

A Factor(s) from a Rat Trophoblast Cell Line Inhibits Prolactin Secretion In Vitro and In Vivo¹

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ABSTRACT

The purpose of the present study was to measure the inhibitory action of secretions from trophoblast cells on prolactin (PRL) secretion in cycling and pregnant rats, and to determine whether factor(s) from trophoblast cells act directly on anterior pituitary cells. A rat choriocarcinoma cell line (Rcho)—a line consisting of trophoblast cells, including differentiated giant cells that secrete members of the placental PRL family—was used. When Rcho cells (1×10^6 cells) were transplanted under the kidney capsule of cycling rats, tumors developed and the rats went into constant diestrus. Eight days after cell injection, plasma progesterone was significantly increased in treated rats compared to controls, whereas plasma and pituitary PRL and pituitary PRL mRNA levels were significantly decreased. Similar PRL results were seen on Day 9 of pregnancy after injection of Rcho cells on Day 0 or Day 1 of pregnancy. To determine whether secretions from Rcho cells had a direct effect on anterior pituitary cells to inhibit PRL release, anterior pituitary cells were enzymatically dispersed and cultured for 4 days. Conditioned medium was obtained from 9-day Rcho cell cultures and concentrated by ultrafiltration. A fraction containing substances with molecular weights greater than 10 000 suppressed PRL release from the pituitary culture after 3 and 24 h. Conditioned medium containing substances with molecular weights between 1000 and 10 000 had no effect on PRL release, nor did conditioned medium from a placental cell line designated HRP-1. HRP-1 also contains trophoblast cells but does not contain the differentiated giant cells. Recombinant rat placental lactogen-I (PL-I) at concentrations of 0.5 and 2.0 $\mu\text{g}/300 \mu\text{l}$ per well did not affect the release of PRL in vitro. To determine whether the inhibitory effect of conditioned medium on spontaneous PRL release from the pituitary cell cultures also blocked thyrotropin-releasing hormone (TRH)-induced PRL release, TRH (100 nM) was added 3 h after conditioned medium was incorporated into the culture. The PRL response to TRH was the same in the presence and absence of conditioned medium.

These results indicate that secretions from Rcho cells, possibly PL-I, may inhibit PRL indirectly, via the hypothalamus, whereas other factors as yet unidentified (but not PL-I) may act directly on the pituitary to inhibit the spontaneous release of PRL. This suggests that the loss of PRL surges at midpregnancy may be due to the secretion of more than one inhibitory factor from the placenta.

INTRODUCTION

During the first half of pregnancy, the pattern of PRL secretion is characterized by twice-daily surges [1, 2]. Elevated PRL levels are necessary for functioning of the corpora lutea for the first 6 days of pregnancy, after which utero-placental secretions maintain progesterone secretion from the ovaries [3–5]. PRL surges abruptly terminate at midpregnancy, and circulating PRL remains low until shortly before parturition [6, 7]. The appearance of and rapid increase in circulating placental lactogen-I (PL-I) suggest that PL-I acts as an inhibitory factor to PRL surges [8, 9]. We previously reported that the occurrence of the nocturnal surge of PRL in the pregnant rat terminates earlier than normal following transplantation of rat choriocarcinoma cells (Rcho cells) under the kidney capsule [10]. Since Rcho cells in vivo express PL-I, but not other identified members of the placen-

tal PRL family [11], PL-I may be responsible for the early termination of the nocturnal PRL surge.

The hypothalamus is probably one site for the suppressive actions of placental secretions on PRL. It has been reported that implantation of human PL in the medial basal hypothalamus [12] and transplantation of Rcho cells into the lateral ventricle block PL surges [13]. Injection of human PL into ovariectomized rats increases hypothalamic activity of PRL-inhibiting hormone and lowers PRL levels in the blood [14]. Furthermore, pregnant rats with Rcho cells have elevated tyrosine hydroxylase activity in the tuberoinfundibular dopamine neurons [13], suggesting that factors secreted by these Rcho cells inhibit PRL by stimulating synthesis and release of dopamine, the tonic inhibitor of PRL [15]. On the other hand, it has been proposed that the anterior pituitary also is one of the sites of action of the inhibitory effects of placental secretions. Extracts of placenta taken from rats on Day 9 through Day 11 of pregnancy and medium obtained from the incubation of Day 11 placentas inhibit prolactin release from pituitary cells or hemipituitaries in vitro [16, 17]. However, the problem with use of placental extracts or conditioned medium from placental incubations is the presence of numerous factors from the blood. Also, the number of cell types and factors found in placental extracts

Accepted September 15, 1992.

Received May 22, 1992.

¹This study was partially financed by NIH grants HD 24190 (J.L.V.), HD 20676 (M.J.S.), HD 02528 (MRRC Center Grant), and NRSA postdoctoral fellowship HD 07368 (L.A.A.).

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is very large. The recent development of the Rcho cell line, which has a trophoblast lineage capable of differentiating into giant cells and of expressing several members of the placental PRL family *in vitro* [18], makes it possible to delineate whether trophoblast cells secrete factor(s) that directly inhibit PRL secretion. Another rat placental cell line, designated HRP-1, also contains trophoblast cells, but not the differentiated giant cells [19]. These cells, which do not express PRL-like proteins, make it possible to determine more specifically which placental cell type may be secreting the inhibitory factor [19]. The availability of recombinant rat PL-I [20, 21] has made it possible to determine whether PL-I is capable of directly inhibiting PRL secretion from anterior pituitary cells.

The objectives of the present study were 1) to determine whether *in vivo* secretions from the Rcho cells inhibit the release of PRL and decrease the amount of PRL and PRL mRNA in the anterior pituitary in cycling and pregnant rats; 2) to evaluate the effect of conditioned medium from Rcho and HRP-1 cell cultures on PRL release from anterior pituitary cell monolayers; 3) to evaluate whether recombinant PL-I has any direct effect on PRL release *in vitro*; and 4) to determine whether the stimulatory effect of thyrotropin-releasing hormone (TRH) on PRL *in vitro* is blocked by conditioned medium from Rcho cells.

MATERIALS AND METHODS

Animals

Female adult Sprague-Dawley rats (Sasco Co., Omaha, NE) were housed in a room with lights-on from 0600 to 1800 h and constant temperature of $24 \pm 2^\circ\text{C}$. Rats were allowed free access to food and water. Vaginal estrous cycles were monitored every morning and mating was accomplished by placing females in proestrus with a male rat overnight. The presence of sperm in the vaginal smear was taken to designate Day 0 of pregnancy. Animal procedures were carried out as approved by the University of Kansas Institutional Animal Care and Use Committee.

Anterior Pituitary Cell Cultures

To obtain dispersed anterior pituitary cells [22], anterior pituitary glands were quickly removed from rats that had been ovariectomized 8 days earlier; the tissues were then placed in a sterilized tube containing 1.5 ml of Krebs' Ringer bicarbonate buffer (KRBGA, pH 7.35) containing 2% Minimum Essential Medium (MEM) amino acids (Gibco, Grand Island, NY), 1% MEM vitamins (Gibco), and 10% BSA. The tissue was cut into pieces (about 1 mm^3) and incubated for 35 min at 37°C in KRBGA containing 0.2% trypsin (Worthington Biochemical Co., Freehold, NJ). DNAase (Sigma Chemical Co., St. Louis, MO) was added during the final 2 min of incubation. The tissue was washed with KRBGA containing lima bean trypsin inhibitor (Worthington). The pieces

were mechanically dispersed by repeated uptake and expulsion through a siliconized Pasteur pipette. The cell suspension was centrifuged for 8 min at $180 \times g$, and the pellet was resuspended in Dulbecco's Modified Eagle's medium (DMEM, pH 7.35; JRH Bioscience, Lenexa, KS) containing 10% horse serum (Gibco), 4% fetal bovine serum (FBS; Whittaker Bioproducts), 1% MEM nonessential amino acids (Gibco), penicillin (100 U/ml)/streptomycin (100 mg/ml) (Gibco), and 25 U/ml Nystatin (Gibco).

Conditioned Medium from Rcho or HRP-1 Cell Cultures

Rcho or HRP-1 cells (1×10^6) were cultured in RPMI-1640 (JRH Bioscience) containing 20% FBS, $50\ \mu\text{M}$ β -mercaptoethanol (Sigma), 1 mM sodium pyruvate, 100 U/ml penicillin, and $100\ \mu\text{g/ml}$ streptomycin at 37°C under a humidified atmosphere of 95% air:5% CO_2 until confluent. At this time the medium was changed to NCTC-135 (Sigma) containing 20% FBS, $50\ \mu\text{M}$ β -mercaptoethanol, 1 mM sodium pyruvate, 10 mM HEPES, and 100 U/ml penicillin, and $100\ \mu\text{g/ml}$ streptomycin [18]. NCTC-135 is an enriched medium that maintains the pH in the confluent culture better than does RPMI-1640. Medium was changed every 2 days, or more frequently, to maintain the pH at 7.2–7.4. After 6 days, the medium was replaced with NCTC-135 containing 1% FBS. FBS from the same lot was used for both Rcho and HRP-1 cell cultures. This medium was collected after 24 h and designated conditioned medium. This conditioned medium was concentrated 10-fold by ultrafiltration using a 10 000 molecular weight cutoff membrane (YM 10 membrane filter, Amicon, Danvers, MA). To obtain a second fraction, the flow-through medium was subjected to a YM 1 membrane filter with a 1000 molecular weight cutoff value.

PRL mRNA Measurement

Total RNA extraction from the pituitary tissue was done according to the method of Puissant and Houdebine [23]. Individual pituitaries were homogenized in $300\ \mu\text{l}$ 4 M guanidium thiocyanate buffer. Sodium acetate (4 M, pH 4.0), water-saturated phenol, and chloroform (1:10:4) were added to the homogenate; the homogenate was vortexed and kept at 4°C for 15 min. Following centrifugation, ethanol was added to the supernatant and RNA was precipitated at -20°C for 1 h. The pellet was resuspended in 10 mM Tris buffer (pH 7.5) and chloroform, followed by centrifugation. The upper phase was collected; 4 M ammonium acetate and ethanol were added and samples were kept at -20°C for 1 h. Following centrifugation, the pellet was dried and dissolved in sterile distilled water. Total RNA was quantitated by absorbance at 260 nm.

Relative concentrations of PRL mRNA were determined by dot-blot analysis as described previously [24]. Three amounts of RNA (62.5, 125, and 250 ng) were spotted on a nitrocellulose membrane. The nitrocellulose membrane was placed in a vacuum oven at 75°C for 4 h and then sealed

in a hybridization bag. Prehybridization, hybridization, and posthybridization treatment of filters was conducted as described previously [24, 25]. A rat PRL cDNA probe, provided by Dr. Richard A. Mauer [26], was labeled with ^{32}P -dCTP by means of a random primers DNA labeling kit (Bethesda Research Laboratories, Gaithersburg, MD). Specific activity of ^{32}P rat PRL cDNA was 3.9×10^7 cpm/ μg DNA, and a saturating amount of probe (2×10^6 cpm/ml) was used [27]. The washed nitrocellulose membrane was enclosed in plastic wrap and autoradiographed with Kodak X-Omat AR x-ray film (Eastman Kodak, Rochester, NY) at -70°C . The autoradiograph spots were scanned with a densitometer. Results showed that when a saturating amount of PRL probe was used, increasing the amount of RNA added per dot resulted in a linear increase in PRL mRNA per dot as determined by densitometer. All samples were measured in the same dot-blot assay. A mean value for each pituitary half was calculated from the three amounts of RNA analyzed.

Hormone Assays

PRL levels were determined using the rat PRL RIA provided by NIDDK with PRL RP-1 as a reference preparation. ^{125}I -PRL was purchased from Dupont (Boston, MA). The limit of sensitivity was 50 pg, and the intra- and interassay coefficients of variation were 8.7% and 14.5%, respectively. Progesterone was measured by RIA using #337 anti-progesterone-11-BSA (Dr. Gordon Niswender, Colorado State University, Fort Collins, CO) as described by Gibori et al. [28]. The limit of sensitivity for the assay was 10 pg, and the intraassay coefficient of variation was 14.1%.

Experimental Procedures

In vivo experiments. To determine the effects of Rcho cells on release of PRL in vivo, cyclic rats (anesthetized with ether) were injected with Rcho cells (1×10^6 cells/50 μl) or medium (50 μl as control) under the kidney capsule on a random day of the cycle, and pregnant rats were injected on Day 0 or Day 1 of pregnancy. The estrous cycle in the cycling rats was monitored until the day rats were killed by decapitation. Cycling controls were killed 6–9 days after injection, on the morning (1000–1200 h) of diestrus Day 2; cyclic rats injected with Rcho cells were killed 8 days after injection. Pregnant rats with or without Rcho cells were killed at 0400 h on Day 9 of pregnancy. Blood was collected into heparinized tubes and plasma was separated by centrifugation. Anterior pituitary glands were removed immediately after decapitation and divided in half. Each half was frozen separately in liquid freon and stored at -70°C until assayed for PRL or PRL mRNA. For PRL measurement, one half of each anterior pituitary was homogenized in 200 μl 50 mM Tris buffer (pH 8.8). To measure PRL concentrations that were within limits of the standard curve of the RIA, an aliquot of the supernatant was diluted with 1% BSA in 10 mM PBS. The homogenate was analyzed for protein content

by the method of Bradford [29] using BSA as the standard. Plasma PRL, pituitary PRL, and plasma progesterone levels were determined by RIA. The concentrations of pituitary PRL and plasma hormones were expressed as $\mu\text{g}/\text{mg}$ protein and ng/ml plasma, respectively. The other half of each anterior pituitary was used for measuring PRL mRNA by dot-blot hybridization [24]. The relative level of PRL mRNA in the pituitary gland was expressed as optical density (O.D.) units per μg RNA.

In vitro experiments. To determine the direct effect of secretions from Rcho cells on the release of PRL, monolayer cell cultures of dispersed anterior pituitary cells were used [22]. The dispersed cells (1.5×10^5 cells/well) were seeded in each well of 24-well plates and cultured for 4 days in a humidified atmosphere of 5% CO_2 :95% air at 37°C . On Day 4 of the incubation, each well was washed three times with RPMI-1640. Cells were incubated in RPMI-1640 containing 20% FBS for 3–24 h in a total of 300 μl medium per well. The pituitary cells were visualized by means of an inverse microscope after exposure to the conditioned medium. No obvious differences in cell number, cell distribution, or change in cell morphology were seen between Rcho-treated and control groups. To determine the amount of PRL secreted into the medium, 10 μl medium was removed for RIA at the specified time in each experiment.

The effects of dopamine or conditioned medium from Rcho cell cultures on PRL release were examined through incubation of pituitary cells with dopamine (50 nM) or 2 doses of conditioned medium (30 μl [single-strength] or 90 μl [triple-strength]). Specificity was established by examining the actions of conditioned medium from a placental cell line, HRP-1 [19], on the pituitary cell cultures.

The effect of recombinant PL-I on PRL release in vitro was examined at two concentrations (0.5 μg or 2.0 $\mu\text{g}/300$ μl). These concentrations were chosen on the basis of plasma PL-I levels at midpregnancy [30–32]. These levels were determined in the rat by Nb_2 assay using ovine PRL as a standard [30, 31] and in the mouse by RIA [32]. It was recently reported that Rcho rPL-I and serum rPL-I were equipotent in the Nb_2 assay, and that recombinant rPL-I was 1.5–2.0 times more potent than ovine PRL in the same assay [33]. After 3 and 24 h of incubation, 10 μl was removed from each well and assayed for PRL.

The final experiment was performed to determine whether conditioned medium from Rcho cells would block the stimulatory effect of TRH (Sigma) on PRL release. Conditioned medium was added to the pituitary cell cultures for 3 h before TRH (100 nM) was added. Samples (10 μl) for PRL assay were collected from each well 0.5 and 1 h after TRH addition.

Statistics

Results are expressed as the mean \pm SE. Student's *t*-test was used when comparisons were made between two groups. A two-way analysis of variance for repeated mea-

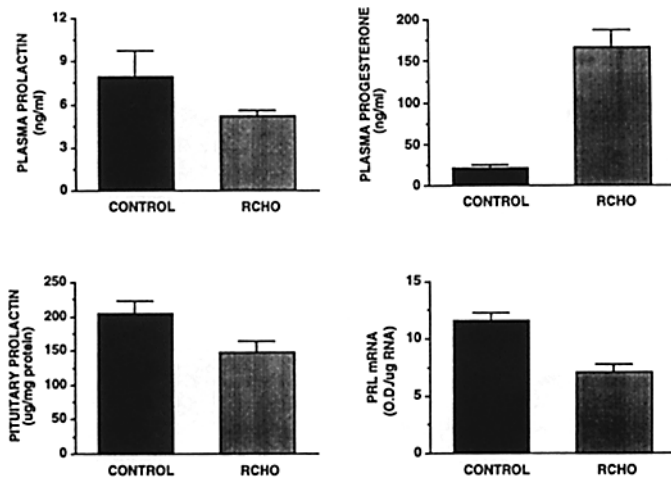


FIG. 1. Effect of Rcho cells on PRL and progesterone in cycling rats. Cells were transplanted under the kidney capsule and the rats were killed 8 days later. Compared to controls, rats with Rcho cells had lower plasma PRL ($p < 0.05$), higher plasma progesterone ($p < 0.05$), lower pituitary PRL ($p < 0.01$), and lower pituitary PRL mRNA levels ($p < 0.01$). Each bar represents the mean \pm SE. Seven control and 10 Rcho cell-transplanted rats were used in this experiment.

tures was used when both incubation time and treatment were variables. Fisher's least-significant procedures and Sheffe's F-test were used for multiple comparisons [34]. Differences between groups were considered statistically significant at $p < 0.05$. Analysis of *in vitro* experiments was performed by combining results from separate incubations. Within each incubation, treatments were replicated in at least 3 wells, yielding one value for use in statistical analysis.

RESULTS

Hormone Levels in Cycling and Pregnant Rats Containing Rcho Cell Transplants

Following transplantation of Rcho cells, cyclic rats showed continuous diestrous vaginal smears for the last 4–7 days before sample collection. Transplants of variable sizes were found under the kidney capsule upon decapitation. Concentration of plasma progesterone was significantly increased ($p < 0.01$) in these rats compared with controls killed on diestrous Day 2 of the estrous cycle (Fig. 1, top panel). Rats receiving Rcho cells had significantly decreased concentrations of plasma PRL ($p < 0.05$). Both pituitary PRL ($p < 0.01$) and PRL mRNA ($p < 0.01$) levels were significantly lower in Rcho cell-treated rats than in controls (Fig. 1, bottom panel).

Similar results were found in pregnant rats injected with Rcho cells on Day 0 or 1 and killed during the nocturnal PRL surge at 0400 h on Day 9. The concentrations of plasma PRL (Fig. 2, top panel, $p < 0.05$) and pituitary PRL (Fig. 2, middle panel, $p < 0.05$) as well as the level of pituitary PRL

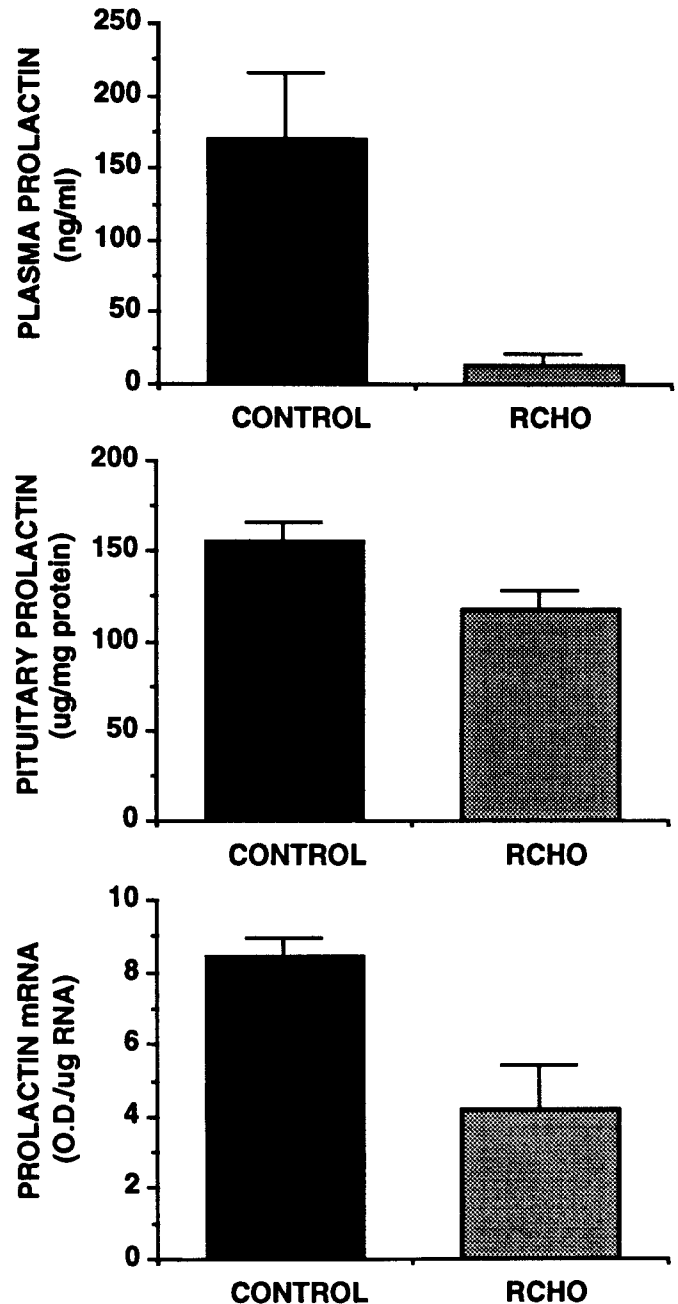


FIG. 2. Effect of Rcho cells on PRL during pregnancy. Rats received cell transplants on Day 0 or 1 of pregnancy and were killed at 0400 h on Day 9, during the nocturnal PRL surge. Compared to controls, rats with Rcho cells had lower plasma PRL ($p < 0.05$), pituitary PRL ($p < 0.05$), and pituitary PRL mRNA ($p < 0.01$) levels. Each bar represents the mean \pm SE. Seven controls and 5 Rcho cell-transplanted rats were used.

mRNA (Fig. 2, bottom panel, $p < 0.01$) were significantly lower than the corresponding values in pregnant controls.

Effect of Conditioned Medium from Rcho Cells on PRL Release from Pituitary Cell Monolayer Cultures

This experiment was performed to determine whether secretions from Rcho cells directly affect PRL release from

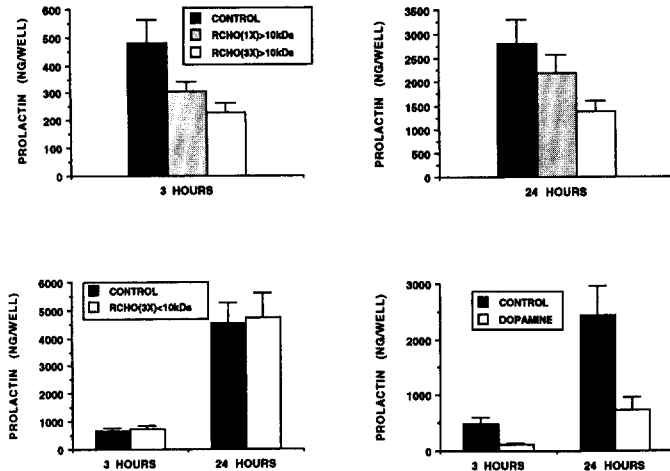


FIG. 3. Effect of dopamine or conditioned medium from Rcho cell cultures on PRL release in vitro. Designations 1X and 3X represent 30 μ l (single-strength) and 90 μ l (triple-strength), respectively, of 10-fold-concentrated Rcho cell-conditioned medium added to each well (300 μ l total volume of medium/well). Dopamine concentration in each well was 50 nmol/L. Both doses of the ultrafiltrate fraction of the conditioned medium containing substances with molecular weights greater than 10 000 (top panel) reduced PRL at 3 h ($p < 0.05$), whereas only the higher dose was effective at 24 h ($p < 0.05$). When the fraction containing substances less than 10 000 in molecular weight was added, there were no effects on PRL. Dopamine significantly reduced PRL at 3 and 24 h ($p < 0.05$). Each bar represents the mean \pm SE. Number of separate incubations was 5–6.

anterior pituitary cells. The dispersed anterior pituitary cells were used after 4 days of culture. The amount of PRL released in control wells was greater after 24 h than after 3 h of incubation (Fig. 3, top panel). PRL release was significantly suppressed ($p < 0.05$) by the addition of dopamine (Fig. 3, right bottom panel). Following ultrafiltration of the conditioned medium from Rcho cells, fractions were separated into those containing substances with molecular weights greater than 10 000 and those containing substances with molecular weights between 1000 and 10 000. As seen in Figure 3, top panel, the amount of PRL released into the medium was significantly less at 3 h after the addition of both concentrations (single-strength and triple-strength) of the higher-molecular-weight fraction than in controls ($p < 0.05$). At 24 h, only the triple-strength conditioned medium significantly reduced PRL release into the medium. There was no significant interaction between treatment and time of incubation. The addition of the fraction containing lower-molecular-weight substances did not alter PRL release (Fig. 3, left bottom panel).

Effect of Conditioned Medium from HRP-1 Cells on PRL Release from Pituitary Cell Monolayer Cultures

To determine whether the effects observed with conditioned medium from Rcho cells was characteristic of other trophoblast cell types, conditioned medium from HRP-1 cells—a trophoblast cell line with a phenotype different from that of Rcho cells—was used. As seen in Figure 4, this conditioned medium had no effect on PRL release.

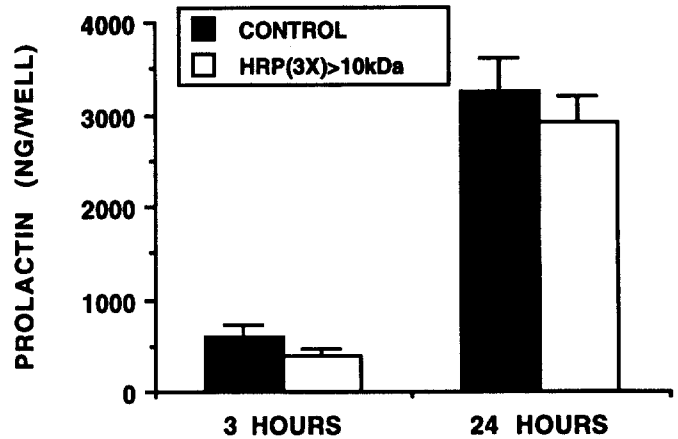


FIG. 4. PRL release in vitro during incubation with conditioned medium from HRP-1 cell cultures. Results from four separate incubations demonstrated that PRL release was unaffected by addition of HRP-1 cell-conditioned medium at either 3 or 24 h. Each bar represents the mean \pm SE.

Effect of Recombinant rPL-I on the Release of PRL

Neither concentration of the recombinant PL-I affected the amount of PRL released into the medium (Fig. 5). There was no cross-reactivity between PL-I and the PRL antibody used in the RIA.

Effect of Conditioned Medium from Rcho Cells on TRH-Induced PRL Release

To determine whether the secretions from Rcho cells affected the responsiveness of pituitary cells to TRH, the monolayer of pituitary cells was exposed to conditioned medium from Rcho cells for 3 h, and then TRH or vehicle was added. An ultrafiltrate fraction of molecular weight greater than 10 000 was used as conditioned medium. As seen before, the amount of PRL released into the medium was significantly less ($p < 0.05$) in the presence of con-

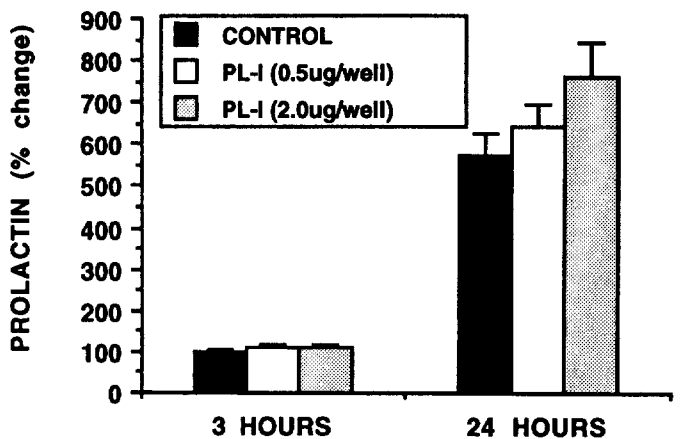


FIG. 5. Effect of recombinant PL-I on PRL release in vitro. Neither 0.5 μ g/300 μ l nor 2.0 μ g/300 μ l PL-I affected the amount of PRL released at 3 or 24 h as compared to controls. Results are presented as percent change from 3-h controls to reduce the amount of variability between the three separate incubations. Each bar represents the mean \pm SE.

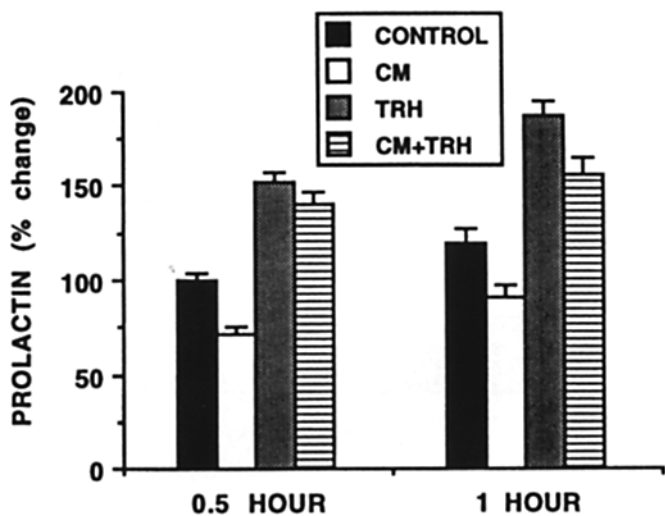


FIG. 6. Effect of conditioned medium from Rcho cell cultures on TRH-induced PRL release in vitro. Pituitary cells were exposed for 3 h to Rcho cell-conditioned medium, to which TRH (100 nM) or vehicle was added for 0.5 or 1.0 h. PRL release was less ($p < 0.05$) at 0.5 h or 1.0 h in the presence of Rcho cell-conditioned medium; TRH significantly ($p < 0.05$) increased PRL as compared to the appropriate control in the presence and absence of conditioned medium. Thus, Rcho cell-conditioned medium failed to affect TRH-induced pituitary cell PRL release. Results are presented as a percent change from 0.5-h controls to reduce the variability between the three separate incubations. Each bar represents the mean \pm SE.

ditioned medium from Rcho cells than in controls (Fig. 6). The addition of TRH into control wells significantly increased ($p < 0.05$) the amount of PRL in the medium within 30 min. However, the presence of conditioned medium did not prevent the stimulatory effect of TRH on PRL release (Fig. 6).

DISCUSSION

The present study reports that secretions from trophoblast cells were inhibitory to PRL secretion both in vivo and in vitro. In both cycling and pregnant rats, Rcho cells transplanted under the kidney capsule suppressed PRL release, decreased pituitary PRL concentration, and reduced PRL mRNA levels. Conditioned medium from Rcho cell cultures inhibited PRL release from pituitary cell monolayers, although recombinant rat PL-I had no effect in this in vitro system.

Previously we reported that pregnant rats with Rcho cells under the kidney capsule or in the lateral ventricle of the brain had significantly lower plasma PRL levels before and during the nocturnal surge than did controls [10, 13]. This effect was specific for Rcho cell transplants, since HRP-1 transplants, which contain trophoblast cells as well, had no effect on PRL [13]. The present study confirms the inhibitory effect of Rcho transplants on plasma PRL, and also reports that either cycling or pregnant rats containing these Rcho cells had significantly lower PRL mRNA levels. This correlates well with the decrease in pituitary PRL content

and plasma PRL, indicating that one mechanism of action for the inhibitory effect of secretions of Rcho cells may be on PRL gene expression. However, these in vivo studies cannot delineate whether the effect is directly on the lactotroph or whether it occurs via a hypothalamic signal that reaches the lactotroph by way of the hypophysial portal blood.

Rcho cells are known to produce PL-I in vivo, but not other identified members of the placental PRL family [11]. Although serum PL-I was not measured in the present study, biological endpoints suggest that it or some other PRL-like hormone was present in physiological amounts in rats with Rcho cell transplants. The luteotrophic effect of secretions from Rcho cells was demonstrated by the significant increase in plasma progesterone in cycling rats, equivalent to that seen in pregnant rats, as well as by the extended period of diestrus. Highly developed mammary glands in the cycling rats with Rcho cells indicate the mammatrophic action of the secreted factor. It is possible that PL-I was responsible for the inhibition of PRL synthesis and release, acting like PRL in exerting a negative short-loop feedback on PRL secretion [12, 35], although other factors secreted by the Rcho cells could have similar effects. Preliminary experiments from our laboratory show that implantation of recombinant PL-I into the hypothalamus abolishes the nocturnal PRL surge in pregnant rats. Given the ability of MMQ cells (a pituitary-derived clonal line secreting PRL [36]) and Rcho cells to increase the catalytic activity of tyrosine hydroxylase in the median eminence [13], it is likely that one site of action of Rcho cell secretions in the present study is the tuberoinfundibular dopamine neurons in the arcuate nuclei of the hypothalamus. Whether PL-I has a direct effect on these neurons in vivo has not yet been determined.

The second major question of this study was whether Rcho cells secreted substance(s) that had a direct inhibitory influence on PRL release, and whether PL-I was one of these substances. Pituitaries from ovariectomized rather than cycling rats were used as the source of cell monolayers to eliminate variability in cell responsiveness due to differences in hormonal background of the cells. The observation that dopamine inhibited while TRH stimulated PRL release from the cell monolayer demonstrated that the cells in culture remained responsive to factors that are either stimulatory or inhibitory to PRL release. Conditioned medium from Rcho cell cultures directly inhibited PRL release. The inhibitory material was contained in a fraction with molecular weight greater than 10 000; however, it is possible that any substance exerting the inhibitory effect was smaller but bound to a larger protein. Conditioned medium into which material with molecular weight above 10 000 had been released by HRP-1 cells did not affect PRL release under the same culture conditions. This suggests that the inhibitory effects were not due to nonspecific factors or death of pituitary cells. Nor is it likely that the effect was due to release of proteolytic enzymes from Rcho cells, since ad-

dition of a trypsin inhibitor to medium containing placental tissue did not augment the amount of prolactin remaining after a 24-h incubation [16]. The difference in the inhibitory action on PRL release between the two cell lines may be attributable to the placental PRLs secreted from the Rcho cells, which have been shown to express PL-I, PL-II, PRL-like protein A, and PRL-like protein C under incubation conditions that were the same as in the present experiment [18]. HRP-1 cells do not express these proteins [19]. Many reports have suggested that PL-I is the initial factor terminating PRL surges in pregnant rats [8, 9, 37, 38]. This lactogen, produced soon after implantation, reaches a peak in circulation by Day 11 of pregnancy [25, 30, 31], at which time its concentration is 1–3 $\mu\text{g/ml}$ plasma, as measured by bioassay using ovine PRL as a standard. Furthermore, when medium conditioned from placental explants containing PL-I was infused into the pregnant rat so that PL-I levels reached 400–2000 ng/ml, the nocturnal PRL surge was completely inhibited [38]. With the recent generation of recombinant PL-I [20, 21], it was possible to test for the first time whether PL-I had a direct inhibitory effect on PRL release in vitro. Neither the 0.5- $\mu\text{g/well}$ dose (equivalent to 1.7 $\mu\text{g/ml}$) nor the 2.0- $\mu\text{g/well}$ dose (equivalent to 6.7 $\mu\text{g/ml}$) had any inhibitory effect on PRL after 3 or 24 h of incubation. This recombinant PL-I has been shown to stimulate Nb₂ lymphoma cells in vitro [21] and is 1.5–2.0 times more potent than ovine PRL in the same assay [33]. The lack of any effect was surprising since Rcho cells, which secrete only PL-I of the PRL family [11], reduced plasma PRL levels in vivo to less than 6 ng/ml. As noted earlier, experiments from our laboratory indicate that when recombinant PL-I is implanted into the hypothalamus, plasma PRL levels are greatly reduced. This suggests that PL-I is one of the placental factors that inhibits PRL secretion, but does so via the hypothalamus. Other trophoblast cell secretory products may act independently at the lactotroph to inhibit PRL secretion.

Voogt [39] reported that the responsiveness of PRL release to TRH declines in the second half of pregnancy compared with the first half of pregnancy. We previously showed that the presence of Rcho cells in cyclic rats does not block TRH-induced PRL release [10]. In this study, TRH was added to the pituitary cell monolayer after 3-h exposure to the conditioned medium from Rcho cells. The results were similar to those in the previous study with Rcho cells in vivo; TRH-induced PRL release was not affected by pre-exposure and continuing presence of the Rcho-conditioned medium. These findings suggest that the decline in the responsiveness of PRL release to TRH in the pregnant rat is likely to be regulated by a set of factors different from those that turn off the PRL surges.

In summary, both the expression and release of PRL in vivo were inhibited by secretions from the Rcho cell line. Recombinant rat PL-I did not act directly on lactotroph cells to inhibit PRL, but another substance (or substances) with molecular weight greater than 10 000, secreted from Rcho

cells, did directly inhibit PRL release from pituitary cells. When the inhibitory substance from the conditioned medium of Rcho cell cultures is identified, its presence in normal placental tissue can be more easily ascertained. These findings taken together with previous results [10, 13] suggest that the loss of PRL surges at midpregnancy probably occurs via different substances acting at two separate sites. Secretions from trophoblast giant cells, possibly PL-I and PL-II, inhibit PRL release via neuronal mechanisms whereas other trophoblast-specific products act on pituitary cells.

ACKNOWLEDGMENTS

We wish to thank the NIDDK and the National Hormone and Pituitary Program (University of Maryland School of Medicine, Baltimore, MD) for the gift of PRL RIA materials. We also acknowledge the receipt of the cDNA probe for rat PRL from Dr. Richard Mauer (University of Iowa, Iowa City, Iowa).

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