köves et al., 1990; Lam et al., 1990) and the stimulatory effects of estradiol on the cellular activity and *in vitro* proliferation of pituitary VIP immunoreactive cells have previously been reported by our laboratory (Carretero et al., 1992). Neonatal androgenization induces an increase in pituitary gland VIP levels (Watanobe and Takebe, 1992) and treatment of both sexes with testosterone decreases the release and pituitary contents of the peptide (Lasaga et al., 1991). Also, inhibitory effects of testosterone on the cellular activity and *in vitro* proliferation of pituitary VIP immunoreactive cells have previously been reported by our laboratory (Carretero et al., 1997).

In previous studies we have demonstrated the *in vivo* and *in vitro* hypothalamic regulation of pituitary VIP and have reported an important inhibitory role for dopamine (Carretero et al., 1992, 1994, 1996). *In vivo*, dopamine levels and the pituitary effects of dopamine are modified by estradiol and testosterone (Ben-Jonathan et al., 1977; Raymond et al., 1978; Ferland et al., 1979; Demarest et al., 1981; Gudelsky and Porter, 1981; Gudelsky et al., 1981). Such changes could be related to the *in vivo* effects of gonadal steroids on pituitary VIP, as mentioned above.

With a view to avoiding other factors involved in the regulation of pituitary VIP, we were prompted to carry out an *in vitro* analysis of the effects of estradiol and testosterone on the release of VIP, on the percentage of VIP-immunoreactive cells, and on the proliferation rate of these cells in monolayer pituitary cultures.

As far as we are aware, no studies have been carried out in pituitary monolayer cultures to evaluate the repercussions of different doses and incubation times of treatment with estradiol or testosterone on the release of pituitary VIP and on the proliferation rate of VIP-immunoreactive pituitary cells. Additionally, the differences or similarities between the response of VIP-immunoreactive cells after treatment with testosterone or estradiol have not yet been studied.

The aim of this study is to elucidate whether estradiol and testosterone modulate the *in vitro* release of VIP, modify the percentage of VIP-immunoreactive cells and the proliferation rate of these cells -expressed as the immunocytochemical PCNA-labelling index of VIP-immunoreactive pituitary cells- and the levels of VIP in the culture media after treatment with estradiol (ranging from 10^{-8}M to 10^{-5}M) or testosterone (ranging from 10^{-7}M to 10^{-5}M) of pituitary monolayer cultures over 1 to 24 hours of treatment.

Materials and methods

Pituitary cultures. Following anaesthesia with isofluorane, male Wistar rats (175-200g) 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 37°C 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 streptomycine (Carretero et al., 1991, 1994; Sánchez et al., 1991). Cells were seeded on culture dishes (30 x 15 mm) at a final concentration of 5 x 10^5 cells/1.5 ml/dish and incubated at 37°C in a 5% CO_2/95% air atmosphere for 7 days. On the 4th day of incubation, the medium was replaced by freshly prepared medium. On the 7th day of incubation, the medium was replaced by fresh medium in the control dishes and fresh medium plus 10^{-8}, 10^{-7}, 10^{-6} or 10^{-5}M 17-ß-estradiol or 10^{-8}, 10^{-7}, 10^{-6} or 10^{-5}M testosterone in the treated dishes and incubated for 1, 3, 6, 12 or 24 hours. Previously, 17-ß-estradiol and testosterone were diluted in ethanol, the final concentration of ethanol in the treated and control dishes being 0.0027%. Five dishes per treatment and time were employed. In order to verify the results obtained, four pituitary cultures under identical experimental conditions were carried out. At the end of each experiment, media were collected in ice-cold glass tubes containing 100µl 1N HCl, boiled for 5 minutes and frozen until assayed. The dishes were carefully washed with Dulbecco's sterile PBS, and the cells were fixed in Somogyi solution for 30 min, followed by careful rinsing in PBS.

Radioimmunoassay of VIP. The levels of VIP in the culture media were determined from duplicate aliquots of media by RIA, as described by Lorenzo et al. (1989; 1992). VIP was quantified by radioimmunoassay using an antiserum raised in rabbits against porcine VIP. VIP was conjugated to BSA with carbodiimide hydrochloride. The dilution of the antiserum was 1:250,000. Assay sensitivity was 9 pg/tube, the within- and between assay variations being 4-6 and 10-15% respectively.

Immunocytochemistry. To study PCNA-positive cells and to determine the PCNA-VIP labelling index, 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 4°C with mouse anti-PCNA monoclonal antibody (PC10, Dako, lot. 121 diluted 1:2000 in TBS). Biotinylated goat anti-mouse IgG (Dako, lot. 061 diluted 1:100) and Avidin-Biotinylated horseradish peroxidase complex (ABC kit, Dako lot. 081 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 (0.025% in TRIS buffer containing 0.03% of H_2O_2). Following PCNA immunolabelling, the peroxidase-antiperoxidase (PAP) reaction was performed for the detection of VIP, using as primary serum anti-VIP rabbit serum at a dilution of 1:800, swine anti-rabbit serum (Dako, diluted 1:100), and rabbit-PAP complex (Dako, diluted 1:100). The characterization and specificity of the primary serum had been checked in a previous study (10). Preabsorption tests with VIP and tests substituting the specific serum by normal rabbit serum abolished the reaction. By ELISA, the specificity of swine anti-rabbit IgG was lower than 1% for rat and Mouse IgG and 100% for rabbit IgG. For the washes and dilutions of the sera, Tris buffer (0.05 M, pH7.4) containing 0.8% NaCl was used. The reaction was developed in freshly prepared 4-chloro-1-naphthol ($1.7x10^{-3}$ M in 3% absolute ethanol and TRIS-buffer containing 0.3% H_2O_2).

Quantification of VIP and PCNA-VIP immunoreactive cells. Four thousand cells per dish 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 dishes. Dishes with a high degree of 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 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. The following parameters were determined: 1) The total number of cells, 2) VIP-positive cells and 3) PCNA- and VIP-positive cells; 2 and 3 were calculated as percentages from the total number of cells analyzed. The percentage of PCNA- and VIP-immunoreactive cells from the VIP-positive cells was also calculated.

Statistical analysis. Four pituitary cultures were grown under similar experimental conditions in order to verify the reproducibility of the results (within-assay error was always < 1.5%). For each parameter evaluated, the values obtained were processed statistically and the differences observed were compared using analysis of variance, accepting p values of <0.05 as significant for the Fisher-PLSD and Scheffé F tests jointly. The results are expressed as arithmetic means ± SD.

Results

VIP levels. Table 1 summarizes the levels of VIP in the media found in all experiments. VIP levels were very similar for all time points assayed in the control dishes, ranging from 78.5 to 90.38 pg/ml. Estradiol always induced a significant increase in VIP levels ($p<0.01$ to $p<0.005$, for all doses and time points analyzed with respect to control dishes). The effects were more evident for higher than for lower doses: VIP levels were higher for 10^{-5}M than for 10^{-6}M estradiol ($p<0.01$). Similar values were found for 10^{-6}M and 10^{-7}M estradiol after 1,3 or 6 hours of treatment and were significantly higher after 12 or 24 hours ($p<0.05$). Except after 1 and 3 hours of treatment, VIP levels after treatment with 10^{-7}M estradiol were higher than those observed after treatment with 10^{-8}M ($p<0.05$). Treatment with testosterone decreased the levels of VIP. The effects of 10^{-5} and 10^{-6}M testosterone were very evident and similar from 1 to 24 hours ($p<0.01$ with respect to control dishes for both doses and all time points assayed). After 1 hour of treatment, the VIP levels found for 10^{-7} and 10^{-8}M testosterone were similar to those

Treatment	1 hour	3 hours	6 hours	12 hours	24 hours
Control	78.50±1.05	87.83±1.20	81.35±1.25	86.45±1.34	90.38±1.43
Estradiol					
10^{-5}M	329.20±16.13	293.70±14.09	311.50±17.44	409.40±21.29	364.90±18.97
10^{-6}M	263.16±10.26	224.70±10.56	199.80±10.79	200.25±10.41	215.38±11.19
10^{-7}M	258.14±10.84	207.08±9.32	202.03±10.30	158.42±8.24	132.61±6.91
10^{-8}M	240.33±12.02	195.86±10.38	176.22±10.04	119.26±6.68	105.47±5.48
Testosterone					
10^{-5}M	56.77±2.72	29.06±1.34	20.59±0.89	18.46±1.87	44.17±2.14
10^{-6}M	57.67±2.77	34.98±1.61	21.95±1.25	34.79±2.22	51.51±2.05
10^{-7}M	72.81±3.42	48.68±2.39	45.34±2.73	44.79±2.31	56.19±2.86
10^{-8}M	79.61±3.74	59.96±2.69	46.83±2.71	48.74±2.60	79.61±3.31

Table 1.– Effect of different doses of estradiol or testosterone and different time points of study on VIP levels in media.

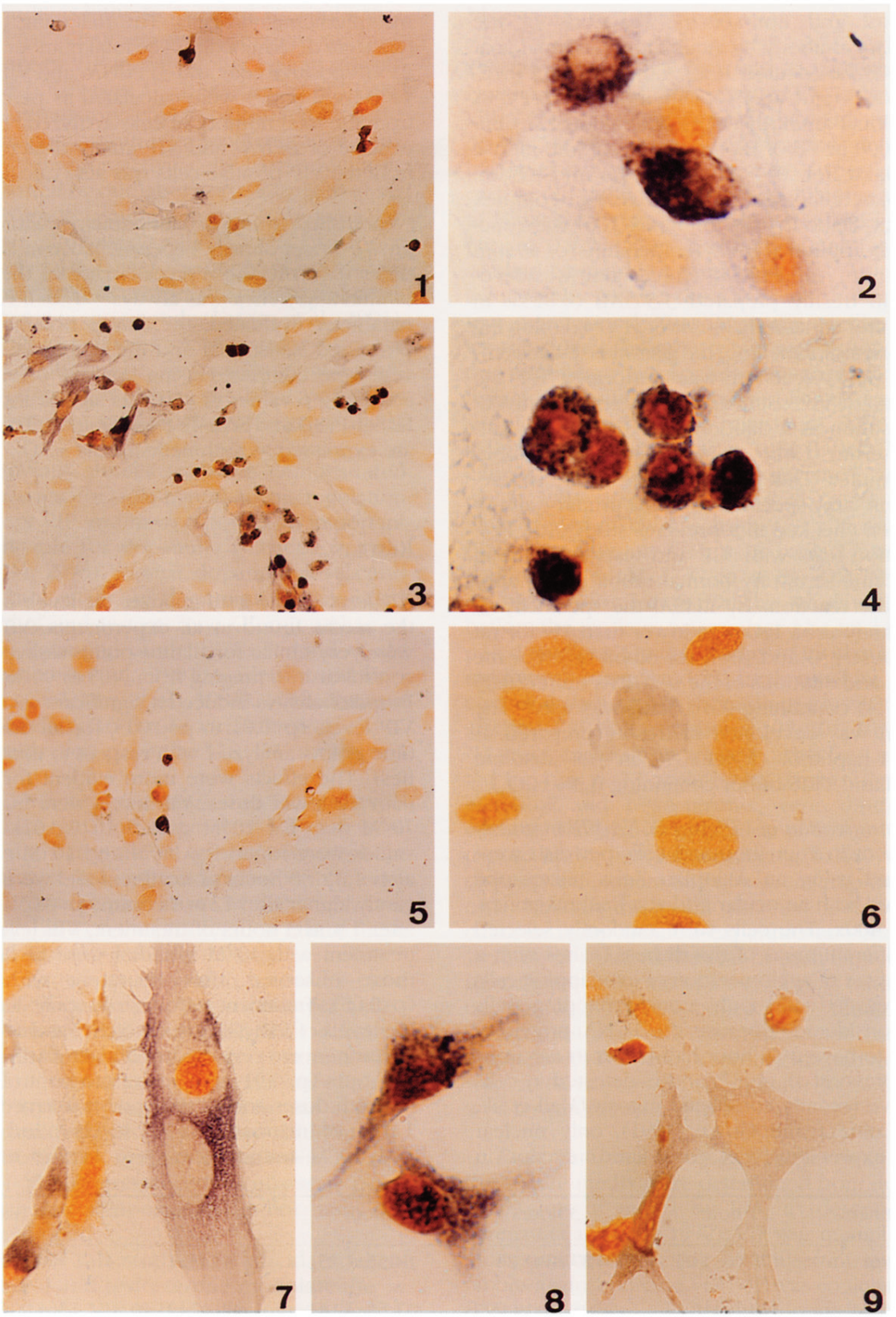

Fig. 1.– Micrograph from a control dish showing scattered VIP-immunoreactive cells (arrows). x 200.
Fig. 2.– Grouped and strongly-stained VIP-immunoreactive cells in a dish treated with estradiol (arrows). x 1000.
Fig. 3.– Scattered, scarce VIP-immunoreactive cells after treatment with testosterone (arrows). x 200.
Fig. 4.– Round or oval strongly-stained and granulated VIP-immunoreactive cells in a control dish. x 1000.
Fig. 5.– Round VIP-immunoreactive cells after treatment with estradiol (arrows). The cells appeared grouped and their cytoplasm showed a very strong and granulated reaction. The nuclear expression for PCNA (brown) was very frequent in these cells. x 200.
Fig. 6.– Oval and weakly-stained VIP-immunoreactive cell after treatment with testosterone (arrow). The granular aspect of the cytoplasm was lost. x 1000
Fig. 7.– Fusiform VIP-immunoreactive cell from a control dish (arrow). x 600.
Fig. 8.– Stellate PCNA- and VIP-immunoreactive cells (arrows) after treatment with estradiol. x 1000.
Fig. 9.– Stellate VIP-immunoreactive cells after treatment with testosterone (arrows). Several stellate cells of this group of treatment were immunoreactive for PCNA. x 600.

obtained in control dishes; from 3 to 12 hours they were significantly lower ($p<0.05$) and after 24 hours of treatment, 10^{-7}M testosterone significantly decreased VIP levels ($p<0.05$) while 10^{-8}M testosterone did not.

Morphological findings. Scattered VIP-immunoreactive cells appeared on the bottom of the dishes (Figs. 1 and 3) and only after treatment with estradiol were groups of clustered VIP-immunoreactive cells found (Fig.2). As reported in a previous study (13), two different types of VIP-immunoreactive cells were found in the pituitary cultures: round or oval cells (Figs. 4 to 6) and fusiform or stellate cells (Figs. 7 to 9). In the controls, and more evident in the estradiol-treated dishes, weakly-stained non-granular cells were found. The reaction for PCNA appeared in the nuclei of both round and stellate cells after treatment with estradiol (Figs. 5 and 8). However, only stellate cells showed a reaction for PCNA following treatment with testosterone (Fig. 9).

Percentage of VIP-immunoreactive cells. Table 2 summarizes the percentages of VIP-immunoreactive cells obtained in the different treatments. In the control dishes, the percentages of VIP-immunoreactive cells observed from 0 to 24 hours of incubation were very similar and no significant differences were found. Estradiol increased the percentage of VIP-immunoreactive cells, each dose assayed significantly increasing this percentage ($p<0.01$). For 1 or 3 hours of treatment, no significant differences among the doses assayed were found; however, after 6,12 or 24 hours of treatment the effects were more evident for high than for low doses.

Testosterone decreased the percentage of VIP-immunoreactive cells for all doses and times studied. These decreases were more prominent for high than for low doses. After 6, 12 and 24 hours of treatment, the decreases induced by 10^{-8}M and 10^{-7}M testosterone were less manifest than those observed for 10^{-6} and 10^{-5}M ($p<0.05$).

Percentages of PCNA- and VIP-immunoreactive cells. Tables 3 and 4 summarize the percentages of PCNA- and VIP-immunoreactive cells obtained in the different treatments. The proliferation rate of VIP-immunoreactive cells in control dishes was very similar for all time points analyzed in the study. Estradiol induced an important

Treatment	1 hour	3 hours	6 hours	12 hours	24 hours
Control	20.93±1.05	23.65±1.20	25.16±1.25	24.71±1.34	25.64±1.43
Estradiol					
10^{-5}M	29.49±1.39	30.53±1.43	38.53±1.81	42.17±2.08	49.31±2.22
10^{-6}M	27.90±1.31	33.59±1.57	37.17±1.75	36.22±1.63	39.17±1.76
10^{-7}M	26.83±1.26	31.96±1.50	30.70±1.43	31.30±1.40	35.05±1.60
10^{-8}M	26.13±1.38	30.84±1.45	31.98±1.47	28.04±1.26	28.07±1.26
Testosterone					
10^{-5}M	4.55±0.22	4.40±0.21	10.14±0.48	10.42±0.47	10.80±0.49
10^{-6}M	7.30±0.34	7.99±0.38	11.02±0.49	10.53±0.47	10.84±0.58
10^{-7}M	8.98±0.42	8.33±0.39	15.03±0.68	14.54±0.65	17.47±0.68
10^{-8}M	10.98±0.52	8.51±0.42	15.38±0.70	14.44±0.64	17.18±0.79

Table 2.– Effect of different doses of estradiol or testosterone and different time points of study on labelling index for VIP, expressed as percentage of VIP-immunoreactive cells from total amount of cells per dish.

Treatment	1 hour	3 hours	6 hours	12 hours	24 hours
Control	8.15±0.41	9.60±0.48	9.03±0.45	8.79±0.43	9.22±0.44
Estradiol					
10^{-5}M	18.86±0.81	21.17±1.29	22.08±1.98	23.30±1.47	19.79±1.11
10^{-6}M	18.10±0.78	18.50±1.13	19.02±0.95	19.82±1.21	18.50±0.91
10^{-7}M	17.29±0.74	20.36±1.24	15.12±1.09	16.60±0.86	17.20±0.91
10^{-8}M	17.36±0.75	16.77±1.52	12.89±0.83	10.32±0.54	11.36±0.55
Testosterone					
10^{-5}M	1.45±0.06	0.80±0.06	3.78±0.23	3.11±0.19	3.38±0.21
10^{-6}M	2.58±0.11	2.38±0.15	3.66±0.22	3.43±0.21	3.51±0.21
10^{-7}M	2.23±0.09	1.29±0.08	6.09±0.42	5.74±0.41	6.19±0.38
10^{-8}M	3.92±0.24	3.32±0.20	6.62±0.40	5.17±0.32	5.23±0.32

Table 3.– Effect of different doses of estradiol or testosterone and different time points of study on labelling index for PCNA and VIP, expressed as percentage of PCNA- and VIP-immunoreactive cells from total amount of cells per dish.

increase in the percentage of PCNA- and VIP-immunoreactive cells (see Table 3). For all doses assayed, after 1 hour of treatment percentages twice as high as those detected in the control dishes ($p<0.01$) were observed. The values found after treatment with estradiol were always higher than in control dishes and the effects of estradiol were more evident for high than for low doses. Estradiol also increased the percentages of PCNA- and VIP-immunoreactive cells when they were calculated only from the VIP-immunoreactive cells (see Table 4).

By contrast, testosterone induced important decreases in the PCNA- and VIP-immunoreactive cells for all doses and time points analyzed (see Table 3). As described above for the percentages of VIP-cells, the inhibitory effects induced by testosterone were more evident for high than for low doses and after 6,12 and 24 hours of treatment the percentages observed for 10^{-8}M and 10^{-7}M testosterone were higher than those observed for 10^{-6} and 10^{-5}M ($p<0.05$), although they were always lower than those found in control dishes ($p<0.01$). Testosterone also decreased the percentages of PCNA- and VIP-immunoreactive cells when only VIP-immunoreactive cells were considered (see Table 4).

Treatment	1 hour	3 hours	6 hours	12 hours	24 hours
Control	8.15±0.41	9.60±0.48	9.03±0.45	8.79±0.43	9.22±0.44
Estradiol					
10^{-5}M	18.86±0.81	21.17±1.29	22.08±1.98	23.30±1.47	19.79±1.11
10^{-6}M	18.10±0.78	18.50±1.13	19.02±0.95	19.82±1.21	18.50±0.91
10^{-7}M	17.29±0.74	20.36±1.24	15.12±1.09	16.60±0.86	17.20±0.91
10^{-8}M	17.36±0.75	16.77±1.52	12.89±0.83	10.32±0.54	11.36±0.55
Testosterone					
10^{-5}M	1.45±0.06	0.80±0.06	3.78±0.23	3.11±0.19	3.38±0.21
10^{-6}M	2.58±0.11	2.38±0.15	3.66±0.22	3.43±0.21	3.51±0.21
10^{-7}M	2.23±0.09	1.29±0.08	6.09±0.42	5.74±0.41	6.19±0.38
10^{-8}M	3.92±0.24	3.32±0.20	6.62±0.40	5.17±0.32	5.23±0.32

Table 4.– Effect of different doses of estradiol or testosterone and different time points of study on labelling index for PCNA and VIP, expressed as percentage of PCNA- and VIP-immunoreactive cells from total of VIP-immunoreactive cells.

Discussion

It has been reported that estrogens increase the pituitary contents of the peptide and increase the pituitary levels of VIP mRNA (Prysor-Jones et al., 1988; Montagne et al., 1989; O'Halloran et al., 1990; Lam et al., 1990; Lam, 1991; Lasaga et al., 1991; Carrillo et al., 1991, 1992; Carretero et al., 1992; Kasper et al., 1992). It has been suggested that the effects of estradiol could be mediated by the hyperprolactinemia induced by treatment with estradiol (Montagne et al., 1989). However, no modifications in pituitary VIP synthesis were found following hyperprolactinemia induced by lactation (Köves et al., 1990). Decreases in pituitary VIP in hyperprolactinemia after dopaminergic inhibition have been found (Prysor-Jones et al., 1987) and estradiol has been reported to induce VIP and prolactin gene expression in the rat anterior pituitary, independently of plasma prolactin levels (Montagne et al., 1995). The present study points to a stimulatory effect of 17ß-estradiol for all doses and time points assayed. The effect was more evident for higher than for lower doses. The decreases in VIP observed in prolonged treatments with lower doses of estradiol can be accounted for by the fact that VIP is internalized inside pituitary cells (Morel et al., 1982) and may be degraded by protease activity (Keltz et al., 1980). The *in vitro* inhibitory effect of testosterone on the release of VIP is consistent with the *in vivo* decreases in the release and pituitary contents of the peptide (Lasaga et al., 1991).

Together with the effects of estradiol and testosterone on the secretion of VIP described by other authors and confirmed *in vitro* in the present study, our findings point to a marked effect of estradiol and testosterone on the percentage and the proliferation rate of VIP-immunoreactive cells. The appearance of VIP-immunoreactive cells in pituitary monolayer cultures is consistent with the evidence for VIP in the pituitary (Segerson et al., 1989; Lam et al., 1990). Although some authors have been unable to detect VIP-reactive cells with immunocytochemistry in the pituitary glands of untreated males (Lam et al., 1989), others have reported that such cells do appear in these animals (Köves et al., 1990; Carrillo and Phelps, 1992; Carretero et al., 1996) and that they are well differentiated in monolayer pituitary cultures (Carretero et al., 1994).

The cell origin of anterior pituitary VIP remain to be determined. There are reports that VIP is synthesized in the rat lactotroph (Nagy et

al., 1988; Morel et al., 1982); however other studies fail to provide convincing evidence for the colocalization of VIP and prolactin in the pituitary of untreated animals these colocalization has been reported to occur in a small number of anterior pituitary cells in hyperestrogenized rats (Lam et al., 1989; Steel et al., 1989; Köves et al., 1990; Carrillo and Phelps, 1992). The existence of different morphological types of in vitro VIP-immunoreactive cells suggest that, without rejecting the possibility of coexistence with prolactin, VIP is expressed in different populations of anterior pituitary cells.

No modifications of VIP-reactive cells after treatment with estradiol benzoate to ovariectomized rats were found when they were compared to normal diestrous female rats (Carrillo and Phelps, 1992) and, *in vivo* , colchicine and haloperidol increase the numerical density of VIP-immunoreactive cells in male rats (Carretero et al., 1992, 1994). However, the results reported by these authors cannot be compared with those of the present study because the findings were calculated from *in vivo* studies and considered the number of VIP cells per section of the pituitary gland or per surface, but not as percentages, as we report in our *in vitro* study.

In a previous work we reported increases in the percentages of VIP-immunoreactive cells and PCNA- and VIP-immunoreactive cells after 3 hours of treatment with 10^{-7}M 17ß-estradiol (Carretero et al., 1995). Very similar values were found for this dose and time point in the present study. Here, we report a stimulatory and sustained effect of estradiol from 1 to 24 hours for this and other doses of 17ß-estradiol. This is well correlated with the release of the peptide into the cultures. In sum, the results obtained with estradiol in the present study as regards the proliferation rate of VIP-immunoreactive cells confirm previous findings and hint at the *in vitro* mitogenic role of estradiol on pituitary VIP-immunoreactive cells.

Decreases in the size and proliferation rate of VIP-immunoreactive cells induced in vitro by testosterone are associated with decreases in VIP release after 3 hours of treatment with 10^{-6}M testosterone, as reported in a previous work (Carretero et al., 1997). Here we report an *in vitro* inhibitory effect of testosterone on VIP. The present results suggest an important inhibitory role for testosterone in the regulation of the proliferation rate of pituitary VIP-immunoreactive cells and suggest that these effects are sustained in *in vitro* studies.

To conclude, our results demonstrate the existence of an *in vitro* antagonic modulation exercised by gonadal steroids on the regulation of the percentage of VIP-immunoreactive cells and their proliferation that could be related to *in vivo* sex differences.

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References

Arnaout MA, Garthwaite TL, Martinson DR and Hagen TC (1986). Vasoactive intestinal polypeptide is synthesized in anterior pituitary tissue. *Endocrinology*, 119: 2052-2057.

Ben-Jonathan N, Oliver C, Weiner HJ, Mical RS and Porter JC (1977). Dopamine in hypophysial portal plasma of the rat during the estrous cycle and throughout pregnancy. *Endocrinology*, 100: 452-458.

Besson J, Rotsztejn W, Laburthe M, Epelbaum J, Beaudet A, Kordon C and Rosselin G (1979). Vasoactive intestinal peptide (VIP): brain distribution, subcellular localization and effect of deafferentation of the hypothalamus in male rats. *Brain Res*, 165: 79-85.

Bravo R, Frank R, Blundell PA and MacDonald-Bravo H (1987). Cyclin-PCNA is the auxiliary protein of DNA polymerase delta. *Nature*, 326: 515-517.

Carrillo AJ and Phelps CJ (1992). Quantification of vasoactive intestinal peptide immunoreactivity in the anterior pituitary glands of intact male and female, ovariectomized, and estradiol benzoate-treated rats. *Endocrinology*, 131: 964-969.

Carrillo AJ, Doherty PC, Guan X, Sturteant JR and Walro DG (1991). Preferential increase of pituitary vasoactive intestinal peptide as a function of estradiol benzoate dose in the ovariectomized rat. *Endocrinology*, 128: 131-138.

Carretero J, Sánchez F, Vázquez R, Cacicedo L, Sánchez-Franco F, Fernández G and Montero M (1991). In vivo and in vitro evidence of growth hormone-releasing factor-like produced locally in the adenohypophyseal cells of the rat. *Neuropeptides*, 19: 223-229.

Carretero J, Sánchez F, Rubio M, Lorenzo MJ, Francos M, Cacicedo L, Sánchez-Franco F and Vázquez R (1992). Immunocytochemical evidence of hypothalamic regulation of adenohypophyseal VIP in the male rat. *Neuropeptides*, 23: 239-243.

Carretero J, Sánchez F, Rubio M, Francos CM, Blanco J and Vázquez R (1994). In vitro and in vivo evidence for direct dopaminergic inhibition of VIP-immunoreactive pituitary cells. *Neuropeptides*, 27: 1-6.

Carretero J, Rubio M, Sánchez F, Vázquez RJ, Santos M, Blanco J and Vázquez R (1995). In vitro morphometric and proliferative variations in VIP-immunoreactive pituitary cells induced by estradiol. *Neuroendocrinology*, 62: 277-282.

Carretero J, Vázquez RJ, Santos M, Cacicedo L, Rubio M, Sánchez-Franco F and Vázquez R (1996). Dopamine inhibits in vitro release of VIP and proliferation of VIP-immunoreactive pituitary cells. *Neuropeptides*, 30: 81-86.

Carretero J, Rubio M, Vázquez-Perfecto RJ, Sánchez F, Torres JL, Pérez RM and Vázquez R (1997). Decreases in the size and proliferation rate of VIP-immunoreactive cells induced in vitro by testosterone are associated with decreases in VIP release. *Neuroendocrinology*, 65: 173-178.

Coltrera MC and Gown AM (1991). PCNA/cyclin expression and BrDU uptake define different subpopulations in different cell lines. *J Histochem Cytochem*, 39: 23-30.

DEMAREST KT, MCKAY DW, RIEGLE GD and MOORE KE (1981). Sexual differences in tuberoinfundibular dopamine nerve activity induced by neonatal androgen exposure. *Neuroendocrinology*, 32: 108-113.

FERLAND L, LABRIE F, EUVRARD C and RAYNAUD JP (1979). Anti-dopaminergic activity of estrogens on prolactin release at the pituitary level in vivo. *Mol Cell Endocrinol*, 14: 199-204.

GUDELSKY GA and PORTER JC (1981). Sex-related difference in the release of dopamine into hypophysial portal blood. *Endocrinology*, 109: 1394-1398.

GUDELSKY GA, NANSEL DD and PORTER JC (1981). Role of estrogen in the dopaminergic control of prolactin secretion. *Endocrinology*, 108: 440-444.

HALL PA, LEVISON DA, WOODS AL, YU CCW, KELLOCK DB, WATKINS JA, BARNES DM, GILLET CE, CAMPLEJOHN R, DOVER R, WASEEM NH and LANE DP (1990). Proliferating cell nuclear antigen (PCNA) immunolocalization in paraffin sections: an index of cell proliferation with evidence of regulated expression in some neoplasms. *J Pathol*, 162: 285-294.

KASPER S, POPESCU RA, TORSELLO A, VRONTAKIS ME, IKEJANI C and FRIESEN HG (1992). Tissue-specific regulation of vasoactive intestinal peptide messenger ribonucleic acid levels by estrogen in the rat. *Endocrinology*, 130: 1796-1801.

KELTZ TN, STRAUS E and YALOW RS (1980). Degradation of vasoactive intestinal polypeptide by tissue homogenates. *Biochem Biophys Res Com*, 92: 669-674.

KÖVES K, GOTTSCHALL PE, GORCS T, SCAMMELL JG and ARIMURA A (1990). Presence of immunoreactive vasoactive intestinal polypeptide in anterior pituitary of normal male and long term estrogen-treated female rats: a light microscopic immunohistochemical study. *Endocrinology*, 126: 1756-1763.

LAM KSL (1991). Vasoactive intestinal peptide in the hypothalamus and pituitary. *Neuroendocrinology*, 53 (S1): 45-51.

LAM KSL, LECHAN RM, MINIMITANI N, SEGERSON P and REICHLIN S (1989). Vasoactive intestinal peptide in the anterior pituitary is increased in hypothyroidism. *Endocrinology*, 124: 1077-1084.

LAM KSL, SRIVASTAVA G, LECHAN RM, LEE T and REICHLIN S (1990). Estrogen regulates the gene expression of vasoactive intestinal peptide in the anterior pituitary. *Neuroendocrinology*, 52: 417-421.

LASAGA M, DUVILANSKI BH, SEILICOVICH A, AFIONE S, DÍAZ MC, PISERA D, TRAKTENBERG R and DEBELJUK L (1991). The effects of gonadal steroids on vasoactive intestinal peptide concentration and release from mediobasal hypothalamus and the anterior pituitary gland. *J Neuroendocrinol*, 3: 75-78.

LORENZO MJ, SÁNCHEZ-FRANCO F, DE LOS FRAILES MT, REICHLIN S, FERNÁNDEZ G and CACICEDO L (1989). Synthesis and secretion of vasoactive intestinal peptide by rat fetal cerebral cortical and hypothalamic cells in culture. *Endocrinology*, 125: 1983-1990.

LORENZO MJ, SÁNCHEZ-FRANCO F, DE LOS FRAILES MT, TOLÓN RM, FERNÁNDEZ G and CACICEDO L (1992). Thyroid hormones regulate release and content of vasoactive intestinal peptide in cultured fetal cerebral cortical cells. *Neuroendocrinology*, 55: 59-65.

MALETTI M, ROTSZTEJN WH, CARR L, SCHERRER H, RETTEN D, KORDON C and ROSSELIN G (1982). Interaction between estradiol and prolactin on vasoactive intestinal peptide concentrations in the hypothalamus and in the anterior pituitary of the female rat. *Neurosci Lett*, 32: 307-313.

MONTAGNE MN, VIAL M, JOUBERT-BRESSION D and ROSTENE W (1989). Hyperprolactinemia-induced modifications in vasoactive intestinal peptide binding site densities: evidence for an interaction between estradiol 17-ß and prolactin effects. *Brain Res*, 485: 258-266.

MONTAGNE MN, DUSSAILLANT M, CHEW LJ, BEROD A, LAMBERTS SJ, CARTER DA and ROSTENE W (1995). Estradiol induces vasoactive intestinal peptide and prolactin gene expression in the rat anterior pituitary independently of plasma prolactin levels. *J Neuroendocrinol*, 7: 225-231.

MOREL G, CHAYVEALLE JA, KERDELHNE B and DUBOIS PM (1982). Ultrastructural evidence for endogenous vasoactive intestinal peptide-like immunoreactivity in the pituitary gland. *Neuroendocrinology*, 34: 85-89.

NAGY G, MULCHAHEY JJ and NEILL JD (1988). Autocrine control of prolactin secretion by vasoactive intestinal peptide. *Endocrinology*, 122: 364-366.

O'HALLORAN DJ, JONES PH, GHATEI MA, DOMIN J and BLOOM SR (1990). The regulation of neuropeptide expression in rat anterior pituitary following chronic manipulation of estrogen status: a comparison between substance P, neuropeptide Y, neurotensin, and vasoactive intestinal peptide. *Endocrinology*, 127: 1463-1469.

PRYSOR-JONES RA, SILVERLIGHT JJ and JENKINS JS (1987). Hyperprolactinemia reduces vasoactive intestinal peptide in the anterior pituitary glands of rats. *Neurosci Lett*, 80: 333-338.

PRYSOR-JONES RA, SILVERLIGHT JJ, KENNEDY SJ and JENKINS JS (1988). Vasoactive intestinal peptide and the stimulation of lactotroph growth by oestradiol in rats. *J Endocrinol*, 116: 259-265.

RAYMOND V, BEAULIEU M and LABRIE F (1978). Potent antidopaminergic activity of estradiol at the pituitary level on prolactin release. *Science*, 200: 1173-1175.

ROSZTEJN WH, BESSON J, BRIAUD GAGNANT L, ROSSELIN G and KORDON C (1980). Effect of steroids on vasoactive intestinal peptide in discrete brain regions and peripheral tissues. *Neuroendocrinology*, 31: 287-291.

SAMSON WK, SAID SF and MCCANN SM (1979). Radioimmunologic localization of vasoactive intestinal peptide in hypothalamic and extrahypothalamic sites in the rat brain. *Neurosci Lett*, 12: 265-269.

SÁNCHEZ F, LARA JI, CACICEDO L, SÁNCHEZ-FRANCO F, CARRETERO J and VÁZQUEZ R (1991). Inhibition by colchicine of immunoreactive vasoactive intestinal polypeptide release from anterior pituitary cells in culture. *Biomedical Res*, 12: 71-75.

SEGERSON TP, LAM KSL, CACICEDO L, MINIMITANI N, FINK S, LECHAN RM and REICHLIN S (1989). Thyroid hormone regulates vasoactive intestinal peptide (VIP) mRNA levels in the rat anterior pituitary gland. *Endocrinology*, 125: 2221-2223.

STEEL JH, GON G, O'HALLORAN DJ, JONES PM, YANAIHARA N, ISHIKAWA H, BLOOM SR and POLAK JM (1989). Galanin and vasoactive intestinal polypeptide are colocalized with classical pituitary hormones and show plasticity of expression. *Histochemistry*, 93: 183-189.

WATANOBE H and TAKEBE K (1992). A comparative study of the effects of neonatal androgenization and estrogenization on vasoactive intestinal peptide levels in the anterior pituitary and the hypothalamus of adult female rats. *Neuroendocrinology*, 56: 653-659.