Regulatory Effects of Dopamine, Oxytocin, and Thyrotropin-Releasing Hormone on the Release of Prolactin Variants From the Adenohypophysis of Lactating Rats

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ABSTRACT

Background: Previous studies used western blotting to show that prolactin (PRL) released from the adenohypophysis (AP) of lactating rats in vitro contains size variants from 7–14 kDa to 70–97 kDa. These variants when eluted from sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and incubated with AP lactotrophs from male rats and rats in other conditions, promoted the selective stimulation and/or inhibition of the in vitro release of PRL variants.
Objectives: In the present study, we determined the regulatory effects of dopamine (DA), thyrotropin-releasing hormone (TRH), and oxytocin (OT) on release of PRL variants from AP lactotrophs.
Materials and Methods: Primary cultures of lactotrophs from lactating rats, which were non-suckled (NS) for 6 h or suckled (S) for 15 min after NS, were incubated with PRL variants that were electroeluted from SDS-PAGE gels along with different doses of DA (0.5, 1.0, 1.5 μM), TRH (0.1, 1.0, 10 μM), or OT (0.1, 1.0, 10 μM). The secretion of PRL from the lactotrophs was determined by enzyme-linked immunosorbant assay.
Results: The results showed stimulatory and/or inhibitory effects of DA, TRH, and OT on the release of PRL variants from AP lactotrophs both by the presence of PRL variants.
Conclusions: These results indicate that PRL variants are released from AP lactotrophs, and, in concert with hypothalamic hormones, they regulate the release of PRL from lactating rat APs.

▸ *Implication for health policy/practice/research/medical education:*
PRL secretion exerted by variants of the PRL in presence of hypothalamic hormones.

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1. Background

The synthesis and release of prolactin (PRL) by lactotrophs in the anterior pituitary (AP) are regulated by autocrine and paracrine signals from the anterior pituitary itself (for a review see 1-19) and by gonadal steroids (20, 21). In addition, it has been reported that total PRL and PRL variants (4, 22) are secreted under different physiological conditions (4, 16-18, 23-25), and interactions with other pituitary cells (4, 7, 11, 16) and hypothalamic hormones (3, 10, 12, 26-30) are known to occur in different circumstances. For instance, the lactotrophs of lactating rats from the central AP region, which is the region surrounding the neurointermediate pituitary lobe (23, 31, 32), 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 periph-

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eral region of the gland (23, 31, 33-39). In these studies, it is possible that the release of PRL variants may have influenced the regulation of PRL release.

In previous reports (37, 38), we showed that reconstituted conditioned media (RCM) and 7–14 kDa and 70–97 kDa PRL variants from lactating rat APs promoted vesicular release of the hormone from preformed, mature PRL granules of male rat APs *in vitro*, and this release was independent of PRL synthesis (37). Autocrine and paracrine actions have also been shown to occur within the AP (4, 6, 9, 11, 14, 19, 34, 37, 38) when the central and peripheral AP regions of lactating rats were incubated with RCM from the pituitaries of lactating, pregnant, and steroid-treated castrated males or females *in vitro*, but not from the pituitaries of untreated castrated rats, intact male rats, or with a PRL standard (37, 38). In addition, more potent effects were observed with the RCM of the APs from early-lactating rats than from mid- or late-lactating rats, and from rats non-suckled for 8 or 16 h than from rats non-suckled for 32 h (38). 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 lactotrophs.

2. Objectives

In the present study, RCM proteins (PRL variants) that were released *in vitro* from the AP regions of lactating rats were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), electroeluted, and tested *in vitro* by using incubation techniques. We sought to determine whether the effects of dopamine (DA), thyrotropin-releasing hormone (TRH), and oxytocin (OT) on PRL release would manifest their effects upon PRL secretion by regulating the release of PRL variants from lactating rat lactotrophs. Our results confirm that PRL variants are released into the conditioned media CM from the central and peripheral AP regions of lactating rats, that they interact with and selectively and specifically regulate the release of other PRL variants from the lactotrophs of lactating rat APs *in vitro* (34, 37, 38), and finally, that hypothalamic hormones selectively regulate and interact with the PRL variants released from AP lactotrophs.

3. Materials and Methods

3.1. General

Animal studies were performed using 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. Primiparous lactating Wistar rats (8–10 pups per litter) were individually housed in a room with a reversed light-dark cycle (14 h light, 10 h darkness) at a constant temperature of 23–25°C, and fed *ad libitum* (Purina Chow; Ralston Purina, 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 either were or were not returned to their mothers to suckle for 15 min. At the end of the suckling period, the mothers were killed by decapitation after light ether anesthesia. The pituitary was removed from all animals (see below) under a dissecting microscope, the posterior lobe was discarded, and, using fine forceps as originally described by Papka *et al.* (32) and Bookfor and Frawley (23), the central region around the neurointermediate lobe and the peripheral region (the remainder of the AP tissue) (23, 31, 33, 34, 36-38), were independently dissected and incubated as described below.

3.2. Preparation of Concentrated Conditioned Media

Conditioned media (CM) were obtained in individual flasks containing 300 μL of Earle's medium by incubating tissue fragments corresponding to the central (CR) and peripheral (PR) pituitary regions (23, 32) from lactating rats. The pituitary fragments were immediately incubated after removal to prevent disruption of hormone storage dynamics (24, 28, 34). Flasks containing the pituitary fragments were gassed with 95% O_2 and 5% CO_2, sealed with rubber stoppers, and incubated at 37°C in a water bath shaker (American Optical, Buffalo, NY, USA) for 1 h. The CM from the pituitary fragments of the rats were concentrated and desalted in a Centricon micro-concentrator (Centripep; Millipore, Bedford, MA, USA), frozen, and stored until assay, along with the corresponding tissue fragments.

3.3. SDS-PAGE

In previous studies (37, 38) as well as in the present study, the amount of PRL released into the media was determined after non-denaturing PAGE. The 12.5% SDS-PAGE gels were 1.0 mm thick, 6 cm long, and were electrophoresed using the buffer system of Laemmli (39) and Bradford (40) in a mini Protean III cell (Bio-Rad, Hercules, CA, USA). CM samples were electrophoresed in non-reducing (NR) conditions or in the presence of 5% (w/v) 2-mercaptoethanol (reducing (R) conditions), and were then divided into 6 fractions, which encompassed the PRL variants from 6 to 97 kDa, as previously shown (37, 38). The proteins in each fraction were electrophoretically eluted, dialyzed, lyophilized, and then assayed by enzyme-linked immunosorbant assay (ELISA) for PRL content as well as their effects on PRL secretion from primary cultures of pituitary cells of lactating rats. The dose-response effects of DA (0.5, 1.0, and 1.5 μM), TRH (0.1, 1.0, and 10 μM), and OT (0.1–10 μM) on the release of PRL variants from NS and S lactating rat APs that were previously exposed to the electroeluted PRL variants *in vitro* were determined by ELISA.

3.4. ELISA

The concentration of PRL in the CM and eluates from the SDS-PAGE gels was determined by the ELISA method

described by Signorella and Hymer (41). Briefly, 96-well microtitre plates (Immulon 2HB; Dynex, Chantilly, VA, USA) were coated with 10 ng of rat PRL in 100 μL of 1 M carbonate buffer, pH 10.3, overnight at 4°C. The plates were then washed with TPBS (0.01 M sodium phosphate, 0.15 mM NaCl, 0.05% v/v Tween-20, pH 7). This washing procedure was performed after each incubation step. For the standard curve, serial dilutions of rat PRL (NHPP-NIH) (0.06–64 ng/mL) in TPBS were incubated with 100 μL of primary anti-rPRL polyclonal antiserum (1:40,000; NHPP-NIH) in TPBS containing 1% (w/v) non-fat dry milk (Bio-Rad, Hercules, CA, USA) for 16 h. Samples and standards (100 μL) were then added to the coated wells and incubated for 2 h at room temperature. Secondary peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad, Hercules, CA, USA) was then added (1:3,000 in TPBS with 1% non-fat dry milk) and incubated at room temperature for 2 h. Bound secondary antibodies were detected by incubation with 2, 2'-azino-di-[3-ethylbenzthiazoline sulfonate] substrate (Roche, Mannheim, Germany). Plates were read at 405 nm 15 min later with an automatic ELISA Microplate Reader (Bio-Rad, Hercules, CA, USA). The assay has a sensitivity of 2 ng/well and inter-assay and intra-assay coefficients of variation of < 6%.

3.5. Primary Cultures of Pituitary Cells

Lactating rat pituitary fragments (n = 5) corresponding to the central and peripheral regions of the anterior pituitary (23, 32, 36) were dissected and processed separately. Primary cultures were prepared as described by Fiordelisio and Hernandez-Cruz (42). Briefly, the central and peripheral regions of the anterior pituitary of NS, S, or male rats were separated, cut into pieces, and digested by incubation with 2.5 mg/mL of trypsin and 0.1 mg/mL of collagenase (Worthington Biochem Co., Lakewood, NJ, USA) in DMEM. The tissue fragments were gently triturated with a Pasteur pipette. Then, the cells were collected by centrifugation for 10 min at 185 × *g* and washed twice with DMEM containing 10% BSA. The pellet was resuspended in DMEM supplemented with 10% horse serum, 2% fetal bovine serum, 10,000 U of penicillin, and 10 mg/mL streptomycin, (Gibco BRL, Grand Island, NY, USA). The cultures were maintained for 24 h at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

3.6. Statistical Analysis

The PRL concentration was calculated by linear regression and the PRL concentration values obtained by ELISA were averaged for each experimental group. Statistical differences were determined by one-way analysis of variance (ANOVA) using Dunnett's test, and were compared to the control (Earle's medium). Comparisons were analyzed using Graph Pad version 5.0 Software (San Diego, CA, USA). The significance level was set at $P < 0.05$. Each control or test compound was assayed in duplicate, and the assays were performed 3 times (n = 3).

4. Results

4.1. Electrophoretic Analysis of RCM From Lactating NS and S Rat APs Incubated With DA, TRH, and OT

The dose-response effects of DA (0.5–1.5 μM), TRH (0.1–10 μM), and OT (0.1–10 μM) on the release of PRL variants from lactotrophs of each AP region of NS and S rats are shown in *Figures 1–6*. In each figure, the electroeluted (EE) PRL released from each AP region of NS and S rats as well as the amount of PRL released in the presence of the EE PRL variants and in the absence of hypothalamic pituitary hormones. Similar to in previous experiments, the PRL content of the eluted fractions and the effects of DA, TRH, and OT on lactotrophs from each AP region of NS and S rats were measured by ELISA.

4.2. Dose-Response Effects of DA on PRL Release From AP Regions of NS Rats

The effects of DA on 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 1 and 2*. Compared with the amount of the PRL variants released without DA, the low dose of DA (0.5 μM) inhibited release of PRL variant 2, stimulated release of PRL variants 3 and 5, and had no effect on release of PRL variants 4 and 6 from the peripheral AP region of NS rats (*Figure 1A*). Higher doses of DA increased release of the 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 only decreased by the highest dose of DA tested (1.5 μM), but not by the lower and intermediate doses. The effects of DA on the release of PRL variants from lactotrophs of the central AP regions of non-suckled rats are shown in *Figure 1B*. The low dose of DA inhibited the release of PRL variant 1, had no effect on variants 2 and 4, and promoted the strong release of variants 3, 5, and 6 from the central AP regions of NS rats; 1.0 μM DA decreased the release of variant 1 and increased the release of variants 2, 3, 5, and 6 from the central AP regions of NS rats. In contrast, 1.5 μM DA, decreased release of variants electroeluted in fractions variant 1 and increased release of variants 3, 5, and 6 from the central AP regions of NS rats. As shown in *Figure 2A,* the low dose of DA inhibited the release of PRL variant 3, stimulated release of variants 2, 5, and 6, and did not affect release of variants 1 and 4 from the peripheral AP region of suckled rats. Higher doses of DA inhibited PRL variant 3, had no effect on variant 4, and stimulated release of variants 2, 5, and 6. As a result of these effects, the total amount of PRL released from the peripheral AP region of S rats was only decreased by high doses of DA (1.0 and 1.5 μM), but not by the lower dose. The effects of DA on the release of PRL variants from lactotrophs of the central AP region of S rats are shown in *Figure 2B*. The low dose of DA inhibited the release of PRL variant 1, had no effect on variant 3, but

Figure 1. Effect of PRL Variants From the Regions of Adenohypophisis of Non-Suckled Rats With DA.
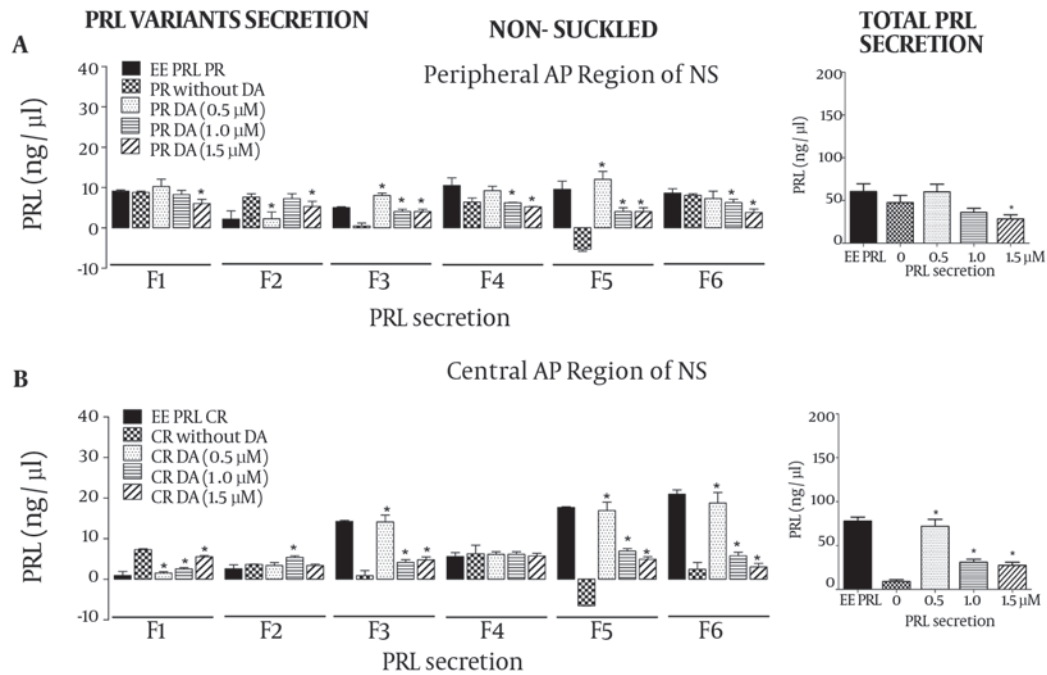
A-B: Effect of prolactin (PRL) variants electroeluted in fractions (F) 1–6 from SDS-PAGE gels of reconstituted conditioned media from the peripheral (PR) and central (CR) regions of adenohypophysis (AP) of non-suckled (NS) lactating rats incubated with DA on the in vitro release of PRL variants from lacto-trophs of the AP regions from NS and suckled (S) lactating rats.
Data shown are the means ± SEM.
* $P < 0.05$ *vs*. the control (electroeluted [EE] PRL).

Figure 2. Effect of PRL Variants From the Regions of Adenohypophisis of Suckled Rats With DA.
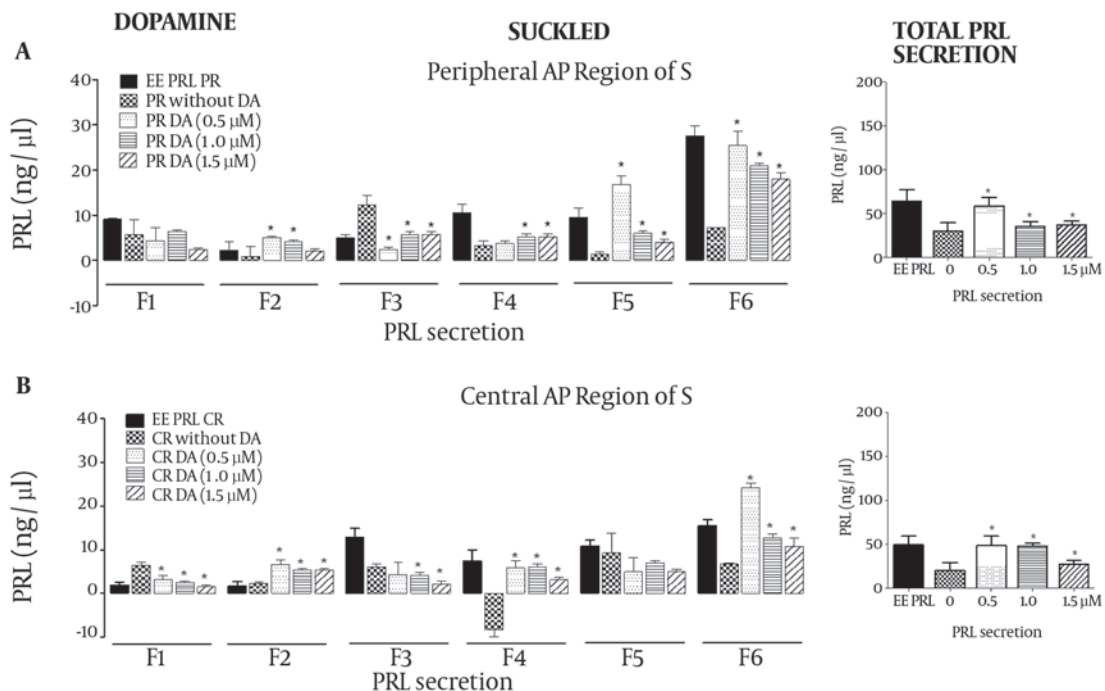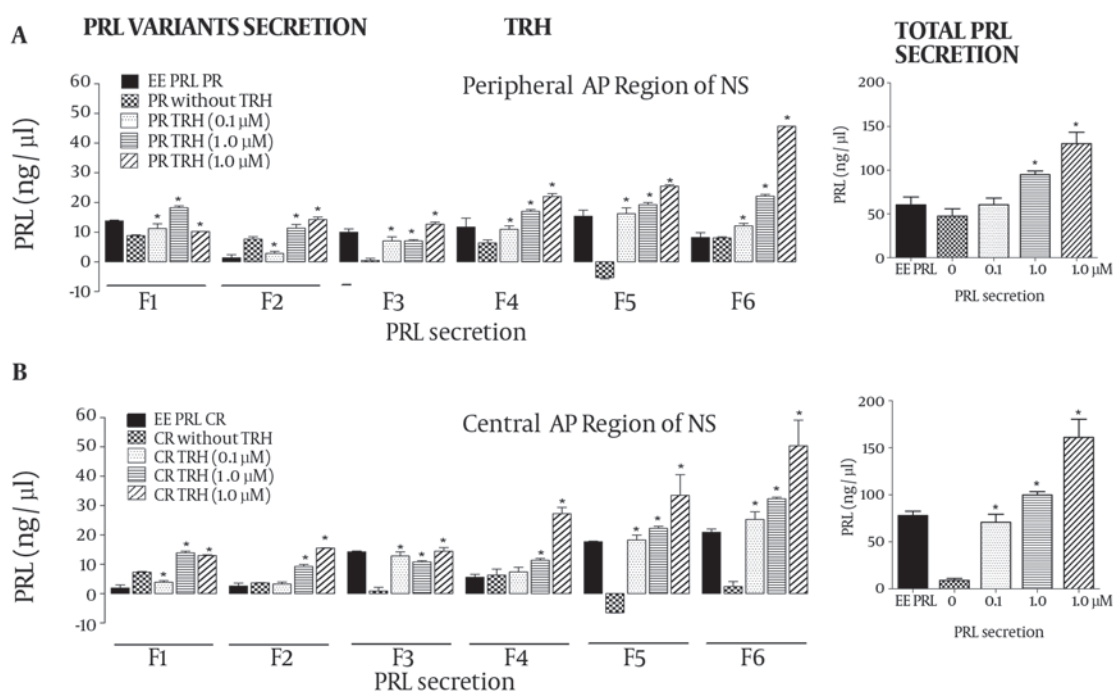

A-B: Effect of prolactin (PRL) variants electroeluted in fractions (F) 1–6 from SDS-PAGE gels of reconstituted conditioned media from the peripheral (PR) and central (CR) regions of adenohypophysis (AP) of suckled (S) lactating rats incubated with dopamine (DA) on the in vitro release of PRL variants from lactotrophs of the AP regions from non-suckled (NS) and S lactating rats.
Data shown are the means ± SEM.
* $P < 0.05$ *vs*. the control (electroeluted [EE] PRL).

promoted the release of PRL variants 2, 4, and 6 from lactotrophs of the central AP region of S rats. The intermediate dose of DA also inhibited the release of PRL variant 1, and promoted the release of variants 2, 4, and 6; 1.5 μM DA inhibited the release of variants 1 and 2 but promoted the release of variants 3–6 from lactotrophs of the central AP region of S rats.

of S rats. The low dose of TRH stimulated release of PRL variants 1, 4, 5, and 6 and did not affect PRL variants 2 and 3. The effect of the low dose of TRH on the release of PRL variants from the central AP region of S rats is shown in *Figure 4B*. The low dose of TRH decreased release of PRL variant 1, increased release of PRL variants 3, 4, and 6, and had no effect on PRL variants 2 and 5.

Figure 3. Effect of PRL Variants From the Regions of Adenohypophisis of Non-Suckled Rats With TRH.

A-B: Effect of prolactin (PRL) variants electroeluted in fractions (F) 1¬–6 from SDS-PAGE gels of reconstituted conditioned media from the peripheral (PR) and central (CR) regions of adenohypophysis (AP) of non-suckled (NS) lactating rats incubated with TRH on the in vitro release of PRL variants from the lactotrophs of AP regions from NS and suckled (S) lactating rats.
Data shown are the means ± SEM.
* $P < 0.05$ *vs.* the control (electroeluted [EE] PRL).

4.3. Dose-Response Effects of TRH

The effects of different doses of TRH (0.1, 1.0, and 10 μM) on the release of PRL variants from the AP regions of NS and S rats are shown in *Figures 3 and* 4, and the PRL values obtained after Incubation with TRH were compared to the PRL released in the absence of TRH (control). Compared with the control PRL values without TRH, 0.1 μM TRH increased the release of PRL variants in fractions 1, and 3–6; and inhibited variants in fraction 2 from the peripheral AP region of NS rats (*Figure 3A*). Higher doses of TRH (1.0 and 10 μM) increased the release of all PRL variants (1–6) from the peripheral AP region of NS rats.

The effect of TRH on the release of PRL variants from the central AP region of NS rats is shown in *Figure 3B*. The low dose of TRH (0.1 μM) decreased release of PRL variants in fraction 1, did not change release of fractions 2 and 4, and increased release of fractions 3, 5, and 6. In addition, higher doses TRH increased release of PRL in all fractions (1–6). *Figure 4A* shows the effect of the low dose of TRH on the release of PRL variants from the peripheral AP region

4.4. Dose-Response Effects of OT

The dose-response effects of OT on the release of PRL variants from NS rat APs are shown in *Figures 5 and 6*. The low dose of OT (0.1 μM) increased the release of PRL variants 3, 5 and 6, from the PR region of NS rat APs, relative to the control; however, the release of PRL variants in fractions 1, 2, and 4 was inhibited relative to the control. The intermediate dose of OT (1.0 μM) increased the release of PRL variants 3, 5, and 6, and inhibited release of PRL variants 1, 2, and 4 from the peripheral AP region of NS rats (*Figure 5A*). The high dose of OT (10 μM) increased release of PRL variants 2, 3, 5, and 6 from the peripheral AP region and release of variants 1–6 from the central AP region of NS rats. This same dose of OT increased release of PRL variants 1, 2, 5, and 6 from the peripheral AP regions of S rats; and had no effect on PRL variants 3 and 4. Finally, as shown in *Figure 5B*, the high dose of OT increased the release of PRL variants in fractions 2, 3, 5, and 6 from the peripheral AP region of NS rats, but had no effect on the release PRL variants in fractions 1 and 3. With the exception

Figure 4. Effect of PRL Variants From the Regions of Adenohypophisis of Suckled Rats With TRH.
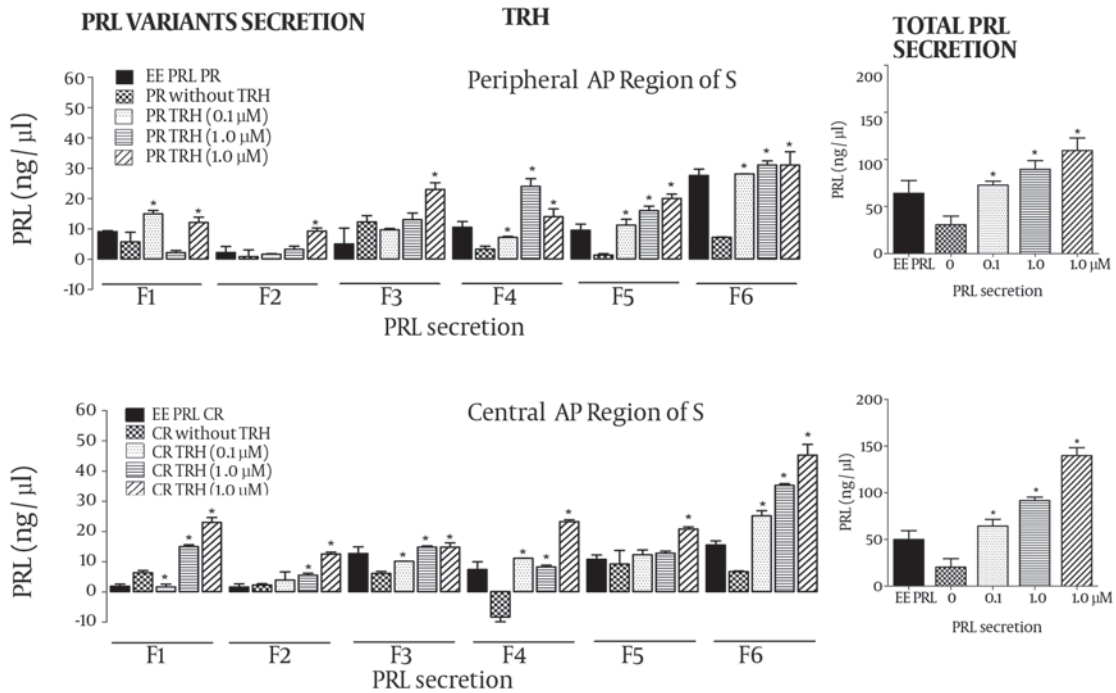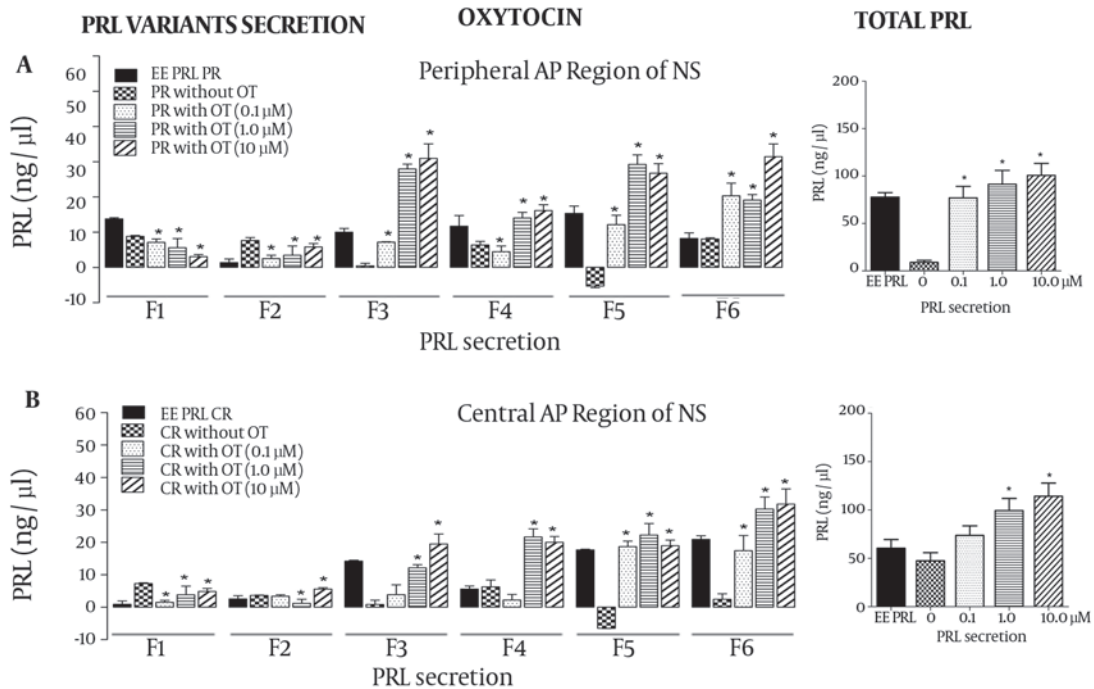

A-B: Effect of prolactin (PRL) variants electroeluted in fractions (F) 1–6 from SDS-PAGE gels of reconstituted conditioned media from the peripheral (PR) and central (CR) regions of adenohypophysis (AP) of suckled (S) lactating rats incubated with TRH on the in vitro release of PRL variants from lactotrophs of AP regions from non-suckled (NS) and S lactating rats.
Data shown are the means ± SEM.
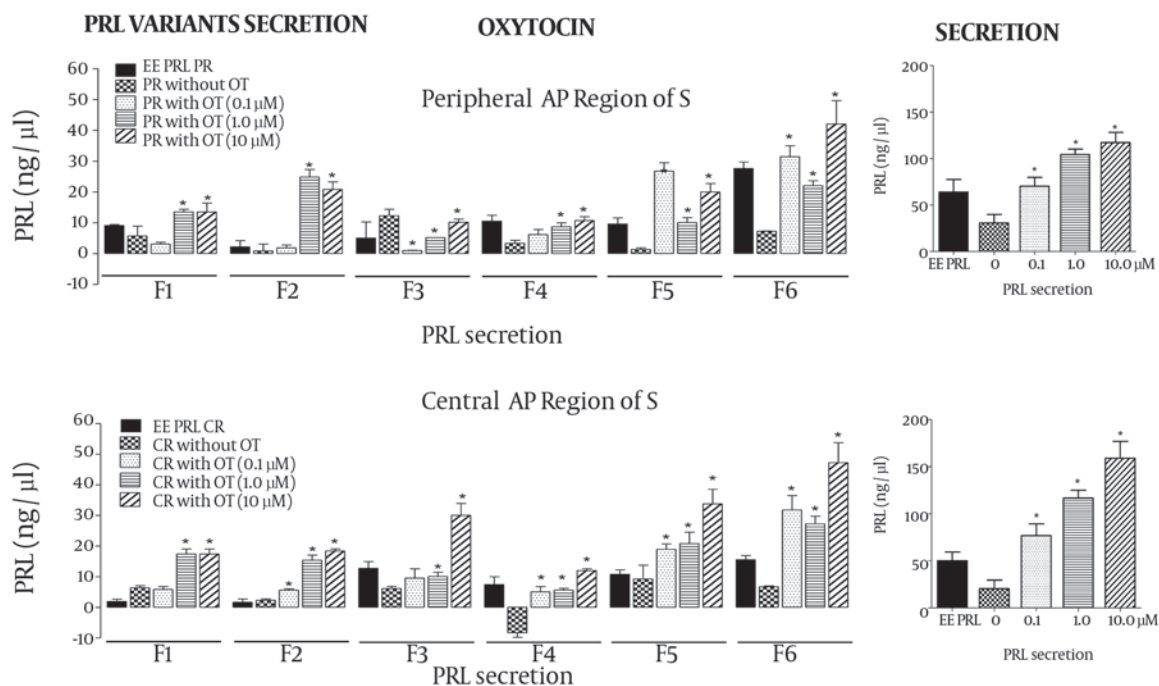* $P < 0.05$ *vs*. the control (electroeluted [EE] PRL).

Figure 5. Effect of PRL Variants From the Regions of Adenohypophisis of Non-Suckled Rats With OT.

A-B: Effect of prolactin (PRL) variants electroeluted in fractions (F) 1–6 from SDS-PAGE gels of reconstituted conditioned media from the peripheral (PR) and central (CR) regions of adenohypophysis (AP) of non-suckled (NS) lactating rats incubated with oxytocin (OT) on the in vitro release of PRL variants from lactotrophs of AP regions from NS and suckled (S) lactating rats.
Data shown are the means ± SEM.
* $P < 0.05$ *vs*. the control (electroeluted [EE] PRL).

Figure 6. Effect of PRL Variants From the Regions of Adenohypophisis of Suckled Rats With OT

A-B: Effect of prolactin (PRL) variants electroeluted in fractions (F) 1–6 from SDS-PAGE gels of reconstituted conditioned media from the peripheral (PR) and central (CR) regions of adenohypophysis (AP) of suckled (S) lactating rats incubated with oxytocin (OT) on the in vitro release of PRL variants from lactotrophs of AP regions from non-suckled (NS) and S lactating rats.
Data shown are the means ± SEM.
* $P < 0.05$ *vs*. the control (electroeluted [EE] PRL).

of fraction 5, in which there was no effect, the high dose of OT increased the release of all other fractions from the central AP region of NS rats, and had the same effect on the release of PRL variants from the peripheral region of S rats (*Figure 6A*). Additionally, increased release of PRL variant 5, inhibited of PRL variant 1, and had no effect on PRL variants 2, 3, 4, and 6. The middle dose of OT (1.0 μM) increased release of PRL variants 1, 2, 5, and 6, and had no effect on PRL variants 3 and 4 from the central AP region of suckled rats (*Figure 6B*, lower panel). This same dose of OT increased the release of PRL variants 1, 2, 5, and 6, and had no effect on PRL variants 3 and 4 from the peripheral AP region of S rats. Finally, the high dose of OT increased the release of PRL variants 1, 2, 5, and 6, and had no effect on the release of PRL variants 3 and 4 from lactotrophs of the PR region from S rats. In contrast, the same dose of OT increased the release of all PRL variants from the lactotrophs of the central AP region.

5. Discussion

Previous studies have shown that DA, OT, and TRH regulate PRL release (3-12, 26, 27, 29, 30, 43). However, most *in vivo* and *in vitro* studies that have determined the effect of hypothalamic hormones on *in vivo* and *in vitro* released PRL used radioimmunoassay and other methods, which detect mainly the 23 kDa PRL variant and not the other variants detected by SDS-PAGE and western blotting. In

the present study, we examined whether the regulation of AP PRL release by the hypothalamic hormones dopamine, TRH, and oxytocin, which has been established by many *in vivo* and *in vitro* studies (see 1, 2, and 4 for review), could occur by their direct action on lactotrophs and interaction with the autocrine actions of PRL variants. In addition, we determined whether these effects would promote or inhibit the release of PRL variants, and thereby regulate the release of the hormone. The results showed that the hypothalamic hormones did affect the lactotrophs, both by interacting with the autocrine actions of the PRL variants and by regulating the release of PRL variants from the PRL cells. Therefore, when a high dose of DA (1.5 μM) was applied directly to lactotrophs from both AP regions of NS and S rats, the secretion of most PRL variants was inhibited, as was previously reported by other studies (35). In contrast, the lower dose of DA (0.5 μM) slightly stimulated the release of PRL variants, mainly from the central AP region of suckled rats. TRH increased the 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 intensely stimulated PRL variant release, particularly the 23–34 kDa variants, from both AP regions of NS and S lactating rats. Therefore, these effects of hypothalamic hormones upon the release of PRL variants may also regulate, and thus interact with the autocrine effects (37, 38) exerted by the PRL variants, leading to integrated

regulation of PRL secretion.

In conclusion, the results of this study and previous studies suggest that in addition to hypothalamic and other influences, PRL variant release from lactating rat APs is regulated by autocrine influences exerted on the gland by previously released PRL variants. Furthermore, in parallel and interacting with this autocrine regulation, the effect on the release of the PRL hormone by hypothalamic hormones is also regulated by the same mechanism: the stimulation or inhibition of PRL variants release from the pituitary gland.

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