Effects of Arg-vasopressin and corticosterone on IL-1β-induced modifications of pituitary ACTH-producing cells

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Summary

Interleukin 1β (IL-1β) stimulates the hypothalamic-pituitary-adrenal axis, however the auto-paracrine effects of IL-1β, on pituitary ACTH-producing cells and the relations among IL-1β and Arg-vasopressin or corticosterone have not yet been analyzed. To address these effects, pituitary monolayer cultures were treated with IL-1β, Arg-vasopressin and corticosterone, either alone or in combined treatment, and an immunocytochemical, morphometric and densitometric study of ACTH-positive cells was carried out. IL-1β significantly increased the percentage and cellular and nuclear size of ACTH-positive cells, and similar effects were found following treatment with Arg-vasopressin. Combined treatment with IL-1β and Arg-vasopressin increased these parameters more than in separate treatments. Corticosterone decreased these parameters and blocked the effects of IL-1β and Arg-vasopressin in the combined treatments. In sum, our results suggest that IL-1β is an auto-paracrine regulator of pituitary ACTH-producing cells, that it reinforces the stimulatory effects of Arg-vasopressin and that corticosterone is an inhibitory regulator of the auto-paracrine effects of IL-1β and Arg-vasopressin.

Key words: Interleukin-1β – ACTH – Pituitary – Auto-paracrine regulation

Introduction

Cytocines are pleotrophic proteins with biological effects on different target cells, acting on specific receptors where they exert autocrine, paracrine, or endocrine effects (Abbas et al., 1995).

Interleukin 1β belongs to the family of cytokines and it has been attributed a regulatory role in stress-induced pituitary ACTH secretion and a role as a response to alterations of the immune system in relation to CRH-release from hypothalamic paraventricular nucleus (Rivest y Rivier, 1991; Fukata et al., 1989; Fagarasan et al., 1989).

Activation of protein kinase A is involved in IL-1β ACTH-secretion (Webster et al., 1991; Gwosdow et al., 1993) as well as in the release of pro-opio-melanocortin-derived peptides (Bernton et al., 1987; Kehrer et al., 1988; Woloski et al., 1985; Fukata et al., 1989; Fagarasan et al., 1989, 1990). In this mechanism, the activation of protooncogens c-fos and/or c-jun could be involved (Brown et al., 1987; Fagarasan et al., 1990), while prostaglandin E_2 mediates the cellular effects of IL-1β on parvocellular neurons in the paraventricular nucleus (Ferri and Ferguson, 2005).

It is well known that interleukin-1β and interleukin-6 are produced locally in the pituitary gland (Vankelecom et al., 1989; Koenig et al., 1990; Arzt et al., 1992; Velkeniers et al., 1994; Rezai et al., 1994; Weigent et al., 1995) and that

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they are involved in the regulation of hormonal secretion. However, the auto-paracrine effects of IL-1β on pituitary ACTH-producing cells have not yet been analyzed, and the involvement of another two important regulators of ACTH, Arg-vasopressin and corticosterone, in this auto-paracrine regulation has not been studied either. In order to analyze the effects of IL-1β, Arg-vasopressin and corticosterone, either alone or combined, on the pituitary ACTH-positive cells, an immunocytochemical, morphometric and densitometric study of ACTH-producing cells of rat pituitary monolayer cultures was carried out.

Materials and Methods

Pituitary cultures. Following anaesthesia with Forene®, male Wistar rats (175-200g) were killed by decapitation (the animals were handled according to guidelines of the European Communities Council Directive: 86/609/EEC and current Spanish legislation for the use and care of laboratory animals: BOE 67/8509-12,1998) and the anterior pituitary glands were removed and washed in Earle's balanced salt solution. Enzymatic dispersion was accomplished 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% 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. The cells were seeded on culture chamber slides (0.5ml) at a final concentration of $2x10^5$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.

Treatments. On the 7th day of incubation, the medium was replaced by fresh DMEM in control dishes and fresh DMEM plus IL-1β 10^{-6}M (Sigma®), 10^{-6}M AVP (Sigma®), 10^{-6}M corticosterone (Sigma®), IL-1β and corticosterone, IL-1β and AVP or AVP and corticosterone, and incubated for 24 hours. Five dishes per treatment were employed. At the end of each experiment, the dishes were carefully washed with Dulbecco's sterile PBS and the cells were fixed in 4% paraformaldehyde in phosphate buffer (0.01M, pH 7.4) for 30 min, followed by careful rinsing in PBS.

Immunocytochemistry for ACTH. To determine ACTH-positive cells, the biotinylated-avidin-peroxidase immunocytochemical method was implemented. Endogenous peroxidase was blocked with H_2O_2 in methanol and non-specific reactions of the secondary antibody were blocked by incubation in normal goat serum (Dako®, diluted 1:30). Cultures were incubated overnight at 4ºC with the rabbit anti-ACTH polyclonal antibody (Dako®, diluted 1:800 in TBS). Biotinylated goat anti-mouse IgG (Dako®, diluted 1:100) and Avidin-Biotinylated horseradish peroxidase complex (ABC kit Dako®, diluted 1:100) were successively applied at room temperature for 40 min and 30 min, respectively. Reactions were developed in freshly prepared 4-1Cl-Naphtol (0.03% in TRIS buffer containing 0.03% of H_2O_2).

Controls included substitution of the primary antibody by normal rabbit serum or TBS, as well as omission of the secondary antibody; after both tests no immunoreactivity was detected. The cross-reaction of goat anti-rabbit antiserum IgG with mouse or rat immunoglobulins was determined by ELISA and was also very low (less than 1%).

Quantification of ACTH positive cells. Four thousand cells per dish and study were evaluated using an Axioplan Zeiss® microscope equipped with an ocular grid at a final magnification of x400. 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. ACTH-immunoreactive cells were determined and the percentages of immunoreactive cells were calculated.

Image analysis: morphometric and densitometric analysis. Cellular and nuclear areas of ACTH-positive cells were calculated using the ImageJ program (NIH, USA) from 100 cells per dish (1000 cells per treatment). Moreover, using the same program, densitometric mean grey values were calculated from the cytoplasm of 100 ACTH-positive cells per dish (1000 cells per treatment) and densitometric mean profiles were obtained. Morphometric and densitometric analyses were carried out from digital microphotographs obtained under homogeneous conditions of colour temperature and following stabilization of the input grey levels from 37 to 223 using the Adobe Photoshop 7.0®.

Statistical analysis. The results obtained were processed statistically using GraphPad Prism 4, and are expressed as arithmetic means ± error standard of the mean. The differences observed were compared using analysis of variance, accepting $p<0.05$ as significant for the Bonfer-

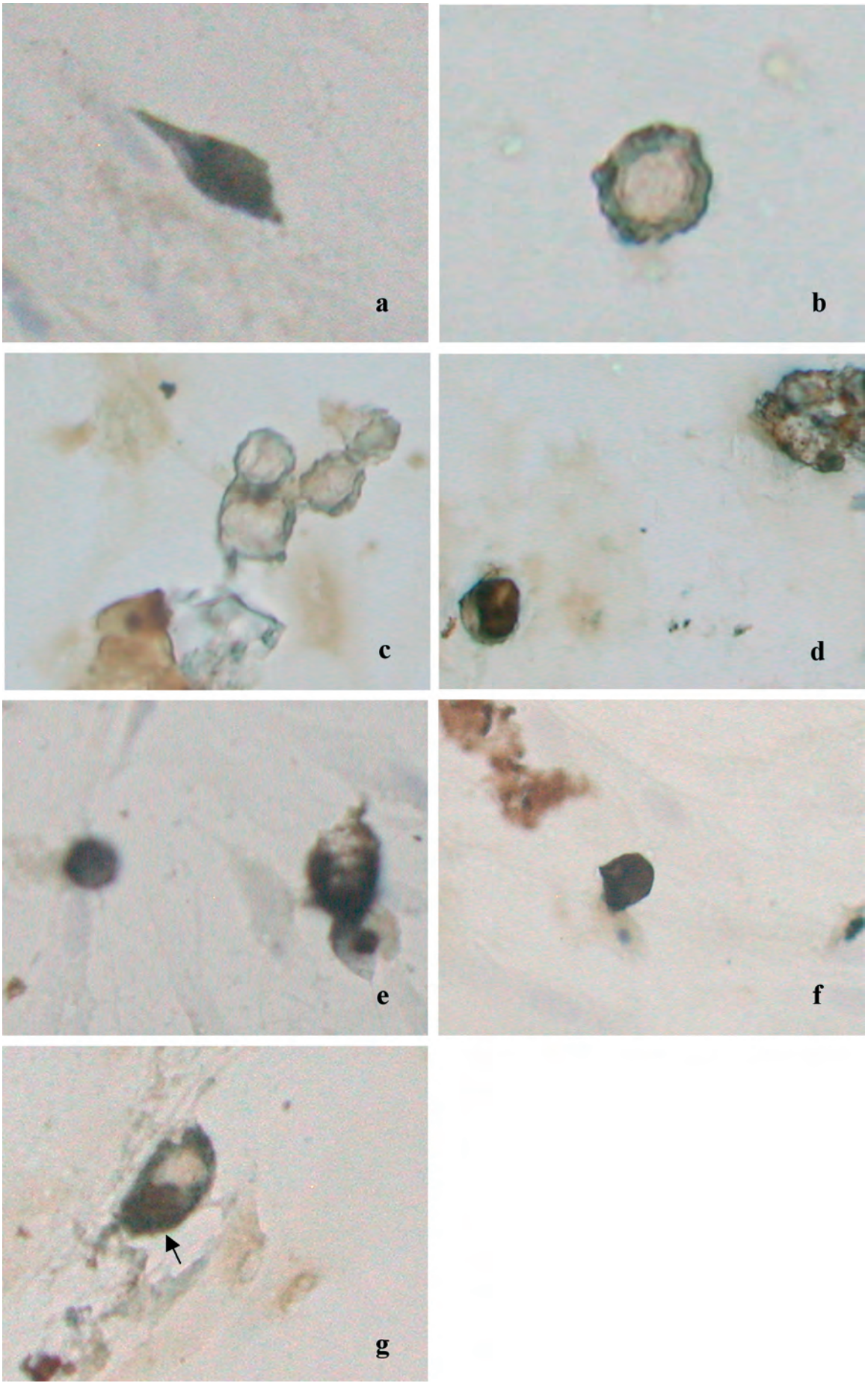

Fig. 1.- Micrographs showing the morphology and reaction for ACTH-positive cells in monolayer pituitary cultures after different treatments: **a**: control dish; **b**: IL-1β-treated dish; **c**: Arg-vasopressin-treated dish; **d**: corticosterone-treated dish; **e**: Arg-vasopressin- and IL-1β-treated dish; **f**: IL-1β- and corticosterone-treated dish; **g**: Arg-vasopressin- and corticosterone-treated dish. a-g = x 1000.

roni test. Two pituitary cultures under similar experimental conditions were made in order to verify the reproducibility of the results (the within-assay error was less than 1.96%).

Results

Control dishes. ACTH-producing cells showed a strong cytoplasmic reaction (Fig. 1a). The mean grey value was calculated as 135.15±4.05 arbitrary densitometric units (Fig. 2). 10.21±0.46% of the glandular cells in the control dishes were ACTH-producing cells (Fig. 3) with a mean size of 94.35±2.83 μm^2 (Fig. 4) and the nuclei mean size was 20.67±1.93 μm^2 (Fig. 5).

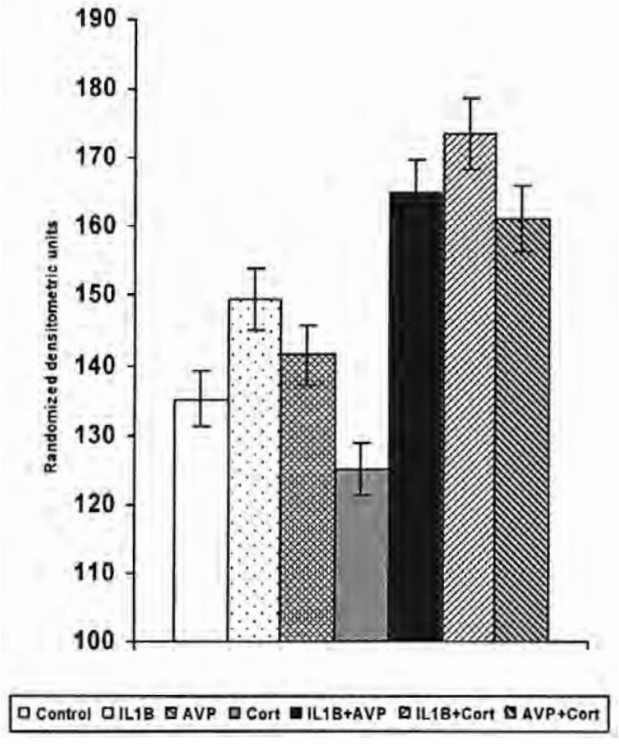

Fig. 2.- Plot showing the variations induced by the different treatments in the grey levels determined by densitometry (units are expressed as transformed arbitrary densitometric units, 0=white).

Arg-vasopressin treated dishes. In this treatment group, the cytoplasmic reaction of ACTH-positive cells was clotted and lighter than in the control or IL-1β treated dishes (Fig. 1c). As result of this reaction pattern, the mean grey value was higher than in the controls, but lower than in the IL-1β treated dishes, with no significant differences between either treatment groups (Fig.2). The percentage of ACTH-producing cells was similar to that observed following treatment with IL-1β (16.66±0.73%, $p<0.01$ with respect to control dishes, Fig. 3). Also, the cellular and nuclear areas were similar to those observed in the IL-1β treated dishes (104.14±2.82 μm^2 and 35.91±1.65 μm^2, respectively, $p<0.05$ and $p<0.01$ with respect to control dishes, Figs. 4 and 5).

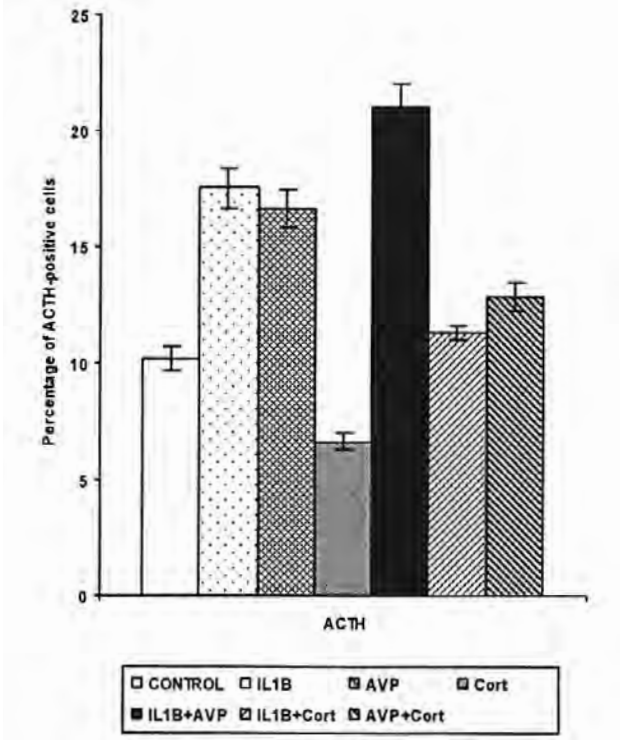

Fig. 3.- Plot showing the variations induced by the different treatments in the percentage of ACTH-positive cells.

IL-1β-treated dishes. Following treatment with IL-1β, the cytoplasmic reaction of ACTH-positive cells appeared as a clotted sediment under the plasmalemma of the cells and was more irregular than in control dishes (Fig. 1b). Treatment with IL-1β induced increases in the densitometric mean grey value (149.35±4.48, $p<0.05$ with respect to control dishes, Fig. 2), the percentage of ACTH-producing cells (17.53±0.85%, $p<0.01$ with respect to control dishes, Fig. 3), the cellular area (109.78±3.29 μm^2, $p<0.01$ with respect to control dishes, Fig. 4) and the nuclear area (36.34±2.08 μm^2, $p<0.01$ with respect to control dishes, Fig. 5).

Corticosterone-treated dishes. Following treatment with corticosterone, the cytoplasmic reaction of ACTH-positive cells appeared homogeneously distributed (Fig. 1d). However, the mean grey value was lower than in the other groups analyzed (Fig.2, 125.15±3.75, $p<0.05$ with respect to control dishes and $p<0.01$ with respect to IL-1β or Arg-vasopressin-treated dishes). After treatment with corticosterone, the percentage of ACTH-positive cells decreased (6.67±0.43%, $p<0.01$, Fig. 3), and significant decreases in the cellular and nuclear areas were found in this treatment group (Cellular area: 68.35±2.05 μm^2, $p<0.001$. Nuclear area: 16.14±1.19 μm^2, $p<0.05$

with respect to control dishes and p<0.001, with respect to dishes treated with IL-1β or Arg-vasopressin, Figs. 4 and 5).

Arg-vasopressin- and IL-1β-treated dishes. Combined treatment with IL-1β and Arg-vasopressin induced the presence of round and large

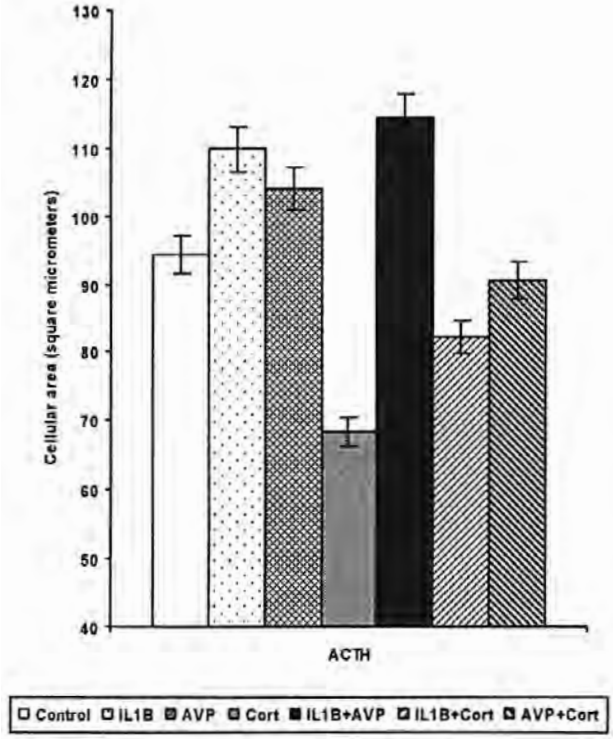

Fig. 4.- Plot showing the variations in the cellular area of ACTH-positive cells in the different treatments assayed.

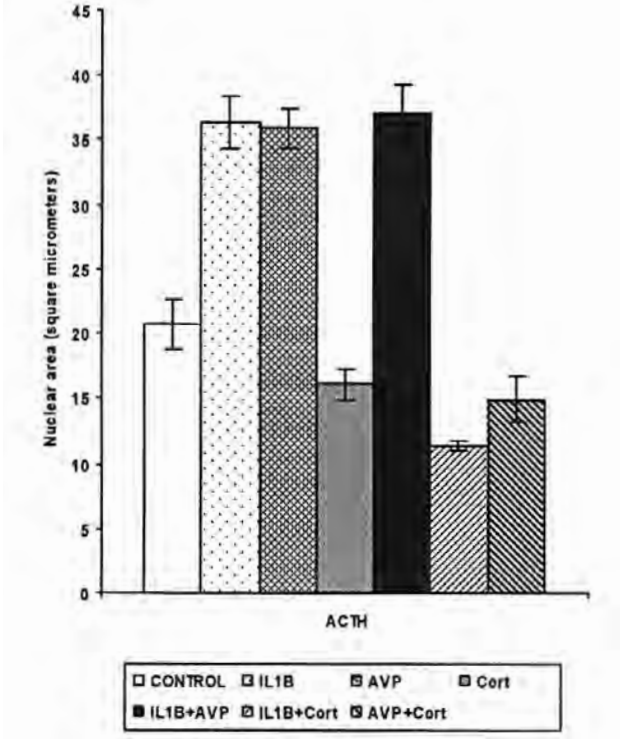

Fig. 5.- Plot showing the variations in the nuclear area of ACTH-positive cells in the different treatments assayed.

ACTH-producing cells with a strong cytoplasmic reaction (Fig. 1e). The mean grey value increased significantly 164.84±4.95, p<0.01 with respect to control and IL-1β or Arg-vasopressin treated dishes and p<0.001 with respect to corticosterone-treated dishes, Fig. 2). Also, the percentage (21.01±1.05%, p<0.01 with respect to control dishes and p<0.05 with respect to IL-1β or Arg-vasopressin treated dishes, Fig. 3) and the cellular area of ACTH-positive cells increased following combined treatment with IL-1β and Arg-vasopressin (114.42±3.43 μm^2, p<0.01 with respect to control dishes and p<0.05 with respect to Arg-vasopressin-treated dishes, Fig. 4). However, the nuclear area (37.08±2.18 μm^2, p<0.01 with respect to control dishes) was similar to those observed in dishes treated only with IL-1β or Arg-vasopressin (Fig. 5).

IL-1β-and corticosterone-treated dishes. Following combined IL-1β and corticosterone treatment, ACTH-positive cells were similar to those observed after treatment with IL-1β alone (Fig. 1f), except for the stronger reaction in the cytoplasm (mean grey value: 173.40±5.20, p<0.01 with respect to dishes treated with IL-1β or corticosterone alone and p<0.05 with respect to dishes treated with IL-1β and Arg-vasopressin, Fig. 2). In this treatment group the percentage of ACTH-positive cells was similar to that observed in control dishes, and lower than in the dishes treated with IL-1β and higher than in corticosterone-treated dishes (11.33±0.34%, p<0.01 with respect to IL-1β or corticosterone treated dishes, Fig. 3). Corticosterone blocked the increases observed in the cellular area after treatment with IL-1β (82.11±2.46 μm^2, p<0.01 with respect to IL-1β and corticosterone-treated dishes, Fig. 4). The combined treatment significantly inhibited the nuclear areas, which decreased significantly in this group (13.33±0.34μm^2, p<0.01 with respect to control dishes, p<0.001 with respect to IL-1β treated dishes and p<0.05 with respect to corticosterone treated dishes, Fig. 5).

Arg-vasopressin- and corticosterone-treated dishes. In this group, the intensity of the cytoplasmic reaction of ACTH-positive cells (Fig. 1g) was stronger (mean grey value: 161.04±4.83, p<0.01 with respect to single treatments, Fig. 2). The percentage of ACTH-positive cells was similar to that observed after combined treatment with IL-1β and corticosterone (12.84±0.64%, p<0.01 with respect to Arg-vasopressin or corticosterone-treated dishes, Fig. 3). Following treatment with Arg-vasopressin and corticosterone,

the cellular area was similar to that observed in control dishes, higher than in corticosterone-treated dishes and lower than in Arg-vasopressin-treated dishes (90.49±2.71 μm^2, $p<0.01$ with respect to Arg-vasopressin- or corticosterone-treated dishes, Fig. 4). Combined treatment with Arg-vasopressin and corticosterone induced similar effects on the nuclear area to those observed following combined treatment with IL-1β and corticosterone (14.96±1.69 μm^2, $p<0.05$ with respect to control dishes, $p<0.001$ with respect to Arg-vasopressin-treated dishes and $p<0.05$ with respect to corticosterone-treated dishes, Fig. 5).

Discussion

Since 1987 has been known that IL-1β directly or indirectly stimulates the secretion of ACTH from the pituitary gland (Uehara et al., 1987a, b; Liege et al., 2000; Philip et al., 2002; Crane et al., 2003; Taishi et al., 2004).

However, the effects of IL-1β on pituitary ACTH-producing cells and the modulation of these effects by Arg-vasopressin or corticosterone have not yet been analyzed. The present study is the first to analyze the *in vitro* effects of IL-1β, Arg-vasopressin and corticosterone, either alone or in combined treatments, on the morphology and percentage of pituitary ACTH-producing cells.

In histochemical studies, Siperstein (1963) defined the population of ACTH cells in 0.13% of total pituitary cells, and with electron microscopy percentages ranging from 2% to 9.2% have been described (Surks and DeFesi, 1977; Takahashi and Kawashima, 1982). With immunocytochemical methods, the percentages ranging from 2.93% to 8.10% have been reported (Childs et al., 1982; Dada et al., 1984).

This *in vitro* study establishes the percentage of pituitary ACTH-producing cells at around 10%, higher than those found *in vivo* by other authors or by us in a previous work (Montero et al., 1990). These differences can be explained because the inhibitory feedback of corticoids was abolished in monolayer pituitary cultures. Moreover, treatment with IL-1β or Arg-vasopressin increased this percentage and treatment with corticosterona decreases the percentage of ACTH-producing cells to values very similar to those described in *in vivo* studies.

IL-1β stimulates the secretion of ACTH and corticosterone (Xiao et al., 2001; Toftegaard et al., 2002-2003; Goshen et al., 2003) and corticosterone inhibits the IL-1β-induced release of ACTH (Philip et al., 2002) and the inflammation-stimulated secretion of IL-1β (Wahl et al., 1975; Besedovsky et al., 1986). Adrenalectomy increases the immunoreactivity of IL-1β in the pituitary gland and this effect is blockaded by corticosterone (Montero et al., 1990). These results suggest that corticosterone would regulate the expression of IL-1β in the pituitary gland in a similar way to how it regulates the secretion of ACTH (Plotsky and Sawchenko, 1987).

Similar to bilateral adrenalectomy, in *in vitro* conditions no glucocorticoids develop their effects on ACTH-producing cells. The effects of IL-1β and vasopressin found in the present study are very similar to those observed after adrenalectomy (Siperstein and Allison, 1965; Kurosumi and Kobayashi, 1966; Pelletier, 1970; Baker and Dummond, 1972; Kraicer et al., 1973; Bowie et al., 1973). Following adrenalectomy, ACTH-immunoreactive cells lose their stellate shape and their cytoplasm displays a granular reaction pattern, with increases in the cellular and nuclear sizes (Sánchez et al., 1988).

Inhibitory feedback is re-established following treatment with corticoids and the increase in the percentages of ACTH-producing cells induced by adrenalectomy disappears after treatment with corticosterone (Hodges et al., 1962; Jobin et al., 1975; Nicholson et al., 1984).

The secretory profiles of IL-1β and corticosterone are inverse, suggesting the existence of a relationship in the regulatory mechanism of secretion (Seres et al., 2004).

Moreover, *in vivo* corticosterone inhibits the stress-estimulated hypothalamic and hypophyseal release of IL-1β (Nguyen et al., 2000) and it blocks the IL-1β-stimulated secretion of ACTH (Holland et al., 2002). Similar effects on cellular size, percentage and cytoplasmic reaction were found in our *in vitro* study, suggesting that corticosterone is a very important inhibitor of the effects of IL-1β on ACTH-producing cells, as seen following combined treatment of corticosterone with IL-1β or Arg-vasopressin.

Arg-vasopressin and CRF stimulate the secretion of pituitary ACTH and IL-1β reinforces the Arg-vasopressin-stimulated release of ACTH (Prickett et al., 2000).

Because combined treatment with IL-1β and Arg-vasopressin induces increases in the values observed for the parameters analyzed with respect to IL-1β or Arg-vasopressin alone, our results suggest that both substances would act through different intracellular pathways and that IL-1β would reinforce the effects of Arg-vasopressin on pituitary ACTH-producing cells.

In sum, our results demonstrate direct effects of IL-1β on pituitary ACTH-producing cells, suggesting that IL-1β is a very important autoparacrine regulator of rat pituitary ACTH-producing cells under modulation by corticosterone together with Arg-vasopressin.

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