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Inhibition of gonadotropin hormone-releasing hormone release by prolactin from GT1 neuronal cell lines through prolactin receptors

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ABSTRACT High levels of prolactin (PRL) are associated with inhibition of luteinizing hormone secretion in several mammalian species. We asked whether this phenomenon could be explained by a direct inhibitory action of PRL on hypothalamic gonadotropin hormone-releasing hormone (GnRH) neurons. The ability of PRL to suppress GnRH release and expression was tested in the highly differentiated GT1 GnRH cell lines. In static culture, nanomolar concentrations of either rat or mouse PRL inhibited the release of GnRH in a dose-dependent fashion. PRL treatment for 24 hr also decreased GnRH mRNA levels determined by Northern analysis. The cells were shown to express the PRL receptor gene, and the mRNAs for both the short and long forms were present by Northern and PCR analysis, although the short form was more abundant. In Western blots with monoclonal antibody against the rat liver PRL receptor, the short 42-kDa form of the receptor was observed. These results demonstrate that PRL inhibits GnRH release and possibly gene expression in GnRH neurons. This action appears to be mediated through prolactin receptors expressed by the cells.

During normal physiological states associated with elevated prolactin (PRL), luteinizing hormone (LH) levels are lowered—e.g., during pregnancy, pseudopregnancy, postpartum, and lactation (1, 2). In women elevated PRL levels resulting from an anterior pituitary tumor or the therapeutic use of dopamine antagonists result in amenorrhea (1, 2). Hyperprolactinemia inhibits the postcastrational rise in LH levels in a dose-dependent fashion in male rats (3). One hypothesis to explain these findings is that elevated PRL levels act centrally to inhibit gonadotropin hormone-releasing hormone (GnRH) release, which in turn results in lowered LH levels.

We used GT1 GnRH neuronal cell lines to test the hypothesis that PRL acts directly on GnRH neurons to modulate GnRH release. The GT1 cell lines were isolated from a GnRH-secreting tumor induced in a transgenic mouse by genetically targeted tumorigenesis (4). GT1 cells appear to be highly differentiated neurosecretory neurons. They have a neuronal phenotype, express neuronal but not glial markers, and have functional fast, tetrodotoxin-sensitive sodium channels (4–6). In addition, they express and process the GnRH gene (6) and release GnRH in response to depolarization (4). Basal release of GnRH from GT1 cells is pulsatile, with a pulse frequency identical to that seen in the mouse (7–9). The cells also have cilia, which is a unique characteristic of hypothalamic GnRH neurons (10).

We demonstrate that PRL at physiological concentrations inhibits GnRH release and mRNA levels. Consistent with this observation, the cells express PRL receptors as shown by Northern, PCR, and Western analysis.

MATERIALS AND METHODS

Two subclones of GT1 cells, GT1-1 and GT1-7, were used in these experiments. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 5% fetal calf serum, 5% horse serum, and penicillin/streptomycin, in an atmosphere of 5% CO₂ in air at 37°C.

Secretion Experiments. Cells were plated on 24-well plates (250,000 cells per well) and, when 50–70% confluent, medium was replaced by defined medium without serum (Opti-MEM, GIBCO). After 1 day, medium was replaced with medium containing various concentrations of mouse or rat PRL (National Institutes of Health) and the incubation was continued for 60 min. The medium was collected, boiled for 5 min to prevent degradation of GnRH by endopeptidases, and stored on –20°C. GnRH concentration in the medium was determined by a radioimmunoassay specific for GnRH using the rabbit polyclonal antibody R1245, provided by T. Nett (Colorado State University; ref. 11).

GnRH mRNA Experiments. Cells were treated in the same way as for secretion experiments, except that they were cultured on 100-mm dishes (5 × 10⁶ cells per dish). Incubation with PRL was continued for 1, 2, 3, 4, 6, or 24 hr.

RNA Extraction. Total RNA was extracted from cells cultured on 100-mm dishes using the procedure of Chomczynski and Sacchi (12). Briefly, cells were lysed in guanidinium solution (4 M guanidinium thiocyanate/25 mM sodium citrate, pH 7/0.5% sarcosyl/0.1 M 2-mercaptoethanol), the lysate was phenol/chloroform extracted, and RNA was precipitated in ethanol. Total tissue RNA was extracted by the urea/lithium chloride method (13). All RNAs were dissolved in diethyl pyrocarbonate-treated water and the integrity of RNA was checked on agarose gels. Poly(A)⁺ RNA was purified directly from cell lysates using oligo(dT) affinity resin (14).

Analysis of RNA. RNA samples for dot blots were applied directly onto nitrocellulose membranes and hybridized using Quickhyb solution (Stratagene). For Northern analysis, RNA samples were electrophoresed in 1% or 1.5% agarose (Pharmacia) gels containing 2.2 M formaldehyde, 20 mM 3-(*N*-morpholino)propanesulfonic acid (Mops, Fisher), 8 mM sodium acetate, and 1 mM EDTA. RNA was transferred onto nylon membranes (N-Hybond, Amersham) and covalently linked by irradiation with 120 mJ of UV light. Hybridization with GnRH and cyclophilin probes was at high stringency (Quickhyb solution) and conditions for hybridization with F3 PRL receptor probe were 20% formamide at 42°C. Quantification of mRNA levels was performed by densitometric scanning of autoradiograms using cyclophilin as an internal control.

cDNA Probes. The cDNA probe for GnRH was a 340-bp *Hind*III/*Bam*HI fragment of SP65 plasmid (15). The probe for cyclophilin was a 700-bp-long *Bam*HI insert of plasmid p1B15 (16). The PRL receptor probe was a 1.6-kb *Eco*RI insert of the

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Abbreviations: GnRH, gonadotropin hormone-releasing hormone; PRL, prolactin; LH, luteinizing hormone.

F3 clone containing the entire open reading frame of the rat PRL receptor plus 5' and 3' nucleotides (17). This probe is 93% identical with the mouse receptor (18). cDNA probes were labeled with [³²P]dCTP (NEN) by random priming (Oligolabeling Kit, Pharmacia) and purified on Quick Spin columns (Boehringer Mannheim).

Reverse Transcriptase-Polymerase Chain Reaction Analysis of the PRL Receptor mRNA. Ten micrograms of total RNA was used for cDNA synthesis with 100 units of mouse liver reverse transcriptase (BRL) primed with 0.1 μg of oligo(dT) primer in 20 μl of 50 mM KCl/20 mM Tris·HCl, pH 8.4/1.5 mM MgCl₂/1 mM (each) dNTP/20 units of RNasin. The reaction mixture was incubated for 1 hr at 37°C. One-fourth of the first strand synthesis reaction mixture was amplified for 30 cycles in 50 mM KCl/20 mM Tris·HCl, pH 8.4/200 μM dNTPs/1.5 mM MgCl₂/50 pmol of each forward and reverse primer/2.5 units of Taq polymerase (Boehringer Mannheim). The amplification conditions were as follows: denaturation at 94°C for 1 min, annealing at 65°C for 2 min, and extension at 72°C for 2 min. After amplification the samples were separated on a 3% agarose gel stained with ethidium bromide. Two different amplification reactions were carried out. In both of them the forward primer was 5'-CATGGATACTG-GAGTAGATGGAGC-3' (A). Reverse primers were 5'-CC-CTCAAAGCCACTGCCCAGAC-3' (B), common for both forms of the receptor, and 5'-CTCAGCAGCTCTCA-GACTTG-3' (C), specific for the long form of the receptor. Location of the primers is shown in Fig. 4. A negative control was included in each amplification reaction to control for possible cross-contamination.

Membrane Preparation. Cells grown on 100-mm dishes (1.5–2 × 10⁷ cells) were washed twice with phosphate-buffered saline, then scraped and resuspended in 25 mM Tris·HCl, pH 7.6/10 μM leupeptin/1 mM phenylmethylsulfonyl fluoride, and lysed in Eppendorf tubes by three freeze-thaw cycles. The lysed cells were microcentrifuged for 10 min, and the pellet was resuspended in 25 mM Tris·HCl, pH 7.5/10 mM MgCl₂/10 μM leupeptin/1 mM phenylmethylsulfonyl fluoride (19). Microsomal membrane fractions from rat and mouse liver were prepared as described (20). Protein concentration was measured by the Bradford method using bovine serum albumin as the standard (21).

Western Analysis. Twenty-five micrograms of the microsome fraction was boiled for 4 min under reducing conditions in 50 mM Tris·HCl (pH 6.8) containing 10% glycerol and 2% SDS and subjected to SDS/polyacrylamide gel electrophoresis (22). Proteins were electrophoretically transferred onto poly(vinylidene difluoride) membranes (Immobilon-P transfer membrane, Millipore) at 40 V, at 4°C overnight. After saturation of nonspecific protein binding sites with 3% bovine serum albumin/0.1% Nonidet P-40 for 2 hr, blots were incubated with the anti-PRL receptor monoclonal antibodies [U5 or U6 (23); dilution, 1:1000]. The bands were revealed by the horseradish peroxidase-coupled anti-mouse antiserum (dilution, 1:4000) and enhanced chemiluminescence Western blotting kit (ECL Western blotting, Amersham).

Data Analysis. Data were analyzed by analysis of variance followed by Student *t* test.

RESULTS

Incubation of GT1 cells with PRL induced a significant inhibition of GnRH release into the medium. As shown in Fig. 1, either mouse PRL (Fig. 1a) or rat PRL (Fig. 1b) decreased the concentration of GnRH released into the medium from either GT1-1 or GT-17 cells during the 60 min of incubation. Maximum inhibition of ≈40% of basal release was achieved with nanomolar concentrations of PRL. Incubation with rat

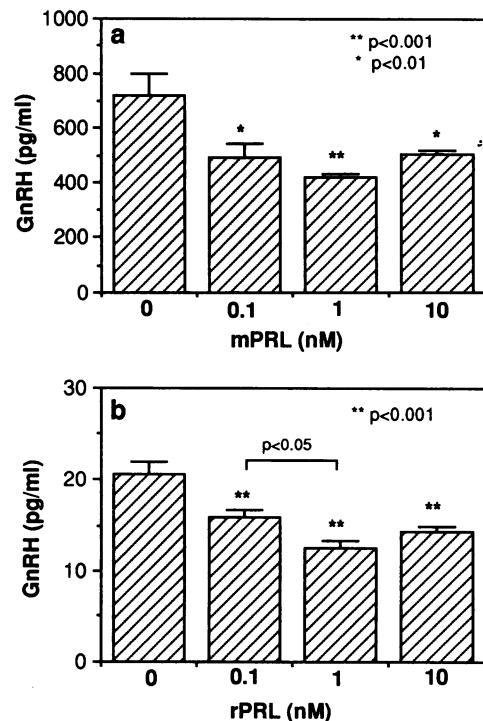


FIG. 1. Dose-dependent inhibition of GnRH release from GT1 cells by PRL (mPRL, mouse PRL; rPRL, rat PRL). Samples were collected after 60 min of incubation of GT1-1 cells with increasing concentrations of mouse PRL (a) or GT1-7 cells with rat PRL (b). Concentration of GnRH (pg/ml) of replicate samples (a, *n* = 6; b, *n* = 12) from each experiment was measured in the same assay, and the results are expressed as mean + SEM.

growth hormone (10 pM–10 nM) in similar experiments had no effect on the secretion of GnRH (data not shown).

Levels of GnRH mRNA transcripts were measured by Northern analysis in cells incubated in PRL for various times. The GnRH probe hybridized to a single band of 510 bp, whereas the cyclophilin probe hybridized to a 900-bp band. We did not observe any change in the level of GnRH mRNA after 1, 2, 3, 4, or 6 hr of incubation with PRL (data not shown). However, prolonged incubation for 24 hr resulted in a decrease in the level of GnRH mRNA, as seen by Northern analysis (Fig. 2a). In experiments in which mRNA levels were measured by dot blots, a 40% decrease (*P* < 0.05) was observed with 10 nM PRL (Fig. 2b).

Hybridization of poly(A)⁺ RNA (30 μg) from GT1-7 cells with the F3 PRL receptor probe, which recognizes short and long forms of the receptor, detected a band at 2.1 kb, similar to that observed for pregnant rat liver RNA (1.8 kb, Fig. 3a).

PCR analysis of reversed transcribed mRNA from GT1-7 cells yielded fragments of 150 bp using primers A and B and 250 bp using primers A and C (Fig. 4). The 150-bp fragment is consistent with the short form of the receptor and was the predominant form seen in the GT1-7 and liver cDNAs. The 250-bp fragment consistent with the long form was detectable in GT1-7 cells and was the predominant form observed in the ovary. These results are semiquantitative since internal standards were not run to control for efficiency of reverse transcription or amplification.

Western analysis of the crude membrane preparations of GT1-7 cells using the U5 monoclonal antibody to rat liver PRL receptor stained a doublet band at 42 kDa. This size corresponds to the expressed posttranslationally modified (glycosylated) short form of the receptor, since the molecular mass of the mature receptor predicted from its sequence is ≈33 kDa (24). This band along with a minor 80-kDa band, indicative of the long form of the receptor, was present in mouse and rat

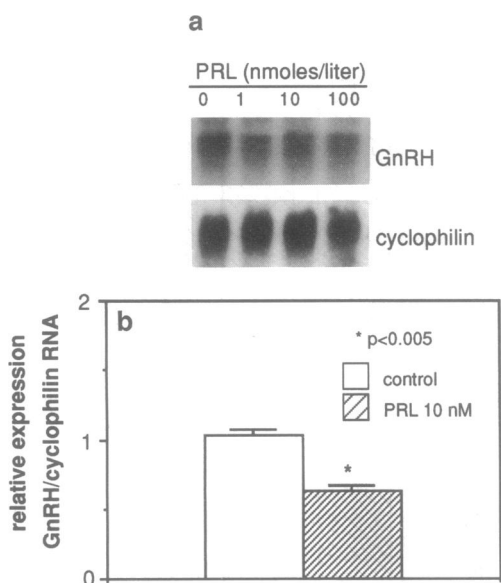


FIG. 2. Decrease of the levels of GnRH mRNA after 24 hr of incubation of GT1 cells with rat PRL. (a) Northern blot of 10 μ g of total RNA from cells incubated with increasing concentrations of PRL (1, 10, and 100 nM) hybridized sequentially with cDNA probes for GnRH and cyclophilin. (b) Histogram from a separate experiment showing the results of scanning densitometry of a dot blot of RNA isolated from cells incubated for 24 hr with 10 nM PRL. Triplicate samples of 1 μ g of total RNA were applied on the blot. The blot was sequentially hybridized with cDNA probes for GnRH and cyclophilin. Density of the GnRH band is expressed relative to that of the cyclophilin band.

liver preparations. Several larger bands were observed with GT1-7 membranes, one of which was 80 kDa. As a control, incubation with monoclonal antibody (U6), which crossreacts with mouse liver PRL receptor with very low affinity, did not stain any band in blots of GT1-7 cell membranes.

DISCUSSION

All of our data support the hypothesis that the reciprocal relationship between LH and PRL levels is mediated through

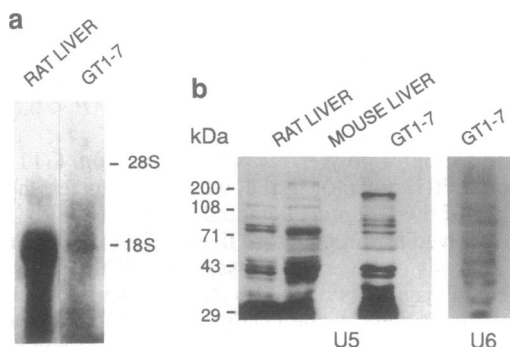


FIG. 3. mRNA and protein for PRL receptor(s) are present in GT1-7 cells. (a) Northern blot of 50 μ g of pregnant rat liver RNA and 30 μ g of GT1-7 cell poly(A)⁺ RNA. The blot was hybridized with F3 PRL receptor probe under low stringency conditions (20% formamide, 42°C overnight). The major transcript for rat liver was 1.8 kb and a minor 2.5-kb transcript was observed, whereas GT1-7 cells showed a transcript at 2.1 kb. Sizes of transcripts were determined by comparison with the RNA size markers and the position of rRNAs. (b) Western blot of crude membrane preparation of GT1 cells and rat and mouse liver. Proteins (25 μ g) were separated on 10% acrylamide gel, electrotransferred onto Immobilon membrane, and hybridized with U5 or U6 monoclonal antibody (dilution, 1:1000). Bands were visualized using horseradish peroxidase-coupled second antibody and an enhanced chemiluminescence Western blotting kit (Amersham).

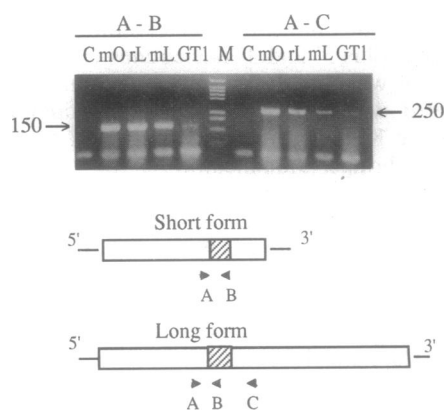


FIG. 4. Short (150 bp) and long (250 bp) forms of the PRL receptor mRNA are expressed in GT1-7 cells. Total RNA from GT1-7 cells, rat liver (rL), mouse liver (mL), and mouse ovary (mO) was reverse transcribed using an oligo(dT) primer and amplified using primers A, B, and C. The relative positions of the primers for the two forms of the receptor are shown by arrowheads. The hatched areas represent the transmembrane domains. Amplified fragments were separated on a 3% agarose gel stained with ethidium bromide. The relative positions for the 150- and 250-bp fragments are labeled.

a direct action on GnRH neurons. Incubation of GT1 GnRH cell lines with physiological levels of PRL suppress the basal release of GnRH. The inhibition of release, although statistically significant, was modest, with a maximal response of 40%. These findings are in close agreement with recent observation that PRL suppresses GnRH release from cultured hypothalamic fragments (25). The maximal response observed in these experiments was 35% inhibition. This effect was specific for PRL in that rat growth hormone had no effect on GnRH secretion.

Inhibitory effects of PRL on GnRH release could be mediated in part by the inhibition of transcription of the GnRH gene. Incubation of GT1 cells with PRL for 24 hr decreased the level of GnRH mRNA by $\approx 40\%$. Further studies are necessary to determine if the decrease in mRNA levels is due to the decrease in transcription or stability of the message. A similar effect on the inhibition of GnRH mRNA levels was detected by *in situ* hybridization in rats bearing a PRL-secreting tumor (26).

Consistent with PRL directly affecting GnRH release and mRNA levels, PRL receptors are expressed on GT1 cells. The mRNA for the short form of the receptor was detected by Northern and PCR analysis, whereas that for the long form was only observed by PCR analysis. The short and possibly long forms of the receptor could also be observed by Western analysis using an antibody that recognizes both forms of the receptor. These findings are consistent with the short form of the receptor being expressed at higher levels. In agreement with these observations, PRL receptors (27, 28) and appropriate mRNAs (29) were shown to be present in the brain. Recently, by PCR analysis, the short and long forms of the PRL receptor were identified in the anterior hypothalamus of the rat, the region in which GnRH cell bodies are located (29). However, it was not possible to determine which cell type expressed the receptor.

The two forms of the PRL receptor, short (17) and long (30), differ in the sequence and length of their cytoplasmic domain. Transcripts coding these two forms are the result of alternative splicing of a common primary transcript (24). The expression of the two forms is tissue specific, with the short form being predominant in the liver and the long form being most abundant in the ovary (30). In the mouse, seven transcripts encoding at least two forms of the PRL receptor with tissue-specific expression and regulation were recently described (31). Potentially the two forms of the PRL receptor

mediate different actions of PRL. In Chinese hamster ovary cells transfected with a reporter gene whose expression is induced by PRL, expression of the long but not the short form of the receptor was capable of transactivating the action of PRL (32). The role of the short form is unknown, although in the liver and in the mammary gland it is the predominant form of the receptor (25). Which form of the receptor is mediating the action of PRL in GT1 cells is not clear at this time. This question is addressable by stably transfecting the GT1 cells with various forms of the PRL receptor and observing the effects on GnRH secretion and mRNA levels.

In conclusion, we have shown that the GT1 GnRH neuronal cell lines respond to the inhibitory action of PRL. By multiple criteria the cells express PRL receptors that have been shown to mediate the action of PRL in other tissues. These observations could help to explain the consistent observation in mammals that high levels of PRL result in the inhibition of reproductive function. Whether PRL secreted from the anterior pituitary or produced in neurons in the brain (33–35) is responsible for the effect on GnRH remains to be elucidated. Effects on GnRH neurons do not eliminate the possibility that PRL could act at additional sites in the brain to inhibit the release of GnRH, possibly through an action on GABAergic (GABA, γ -aminobutyric acid) neurons (36).

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