

A Factor(s) from a Trophoblast Cell Line Increases Tyrosine Hydroxylase Activity in Fetal Hypothalamic Cell Cultures*

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ABSTRACT

We previously reported that a factor(s) from rat choriocarcinoma (Rcho-1) cells suppresses circulating PRL levels and increases tyrosine hydroxylase activity in tuberoinfundibular dopaminergic neurons *in vivo*. The purposes of this study were to determine whether this factor(s) increases tyrosine hydroxylase activity in fetal hypothalamic cells *in vitro* and to evaluate its chemical nature. The Rcho-1 cells are of placental origin and have the capacity to differentiate into giant cells and produce members of the placental PRL family. MMQ cells, a pituitary cell line that secretes PRL, and HRP-1, a placental cell line that does not produce any known members of the PRL family, were used as control cells. Tyrosine hydroxylase activity was assessed by incubation of hypothalamic cells for 1 h with 100 μ M brocresine, an inhibitor of aromatic L-amino acid decarboxylase. Tyrosine hydroxylase activity was increased in a density-dependent manner when Rcho-1, but not HRP-1 or MMQ, cells were cocultured with hypothalamic cells for 24 h. Control and Rcho-1-stimulated tyrosine hydroxylase activities were markedly reduced with 1 mM α -methyl-*p*-tyrosine, a specific inhibitor of tyrosine hydroxylase. Tyrosine hydroxylase activity was not altered when hypothalamic cells were incubated for 24 h with rat PRL or recombinant rat placental lactogen-I, whereas a 24-h stimulation with 100,000 Rcho-1 cells and a 1-h stimulation with 5 mM (Bu)₂cAMP increased tyrosine hydroxylase activity 3.7- and 3-fold, respectively. The magnitudes of the increase in tyrosine hydroxylase

activity were similar when hypothalamic cells were cocultured with Rcho-1 cells for 1 and 24 h. Acetic acid extracts of Rcho-1, but not HRP-1 or MMQ, cells increased tyrosine hydroxylase activity within 1 h in a concentration-dependent manner. The 3-fold increase in tyrosine hydroxylase activity observed with 500,000 Rcho-1 cell equivalents was markedly reduced with 1 mM α -methyl-*p*-tyrosine. The mol wt range of the tyrosine hydroxylase-activating factor(s) (THAF) was estimated using ultrafiltration membranes. The majority of activity was found in the eluate from a 1,000 mol wt cut-off membrane. THAF activity in Rcho-1 cell extracts was decreased by preincubation with pronase, a nonspecific proteolytic enzyme, suggesting that the factor(s) is a peptide. THAF was resistant to inactivation by trypsin or chymotrypsin pretreatment. However, both enzymes destroyed the ability of pituitary adenylate cyclase-activating peptide, either alone or with Rcho-1 cell extracts, to increase tyrosine hydroxylase activity. Oxidation of Rcho-1 cell extracts with performic acid abolished THAF activity. Conditioned medium from Rcho-1, but not HRP-1 or MMQ, cells increased tyrosine hydroxylase activity, indicating that the factor(s) may be secreted. These data indicate that Rcho-1 cells produce a factor(s) capable of increasing tyrosine hydroxylase activity in fetal hypothalamic cell cultures in a rapid, specific, and concentration-dependent manner. The activity of THAF *in vitro* is probably not due to members of the placental PRL family, but appears to be a low mol wt secreted peptide. (*Endocrinology* 133: 111-120, 1993)

THE TUBEROINFUNDIBULAR dopaminergic neurons play a major role in the tonic inhibition of PRL secretion, and the function of these dopamine neurons is regulated by a variety of hormones, including PRL itself (for reviews, see Refs. 1 and 2). The rat placenta produces a family of proteins that are similar in amino acid sequence to pituitary PRL (for review, see Ref. 3). The role of these extrapituitary PRLs in feedback to the maternal hypothalamus and/or pituitary is unclear. A uterine-placental input is important in the cessation of the twice daily (nocturnal and diurnal) PRL surges at midpregnancy and the extinction of the biphasic pattern of tuberoinfundibular dopaminergic activity (4-7). Since these events are temporally correlated with the onset of placental

production of members of the PRL family (4, 8), it is hypothesized that these placental PRLs may activate a negative feedback loop involving the tuberoinfundibular dopaminergic neurons, resulting in suppression of circulating PRL during the last half of gestation.

Given that a finite amount of PRL appears to be essential for the normal development and function of the tuberoinfundibular dopaminergic neurons during the neonatal and adult periods (9-12), consideration should also be given to a potential role for placental hormones in the development of these neurons in the fetus. Members of the placental PRL family are produced in a temporal and tissue-specific pattern during middle and late pregnancy, and some have been found in the fetal circulation (3, 13). However, virtually nothing is known about the effects of the placental PRLs or other placental hormones on hypothalamic development, even though tyrosine hydroxylase-containing neurons in the fetus develop significantly during the last half of gestation (14-17).

The recent establishment of a rat choriocarcinoma cell line (Rcho-1) (18, 19) provides a unique tool to examine feedback

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to the maternal hypothalamo-pituitary axis or effects on the fetal hypothalamus and/or pituitary by a specialized placental cell type. The Rcho-1 cells develop along the trophoblast cell lineage and have the capacity to differentiate into giant cells, which are morphologically and hormonally similar to the giant cells of the normal rat placenta. The Rcho-1 cells produce placental lactogen-I (PL-I) *in vivo* and also express PL-II, PRL-like protein-A, and PRL-like protein-C *in vitro*.

We recently reported that a factor(s) from Rcho-1 cells suppresses circulating PRL levels and increases tyrosine hydroxylase activity in tuberoinfundibular dopaminergic neurons *in vivo* (20, 21). This factor(s) abolishes the PRL surges of early pregnancy, coincident with increases in tyrosine hydroxylase activity in the stalk-median eminence during these times. Since the action of the Rcho-1 factor(s) was similar to that of PRL under some conditions (9, 21, 22), we postulated that one of the members of the placental PRL family, possibly PL-I, may be the hormone responsible for these effects. Because of the difficulty in characterizing the factor(s) using a complex *in vivo* system, a fetal hypothalamic cell culture system was used to characterize the Rcho-1 factor(s). The objectives of this study were 1) to determine if the factor(s) increases tyrosine hydroxylase activity *in vitro* by coculturing fetal hypothalamic cells with trophoblast and pituitary cell lines; 2) to assess the ability of lactogenic hormones, pituitary PRL, and PL-I to alter tyrosine hydroxylase activity in hypothalamic cell cultures; and 3) to estimate the mol wt and characterize the chemical nature of the factor(s) from acetic acid extracts of Rcho cells.

Materials and Methods

Animals

Adult female (200–250 g) and male Sprague-Dawley (300–350 g) rats were obtained from Sasco (Omaha, NE). Rats were housed under controlled temperature (22 C) and lighting (lights on from 0600–1800 h) and supplied with food and water *ad libitum*. The estrous cycles of female rats were followed by daily vaginal lavage. Each female was placed with a single male on the day of proestrus for mating purposes. If sperm were present on the following day, it was designated day 0 of pregnancy.

Fetal hypothalamic cell cultures

Fetal hypothalamic cells were cultured using a modification of the method described by Ahmed *et al.* (23) and used by Porter *et al.* (24, 25) for TH-containing neurons. On day 19 or 20 of pregnancy, 3–4 rats were anesthetized with ether, and the fetuses were removed through a midline abdominal incision under aseptic conditions. The ventral hypothalamus was excised and immediately placed in sterile dispersion buffer, pH 7.4, at 4–10 C. The dispersion buffer contained 137 mM sodium chloride, 5.4 mM potassium chloride, 0.2 mM dibasic sodium phosphate, 0.2 mM monobasic potassium phosphate, 5.5 mM glucose, 59 mM sucrose, 0.002% phenol red, 100 U/ml penicillin G, 100 µg/ml streptomycin, and 0.25 µg/ml fungizone. The hypothalamic fragments were cut into small pieces (<1 mm³) with a scalpel. The tissue pieces were incubated for 15 min at 37 C in 10 ml dispersion buffer containing 0.25% trypsin with DNAase (0.08 mg/ml) added during the final 5 min. An equal volume of dispersion buffer containing soybean trypsin inhibitor (0.3 mg/ml) was added, and the mixture was centrifuged (500 × g for 5 min). The pellet was resuspended in dispersion medium, dispersed mechanically by repeated up and down pipetting with a siliconized transfer pipette, and centrifuged (1,000 × g for 10 min). The dispersed

cells were resuspended in high glucose Dulbecco's medium, modified as described below, supplemented just before use with 5.4 µM linoleic acid, 3.6 µM linolenic acid, 12 µM fatty acid-free BSA, 2.5% fetal bovine serum, 5% horse serum, and 100 U/ml Nystatin. This high glucose Dulbecco's medium was modified by adding 25 mM HEPES, 20 mM potassium chloride, 0.2 mM magnesium chloride, 40 µM L-alanine, 30 µM L-asparagine, 30 µM L-aspartic acid, 30 µM L-glutamic acid, 70 µM L-proline, 0.1 µM D-biotin, 5.8 µM lipoic acid, 1 µM vitamin B₁₂, 1 nM chromium chloride, 1 nM cupric sulfate, 1 nM manganese sulfate, 1 nM molybdenum trioxide, 1 nM zinc sulfate, 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml fungizone. The cell suspension was filtered through a nylon mesh (60 µm) and plated at a density of 200,000 cells/0.2 ml well in 96-well plates previously coated with poly-D-lysine (100 µg/ml). The cells were maintained in culture for 14–16 days at 37 C under a humidified atmosphere of 95% air-5% carbon dioxide. Eighteen to 24 h after plating the cells, the medium was replaced with serum-free modified high glucose Dulbecco's medium, supplemented with ITS⁺ (1:100; Collaborative Biomedical Products, Bedford, MA), 3.6 µM linolenic acid, 10 µM putrescine, and 100 U/ml nystatin. The medium was changed every 1–2 days as needed.

Some hypothalamic cells were cultured in Lab-Tek chamber slides (Baxter Scientific, McGaw Park, IL) and maintained as described above. After 14 days in culture, the cells were fixed in 4% paraformaldehyde, and immunocytochemistry for tyrosine hydroxylase was performed as described previously (7, 9, 21).

Maintenance of Rcho, HRP, and MMQ cells in culture

A Rcho-1 cell line established by Faria and Soares (19) and a HRP-1 cell line described by Soares *et al.* (26) were maintained in culture as described previously by these investigators. Preconfluent and postconfluent Rcho cultures were generated as previously described (19). HRP-1 cells are of trophoblast origin, but unlike Rcho cells, do not differentiate into giant cells or produce any known members of the placental PRL family. Thus, they were used as control cells. MMQ cells, a pituitary-derived PRL-secreting line, were generously provided by Drs. R. M. MacLeod and A. M. Judd (University of Virginia, Charlottesville, VA) and maintained in culture as described by Judd *et al.* (27).

Tyrosine hydroxylase activity and dopamine secretion

The hypothalamic cells were preincubated for 15 min in Earle's Balanced Salt Solution to which 20 µM tyrosine and 20 mM potassium chloride were added. The preincubation medium was removed, and 100 µl medium containing 100 µM brocresine (4-bromo-hydroxybenzylxyamine; gift from American Cyanamid Co., Pearl River, NY), an aromatic L-amino acid decarboxylase inhibitor, were added. The cells were then incubated for 0, 30, 60, and 120 min under a humidified atmosphere of 90% air-10% carbon dioxide. A 60-min incubation period was used for the experiments characterizing the factor(s) from Rcho-1. At the end of the incubation period, the medium was removed, acidified with 10 µl 1 N perchloric acid, and frozen until samples were analyzed for medium DOPA accumulation. Lyophilized extracts of Rcho-1, HRP-1, or MMQ cells; 1 mM α-methyl-p-tyrosine; or 5 mM (Bu)₂cAMP were included in the modified Earle's Balanced Salt Solution, as indicated. Dopamine secretion was determined by incubating the hypothalamic cells in 100 µl Earle's Balanced Salt Solution without brocresine for 120 min. The medium was acidified with 10 µl 1 N perchloric acid until samples were analyzed for dopamine. Some hypothalamic cells were scraped from the culture plates, homogenized in 100 µl 0.1 N perchloric acid, and centrifuged at 10,000 × g for 2 min for determination of cellular catecholamine content. The cellular contents of DOPA, dopamine, and L-dihydroxyphenylacetic acid (DOPAC) and the medium contents of DOPA and dopamine were determined by HPLC (CR4A, Shimadzu, Kyoto, Japan), as described previously (7, 9, 21).

Coculture with trophoblast and pituitary cell lines and incubation with hormones

24-h coculture. HRP-1 and preconfluent or postconfluent Rcho-1 cells were briefly exposed to 0.25% trypsin and mechanically scraped from

the flask. Rcho-1, HRP-1, and MMQ cells were centrifuged at $1,000 \times g$ for 5 min and resuspended in modified Dulbecco's medium at densities of 25,000, 50,000, or 100,000 cell/200 μ l as indicated, and 200 μ l of the cell suspension were added to individual wells of hypothalamic cells. After a 24-h incubation period, the medium was removed, while the Rcho-1, HRP-1 or MMQ cells were retained with the hypothalamic cells. DOPA accumulation was determined as described above.

24-h coinubation. Rat PRL (B-6; National Hormone and Pituitary Program) or recombinant rat PL-1, as described Robertson *et al.* (28, 29), was included for 24 h with the hypothalamic cells in modified high glucose Dulbecco's medium at a concentration of 1 μ g/ml. Rat PRL or rat PL-1 (1 μ g/ml) was included in Earle's Balanced Salt Solution during the 15-min preincubation period and the 60-min incubation period for determining tyrosine hydroxylase activity.

1-h coculture. Preconfluent or postconfluent Rcho-1 cells were briefly exposed to 0.25% trypsin and mechanically scraped from the flask. Rcho-1 cells were centrifuged at $1,000 \times g$ for 5 min, washed twice in Earle's Balanced Salt Solution, and resuspended in Earle's Balanced Salt Solution containing 20 μ M tyrosine, 20 mM potassium chloride, and 100 μ M brocresine at a density of 100,000 cells/100 μ l. After a 15-min preincubation of the hypothalamic cells, the Rcho-1 cells (100,000 cells/well) were included during the final 1-h incubation for the determination of DOPA accumulation.

Preparation of cell extracts

After dislodging Rcho-1 and HRP-1 cells with mild trypsinization, as described above, Rcho-1, HRP-1, and MMQ cells were washed twice in Hanks' Balanced Salt Solution (2×10^7 cells/50 ml) and homogenized by sonication at a concentration of 1×10^7 cells/ml in 0.1 N acetic acid, 1 N formic acid, 100 mM ammonium bicarbonate (pH 8.8), or methanol. The extracts were centrifuged at $10,000 \times g$ for 5 min, and the supernatants were removed. Some acetic acid extracts were then heated in a boiling water bath for 15 min. Acetic acid, formic acid, and ammonium bicarbonate supernatants were lyophilized, and the methanol supernatant was dried under a stream of nitrogen gas. The lyophilized or dried extracts were reconstituted in Earle's Balanced Salt Solution containing 20 μ M tyrosine, 20 mM potassium chloride, and 100 μ M brocresine; filtered through a 0.22- μ m syringe filter; and added to the hypothalamic cells during the 60-min period for determination of tyrosine hydroxylase activity.

Molecular filtration

The molecular filtration of Rcho-1 cell extracts was performed under nitrogen pressure (30–40 psi) with constant stirring, using a 10-ml (25-mm diameter) ultrafiltration cell (Amicon Corp., Danvers, MA). Diaflo ultrafiltration membranes (Amicon Corp.) of 30,000 (YM30), 10,000 (YM10), and 1,000 (YM1) mol wt cut-off (MWCO) limits were washed extensively with 0.1 N acetic acid before use. Acetic acid extracts of Rcho cells (5×10^7 cell eq/5 ml) were ultrafiltered through the 30,000 MWCO membrane, followed by two successive washes of 3 and 2 ml 0.1 N acetic acid. The eluate from the 30,000 MWCO membrane was then ultrafiltered and washed sequentially through 10,000 and 1,000 MWCO membranes. The retentate from all three membranes and the eluate from the 1,000 MWCO membrane were lyophilized and reconstituted in Earle's Balanced Salt Solution containing 20 μ M tyrosine, 20 mM potassium chloride, and 100 μ M brocresine before addition to the hypothalamic cells for determining tyrosine hydroxylase activity.

Treatment with proteolytic enzymes

To determine whether the factor(s) activity was peptidergic in nature, Rcho-1 cell extracts were exposed to proteolytic enzymes. Lyophilized acetic acid extracts of Rcho-1 cells (2.5×10^6 cell eq), 10 μ M pituitary adenylate cyclase-activating peptide (PACAP), the combination of Rcho-1 cell extract and PACAP, or the medium alone were incubated for 18 h at 37 C in 100 μ l Earle's Balanced Salt Solution with 25 mM HEPES, pH 8.0, containing trypsin (50 μ g/ml; L-1-tosylamide-2-phenylethyl chloromethyl ketone-treated; Worthington Biochemical Corp., Free-

hold, NJ) or chymotrypsin (50 μ g/ml; *N* α -*p*-tosyl-L-lysine chloromethyl ketone-treated; Sigma Chemical Co., St. Louis, MO). Controls were incubated in the medium described without enzymes for 18 h at 37 C and compared to unincubated controls at the same concentration. All samples were diluted 10-fold with Earle's Balanced Salt Solution containing 20 μ M tyrosine, 20 mM potassium chloride, and 100 μ M brocresine before addition to hypothalamic cell cultures to determine the effects on tyrosine hydroxylase activity.

Lyophilized acetic acid extracts of Rcho-1 cells (5×10^6 cell eq/100 μ l), 10 μ M PACAP, 100 μ M morphine, or medium alone were incubated for 18 h at 37 C in Earle's Balanced Salt Solution with 25 mM HEPES, pH 7.5, containing pronase-E (250 μ g/ml; Sigma Chemical Co.). At the end of the 18-h incubation, the samples were placed in boiling water for 10 min to inactivate the pronase activity. After control samples were incubated for 18 h and subjected to boiling, heat-inactivated pronase was added. All samples were diluted 10-fold with Earle's Balanced Salt Solution containing 20 μ M tyrosine, 20 mM potassium chloride, and 100 μ M brocresine.

Oxidation with performic acid

Performic acid was prepared immediately before use, as described by Hirs (30). Formic acid (99%) and hydrogen peroxide (30%) were mixed (95:5, vol/vol) and allowed to stand at 22 C for 2 h. Lyophilized acetic acid extracts of Rcho-1 cells (5×10^6 cell eq/tube) or PACAP (4.5 μ g/tube) were dissolved in 0.2 ml performic acid and placed on ice for 2 h. The samples were diluted 20-fold with water, lyophilized, and reconstituted in 1 ml Earle's Balanced Salt Solution containing 20 μ M tyrosine, 20 mM potassium chloride, and 100 μ M brocresine. Controls were incubated with water instead of performic acid and then treated in an identical manner.

Preparation of conditioned medium

Rcho-1 and HRP-1 cells ($\sim 5\text{--}6 \times 10^6$ cells/75 cm²) that remained attached to the flask and MMQ cells (6×10^6 cells) were washed (twice, 1 h each time) with 20 ml Earle's Balanced Salt Solution. For generation of conditioned medium, the cells were incubated in 2.5 ml Earle's Balanced Salt solution containing 20 μ M tyrosine and 20 mM potassium chloride for 8 h at 37 C under an atmosphere of 95% air-5% carbon dioxide. After the 1,000 MWCO membrane was prewashed extensively with Earle's Balanced Salt Solution, conditioned media from two flasks (5 ml) were ultrafiltered through a 1,000 MWCO membrane, followed by two successive washes in 3 and 2 ml modified Earle's Balanced Salt Solution. Brocresine (100 μ M) was added to the eluate from the 1,000 MWCO membrane, and the effect on tyrosine hydroxylase activity was determined.

PRL determination

PRL in MMQ cell extracts and media was determined by rat PRL RIA provided by the NIDDK. PRL RP-3 was used as a reference preparation, and the limit of sensitivity for the assay was 50 pg. The intraassay coefficient of variation was 9.1%.

Statistical analysis

Results are expressed as the mean \pm SE of determinations from three to six different hypothalamic cultures. The value for each hypothalamic culture is a mean of triplicate wells. The n for all experiments refers to the number of different hypothalamic cultures. When cell extracts or conditioned medium were used, three to five different preparations were used. Data were evaluated by *t* test when two groups were being compared (31). Data were evaluated by analysis of variance when three or more groups were being compared, and multiple comparisons were made with Fisher's least significant procedures (31, 32).

Results

Dopaminergic neurons in the hypothalamic cell cultures

Cells from the ventral hypothalamus of 19- to 20-day-old rat fetuses cultured for 14–16 days contained tyrosine hydroxylase-immunopositive neurons with long axons (Fig. 1). The contents of dopamine and DOPA in perchloric acid extracts of 200,000 hypothalamic cells were approximately 183 and 50 pg, respectively. The amounts (picograms per 200,000 cells/2 h) of DOPA, dopamine, and L-dihydroxyphenylacetic acid released into the Earle's Balanced Salt Solution without brocresine were 257 ± 20 , 72 ± 10 , and 44 ± 5 , respectively. Norepinephrine and epinephrine were not detectable in the hypothalamic cell extracts or the medium, indicating the tyrosine hydroxylase-containing cells were dopaminergic neurons.

The hypothalamic cells were incubated in medium containing $100 \mu\text{M}$ brocresine for the determination of tyrosine hydroxylase activity. The accumulation of DOPA in the medium was linear for 120 min with or without brocresine, but was approximately 3-fold higher with brocresine (Fig. 2). Thus, a 60-min exposure to brocresine was used to assess tyrosine hydroxylase activity in all experiments.

Coculture of fetal hypothalamic cells with trophoblast and pituitary cell lines

The hypothalamic cells were cocultured with HRP-1, MMQ, and postconfluent Rcho-1 cells for 24 h before deter-

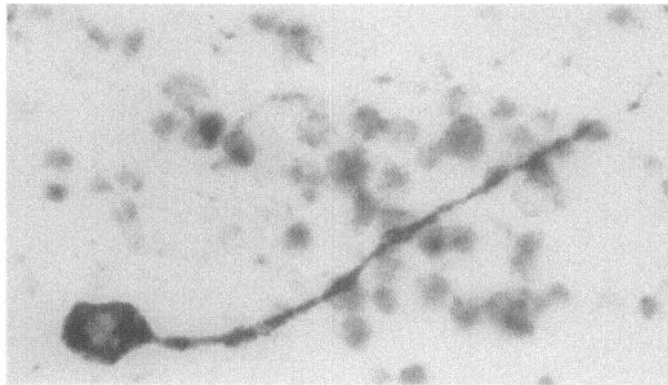


FIG. 1. Representative tyrosine hydroxylase-immunopositive neuron in hypothalamic cell cultures maintained *in vitro* for 14 days.

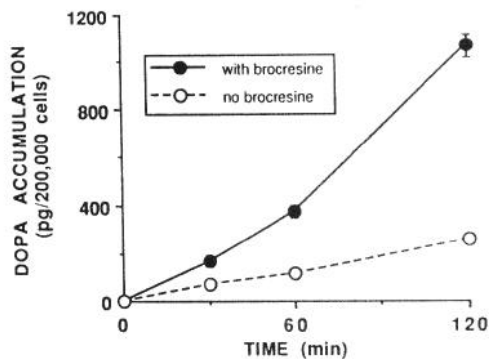


FIG. 2. Medium DOPA accumulation in fetal hypothalamic cell cultures incubated with (●) or without (○) $100 \mu\text{M}$ brocresine, an aromatic L-amino acid decarboxylase inhibitor, for 0–120 min.

mining tyrosine hydroxylase activity (Fig. 3). A density-dependent increase in tyrosine hydroxylase activity was observed when 25,000, 50,000, or 100,000 Rcho-1 cells were cocultured with hypothalamic cells. Coculture of 100,000 Rcho-1 cells/well with hypothalamic cells resulted in a 4-fold increase ($P < 0.05$) in tyrosine hydroxylase activity. Neither HRP-1 nor MMQ cells altered tyrosine hydroxylase activity when they were cocultured at the same density. The medium concentration of PRL in wells containing the MMQ cells after 24 h in culture was $275 \pm 37 \text{ ng}/100,000 \text{ cells} \cdot 100 \mu\text{l}$. Both control and Rcho-1-stimulated DOPA accumulation were decreased ($P < 0.05$) by 1 mM α -methyl-*p*-tyrosine, a specific inhibitor of tyrosine hydroxylase activity. When 100,000 Rcho-1, HRP-1, or MMQ cells were cultured without hypothalamic cells, DOPA was not observed in the medium. Since the Rcho-1 factor(s) increased the activity of tyrosine hydroxylase, it is called tyrosine hydroxylase-activating factor(s) (THAF).

Given that the PRL-induced increase in tuberoinfundibular dopaminergic activity *in vivo* may require at least 12 h (2, 33, 34), the next experiment was performed to determine whether it was necessary to have the Rcho-1 cells present for 24 h or whether the Rcho-1 cells could acutely stimulate tyrosine hydroxylase activity when they were added only during the final 1-h incubation. The magnitudes of the Rcho-1 cell-induced increase in tyrosine hydroxylase activity were similar in the 1-h and 24-h periods, indicating that 1 h with THAF was sufficient (Fig. 4). In addition, since the maintenance of Rcho-1 cells in postconfluent culture conditions results in increased differentiation into giant cells and augmented production of members of the placental PRL family compared to preconfluent cultures (19), THAF activity of Rcho cells from preconfluent and postconfluent cultures was examined. Tyrosine hydroxylase activity was increased similarly 2- to 2.5-fold by Rcho-1 cells from both preconfluent and postconfluent cultures, suggesting that production of higher levels of members of the placental PRL family does

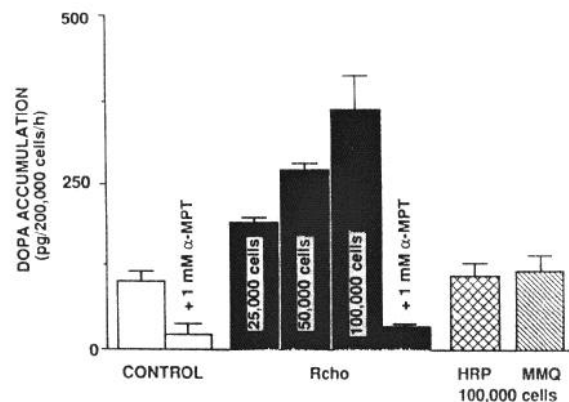


FIG. 3. Differential effects of coculturing fetal hypothalamic cells with Rcho-1, HRP-1, or MMQ cells for 24 h on tyrosine hydroxylase activity. Inclusion of 1 mM α -methyl-*p*-tyrosine (α -MPT) in control and Rcho-1 cell-containing wells is indicated above the respective bars. The number of Rcho-1 cells per well is indicated within the bar, and the number of HRP-1 and MMQ cells is indicated below the axis. Each value is a mean \pm SE of determinations from four different hypothalamic cell cultures.

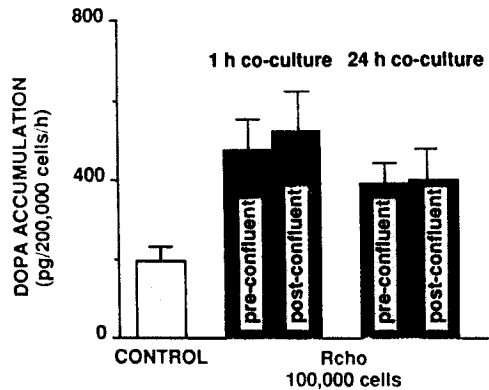


FIG. 4. Comparison of coculturing pre- or postconfluent Rcho-1 cells, as indicated within the bars, with hypothalamic cells on tyrosine hydroxylase activity. The duration of coculture is represented above the bars. Each value is a mean \pm SE of determinations from four different hypothalamic cell cultures.

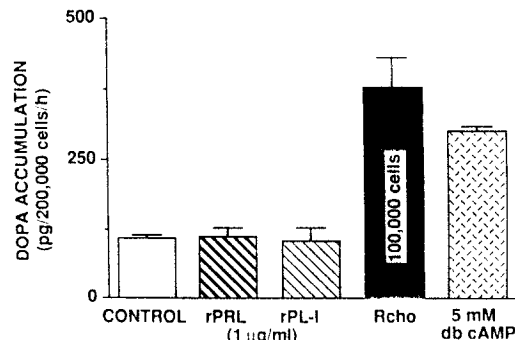


FIG. 5. Inability of rat (r) PRL or rat PL-I to alter tyrosine hydroxylase activity when incubated with hypothalamic cells for 24 h at a concentration of 1 µg/ml, whereas 1-h incubation with 5 mM (Bu)₂cAMP (db cAMP) or 24-h coculture with 100,000 Rcho-1 cells increased tyrosine hydroxylase activity. Each value is a mean \pm SE of determinations from three different hypothalamic cell cultures.

not enhance the stimulatory effects of THAF on tyrosine hydroxylase activity (Fig. 4).

Effect of lactogenic hormones on tyrosine hydroxylase activity

The hypothalamic cells were incubated with either rat PRL or rat PL-I at a concentration of 1 µg/ml for 24 h (Fig. 5). Neither of these lactogenic hormones increased tyrosine hydroxylase activity. However, the cells were responsive to a 24-h stimulation with Rcho-1 cells or an acute 1-h stimulation with 5 mM (Bu)₂cAMP. A 10-fold higher concentration (10 µg/ml) of PRL or rat PL-I was also ineffective in altering tyrosine hydroxylase activity (data not shown).

Extraction of THAF from Rcho cells

The efficacy of extracting THAF from preconfluent Rcho-1 cells by several methods was examined. The accumulation of DOPA (picograms per 200,000 cells/h) in hypothalamic cell cultures was increased from control levels (318 \pm 64) to 743 \pm 149, 880 \pm 132, or 666 \pm 94 by Rcho-1 cells extracted with 0.1 M ammonium bicarbonate adjusted to pH 8.8, 0.1 N acetic acid, or 1 N formic acid, respectively. THAF activity

was not extracted from the acetic acid extracts of Rcho-1 cells by either diethyl ether or chloroform (data not shown). However, methanol extracts of Rcho-1 cells stimulated ($P < 0.05$) tyrosine hydroxylase activity approximately 2.8-fold, and this increase was markedly decreased by 1 mM α -methyl-*p*-tyrosine (Fig. 6). Methanol extracts of HRP-1 and MMQ cells did not alter tyrosine hydroxylase activity. There was no detectable DOPA in any of the trophoblast or pituitary cell extracts. Acetic acid appeared to be the most efficient in extracting THAF and, thus, was used in all subsequent experiments. In contrast, ammonium bicarbonate was a better extraction method for lactogenic hormones. Although no extract of MMQ cells significantly altered tyrosine hydroxylase activity, 0.1 M ammonium bicarbonate extracts of MMQ cells contained 276 \pm 7 ng PRL/500,000 cell eq, whereas 0.1 N acetic acid extracts contained 17 \pm 1 ng PRL/500,000 cell eq.

There was a concentration-dependent increase in tyrosine hydroxylase activity with Rcho-1 cell extracts (Fig. 7). A 3-fold increase ($P < 0.05$) in tyrosine hydroxylase activity was observed with 500,000 cell eq, and this increase was markedly decreased with 1 mM α -methyl-*p*-tyrosine. In agreement with the coculture experiment, acetic acid extracts of the same number of HRP or MMQ cells did not significantly stimulate tyrosine hydroxylase activity, supporting the view that THAF is found only in Rcho-1 cells. To determine whether the factor(s) stimulating tyrosine hydroxylase activity was heat stable, the acetic acid extract was boiled. Rcho-1 cell extract increased ($P < 0.05$) tyrosine hydroxylase activity (picograms per 200,000 cells/h) to 1116 \pm 80 from a control level of 341 \pm 53, but boiling reduced ($P < 0.05$) this increase 35% to 708 \pm 91. In addition to the augmentation of tyrosine hydroxylase activity, acetic acid extracts of Rcho-1 cells increased ($P < 0.05$) dopamine secretion from 89 \pm 15 to 241 \pm 59 pg/200,000 cells \cdot 2 h.

Estimation of mol wt range of THAF

The mol wt range of THAF was estimated using a sequential series of ultrafiltration membranes (Fig. 8). A greater than

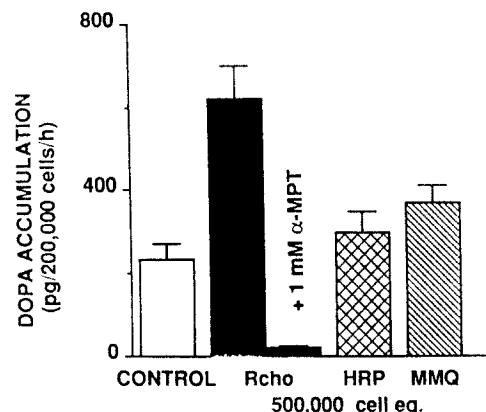


FIG. 6. The ability of methanol extracts of Rcho-1, but not HRP-1 or MMQ, cells to increase hypothalamic tyrosine hydroxylase *in vitro*. α -Methyl-*p*-tyrosine (α -MPT) was included with the Rcho-1 cells when indicated above the bar. Each value is a mean \pm SE of determinations from six different hypothalamic cell cultures.

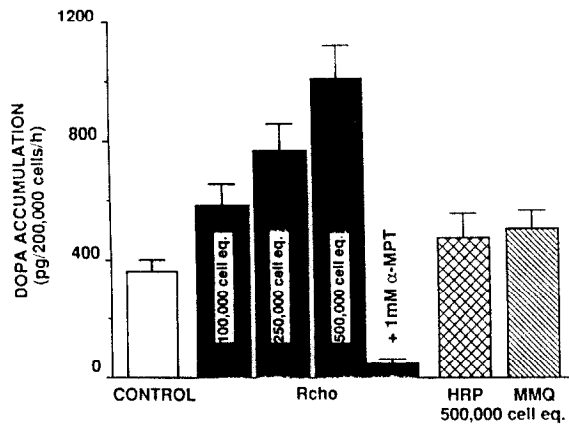


FIG. 7. The ability of acetic acid extracts of Rcho-1, but not HRP-1 or MMQ, cells to increase tyrosine hydroxylase activity in a concentration-dependent manner. The number of Rcho-1 cell equivalents per well is shown *within the bar*, and the number of HRP-1 or MMQ cell equivalents is *below the axis*. Inclusion of 1 mM α -methyl-*p*-tyrosine (α -MPT) is indicated *above the bar*. Each value is a mean \pm SE of determinations from six different hypothalamic cell cultures.

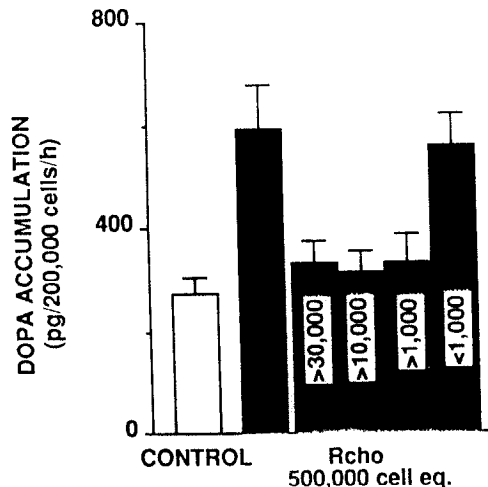


FIG. 8. Elution of THAF(s) through the 1,000 mol wt ultrafiltration membrane. Acetic acid extracts of Rcho cells were ultrafiltered sequentially through 30,000, 10,000, and 1,000 MWCO membranes. ■, THAF(s) activity in an aliquot of Rcho cell extract that was not ultrafiltered. After ultrafiltration, the retentates (>30,000, >10,000, and >1,000) and eluate (<1,000), as indicated *within the bars*, were tested for their ability to increase tyrosine hydroxylase activity. Each value is a mean \pm SE of determinations from six different hypothalamic cell cultures.

2-fold increase ($P < 0.05$) in tyrosine hydroxylase activity was observed in an aliquot of Rcho-1 cell extract that was not ultrafiltered. There was little THAF activity in the retentates from the 30,000, 10,000, and 1,000 MWCO membranes. The majority of activity was found in the eluate from the 1,000 MWCO membrane, suggesting that THAF is a relatively low mol wt compound.

Analysis of the peptidergic nature of THAF with proteolytic enzymes

To determine whether THAF is a peptide, we conducted a series of experiments using proteolytic enzymes. We first

compared the ability of the THAF from Rcho-1 cell extracts to retain its tyrosine hydroxylase stimulatory activity during the incubation period to that of PACAP used as a control peptide (Fig. 9, upper panel). The 18-h incubation at 37 C did not alter the magnitude of the increase in tyrosine hydroxylase activity induced by Rcho-1 cell extract and PACAP alone. Since cell extracts may contain many peptides/proteins that may decrease the enzyme efficiency, the Rcho-1 cell extract was coincubated with PACAP. The increase (control) in tyrosine hydroxylase activity was additive when the Rcho-1 cell extract (250,000 cell eq) and 1 μ M PACAP were added simultaneously during the final 1-h incubation. However, when the Rcho-1 cell extract and PACAP were preincubated together for 18 h at 37 C, the increase in tyrosine hydroxylase activity was decreased ($P < 0.06$) 20% compared to that in the unincubated control, but was significantly greater ($P < 0.05$) than that in preincubated Rcho-1 cell extract or PACAP alone. Thus, it is likely that the Rcho-1 cell extract contained protease activity to decrease the additive effect of PACAP.

Rcho-1 cell extract, PACAP, and morphine were preincubated with pronase, a nonspecific proteolytic enzyme (Fig. 10). The Rcho-1 cell-induced increase in tyrosine hydroxylase

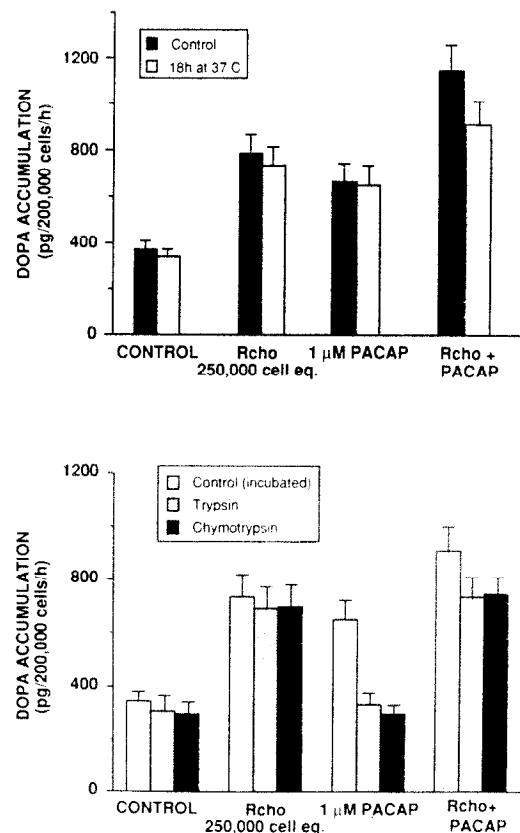


FIG. 9. Stability of THAF(s) and PACAP alone, but not PACAP with Rcho-1 cell extract, during 18-h incubation at 37 C (*top panel*). Resistance of THAF(s), but not PACAP, to inactivation by trypsin or chymotrypsin (*bottom panel*). Medium (control), Rcho-1 cell extract, PACAP, or Rcho-1 cell extract plus PACAP were preincubated for 18 h at 37 C with or without (control) trypsin or chymotrypsin and then included with hypothalamic cells to determine their ability to stimulate tyrosine hydroxylase activity. Each value is a mean \pm SE of determinations from five different hypothalamic cell cultures.

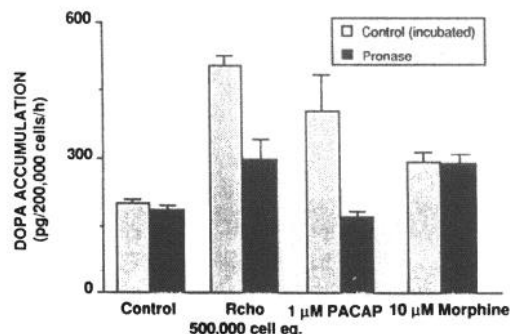


FIG. 10. Inactivation of THAF(s) and PACAP, but not morphine, by pronase. Medium (control), Rcho-1 cell extract, PACAP, or morphine was preincubated with or without (control) pronase for 18 h at 37 C and then included with hypothalamic cells to determine their ability to stimulate tyrosine hydroxylase activity. Each value is a mean \pm SE of determinations from four different hypothalamic cell cultures.

activity was reduced ($P < 0.05$) by pronase pretreatment of Rcho cell extracts, indicating that THAF may be a peptide. The 2-fold increase in tyrosine hydroxylase activity elicited by 1 μ M PACAP was abolished by pronase, whereas the 40% increase ($P < 0.05$) induced by the nonpeptidergic morphine was not altered by pronase. These data suggest that the effect of pronase is probably due to its proteolytic activity and not a result of nonspecific effects.

Rcho-1 cell extracts and/or PACAP were incubated without (controls) or with trypsin or chymotrypsin for 18 h at 37 C (Fig. 9, lower panel). Basal (control) tyrosine hydroxylase activity in the hypothalamic cell cultures was similar in the presence of preincubated medium alone, trypsin, or chymotrypsin. Neither trypsin nor chymotrypsin pretreatment altered the activity of THAF in Rcho-1 cell extracts, whereas both enzymes abolished ($P < 0.05$) the 2-fold increase in tyrosine hydroxylase activity elicited by 1 μ M PACAP. Compared with the incubated Rcho-1 and PACAP control, both trypsin and chymotrypsin decreased ($P < 0.10$) by 20% the Rcho-1- and PACAP-induced increase in tyrosine hydroxylase activity to a level identical to the Rcho-1 cell-stimulated value. These data indicate that the enzymes were effective in suppressing PACAP's ability to stimulate tyrosine hydroxylase activity even in the presence of Rcho-1 cell extract. Moreover, the resistance of THAF to trypsin and chymotrypsin digestion was confirmed with 10-fold higher (500 μ g/ml) concentrations of these enzymes (data not shown).

Chemical modification of THAF

Performic acid oxidation cleaves disulfide bonds as well as modifies cystine, methionine, and tryptophan residues (30). Tyrosine hydroxylase activity in control wells was not altered by performic acid treatment (Fig. 11). Performic acid oxidation decreased ($P < 0.05$) the 3-fold Rcho-1-induced increase in tyrosine hydroxylase activity to control levels. PACAP, which does not have a disulfide bond, was used as a control peptide. As expected, the 2-fold increase in tyrosine hydroxylase activity induced by PACAP was not significantly altered by performic acid oxidation.

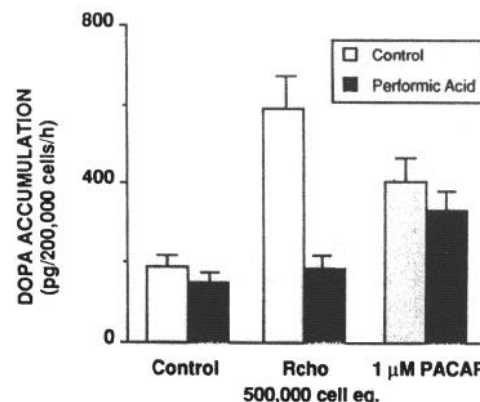


FIG. 11. Inactivation of THAF(s), but not PACAP, by performic acid oxidation. Rcho cell extract or PACAP was included with hypothalamic cells to test their ability to stimulate tyrosine hydroxylase activity after 2-h treatment with or without (control) performic acid. Each value is a mean \pm SE of determinations from six different hypothalamic cell cultures.

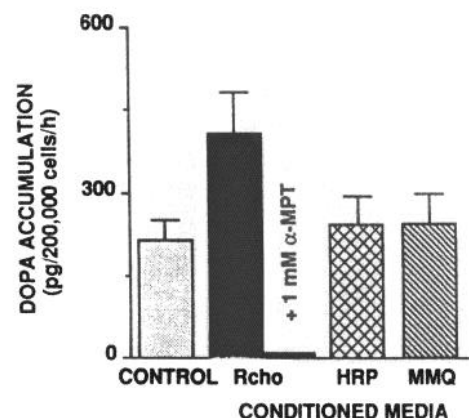


FIG. 12. The ability of conditioned medium from Rcho-1, but not HRP-1 or MMQ, cells to increase tyrosine hydroxylase activity in fetal hypothalamic cell cultures. Inclusion of 1 mM α -methyl-*p*-tyrosine (α -MPT) is indicated above the bar. Conditioned medium (Earle's Balanced Salt Solution with 20 μ M tyrosine and 20 mM potassium chloride) was collected after 8 h with Rcho-1, HRP-1, or MMQ cells; ultrafiltered through a 1000 MWCO membrane; added to brocresine (100 μ M); and included with the hypothalamic cells. Each value is a mean \pm SE of determinations from five different hypothalamic cell cultures.

Evaluation of the secretion of the THAF into conditioned medium

To determine whether the THAF is secreted, the effect of conditioned medium from Rcho-1 cell cultures on tyrosine hydroxylase activity was examined. The less than 1,000 mol wt fractions of the conditioned media from confluent Rcho-1, HRP-1, and MMQ cells were collected and diluted 1:2 with fresh medium. Conditioned medium from Rcho-1 cells increased ($P < 0.05$) tyrosine hydroxylase activity 2-fold, indicating that the THAF was secreted into the medium (Fig. 12). This was unique to conditioned medium from the Rcho-1 cells, since the less than 1,000 mol wt fraction of conditioned medium from HRP-1 and MMQ cells did not alter tyrosine hydroxylase activity. There was no DOPA in the conditioned medium alone from Rcho-1, HRP-1, or

MMQ cells, and 1 mM α -methyl-*p*-tyrosine markedly decreased the Rcho-1 cell-stimulated DOPA accumulation, indicating that the medium DOPA accumulation was from tyrosine hydroxylase in the hypothalamic cell cultures.

Discussion

We are describing a factor(s), produced by Rcho cells, that markedly increased tyrosine hydroxylase activity in a specific and concentration-dependent manner and increased dopamine secretion in fetal hypothalamic cell cultures. THAF is probably not PL-I, but appears to be a small mol wt secreted peptide.

The cultured cells were from the medioventral hypothalamus of day 19 or 20 rat fetuses. Our dissection probably did not include the tyrosine hydroxylase-positive cell group from the dorsal hypothalamus (15–17). In the adult rat, this ventral region contains perikarya of the tuberoinfundibular dopaminergic (A12) neurons, but not the incertodopaminergic (A13) neurons (35). We observed tyrosine hydroxylase-immunopositive neurons with neurite outgrowth in the cultures. In addition, the cultures contained unidentified neuronal and nonneuronal cell types. The tyrosine hydroxylase-containing neurons were entirely dopaminergic, since DOPA, DOPAC, and dopamine, but not norepinephrine or epinephrine, were detectable in the media and extracts of the cultured hypothalamic cells. The cells contained approximately 4-fold more dopamine than DOPA, whereas approximately 3- to 4-fold more DOPA than dopamine was released into the medium without brocresine. This may indicate that dopamine is stored and its secretion regulated, whereas DOPA may readily diffuse into medium. The fact that DOPA was from the tyrosine hydroxylase-containing cells was confirmed by the observation that α -methyl-*p*-tyrosine, a specific inhibitor of tyrosine hydroxylase, markedly decreased DOPA production.

This study characterizes a factor(s) that profoundly stimulates hypothalamic tyrosine hydroxylase when Rcho-1 cells were cocultured with hypothalamic cells. THAF activity was also found in Rcho-1 cell extracts and conditioned medium from Rcho-1 cells. In contrast, HRP-1 and MMQ cells cocultured with hypothalamic cells, cell extracts of HRP-1 and MMQ cells, and conditioned media from HRP-1 and MMQ cells did not alter hypothalamic tyrosine hydroxylase activity. Rcho-1 cells have the capacity of differentiate into trophoblast giant cells and subsequently produce members of the placental PRL family (18, 19), whereas HRP-1 cells, which are also of trophoblast origin, lack this differentiation capacity (26). Although it is not known whether the rat placenta contains a substance(s) that increases tyrosine hydroxylase activity, it is reasonable to hypothesize that THAF may be found in stem cells committed to differentiation into trophoblast giant cells. The MMQ cells are of pituitary origin, and they secrete PRL (27). Thus, THAF was not common to a tumor cell line(s) or to cell lines that produce lactogenic hormones.

THAF does not appear to be one of the members of the placental PRL family. First, recombinant PL-I was not effective in increasing tyrosine hydroxylase in the fetal hypothalamic culture.

Second, several lines of evidence with the Rcho-1 cells indicate that the factor(s) described in this study was not one of the placental PRLs. When Rcho-1 cells were maintained in postconfluent cultures to increase the number of giant cells and augment production of the placental PRLs (19), the increase in tyrosine hydroxylase activity was not different from that in Rcho-1 cells in preconfluent cultures. Moreover, activation of tyrosine hydroxylase was not enhanced when the Rcho-1 cells were extracted with ammonium bicarbonate, which extracts lactogenic hormones more efficiently than acetic acid. The placental PRLs have a higher mol wt, with the monomeric forms ranging from 25–40 kilodaltons (3), whereas the tyrosine hydroxylase-inducing activity was found in the less than 1000 mol wt fraction. In addition, the placental PRLs would not be resistant to digestion by trypsin and chymotrypsin, as was THAF from Rcho-1 cells. In contrast to the delayed action of PRL *in vivo*, THAF increased tyrosine hydroxylase activity *in vitro* in a rapid concentration-dependent manner. The magnitude of the increase induced by Rcho-1 cells was similar whether the cells were included for only 1 h or for 24 h. This suggests that the synthesis of new enzyme was not necessary, but, rather, existing enzyme was activated.

THAF described in the present study does not appear to be PL-I, because this lactogenic hormone did not stimulate tyrosine hydroxylase activity *in vitro*. However, we cannot rule out a role of PL-I *in vivo*. Similar to results reported by Porter and colleagues (24), rat PRL also did not alter tyrosine hydroxylase in fetal hypothalamic cell culture. However, hyperprolactinemia clearly stimulates tyrosine hydroxylase activity in tuberoinfundibular dopaminergic neurons *in vivo* (2, 9, 34, 36). Indeed, the factor(s) from Rcho-1 cells resembles the action of PRL in pregnant rats and appears capable of reversing the hypoprolactemia-induced decrease in tyrosine hydroxylase activity and mRNA levels in bromocriptine-treated ovariectomized rats (21). The relative contributions of the placental PRLs and other trophoblast factor(s) *in vivo* requires further study. The failure of lactogenic hormones to increase tyrosine hydroxylase *in vitro* may indicate that these hormones do not have a direct effect on dopaminergic neurons, but act via an intermediate neuron(s). Alternatively, this may reflect the immaturity of fetal compared to adult dopaminergic neurons or differential development *in vitro* compared to *in vivo*. These possibilities warrant further study.

THAF activity was extracted from Rcho-1 cells by several methods. Although acetic acid was the most efficient, THAF activity was extracted under both acidic and basic conditions. Given the ability of pronase to decrease THAF in Rcho-1 cell extract, the factor(s) appears to be a peptide. Pronase exhibits the broadest specificity of any known proteolytic enzymes and will hydrolyze most peptide bonds (37). The effect of pronase appeared to be specific to peptides, since the PACAP (peptide control)-induced increase was abolished, but the morphine (nonpeptide control)-induced increase was unaltered. THAF activity was resistant to inactivation by trypsin and chymotrypsin, whereas the increase in tyrosine hydroxylase activity elicited by PACAP was abolished. Given the heterogeneous nature of the Rcho-1 cell extract, the possibil-

ity exists that other proteins/peptides in the extract interfered with the proteolytic action of these enzymes. This difficulty was examined by coincubating PACAP and Rcho-1 cell extract with the enzymes. Although the effect of PACAP is additive to that of Rcho-1 cell extract when both are added together acutely, the additive increase is somewhat decreased after the 18-h incubation. However, trypsin and chymotrypsin decreased the additive effect of PACAP to levels identical to those in the Rcho-1 cell extract alone, suggesting that these enzymes are inactivating PACAP's ability to stimulate tyrosine hydroxylase activity even in the presence of the Rcho-1 cell extract. Although we cannot completely rule out that certain peptide bonds may be inaccessible to proteolytic digestion, the data indicate that THAF may not contain trypsin-sensitive lysine or arginine residues or chymotrypsin-sensitive phenylalanine, tyrosine, or tryptophan residues. However, if THAF is a peptide, it may contain some hydrophobic amino acid residues, given the ability of methanol to extract it.

THAF appears to be a low mol wt peptide. The mol wt range was estimated using ultrafiltration membranes. The majority of the tyrosine hydroxylase-increasing activity was recovered in the less than 1000 mol wt fraction. THAF appears to contain a disulfide bond and cystine and/or methionine residues, since its activity was abolished when Rcho-1 cell extracts were treated with performic acid.

Although PACAP and morphine were used as controls in this study, their actions on tyrosine hydroxylase activity in fetal hypothalamic cell cultures are noteworthy. PACAP is a member of the secretin/glucagon/vasoactive intestinal peptide family and is found in high concentrations in the hypothalamus (38). PACAP acts to increase cAMP accumulation in pituitary cells (39) and increase tyrosine hydroxylase activity in adrenal chromaffin cells (40). In the present study, PACAP increased tyrosine hydroxylase activity in fetal hypothalamic cell cultures. However, its role in regulating tyrosine hydroxylase activity in tuberoinfundibular dopaminergic neurons *in vivo* remains to be determined. The stimulatory effect of morphine on tyrosine hydroxylase activity was somewhat surprising. This contrasted with the effect of β -endorphin, which was ineffective or modestly inhibitory in concentrations from 10 nM to 10 μ M (our unpublished data). Morphine decreases tuberoinfundibular dopaminergic neuronal activity in adult rats, although it is stimulatory to dopaminergic terminals in the striatum (41–43). Endogenous opioid peptides are also inhibitory to hypothalamic dopaminergic activity in adults (43–45). To our knowledge, it is not known whether the effects of morphine *in vivo* are similar during the fetal/neonatal period and adulthood. However, the opiate receptor subtype involved in PRL regulation appears to change during the early neonatal period (46).

The identification of THAF activity in Rcho-1 cells, but not HRP-1 and MMQ cells, does not preclude its localization in other tissues as well. Indeed, the brain itself could express THAF, where it might function as a regulator of dopaminergic neurons. Recently, Porter and colleagues (24, 25, 36) described a trypsin-resistant cytotropic factor in the pituitary gland that increased tyrosine hydroxylase activity and tyro-

sine hydroxylase gene expression in fetal hypothalamic-midbrain cell cultures. Whether THAF and the pituitary cytotropic factor share any structural as well as functional properties remains to be determined. Other substances have also been shown to affect dopaminergic activity in other systems. Iacovitti *et al.* (47) reported that muscle-derived differentiation factor(s) increased tyrosine hydroxylase mRNA and protein in neurons of cultured rat cerebral cortex. Denis-Donini (48) observed differentiation of dopaminergic olfactory bulb neurons by calcitonin gene-related peptide. Chang and Ramirez (49) found a dopamine-releasing protein in the rat adrenal gland. However, since these activities were identified in other systems, the relationship of these to THAF is unknown.

We speculate that if THAF is found in the trophoblast cells of the placenta, it may play a role in placental feedback to the hypothalamus/pituitary at midpregnancy. Alternatively, it may influence the development of the tuberoinfundibular dopaminergic neurons in the fetus.

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References

1. Ben-Jonathan N 1985 Dopamine: a prolactin-inhibiting hormone. *Endocr Rev* 6:564–589
2. Moore KE 1987 Interactions between prolactin and dopaminergic neurons. *Biol Reprod* 36:47–58
3. Soares MJ, Faria TN, Roby KF, Deb S 1991 Pregnancy and the prolactin family of hormones: coordination of anterior pituitary, uterine, and placental expression. *Endocr Rev* 12:402–423
4. Tonkowicz P, Robertson M, Voogt J 1983 Secretion of rat placental lactogen by the fetal placenta and its inhibitory effect on prolactin surges. *Biol Reprod* 28:707–716
5. Demarest KT, Moore KE, Riegler GD 1983 Role of a uterine-placental factor in the cessation of the semicircadian rhythm of tuberoinfundibular dopaminergic neuronal activity at midpregnancy in the rat. *Neuroendocrinology* 36:401–414
6. Yogev L, Terkel J 1978 The temporal relationship between implantation and termination of nocturnal prolactin surges in pregnant lactating rats. *Endocrinology* 102:160–165
7. Arbogast LA, Voogt JL 1991 Mechanisms of tyrosine hydroxylase regulation during pregnancy: evidence for protein dephosphorylation during the prolactin surges. *Endocrinology* 129:2575–2582
8. Robertson MC, Gillespie B, Friesen HG 1982 Characterization of the two forms of rat placental lactogen (rPL): rPL-I and rPL-II. *Endocrinology* 111:1862–1866
9. Arbogast LA, Voogt JL 1991 Hyperprolactinemia increases and hypoprolactinemia decreases tyrosine hydroxylase messenger ribonucleic acid levels in the arcuate nuclei, but not the substantia nigra or zona incerta. *Endocrinology* 128:997–1005
10. Demarest KT, Riegler GD, Moore KE 1985 Hypoprolactinemia induced by hypophysectomy and long-term bromocriptine treatment decreases tuberoinfundibular dopaminergic neuronal activity and the responsiveness of these neurons to prolactin. *Neuroendocrinology* 40:369–376
11. Shyr SW, Crowley WR, Grosvenor CE 1986 Effect of neonatal prolactin deficiency on prepubertal tuberoinfundibular and tubero-

- hypophyseal dopaminergic neuronal activity. *Endocrinology* 119:1217-1221
12. **Morgan WW, Besch KC** 1990 Effect of prolactin replacement on the number of tyrosine hydroxylase expressing neurons in the arcuate nuclei of Ames dwarf and normal mice. *Neuroendocrinology* 52:70-74
 13. **Deb S, Youngblood T, Rawitch A, Soares MJ** 1989 Placental prolactin-like protein-A: identification and characterization of two major glycoprotein species with antipeptide antibodies. *J Biol Chem* 264:14348-14353
 14. **Coulon JF, Faucon Biguet N, Cavoy A, Delacour J, Mallet J, David JC** 1990 Gene expression of tyrosine hydroxylase in the developing fetal brain. *J Neurochem* 55:1412-1417
 15. **Arbogast LA, Voogt JL** 1991 Ontogeny of tyrosine hydroxylase mRNA signal levels in central dopaminergic neurons: development of a gender difference in the arcuate nuclei. *Dev Brain Res* 63:151-161
 16. **Specht LA, Pickel VM, Joh TH, Reis DJ** 1981 Light-microscopic immunocytochemical localization of tyrosine hydroxylase in prenatal rat brain. I. Early ontogeny. *J Comp Neurol* 199:233-253
 17. **Ugrumov MV, Taxi J, Tixier-Vidal A, Thibault J, Mitskevich MS** 1989 Ontogenesis of tyrosine hydroxylase-immunopositive structures in the rat hypothalamus. An atlas of neuronal cell bodies. *Neuroscience* 29:135-156
 18. **Faria TN, Deb S, Kwok SCM, Vandeputte M, Talamantes F, Soares MJ** 1990 Transplantable rat choriocarcinoma cells express placental lactogen: identification of placental lactogen-I immunoreactive protein and messenger ribonucleic acid. *Endocrinology* 127:3131-3137
 19. **Faria TN, Soares MJ** 1991 Trophoblast cell differentiation: establishment, characterization and modulation of a rat trophoblast cell line expressing members of the placental prolactin family. *Endocrinology* 129:2895-2906
 20. **Tomogane H, Mistry AM, Voogt JL** 1992 Late pregnancy and rat choriocarcinoma cells inhibit nocturnal prolactin surges and serotonin-induced prolactin release. *Endocrinology* 130:23-28
 21. **Arbogast LA, Soares MJ, Tomogane H, Voogt JL** 1992 A trophoblast-specific factor(s) suppresses circulating prolactin levels and increases tyrosine hydroxylase in tuberoinfundibular dopaminergic neurons. *Endocrinology* 131:105-113
 22. **Vidal G, Mathiasen JR, Voogt JL** 1991 Prolactin implants in the hypothalamus inhibit prolactin surges during pregnancy and alter prolactin release in response to dopamine receptor blockade. *J Neuroendocrinol* 3:249-252
 23. **Ahmed Z, Walker PS, Fellows RE** 1983 Properties of neurons from dissociated fetal rat brain in serum-free culture. *J Neurosci* 3:2448-2462
 24. **Porter JC, Kedzierski W, Aguila-Mansilla N, Jorquera BA** 1990 Expression of tyrosine hydroxylase in cultured brain cells: stimulation with an extractable pituitary cytotropic factor. *Endocrinology* 126:2474-2481
 25. **Porter JC, Aguila-Mansilla N, Ramin SM, Kozlowski GP, Kedzierski W** 1991 Tyrosine hydroxylase expression in hypothalamic cells: analysis of the roles of adenosine 3',5'-monophosphate- and Ca²⁺/calmodulin-dependent protein kinases in the action of pituitary cytotropic factor. *Endocrinology* 129:2477-2485
 26. **Soares MJ, Schaberg KD, Pinal CS, De SK, Bhatia P, Andrews GK** 1987 Establishment of a rat placental cell line expressing characteristics of extraembryonic membranes. *Dev Biol* 124:134-144
 27. **Judd AM, Login IS, Kovacs K, Ross PC, Spangelo BL, Jarvis WD, MacLeod RM** 1988 Characterization of the MMQ cell, a prolactin-secreting clonal cell line that is responsive to dopamine. *Endocrinology* 123:2341-2350
 28. **Robertson MC, Croze F, Schroedter IC, Friesen HG** 1990 Molecular cloning and expression of rat placental lactogen-I complementary deoxyribonucleic acid. *Endocrinology* 127:702-710
 29. **Robertson MC, Cosby H, Cattin P, Friesen HG** Characterization of recombinant rat placental lactogen-I. 73rd Annual Meeting of The Endocrine Society, Washington DC, 1991 (Abstract 109)
 30. **Hirs CHW** 1967 Performic acid oxidation. *Methods Enzymol* 11:197-199
 31. **Zar JH** 1984 *Biostatistical Analysis*, ed 2. Prentice-Hall, Englewood Cliffs
 32. **Gerald GB** 1990 Common multiple comparison procedures. *Nurse Anesthesia* 1:162-165
 33. **Nicholson G, Greeley GH, Humm J, Youngblood WW, Kizer JS** 1980 Prolactin in cerebrospinal fluid: a probable site of prolactin autoregulation. *Brain Res* 190:447-457
 34. **Demarest KT, Riegler GD, Moore KE** 1984 Prolactin-induced activation of tuberoinfundibular dopaminergic neurons: evidence for both a rapid 'tonic' and a delayed 'induction' component. *Neuroendocrinology* 38:467-475
 35. **Björklund A, Moore RY, Nobin A, Stenevi U** 1973 The organization of tuber-hypophyseal and reticulo-infundibular catecholamine neuron systems in the rat brain. *Brain Res* 51:171-191
 36. **Gonzalez HA, Kedzierski W, Aguila-Mansilla N, Porter JC** 1989 Hormonal control of tyrosine hydroxylase in the median eminence: demonstration of a central role for the pituitary gland. *Endocrinology* 124:2122-2127
 37. **Smyth DG** 1967 Techniques in enzymic hydrolysis. *Methods Enzymol* 11:214-231
 38. **Arimura A, Somogyvári-Vigh A, Miyata A, Mizuno K, Coy DH, Kitada C** 1991 Tissue Distribution of PACAP as determined by RIA: highly abundant in the rat brain and testes. *Endocrinology* 129:2787-2789
 39. **Miyata A, Arimura A, Dahl RR, Minamino N, Uehara A, Jiang L, Culler MD, Coy DH** 1989 Isolation of a novel 38 residue-hypothalamic polypeptide which stimulates adenylate cyclase in pituitary cells. *Biochem Biophys Res Commun* 164:567-574
 40. **Waymire JC, Hemelt V, Marshak D** Evidence that pituitary adenylate cyclase activator peptide (PACAP) is a presynaptic neurotransmitter in the bovine adrenal medulla. 22nd Annual Meeting of the Society for Neuroscience, Anaheim, CA, 1992, p 990 (Abstract)
 41. **Alper RH, Demarest KT, Moore KE** 1980 Morphine differentially alter synthesis and turnover in dopamine in central neuronal systems. *J Neural Transm* 48:157-165
 42. **Reymond MJ, Kaur C, Porter JC** 1983 An inhibitory role for morphine on the release of dopamine into hypophysial portal blood and on the synthesis of dopamine in tuberoinfundibular neurons. *Brain Res* 262:253-258
 43. **Deyo SN, Swift RM, Miller RJ** 1979 Morphine and endorphins modulate dopamine turnover in rat median eminence. *Proc Natl Acad Sci USA* 76:3006-3009
 44. **Ferland L, Fuxe K, Eneroth P, Gustafsson J-A, Skett P** 1977 Effects of methionine-enkephalin on prolactin release and catecholamine levels and turnover in the median eminence. *Eur J Pharmacol* 43:89-90
 45. **Van Loon GR, Ho D, Kim C** 1980 β -Endorphin-induced decrease in hypothalamic dopamine turnover. *Endocrinology* 106:76-80
 46. **Bero LA, Lurie SN, Kuhn CM** 1987 Early ontogeny of κ -opioid receptor regulation of prolactin secretion in the rat. *Dev Brain Res* 37:189-196
 47. **Iacovitti L, Evinger MJ, Joh TH, Reis DJ** 1989 A muscle-derived factor(s) induces expression of catecholamine phenotype in neurons of cultured rat cerebral cortex. *J Neurosci* 9:3529-3537
 48. **Denis-Donini S** 1989 Expression of dopaminergic phenotypes in the mouse olfactory bulb induced by the calcitonin gene-related peptide. *Nature* 339:701-703
 49. **Chang GD, Ramirez VD** 1988 A potent dopamine-releasing factor is present in high concentrations in the rat adrenal gland. *Brain Res* 463:385-389