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Different Ultrastructural Localization of VIP and Prolactin in Anterior Pituitary Cells of Rats Chronically Treated With Estrogen

K. Köves,[1] I-Li Chen,[2] T. J. Görcs,[3] J. G. Scammell,[4] and A. Arimura[5]

[1]*Department of Human Morphology and Developmental Biology, Semmelweis University Medical School, Budapest, Hungary;* [2]*Department of Anatomy, Tulane University School of Medicine, New Orleans, LA;* [3]*Laboratory of Neurobiology, Department of Anatomy, Semmelweis University Medical School, Budapest, Hungary;* [4]*Department of Pharmacology, College of Medicine, University of South Alabama, Mobile, AL;* [5]*US-Japan Biomedical Research Laboratories, Tulane University Hebert Center, Belle Chasse, LA and Department of Anatomy, Tulane University School of Medicine, New Orleans, LA*

In the present study we investigated the effect of a long-term estrogen treatment on the intracellular distribution of VIP immunoreactivity in pituitary prolactin cells using double-labeling immunocytochemistry. With the use of pre-embedding ABC method it was found that VIP immunoreactivity was associated with the outer surface of membrane-bound organelles, and was not found in secretory granules. However, prolactin immunoreactivity demonstrated by postembedding immunogold technique was mainly associated within the secretory granules of the same cells. The discrepancy between our and Hsu et al.'s results (1989), who observed VIP immunoreactivity in secretory granules of human anterior pituitary cells, may be owing to the overstimulation of VIP cells by estrogen. It is possible that estrogen treatment depleted the VIP content of the secretory granules and enhanced the cytosolic VIP. The appearance of an alternative form of VIP in estrogen-treated rats with preferential distribution in the cytosol cannot be excluded.

Key Words: VIP; prolactin; pituitary; rat; estrogen; immunocytochemistry; electron microscopy; double-labeling.

Introduction

In rats vasoactive intestinal polypeptide (VIP) is present and is synthesized by the central and peripheral nervous systems and the anterior lobe of the pituitary gland (Said and Mutt, 1970; Larsson et al., 1976; Said and Rosenberg, 1976; Rotsztejn et al., 1980; Maletti et al., 1982; Arnaout et al., 1986). Hypothyroidism in male rats enhances pituitary VIP immunoreactivity as detected by light-microscopic immunohistochemistry (Lam et al., 1989). Likewise, estrogen treatment exerts dramatic effect on the expression of VIP in the pituitary. It increases the content of VIP mRNA (Lam et al., 1990; O'Halloran et al., 1990) and the concentration of VIP in the anterior pituitary (Prysor-Jones et al., 1988). We demonstrated that long-term exposure to high levels of estrogen, produced by diethylstilbestrol (DES) implantation, elevated the number of VIP-immunolabeled cells, which were scattered or arranged in groups suggestive of VIP-omas in the anterior pituitary of female rats (Köves et al., 1990). In untreated female rats only few immunopositive cells were observed (Köves et al., 1990; Léránth et al., 1991). However, Carillo et al. (1992) have found a considerable number of VIP cells in intact animals using a VIP antiserum different from ours. VIP-immunopositive cells have also been demonstrated in the anterior pituitaries of other species than rats, such as monkey and human (Reichlin, 1988; Hsu et al., 1989). It was found that VIP colocalized with all the classical hormone immunoreactivities in human pituitaries.

In estrogen-treated rats VIP immunoreactivity partially colocalized only with prolactin (PRL) immunoreactivity, when studied by light-microscopic double-labeling immunohistochemistry. However, no colocalization was observed between VIP and other pituitary hormone immunoreactivities (Köves et al., 1990).

There are several studies investigating the effect of chronic estrogen treatment on the distribution and morphological features of pituitary hormone secreting cells. However, no data are available on the ultrastructural distribution of VIP immunoreactivity in rat pituitaries after chronic estrogen treatment.

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Author to whom all correspondence and reprint requests should be addressed: Katalin Köves, Department of Human Morphology and Developmental Biology, Semmelweis University Medical School, H- 1094 Budapest, Hungary, E-mail: koves@ana2.sote.hu

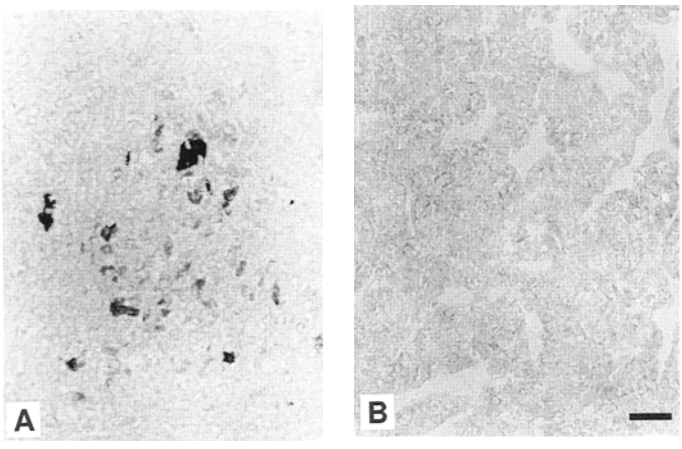

Fig. 1. Light-microscopic photographs of anterior pituitary of a DES-implanted female rat. **(A)** VIP immunoreactive cells form a group suggestive of VIP-oma. **(B)** The preabsorbtion of VIP antiserum with VIP abolished the immunostaining. Scale bar represents 100 μm.

Therefore the aim of our present study was to investigate the intracellular distribution of VIP and PRL immunoreactivities in the rat anterior pituitary using double-labeling immunocytochemistry at ultrastructural level in estrogen-treated rats and to compare the distribution of VIP to that which was found in human nonfunctioning adenomas (Hsu et al., 1989).

Results

Prolactinomas and VIP-omas were observed in the anterior pituitary of DES-implanted male and female rats. Many prolactinomas (not shown) and 1–3 VIP-omas (Fig. 1A) were present in every pituitary gland. Scattered VIP cells were also present in the pituitaries. The appearance of both immunoreactivities in estrogen-treated rats was very similar to that described previously (Köves et al., 1990).

VIP immunostaining was abolished when the sections were incubated with VIP antiserum preabsorbed with VIP (Fig. 1B) or when primary antiserum was omitted or substituted with normal rabbit serum.

Ultrastructurally, VIP-immunoreactive cells in DES-implanted rats showed many characteristics of hypertrophic PRL cells, which are normally present in pregnant (Merchant, 1974; Ozawa et al., 1989) or estrogen-stimulated (Shiino and Rennels, 1976; Lloyd, 1983; Nogami, 1984) rat pituitary glands. These cells contained predominantly spherical secretory granules as well as hypertrophic Golgi complex and endoplasmic reticulum. The size of the secretory granules ranged from 200–500 nm in diameter and the endoplasmic reticulum formed multiple layers parallel to the plasmalemma. The DAB reaction product indicating VIP immunoreactivity was present diffusely in the cytoplasm (Fig. 2A, B and Fig. 3). It was not observed within the secretory granules, but the presence of it in the nucleus was not excluded (Fig. 2). Patches of electron-opaque DAB

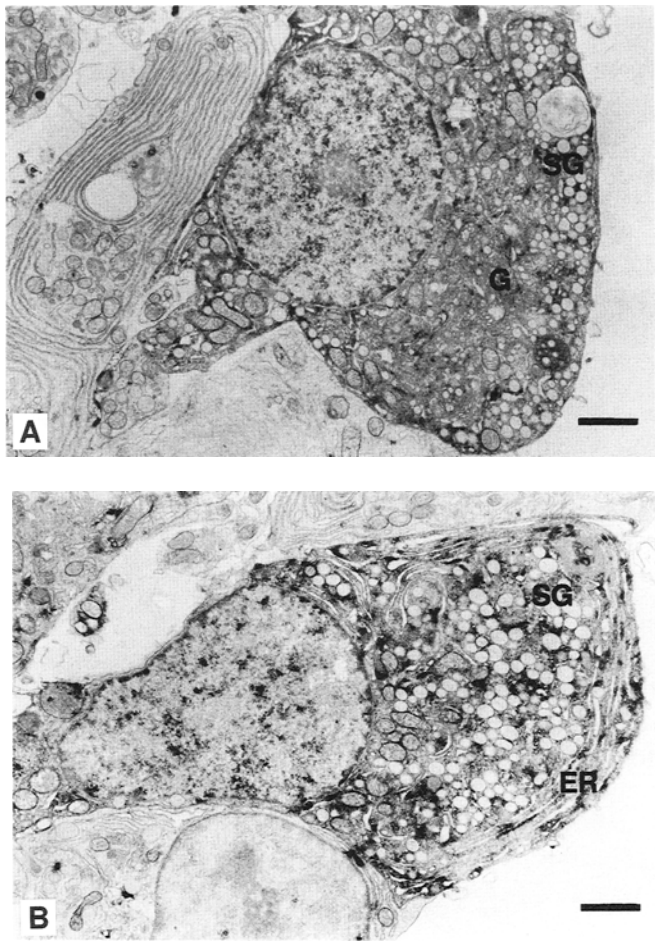

Fig. 2. Electron micrographs showing VIP cells (VIP) in the anterior pituitary of a DES-implanted female rat. The DAB reaction product appears as patches of electron opaque deposits in the Golgi region (G) **(A)**, in the cytoplasmic matrix in the vicinity of secretory granules (SG) and between cisterns of the endoplasmic reticulum (ER) **(B)**. The scale bar represents 1 μm.

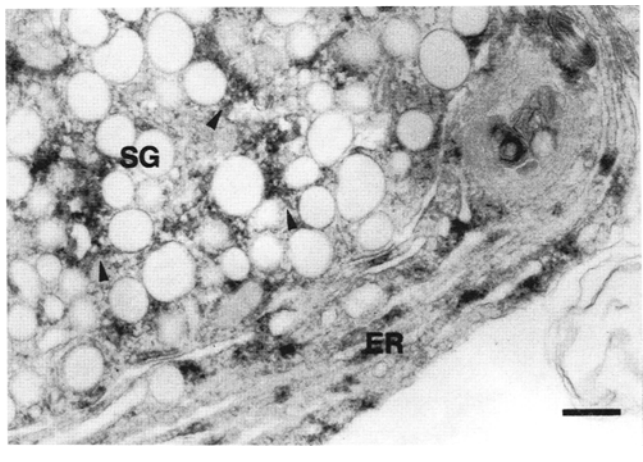

Fig. 3. A high power detail of Fig. 2B. Note that DAB deposit (indicated by arrowheads) is present in the immediate vicinity, but not in the core of the secretory granules (SG). The scale bar represents 0.5 μm.

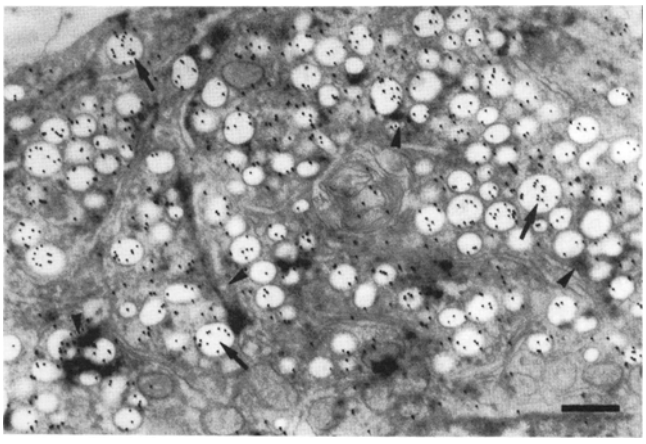

Fig. 4. Electron micrograph demonstrating a high power detail of a double-stained cell in the anterior pituitary of a DES-implanted female rat. Electron opacity of the DAB reaction product (arrowheads), indicating VIP immunoreactivity, is somewhat reduced by the bleaching effect of the sodium-m-periodate treatment. The gold particles, indicating PRL immunoreactivity, are mainly present above the secretory granules (indicated by arrows). The scale bar represents 0.75 μm.

deposits were evident in the cytoplasmic matrix between secretory granules, the cisternae of the endoplasmic reticulum, and on the outer surface of other membrane-bound organelles.

In double-labeled cells VIP immunoreactivity, indicated by the DAB reaction product, was observed in the cytoplasmic matrix; however, gold particles indicating PRL immunoreactivity were mainly localized within the core of the secretory granules (Fig. 4).

Discussion

VIP-immunoreactive cells have been identified in the anterior pituitary gland using light-microscopic immunocytochemistry (Reichlin, 1988; Hsu et al., 1989; Lam et al., 1989, Köves et al., 1990; Carillo and Phelps, 1992). In intact rats the number of VIP cells is less than in those animals which received a long-term estrogen treatment. Therefore in our experiments estrogen treatment was used to enhance the number of VIP immunopositive cells in the anterior pituitary. With the use of a double-labeling technique, the present study indicates that VIP immunoreactivity in the anterior pituitary of this model is present in the cytoplasmic matrix of hypertrophic PRL cells and was not observed in the secretory vesicles. However, PRL immunoreactivity as indicated by immunogold particles was concentrated mainly over the secretory granules. It is well known from the literature that immunoreactivities of anterior pituitary hormones are mainly found in secretory granules independent of the methods applied and the models used (Nakane, 1975; Childs, 1986).

There are only a few publications about the ultrastructural features of VIP immunoreactive elements in various tissues. In the hypothalamic suprachiasmatic nucleus VIP immunoreactivity was identified on the outer surface of the membranes of several cellular organelles in perikarya (Card et al., 1981). Although, by using pre-embedding immunostaining in normal and adenomatous anterior pituitaries, VIP immunoreactivity was observed in the secretory granules. In this material VIP immunoreactivity partially colocalized with all pituitary hormone (except of growth hormone) immunoreactivities (Hsu et al., 1989). In the anterior pituitary of male and female rats Morel et al. (1982) have found small round-shaped VIP-granules as small as 30 nm in diameter in both the cytoplasm and, unexpectedly, in the nucleus. These granules in the cytoplasm were never associated with cell organelles and in the nucleus they were primarily localized in the euchromatin. The authors suggested that VIP present in small granules is not synthesized by these cells but they may be associated with internalized VIP receptors occupied by VIP.

In our experiments pre-embedding immunohistochemistry was chosen to study the distribution of VIP immunoreactivity at the ultrastructural level because of the relatively low number of VIP cells even in estrogen-treated rats. The other reason to use pre-embedding VIP staining was that the demonstration of VIP immunoreactivity by immunogold technique is very difficult, which is probably due to the low amount of this neuropeptide in the pituitary gland compared to the amount of classic pituitary hormones.

With the use of the above-mentioned techniques we have found different ultrastructural localization of VIP and prolactin. VIP immunoreactivity was associated with several cytoplasmic organelles but was not observed within secretory granules as the prolactin immunoreactivity. With the use of the chronic estrogen-treated model we could not confirm the observations of Hsu et al. (1989), who demonstrated VIP immunoreactivity in secretory granules in normal and adenomatous human pituitaries.

In our experiments the animals were treated with estrogen, which is a well known stimulator of pituitary VIP secretion and release even in in vitro system (Carretero et al, 1995). It cannot be excluded that the discrepancy between our and Hsu et al.'s results may be owing to the overstimulation of VIP cells by estrogen. It is possible that the cells depleted VIP during the prolonged estrogen stimulation and therefore the secretory granules did not contain VIP immunoreactivity by the end of the experimental period. To resolve the discrepancy we plan to examine the effect of shorter estrogen treatments on the ultrastructural features of pituitary VIP cells.

We may hypothesize that the depletion of VIP from secretory granules stimulates the synthesis of VIP, therefore cytoplasmic VIP immunoreactivity becomes detectable. Unfortunately no data are available concerning the dynamics of the uptake of freshly synthesized VIP by secretory granules.

According to our results the presence of VIP immunoreactivity in a population of PRL cells, determined at the ultrastructural level, supports the view that pituitary VIP may play a role in regulating PRL secretion either in an autocrine or a paracrine manner.

Antiserum raised against VIP has been shown to inhibit PRL release from cultured pituitary cells studied by RIA (Hagen et al., 1986) and from GH3 cell line studied by reverse hemolytic plaque assay (Nagy et al., 1988). On the basis of our results it is difficult to explain how cytoplasmic VIP can be released into the extracellular space. In the above-mentioned in vitro systems Hagen et al. (1986) and Carratero et al. (1995) have demonstrated that the pituitary VIP cells release VIP into the culture medium. This phenomenon should occur by the release of VIP from secretory granules. Therefore it is worth to note that our results do not contradict the results of Hsu et al. (1989). Chronic estrogen treatment may dramatically alter the distributional pattern of VIP immunoreactivity at the ultrastructural level in estrogen-treated rats. In order to obtain data concerning the dynamism of the depletion of VIP by secretory granules and the parallel appearance of cytoplasmic VIP immunoreactivity needs further studies using pituitary cell cultures.

Recently it was demonstrated by Chew et al. (1994) that beside the common form, an alternative VIP mRNA may be present in pituitary cells. The two forms are differentially regulated by estrogen. Therefore the possibility that the alternative form of VIP is preferentially synthesized in the pituitaries of estrogen-treated rats, where VIP immunoreactivity shows different morphological and distributional pattern, cannot be excluded.

Materials and Methods

Animals

Twenty-day-old rats of both sexes (Charles River Breeding Laboratories, Wilmington, MA) were weaned from their mothers and housed in a light (lights on at 600 and lights off at 1600) and temperature-controlled room (22 ± 2°C). When the rats were 25-d old, Silastic capsules (10 mm in length; id 1.55 mm, od 3.13 mm) containing DES were implanted subcutaneously. The pituitary glands of three male and three female rats bearing DES capsule were processed for electron microscopic immunocytochemistry, 3–6 mo after the implantation of the capsules.

Electron Microscopic Immunocytochemistry

Animals were anesthesized with sodium pentobarbital and perfused sequentially with cold saline and 4% paraformaldehyde containing 0.2% glutaraldehyde via the ascending aorta. Thirty min later, the pituitary glands were removed, fixed overnight in 4% paraformaldehyde without glutaraldehyde, and 50-μm-thick sections were cut horizontally on a Lancer Vibratome (St. Louis, MO). The sections were washed 10 times, soaked in graded sucrose solutions (15–20–30% in buffer), and frozen in order to enhance the penetration of immunoreagents. The sections were incubated sequentially with VIP antiserum (raised in rabbit) at 1:10,000 dilution at 4°C for 72 h, with biotinylated second antibody for 8 h, and with avidin–biotin complex (ABC) overnight (Vectastain ABC rabbit kit, Vector Laboratories, Burlingame, CA). The final complex was visualized with the nickel-DAB chromogen (400 mg nickel ammonium sulfate, 10 mg DAB, 10 μL 30% hydrogen peroxide in 100 mL Tris buffer, pH 7.6). Immunostained sections were postosmicated and embedded in Epon for electron microscopy. Thin sections were cut on Porter-Blum Bll Ultramicrotome. Several ultrathin sections were selected for PRL immunostaining using immunogold technique. These sections were treated with 10% sodium-m-periodate, throughly washed with distilled water incubated with a monoclonal anti-PRL antibody solution at a 1:5000 dilution overnight at room temperature, rinsed with Tris-buffered saline and treated with goat antimouse IgG conjugated to colloidal gold (1:100 dilution with Tris-buffered saline containing 0.05% polyethylene glycol, MW 8000). The sections were washed with distilled water, air-dried, and examined with a JEM 100CX electron microscope at 40 kV accelerating voltage without further heavy metal staining.

VIP antiserum was previously characterized in both solid-phase (ELISA) and liquid-phase systems by Gulyás et al. (1990). In ELISA VIP antiserum reacted with VIP and in lesser extent with PHI; however, there was no crossreactivity with ACTH, angiotensin II, atrial natriuretic peptide, bombesin, bradykinin, corticotropin releasing factor, diazepam binding inhibitor fragment, B-endorphin, methionin and leu-enkephalin, galanin, growth hormone releasing factor, a-melanocyte-stimulating hormone , neuropeptide Y, neurotensin, oxytocin, vasopressin, secretin, substance P, thyrotropin releasing hormone and serum thymus factor. Prolactin antiserum was produced and characterized by Scammell et al. (1990).

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