1. Introduction

The synthesis and release of prolactin (PRL) by lactotrophs in the anterior pituitary (AP) are regulated by factors produced in the hypothalamus as well as in the posterior and neurointermediate pituitary lobes, by autocrine and paracrine signals from the anterior pituitary itself (Ben-Jonathan & Hnasko, 2001; Kordon, 1985; Denef, 1988; Denef, 2008; Freeman et al., 2000; Lorenson and Walker, 2001; Schwartz & Cherny, 1992; Schwartz, 2000; Wang & Walker, 1993; Sinha, 1992; Sinha 1995; Moore et al., 2002; Bollengier et al., 1989; Bollengier et al., 1996; Kadowaki et al., 1984; MacLeod et al., 1966; Sgouris & Meites, 1953; Chen et al., 1968; Welsch et al., 1968) and also by gonadal steroids (Maurer & Gorski, 1977; Maurer, 1982). In addition, it has been reported that total PRL and PRL variants (Denef, 2008; Shah & Hymey, 1989) are secreted under different physiological conditions (Denef, 2008; Wang & Walker, 1993; Sinha, 1992; Sinha 1995; Mena et al., 1984; Mena et al., 1992; Boockfor & Frawley, 1987). And, it is known that functional interactions and cytological differences exist among pituitary lactotrophs within the anterior pituitary gland (Denef, 1988; Schwartz & Cherny, 1992; Schwartz, 2000; Boockfor & Frawley, 1987) and that functional variations (Boockfor & Frawley, 1987; Boockfor et al., 1986; Frawley & Boockfor, 1991; Nagy & Frawley 1990), as well as autoregulation (Nagy et al., 1991) and interactions with other pituitary cells (Denef, 2008; Sinha, 1992; Moore et al., 2002; Kadowaki et al., 1984) and with hypothalamic hormones (Ben-Jonathan & Hnasko, 2001; Chen et al., 1968) occur in different circumstances. For instance, lactotrophs from the central AP region of lactating rats, i.e., the region surrounding the neurointermediate pituitary lobe (Boockfor & Frawley, 1987; Frawley & Boockfor, 1991; Papka et al., 1986) are bigger, secrete more PRL than those of the peripheral AP region and after a short period of suckling become more sensitive to the PRL-stimulatory agents, TRH and angiotensin II; moreover, they become unresponsive...
to dopamine; and interact with lactotrophs in the peripheral region of the gland (Boockfor & Frawley, 1987; Boockfor et al., 1986; Frawley & Boockfor, 1991; Nagy et al., 1991; Nagy & Frawley 1990; Diaz et al., 2002). In these studies, it is possible that the release of PRL variants may have influenced the regulation of PRL release.

In previous reports (Huerta-Ocampo et al., 2007; Mena et al., 2010) we showed that conditioned media (CM) and PRL variants i.e., from 7-14 and 70-97 kDa, from lactating rat APs, characterized by Western blotting and eluted from SDS-PAGE, promoted the in vitro vesicular release of the hormone from preformed, mature PRL granules of male rat APs, and that such release was independent of PRL synthesis (Huerta-Ocampo et al., 2007). Autocrine and paracrine types of actions have also been shown to occur within the AP (Denef, 2008; Freeman et al., 2000; Lorenson and Walker, 2001; Schwartz & Cherny, 1992; Schwartz, 2000; Welsch et al., 1968; Diaz, et al., 2002; Huerta-Ocampo et al., 2007; Mena et al., 2010), and were demonstrated when the central and peripheral AP regions of lactating rats were incubated in vitro with CM from pituitaries of lactating, pregnant and steroid-treated castrated males or females, but not from untreated castrated rats, intact male rats or by a PRL Standard (Huerta-Ocampo et al., 2007; Mena et al., 2010). Also, more potent effects occurred with CM from APs of early- than from mid- or late- lactating rats and from rats non-suckled for 8 or 16 h than from those non-suckled for 32 h (Mena et al., 2010). These results suggest that, under certain conditions, PRL variants released from lactating and non-lactating rat APs may regulate the release of PRL variants from the lactotrophs.

In the present study, CM proteins, i.e., PRL variants, that were released in vitro from the AP regions of lactating rats were separated and electroeluted from SDS-PAGE and tested using in vitro incubation techniques. We sought to determine first, whether PRL variants, which are known to occur within the AP (Schwartz & Cherny, 1992; Schwartz, 2000; Wang & Walker, 1993; Sinha, 1992; Bollengier et al., 1989; Huerta-Ocampo et al., 2007; Mena et al., 2010; Mena & Grosvenor, 1972; Asawaroengchai et al., 1978; Nicoll et al., 1969; Mansur & Hymer, 1985), and are released in vitro (Mena et al, 1984; Mena et al., 1992; Huerta-Ocampo et al., 2007; Mena et al., 2010; Mena & Grosvenor, 1972; Asawaroengchai et al., 1978; Nicoll et al., 1969; Mansur & Hymer, 1985), and are released in vitro (Mena et al, 1984; Mena et al., 1992; Huerta-Ocampo et al., 2007; Mena et al., 2010; Mena & Grosvenor, 1972; Asawaroengchai et al., 1978; Nicoll et al., 1969; Mansur & Hymer, 1985), and are released in vitro (Mena et al, 1984; Mena et al., 1992; Huerta-Ocampo et al., 2007; Mena et al., 2010; Mena & Grosvenor, 1972; Asawaroengchai et al., 1978; Nicoll et al., 1969; Mansur & Hymer, 1985) after the suckling-induced PRL transformation i.e., the transfer of the hormone from a pre-releasable to a releasable state (Mena & Grosvenor, 1972; Asawaroengchai et al., 1978; Nicoll et al., 1969; Mansur & Hymer, 1985; Mena et al., 1993) after the suckling-induced PRL transformation i.e., the transfer of the hormone from a pre-releasable to a releasable state (Mena & Grosvenor, 1972; Asawaroengchai et al., 1978; Nicoll et al., 1969; Mansur & Hymer, 1985; Mena et al., 1993) would influence the release of PRL variants from lactating rat lactotrophs; and second, whether the effects of dopamine (DA), thyrotropin-releasing hormone (TRH) and oxytocin (OT) upon PRL release would manifest their effects upon PRL secretion by regulating the release of PRL variants from lactating rat lactotrophs (Mena et al., 2011).

Several reports indicate that PRL has some neuro and gliatrophic properties, and that it mediates the development and maturation of dopaminergic neurons in the hypothalamo-pituitary system (Möderscheim et al., 2007). We showed previously (Morales et al., 2001) that intrathecal injection of PRL in the spinal cord promoted the sympathetic inhibition of milk ejection in lactating rats, and that prolactin variants in CM from the central and peripheral regions of the anterior pituitary from lactating, but not from male rats promoted
the \textit{in vitro} release of PRL from pituitary glands of male rats in a dose-dependent manner (Huerta-Ocampo et al., 2007; Mena et al., 2010).

Our results suggest that PRL variants are released into the CM from the central and peripheral AP regions of lactating rats, that they interact and selectively and specifically stimulate or inhibit the \textit{in vitro} release of other PRL variants from lactotrophs of lactating rat APs; that hypothalamic hormones selectively regulate and interact with PRL variants released from AP lactotrophs, and finally, whether in response to CM’s from lactating rats, changes in electrical activity (EA) occur in male lactotrophs, as well as in astrocytes from the central nervous system, and in intracellular calcium concentration in sympathetic neurons (Mena et al, 2012b).

2. Materials and methods

2.1 General

Animal studies were performed under a protocol similar to the USPHS Guide for the Care and Use of Laboratory Animals and the Official Mexican Guide from Secretary of Agriculture (SAGARPA NOM-062-Z00-1999) published in 2001. Wistar primiparous lactating rats (8-10 pups per litter) were housed individually in a room with a reversed light-dark cycle (14 h light, 10 h darkness) and constant temperature (23-25°C) and were fed \textit{ad libitum} (Purina Chow, Ralston Purina Co., Chicago IL, USA). On postpartum days 10-12 (7 am, local time) groups of mothers had their pups removed, and 6 h later their pups were or were not returned to the mothers and suckled for 15 min. At the end of the suckling or non-suckling periods, the mothers were killed by decapitation after light ether anesthesia. From all animals employed (see below), the pituitary was removed under a dissecting microscope, the posterior lobe was discarded and, using fine forceps as originally described by Papka et al. 1986, and by Bookfor & Frawley, 1987, the central region around the neurointermediate lobe and the peripheral region i.e., the rest of the AP tissue (Boockfor & Frawley, 1987; Diaz et al., 2002) were dissected independently, and incubated in Earle’s medium as described below.

2.2. Preparation of concentrated conditioned media

In individual flasks containing 300 μl of Earle’s medium, media were conditioned by incubating tissue fragments corresponding to the central (CR) and peripheral (PR) pituitary regions from lactating rats. The pituitary fragments were incubated immediately after removal to prevent disruption of hormone storage dynamics (Mena et al., 1992; Diaz et al., 2002). Flasks containing the pituitary fragments were gassed with 95% O₂, 5% CO₂, sealed with rubber stoppers and incubated at 37°C in a water bath shaker (American Optical, Buffalo NY, USA) for 1h. CM from pituitary fragments of each group of rats employed was concentrated and desalted in a Centricon micro-concentrator (Centriprep, Millipore, Bredford MA, USA) and stored frozen until assayed, along with the corresponding primary cultures of pituitary cells or with cultures of sympathetic neurons.
The dose-response effects of DA (0.5, 1.0, 1.5 μM), TRH (0.1, 1.0, 10 μM) and OT (0.1-10 μM), upon the in vitro release of PRL variants previously exposed to the electroeluted PRL variants from NS and S lactating rat APs were determined by Enzyme-linked immunoabsorbant assay (ELISA).

2.3. SDS-PAGE

The PRL released into the media was determined by non-denaturing SDS-PAGE (12.5% gels) and Western blotting. The gels of SDS-PAGE were divided into 6 fractions which encompassed PRL variants from 6 to 97 kDa. The proteins in each fraction were electrophoretically eluted, dialyzed, lyophilized, and then assayed by ELISA for PRL content, as well as for their effects upon PRL secretion on primary culture of pituitary cells from the lactating rats, different concentrations of hypothalamic hormones, and in central and peripheral cultures of sympathetic neurons and astrocytes from hippocampus.

2.4. Primary cultures of pituitary cells

Pituitary fragments from male APs, or from NS and S lactating rats (n=5) corresponding to the central and peripheral regions of the anterior pituitary were dissected and processed separately. Primary cultures (lactotrophs) were prepared as described by Fiordelisio & Hernandez-Cruz, 2002. The tissue fragments were gently triturated with a Pasteur pipette; the cells were collected by centrifuging for 10 min at 185 x g, and washed twice with Dulbecco’s Modified Eagle’s Medium (DMEM) containing 10% BSA. The pellet was resuspended in DMEM medium, supplemented with 10% horse serum, 2% Fetal Bovine Serum, 10,000 U penicillin, 10 mg/ml streptomycin, all from Gibco BRL, Grand Island NY, USA. The cultures were maintained for 24 h at 37°C in a humidified atmosphere (95% air and 5% CO2). The primary cultures were placed in the bottom of 24-multiwell culture plates (Costar, Cambridge, MA, USA) at a density of 2 x10⁴ cells per well and three replicates were used in each experiment.

2.5. Cultures of astrocytes from the hippocampal and medial preoptic areas

Astrocyte cultures were obtained as shown previously by Hernández-Morales and García-Colunga, 2009. Two newborn Wistar rats were killed by decapitation and their brains removed. Slices from hippocampal CA1 region, or medial preoptic area were dissected and then the tissue was dissociated (~5000 cells/mL). The suspended cells were placed on a glass coverslip in a 35-mm Petri dish coated with poly-L-ornithine with DMEM supplemented with 10% FBS, 100 U/mL penicillin, 0.1 mg/mL streptomycin and 11 μg/mL piruvate was added. After 24 h, the medium was changed by Neurobasal medium supplemented with G5 (specific for growing astrocytes) and 100 U/mL penicillin, and 0.1 mg/mL streptomycin and 2 mM of L-glutamine. Cultures were kept under controlled air and temperature.
2.6. Cultures of sympathetic neurons

Cultures of sympathetic neurons were obtained as previously reported by Fiordelisio & Hernandez-Cruz, 2002. Briefly, ganglia from 10-day-old rats were removed under aseptic conditions after ether anaesthesia and cervical dislocation. After cleaning and chopping, the neurons were incubated in Ca2+/Mg2+-free Hanks solution with 1 mg/ml trypsin (Worthington Biochem Co.) and 2 mg/ml DNAse I for 30 min at 37°C. After digestion, trypsin was inactivated by dilution in DMEM containing 10% fetal bovine serum (FBS) and 1 mg/ml trypsin inhibitor (Sigma) and the tissue was incubated in Hanks solution with 2 mg/ml collagenase and 2 mg/ml DNAse I for 30 min at 37°C. After trituration with a Pasteur pipette, the cell suspension was centrifuged, washed twice in Hanks solution, and resuspended in fresh control culture medium. Cells were seeded on poly L-lysine-treated #1 round glass coverslips (1r105 cells per well), and maintained in control culture medium supplemented with 30 ng/ml of 7S NGF (Sigma) at 37°C in a humidified atmosphere of 95% air and 5% CO2. Culture medium was changed three times per week. All experiments were carried out with cultures less than 5 days old.

2.7. Electrophysiology

Astrocyte ion currents were recorded using the whole-cell voltage-clamp technique, as shown previously by Hernández-Morales and García-Colunga, 2009. Primary astrocytes between 4-7 days of culture were placed in a recording chamber and continuously superfused with control solution containing (mM): 136 NaCl, 2.5 KCl, 10 HEPES, 4 CaCl2, 0.5 MgCl2, 10 glucose, pH 7.2. Pipettes were filled with a solution containing (mM): 130 K-glucuronate, 10 NaCl, 10 EGTA, 10 HEPES, 2 ATP, 0.2 GTP, pH 7.2, having a resistance of 3-5 MΩ. Astrocytes were held at a potential of -60 mV. The data were analyzed with pClamp 8.2 software and Origin 7. We determined changes in electrical activity (EA) in response to CM’s from lactating and from male rats in male lactotrophs, as well as in neurons from the central nervous system.

2.8. Measurements of intracellular Ca2+ concentration

Primary astrocytes from hippocampus, medial preoptic area, sympathetic neurons or male rat lactotrophs, between 4-7 days of culture, were incubated for 30 min with the Ca2+ indicator Fluo 4-AM. Following this, cells were washed out three times for 10 min with solution containing (mM): 136 NaCl, 2.5 KCl, 10 HEPES, 4 CaCl2, 0.5 MgCl2, 10 glucose, pH 7.2. Cells were recorded on a Confocal Microscope LSM-510. Local application of CM was carried on with U-tube system placed at ~250 μm from the recorded cell. Cells were excited at 488 nm-Ar laser. Fluorescent intensity represents the change in intracellular calcium concentration and is measured in the whole soma. Data are expressed as a change from basal intensity (F0) to maximum intensity reached (%ΔF), %ΔF/F0. Then, we determined fluctuations in intracellular Ca2+ concentration, [Ca2+]i, in male lactotrophs in response to CM from lactating and from male rats.
3. Statistical analysis

The PRL concentration was calculated by linear regression and values of PRL concentration obtained by ELISA were averaged for each experimental group. Statistical differences were determined by a one-way analysis of variance (ANOVA), using Dunnett’s test, and all treatments were compared versus the control (Earle’s medium or Total PRL). Comparisons were analysed with the Graph Pad 5.0 Software, Inc. (San Diego, CA). The significance level was set at p<0.05. Each control or test compound was assayed in duplicate, and the assays were performed three times (n=3).

4. Experiments

4.1. Autocrine regulation of prolactin secretion

4.1.1. PRL content of electroeluted (EE) PRL variants released from AP regions of lactating non-suckled (NS) and suckled (S) rats

The PRL variants released from each AP region of NS and S rats were analyzed previously by SDS-PAGE and by Western blotting (32, 33). In the present study these PRL variants were electroeluted i.e., EE PRL from fractions 1-6 after SDS-PAGE; and the PRL content of each fraction was determined by ELISA, as shown in Fig. 1 A-D, and subsequently, to determine whether their increased and/or decreased release of PRL variants, each electroeluted PRL fraction (EE PRL) was incubated with lactotrophs of each AP region of non-suckled and suckled rat APs i.e., Figs. 2-3 (A-D). The fractions contained bands of 7-23 to 97 kDa under NR conditions, and 7-16 to 42 kDa under R conditions, and between 1 and more than 20 ng/μl of PRL variant protein. However, in spite of these variations, similar amounts of total PRL (about 60 ng/μl right panels), were released from each AP regions of NS and S rat, except for the higher amount (80 ng/μl) released from the central AP region of NS rats.

4.1.2. Effects of electroeluted PRL variants (EE PRL), released from AP regions of lactating non-suckled (NS) rats, upon the in vitro release of PRL variants from lactotrophs of NS rats

The EE PRL variants that were released from each AP region of NS rats (Figs. 1 A-B) were tested for their effects upon the amount of PRL release by lactotrophs from AP regions of NS rats, and the results are shown in Fig. 2A-B. CM from the PR region of NS rat APs (Fig. 1 A) contained a low concentration of PRL (< 3 ng/μl) in fraction 2 and high (about 10 ng/μl) in fractions 1 and 3-6; after incubation with the EE PRL variants, lactotrophs of the same region, i.e., the peripheral AP region of NS rats, exhibited increased release i.e., 5-7 ng/μl of PRL variants 1, 2, 4, and 6, and decreased release of PRL variants 3 and 5. When incubated with EE fractions from CM of the central AP region of NS rats, lactotrophs from the same region showed increased release of PRL variants in fractions 1 and 6, and lower release in fractions 2, 3, 4 and 5. Thus, except for the amounts of PRL released from fractions 1 and 6, whose levels were higher or similar to those of the other EE PRL variants, the amounts of other PRL variants released were significantly lower than those of the EE variants. When the central AP
Figure 1. (A-B). SDS-PAGE (left panels) and prolactin (PRL) content (ng/μl) of PRL variants (middle and right panels) released from the peripheral (PR) and central (CR) adenohypophyseal regions of non-suckled (NS) rats in conditioned medium (CM) electroeluted (EE) from SDS-PAGE.
(AP) regions of non-suckled (NS) rats, and electroeluted (EE PRL) from fractions 1-6 of SDS-PAGE. Data are means ± SEM. Letters (a-d) indicates $P < 0.05$ difference between fractions of EE PRL.

(C-D). SDS-PAGE (left panels) and prolactin (PRL) content (ng/μl) of PRL variants (middle and right panels) released from peripheral (PR) and central (CR) AP regions of suckled (S) rats and electroeluted from fractions 1-6 of SDS-PAGE. Data are means ± SEM. Letters (a-d) indicates $P < 0.05$ for the difference of PRL content between electroeluted fractions.

region of NS rats was incubated with the EE PRL variants from the peripheral AP region of NS rats, medium levels (about 10 ng/μl) of PRL variants 1 and 6, and medium to low levels to PRL variants 2, 3, 4, and 5 were released. As a result of these effects, high levels of total PRL were released from the peripheral but not from the central AP region; indeed, total PRL release from the central AP region was significantly depressed, below the initial level.

In figure 2B, the PRL content of the EE control PRL variants released from the central AP region of NS rats was low in fractions 1, 2 and 4 of CM, and high (>10 ng/μl) in fractions 3, 5 and 6. Also, with respect to the effect of incubating lactotrophs from the central AP region of NS rats with the EE PRL variants from the peripheral AP region, increased release occurred only of PRL variants 1 and 6; and low levels occurred to PRL variants 2-5 from the central AP region; and only the PRL variant 6 was above the zero level, i.e., about 10 ng/μl. Overall, significantly lower levels of PRL than those contained both in the EE PRL variants and in those released from the peripheral region, were released from lactotrophs of both the central and peripheral AP regions.

4.1.3 Effects of EE PRL variants released from AP regions of lactating non-suckled (NS) rats upon the in vitro release of PRL variants from lactotrophs of suckled (S) rat APs

The effect of incubating lactotrophs from AP regions of S rats with EE PRL variants released from AP regions of NS rats is shown in figures 2 C-D. In figure 2 C the PRL level was low in fraction 2, but medium to high levels, (about 10 ng/μl) in fractions 1, 3-6; the amount of PRL released from the same AP region of S rats was around zero in fractions 2-5; and low in fractions 1 and 6. Overall, significantly lower amounts of PRL were released from the peripheral AP region of S rats. Also, as shown in figure 2D, the amount of EE PRL released from the central AP region of NS rat APs was low in fractions 1, 2 and 4 and high in fractions 3, 5 and 6; the total amount of released PRL from the peripheral AP region was zero in all fractions, and thus, it was significantly lower than that of the EE PRL. With respect to the amount of PRL released from the central AP region, only fractions 2 and 6 showed high levels and the levels of the other fractions were much lowers (1-3 ng/μl). Thus, as with the effect of CM from the PR of NS rats, the amount of released PRL from the peripheral AP region of S rats, was less than zero, i.e., lower than that of the EE control PRL, and of the still lower amount of PRL released from the central AP region, whose levels also were lower than those of the EE PRL.
Figure 2. (A-B). Effect of the PRL variants electroeluted (EE PRL) in fractions 1-6 from SDS-PAGE of CM from the peripheral (PR) (panel A), and central (CR) (panel B), AP regions of non-suckled (NS) rats.
lactating rats upon the *in vitro* release of PRL variants by lactotrophs from AP regions of NS lactating rats. Data are means ± SEM. *Differences \( p < 0.05 \) versus control (EE PRL). (C-D). Effect of the PRL variants electroeluted (EE PRL) in fractions 1-6 from SDS-PAGE of CM from the peripheral (PR) (panel A), and central (CR) (panel B), AP regions of non-suckled (NS) lactating rats upon the *in vitro* release of PRL variants by lactotrophs from AP regions of suckled (S) lactating rats. Data are means ± SEM. *Differences \( p < 0.05 \) versus control (EE PRL).

### 4.1.4. Effects of electroeluted PRL variants released from AP regions of lactating suckled (S) rats upon the *in vitro* release of PRL variants from lactotrophs of suckled (S) rat APs

The effect of incubating lactotrophs from AP regions of S rats, with EE PRL variants released from AP regions of S rats is shown in figures 3 A-B. As shown in figure 3A, the EE PRL content from the peripheral AP region (c.f. Fig. 1C-D) showed medium to high levels (5-20 ng/μl), in all fractions except fraction 2; the amount of PRL released from the peripheral AP region of S rats was medium to high in fractions 1, 3 and 6 and medium to low in fractions 2, 4 and 5; and with respect to the amount of EE PRL released from the central AP region of S rats, (figure 3B), low and medium levels (2 and 8-10 ng/μl), were found in fractions 1, 2 and 3-6, respectively; and with respect to the effect of incubating lactotrophs from the central AP region of S rats with EE PRL variants from the same AP region of S rats, the levels were low in fractions 1 and 2 and higher (around 10 ng/μl) in fractions 3-6; and after incubation with the EE PRL, medium to low levels (1-6 ng/μl) occurred in fractions 1 and 2, reduced levels (-10 ng) in fraction 4 and higher levels (8-10 ng/μl) in fractions 3, 5 and 6 of PRL variants were released from the peripheral AP region of S rats, below zero levels were released from fraction 4 of the same AP region; and there was only a small stimulatory effect on PRL variants released from the central AP region to below zero levels in all fractions. As a result of these effects, the total amount of PRL released from the peripheral AP region, and particularly from the central AP region, was significantly lower than that of both the EE PRL variants and of the amount released from the peripheral AP region.

### 4.1.5. Effects of electroeluted PRL variants, released from AP regions of lactating, suckled (S) rats, upon the *in vitro* release of PRL variants from lactotrophs of non-suckled (NS) rat APs

The effect of PRL variants released from AP regions of suckled rats upon the release of PRL variants from lactotrophs of the peripheral and the central AP regions of non-suckled rats is shown in figures 3 C-D. In Fig. 3C, the PRL content of the EE fractions from the CM of the peripheral AP regions of S rats was low to medium in fractions 2 and 3, medium to high in fractions 1, 4 and 5, and particularly high in fraction 6; and, as a result of incubation, the amount of PRL released from lactotrophs of the peripheral AP region of NS rats was low in fractions 1, 4-6, and high only in fractions 2 and 3; and from the central AP region of these rats, the amount of PRL released was particularly high in fractions 1 and 6; medium in fractions 2 and 3, and low in fractions 1 and 4-5. As a result of these interactions, the amount of total PRL released from both AP regions was significantly lower than the amount electroeluted from SDS-PAGE, but higher than that shown from NS and S rat AP regions, (c.f. Figs. 2 A-B), due to the effect of CM from NS and S rats.
Figure 3. (A-B). Effect of the PRL variants electroeluted (EE PRL) in fractions 1-6 from SDS-PAGE of CM from the peripheral (PR) (panel A), and central (CR) (panel B), AP regions of suckled (S) lactating rats.
rats upon the in vitro release of PRL variants by lactotrophs from AP regions of suckled (S) lactating rats. Data are means ± SEM. *Differences *P* < 0.05 versus control (EE PRL).

(C-D). Effect of the PRL variants electroeluted (EE PRL) in fractions 1-6 from SDS-PAGE of CM from the peripheral (PR) (panel A), and central (CR) (panel B), AP regions of suckled (S) lactating rats upon the in vitro release of PRL variants by lactotrophs from AP regions of non-suckled (NS) lactating rats. Data are means ± SEM. *Differences *P* < 0.05 versus control (EE PRL).

The PRL content of the electroeluted fractions from the central AP region of S rats was low in fractions 1 and 2, and high in fractions 3-6 of CM (Figure 3D). However, the amount of PRL released mainly from the peripheral, and in part also by the central AP region, was higher by the peripheral than the EE PRL variants in fractions 1, 2 and 5, 6; this level was about the same in fraction 3, and lower in fraction 4; and with respect to the amount of PRL released from the central AP region it was higher than the electroeluted hormone in fractions 1 and 2, lower in fraction 3 and 4, and about the same high level in fractions 5 and 6. As a result of these effects, an increased release of the hormone occurred from both AP regions, particularly from the peripheral region, whose levels, except from that of fraction 3, were significantly higher than those of the EE PRL as well as of that released from the central AP region, whose levels in fractions 1 and 2 were also higher than those of the EE PRL.

4.2. Effects of hypothalamic hormones upon the release of PRL variants from AP regions of NS and S rats

4.2.1. Dose-response effects of dopamine

The effects of dopamine (DA) upon the release of PRL variants 1-6 from the lactotrophs of the peripheral and central AP regions of non-suckled (NS) and suckled (S) rats are shown in figures 4 A-D. As shown in Fig. 4 A, as compared with the amount of PRL released without DA, the low dose of DA (0.5 μM) inhibited the release of PRL variant 2 and stimulated PRL variants 3 and 5 of the peripheral AP regions of NS rats, and showed no effect upon the release of PRL variants 4 and 6 from the same AP region. Higher doses of DA increased the release of PRL variants in fractions 3 and 5, but 1.5 μM DA inhibited the release of PRL variants 1 and 6. As a result of these effects, the total amount of released PRL from the peripheral AP region of NS rats was decreased only by the highest dose of DA (1.5 μM) but not by the lower and intermediate doses.

The effects of dopamine upon the release of PRL variants from lactotrophs of the central AP regions of non-suckled rats are shown in figure 4 B. The low dose of DA inhibited the release of the PRL variant 1 and showed no effect upon PRL variants 2 and 4, but it promoted a strong release of PRL variants 3, 5 and 6 from the central AP regions of NS rats; 1.0 μM dopamine provoked decreased release of PRL variant 1, and increased release of PRL variants 2, 3, 5 and 6. With 1.5 μM DA, decreased release occurred in fraction 1, and increased release in fractions 3, 5 and 6.

With respect to the effects of DA upon the release of PRL variants from the peripheral AP region of suckled rats APs, as shown in figure 4 C, at the low dose of DA inhibition occurred in fraction 3, stimulation of PRL in fractions 2, 5 and 6, and no effect on PRL variants in fractions
1 and 4. In the presence of 1.0 μM DA there was inhibition of PRL variants in fraction 3, no effect in fraction 4, and stimulation in fractions 2 and 5, 6. The total amount of PRL released from the peripheral AP region of S rats was decreased only by 1.0 and 1.5 μM DA, but not by the lower dose. The effects of DA upon the release of PRL variants from lactotrophs of

Figure 4. A-D. Effect of dose-response of the PRL variants electroeluted in fractions 1-6 from SDS-PAGE of CM from the peripheral (PR) and the central (CR) regions of adenohypophysis (AP) of suckled (S) and non-suckled (NS) lactating rats incubated with 0.5, 1.0 and 1.5 μM of dopamine (DA), upon the in vitro release of PRL variants by lactotrophs from AP regions of NS and S lactating rats. Data are means ± SEM. *Differences $P < 0.05$ versus control (PRL content without DA).
the central AP region of suckled rats are shown in figure 4D. The low dose of DA inhibited the release of PRL in fraction 1, had no effect upon the release of the PRL variant 3, but it promoted the release of PRL variants 2, 4 and 6; the intermediate dose of DA also inhibited the release of PRL from fraction 1, and promoted the release of fractions 2, 4 and 6; 1.5 μM DA inhibited the release of PRL fractions 1 and 2, but it promoted the release of fractions 3-6.

4.2.2. Dose-response effects of TRH

The effects of different doses of TRH, i.e., 0.1, 1.0 and 10 μM, upon the release of PRL variants from AP regions of NS and S rats are shown in Figs. 5 A-D and the values obtained after TRH, are compared with the control values of PRL that were released in the absence of TRH. As compared with control values, without TRH, 0.1 μM TRH provoked increased release, i.e., stimulation of PRL, in fractions 1, 3-6; and inhibition in fraction 2 from the peripheral AP region of NS rats (Fig. 5 A). The effect of 1.0 and 10 μM TRH was to increase the release of all PRL variants 1-6.

The effect of TRH upon the release of PRL variants from the central AP region of NS rats is shown in Fig. 5 B. With 0.1 μM TRH, decreased release of PRL occurred in fraction 1, no change in fractions 2 and 4, and increased release in fractions 3, 5 and 6. Also, with respect to the effect of the higher doses of TRH, increased release occurred to PRL in all fractions 1-6. Fig. 5 C, shows the effect of the low dose of TRH upon the release of PRL variants from the peripheral AP region of S rats. As shown there, release of PRL variants 1, 4, 5 and 6 was stimulated, and there was no effect on PRL variants 2 and 3; and with respect to the effect of the low dose of TRH upon the release of PRL variants from the central AP region of S rats (Fig. 5 D) a decreased release of PRL variant 1; increased release of PRL variants 3, 4 and 6, and no effect upon PRL variants 2 and 5 were observed.

4.2.3. Dose-response effects of oxytocin

The dose-response effects of oxytocin (OT) upon the release of PRL variants from NS rat APs are shown in Figs. 6 A-D. With 0.1 μM oxytocin, increased release of PRL, relative to the control, occurred only for PRL variants 3, 5 and 6 of the PR region of NS rat APs, but the release of PRL in fractions 1, 2 and 4 was inhibited. With respect to the effect of 1.0 μM of oxytocin upon the release of PRL variants from the peripheral AP region of NS rats (Fig. 6 A), there was increased release of PRL variants 3, 5 and 6 and reduced release of PRL variants 1, 2 and 4 from lactotrophs of the peripheral AP region. The high dose of OT, i.e., 10 μM resulted in increased release of PRL variants 2, 3, 5 and 6 from the peripheral AP region and to variants 1-6 from the central AP region of NS rats (Fig. 6 B); and with respect to the effect of the 10 μM OT upon the release of PRL from AP regions of S rats, increased release from the peripheral region occurred for PRL variants 1, 2, 5 and 6; with no effect on release of PRL variants 3 and 4. Finally, with respect to the effect of the high dose of OT upon AP regions of NS rats, there was increased release of fractions 2, 3, 5 and 6 and no effect on fractions 1 and 3 from the peripheral AP region of NS rats; and with the exception of fraction 5 in which there was no effect, increased release occurred in all the other fractions from the central AP region of the NS rats; and upon the effect of the same dose of oxytocin upon the release of PRL variants from the peripheral AP region of S rats, i.e., Fig. 6 C, also, increased release
occurred to PRL variant 5; inhibition to PRL variant 1, and no effect upon PRL variants 2, 3, 4 and 6. And with respect to the effect of the same dose of OT i.e., 1.0 μM upon the release of PRL variants from the central AP region of suckled rats, i.e., Fig. 6 D, lower panel, increased release occurred to PRL variants 1, 2, 5 and 6, and no effect upon PRL variants 3 and 4; and with respect to the effect of the same dose of OT upon the release of PRL variants from the

Figure 5. A-D. Effect of dose-response of the PRL variants electroeluted in fractions 1-6 from SDS-PAGE of CM from the peripheral (PR) and the central (CR) regions of adenohypophysis (AP) of suckled (S) and non-suckled (NS) lactating rats incubated with 0.1, 1.0 and 10 μM of TRH, upon the in vitro release of PRL variants by lactotrophs from AP regions of NS and S lactating rats. Data are means ± SEM. *Differences P < 0.05 versus control (PRL content without TRH).
same, i.e., peripheral AP region of S rats, increased release occurred to PRL variants 1, 2 and 5, 6; and no effect upon PRL variants 3 and 4. Finally, with respect to the effect of the high dose of OT upon the release of PRL from lactotrophs of the PR region of S rats, increased release occurred to PRL variants 1, 2, 5 and 6, with no change of PRL variants 3 and 4; the high dose of OT resulted in increased release of all PRL variant from lactotrophs of the central AP region.

**Figure 6. A-D.** Effect of dose-response of the PRL variants electroeluted in fractions 1-6 from SDS-PAGE of CM from the peripheral (PR) and the central (CR) regions of adenohypophysis (AP) of suckled (S) and non-suckled (NS) lactating rats incubated with 0.1, 1.0 and 10 μM of oxytocin (OT), upon the *in vitro* release of PRL variants by lactotrophs from AP regions of NS and S lactating rats. Data are means ± SEM. *Differences P < 0.05 versus control (PRL content without OT).
4.3. Electrical activity upon neurons

4.3.1. Effect of conditioned medium from the lateral and central AP regions of S rats upon cultured sympathetic neurons

Cultured sympathetic neurons, previously incubated with Fluo 4, to record variations of intracellular [Ca^{2+}], showed clear increases of [Ca^{2+}] within 60 sec of adding conditioned medium (CM) from lactating rats, thus indicating that these neurons were activated by the CM (Figure 7). These effects did not occurred when the neurons were incubated with medium from male rat APs (data not shown).

Figure 7. Effect of 60 s exposure of conditioned medium from the peripheral region of suckled rat APs (CM PRS) upon intracellular calcium concentration in cultured sympathetic neurons.

4.3.2. Effect of conditioned medium from the lateral AP region of suckled and of male rat APs upon electrical activity of male rat lactotrophs, and of astrocytes from the hippocampus and medial preoptic area

As shown in Figures 8 A-D, CM of the peripheral region (CMPR) from male rat APs had no effect upon electrical activity of hippocampal astrocytes (Fig. 8A) whereas application of CM from lactating, suckled rats provoked a cationic inward current, shown as a downward deflection in male lactotrophs (Fig. 8C), as well as in astrocytes from the hippocampus and medial preoptic area (Figs. 8B, D). These responses remained for several seconds after washing CMPR out.

4.3.3. Effect of conditioned medium from lactating and male rat APs upon [Ca^{2+}] concentration in astrocytes from the hippocampus

As shown in Figure 9, CM from lactating, suckled rats (Fig. 9A), but not from male rat APs (Fig. 9B) or from non-suckled rats (data not shown) provoked an increased [Ca^{2+}] in hippocampal astrocytes similar to that induced by application of 120 mM K+ (see Fig. 9C).
Figure 8. (A-D). Effect of a 1 h incubation with conditioned medium (CM), from the peripheral region (PR) of male rats (A), and from suckled lactating rat APs (B-D) upon hippocampal astrocytes (B), neurons from the preoptic area (D) and upon male rat lactotrophs (C).

Figure 9. (A-C). Effect of conditioned media from the peripheral AP region of suckled lactating rats (CMPR) and of male rats upon intracellular calcium concentration in hippocampal astrocytes (A-B). Arrow in C indicates the application of 120 mM K⁺. Images in D are from astrocytes in A-C.
5. Conclusions

This study confirms that PRL variants ranging from 7 to 97 kDa are released from tissue fragment of each AP region of the anterior pituitary gland of non-suckled and suckled lactating rats. When these variants are electroeluted from SDS-PAGE and then incubated with lactotrophs from each AP region of the same type of rats, they exert different effects (promotion, inhibition or no effect) upon the release of PRL variants from lactotrophs of both AP regions of NS and S lactating rats. In support of this was the fact that the immunoprecipitation of PRL contained in the CM from lactating rats, prevented the effects of PRL variants upon PRL release. Thus, these results indicate that autocrine regulatory effects are exerted by PRL variants upon the release of other variants of the hormone from lactating rat APs, and they are in accord with previous studies showing autoregulation of PRL secretion (Nagy et al., 1991; Nagy & Frawley, 1990; Diaz et al., 2002; Spies & Clegg, 1971; Hebert et al., 1979; Melmed et al., 1980). Similar effects on male lactotrophs by CM from pregnant and lactating females and steroid-treated castrated males or females, but not by CM from intact males or by a PRL standard were reported previously (Huerta-Ocampo et al., 2007; Mena et al., 2010).

Prior to fractionation the total PRL variants from both, the central and peripheral AP regions of NS rats stimulated the release of PRL from the peripheral region, but they inhibited its release from the central AP region. However, when CM from lactating rats was fractionated by SDS-PAGE, eluates of fractions 5 and 6 containing 23-25 kDa PRL had the greatest effect on PRL release, although weaker immunoreactive bands with lower, or even inhibitory activity, were also detected in the upper gel fractions. In addition, separation by SDS-PAGE and electroelution of PRL variants indicated that CM from the lactating rat pituitary contains 37 to 46 kDa PRL variants as well as 23 to 25 kDa PRL variants, that exert different effects upon the release of other PRL variants from the lactating rat pituitary and from APs of rats in different conditions Huerta-Ocampo et al., 2007; Mena et al., 2010). Therefore, the present study shows that the lactating rat pituitary produces PRL variants that are absent or deficient in the male pituitary gland and in the PRL Standard, even though the AP of male and of other types of rats, do respond to stimulatory factors released from the anterior pituitary of lactating rats (Mena et al., 2010). The results presented here, together with those in our previous study, also indicate that several PRL variants are produced and released by the lactating rat pituitary (Denef, 1988; Sinha 1992; Asawaroengchai et al., 1978); this hormonal heterogeneity may be physiologically very relevant in the context of autoregulatory mechanisms determining the wide range of PRL effects under different physiological conditions (Schwartz & Cherny, 1992; Schwartz, 2000; Sinha, 1992) and upon different structures (Ben-Jonathan et al., 2001; Lorenson & Walker, 2001; Ho et al., 1993; Celotti et al., 1997).

In addition to the regulatory effects of PRL variants from lactating rats upon the release of the hormone, further evidence of these effects was obtained when CM’s from lactating rats were treated with phosphatase or with endoglycosidase which increased their ability, i.e., that of the PRL variants present in them to stimulate PRL release from lactating rat APs, similar to the effect shown previously upon male rat lactotrophs (Mena et al., 2010). These effects of dephosphorylation and deglycosylation of CM provide additional evidence that PRL variants in CM are responsible for the effects upon lactating rat lactotrophs. PRL released from the AP of lactating and non-lactating rats is phosphorylated and glycosylated...
and thus, it is less bioactive than the dephosphorylated and deglycosylated variants (Ho et al., 1993a; Sinha, 1995, Ho et al., 1993b).

In the present study we also analyzed whether the ability of the hypothalamic hormones dopamine, TRH and oxytocin, as established by many previous in vivo and in vitro studies (Mena et al., 1989), to regulate the release of AP PRL, would be manifest by their direct action upon the lactotrophs and interaction with the autocrine actions of PRL variants; and whether these effects would finally promote or inhibit the release of PRL variants, thereby regulating the release of the hormone. The results obtained showed that the effects of the hypothalamic hormones were exerted upon the lactotrophs both by interacting with the autocrine actions of the PRL variants, and also by regulating the release of PRL variants from these cells. Thus, when a high dose (1.5 μM) of DA was applied directly upon the lactotrophs of NS and S rats, the secretion of most PRL variants from both AP regions of non-suckled and suckled rats was inhibited, as previously reported by others (Nagy et al., 1991), whereas the lower dose (0.5 μM) slightly stimulated the release of PRL variants, mainly from the central AP region of suckled rats. TRH provoked an increased release of some PRL variants from both the central and peripheral AP regions of NS and S rats, and OT at 1 and 10 μM, showed an intense stimulatory effect, particularly of 23-34 kDa PRL, from both AP regions of non-suckled and suckled lactating rat APs. Thus, these effects of hypothalamic hormones upon the release of PRL variants may also regulate, and thus interact with, the autocrine effects exerted by the PRL variants and lead to an integrative regulation of PRL secretion (Mena et al., 1989; Mena et al., 2011).

This study shows that when pituitaries from male rats are incubated for a short period of time in conditioned media from each pituitary region of lactating rats, either suckled or non-suckled, there is a significant dose-dependent increase of PRL release from male rat lactotrophs. Also, as shown previously (Mena et al., 2010) our results obtained by Western blotting confirm that CM from lactating rat pituitary contains several prolactin variants, i.e., 37-46 kDa as well as 16-25 kDa, capable of stimulating PRL release from male rat pituitary. In addition, CM obtained from male rat pituitary regions as well as PRL standard have no significant stimulatory effect on PRL secretion of pituitary regions from either lactating or male rats (Huerta-Ocampo, 2007; Mena et al., 2010), this suggests that the male pituitary gland is deficient in the same PRL variants, and of other possible factors, that are released by the lactating rat AP, even though it does contain receptors for stimulating factors released from the anterior pituitary of lactating rats (Mena et al., 2011; Mena et al., 2012a).

Based upon these results, it was of interest to determine whether production and release of pituitary prolactin variants contained in CM from lactating rats under different conditions, and their effects upon PRL release from incubated male AP regions, vary depending on the animal’s physiological condition, and whether CM’s from lactating or male rats exert effect upon brain structures, i.e., astrocytes in the hippocampus and medial preoptic area, and of sympathetic neurons (Fiordelisio & Hernandez-Cruz, 2002). The results obtained from Electrical recording of these astrocytes showed that astrocytic activation occurred upon exposure to CM from the lateral and central regions of lactating suckled rat APs (Hernández-Morales M & García-Colunga, 2009).
In conclusion, the results of the present and previous studies suggest that, in addition to regulation by hypothalamic and other influences, the release of PRL variants from the lactating rat AP is also regulated by autocrine influences exerted upon the gland by the previously released PRL variants; furthermore in parallel and interacting with such autocrine regulation, the effect of the hypothalamic hormones on PRL release is also regulated through the same mechanism i.e., the stimulation or inhibition of the release of PRL variants from the pituitary gland. Moreover, the present results confirm previous findings (Huerta-Ocampo, 2007; Mena et al., 2010; Mena et al., 2011; Möderscheim et al., 2007, Mena et al., 2012a), that CM from lactating rats, contain prolactin variants capable of inducing rapid release of PRL from the untreated male rat pituitary, and that they can also activate of astrocytes and neurons in different areas of the central and peripheral nervous system (Mena et al, 2012b).

**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>AP</td>
<td>Anterior pituitary</td>
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<tr>
<td>[Ca(^{2+})]</td>
<td>Intracellular calcium concentration</td>
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<tr>
<td>CM</td>
<td>Conditioned media</td>
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<tr>
<td>CR</td>
<td>Central region</td>
</tr>
<tr>
<td>DA</td>
<td>Dopamina</td>
</tr>
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<td>EE</td>
<td>Electroeluted</td>
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<tr>
<td>EA</td>
<td>Electrical activity</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunoabsorbant assay</td>
</tr>
<tr>
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<td>Kilodalton</td>
</tr>
<tr>
<td>NR</td>
<td>Non-reducing</td>
</tr>
<tr>
<td>NS</td>
<td>Non-suckled</td>
</tr>
<tr>
<td>OT</td>
<td>Oxytocin</td>
</tr>
<tr>
<td>PR</td>
<td>Peripheral region</td>
</tr>
<tr>
<td>PRL</td>
<td>Prolactin</td>
</tr>
<tr>
<td>S</td>
<td>Suckled</td>
</tr>
<tr>
<td>TRH</td>
<td>Thyrotropin releasing hormone</td>
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6. References


