

Local Regulation of Gonadotroph Function by Pituitary Gonadotropin-Releasing Hormone

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ABSTRACT

Cultured rat pituitary cells and immortalized pituitary gonadotrophs (α T3-1 cells) express specific messenger RNA transcripts for GnRH and exhibit positive immunostaining for the GnRH peptide. Each cell type released GnRH during both static culture and perfusion, albeit in lesser amounts than cultured hypothalamic cells and GT1-7 neurons. In perfused pituitary cells, exposure to a GnRH agonist stimulated the release of GnRH as well as LH. In contrast, treatment with a GnRH receptor antagonist or with GnRH antiserum decreased basal LH release. In pituitary cell cultures, a small proportion of gonadotrophs exhibited high amplitude and low frequency baseline Ca^{2+} oscillations in the absence of GnRH stimulation. Such spontaneous oscillations were comparable to those induced by picomolar concentrations of GnRH and could be abolished by treatment with a GnRH antagonist. These *in vitro* findings indicate that locally produced GnRH causes low level activation of pituitary GnRH receptors, induces spontaneous intracellular Ca^{2+} oscillations, and contributes to basal LH secretion in cultured pituitary cells. *In vivo*, such autocrine or paracrine actions of pituitary-derived GnRH could provide a mechanism for the maintenance of optimal responsiveness of the gonadotrophs to pulses of GnRH arising in the hypothalamus. The presence and actions of GnRH in the anterior pituitary gland, the major site of expression of GnRH receptors, suggest that local regulatory effects of the neuropeptide could supplement the primary hypothalamic mechanism for the control of episodic gonadotropin secretion. (*Endocrinology* **141**: 1187–1195, 2000)

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THE HYPOTHALAMIC GnRH system sends axonal projections to the median eminence, where the pulsatile release of GnRH controls the secretion of gonadotropins from the anterior pituitary gland (1, 2). Temporal and functional links among hypothalamic multiunit electrical activity, GnRH release, and pituitary gonadotropin secretion have been demonstrated (3). The intrinsic pulsatile activity of the GnRH-producing neurons provides the basis for pulsatile GnRH release, and its modulation by peripheral hormones and neurotransmitters leads to the synchronous discharge of gonadotropins from the pituitary gland (4). These and earlier observations have identified the hypothalamus as the major site that drives pulsatile hormone release from the pituitary gland (5).

In addition to the hypothalamic GnRH system, there is evidence for the production of GnRH at other sites in the brain (6) as well as in rat ovarian granulosa (7) and testicular cells (8), the human placenta (9), the immune system (10), and the pituitary gland (11). The presence of GnRH-producing cells in the pituitary gland and other extrahypothalamic tissues suggests that the autocrine actions of locally produced GnRH could have a physiological role in such processes as self-priming, inter- and intracellular signaling, and hormone secretion. Earlier studies have demonstrated the presence of GnRH immunostaining in long-term cultures of dispersed

pituitary cells (11–13). Also, expression of GnRH messenger RNA (mRNA) in pituitary cells (14, 15) and enzymatic processing and release of GnRH from the pituitary gland (16, 17) have been reported. However, the physiological relevance of this locally produced GnRH has not been investigated, and its potential role in the pituitary gland has not been defined.

Materials and Methods

Hypothalamic and pituitary tissue

Hypothalamic tissue was removed from fetuses of 18-day pregnant Sprague Dawley rats. The borders of the excised hypothalami were delineated by the anterior margin of the optic chiasm, the posterior margin of the mammillary bodies, and laterally by the hypothalamic sulci. Pituitary glands were removed from adult female rats at random stages of the estrous cycle, and the posterior lobes were discarded. Hypothalamic and anterior pituitary tissues were placed in ice-cold medium 199 (Life Technologies, Inc., Gaithersburg, MD), pH 7.4, containing 25 mM HEPES and 0.1% BSA (Sigma, St. Louis, MO).

Primary culture of hypothalamic and pituitary cells

Fetal rat hypothalamic tissue was dispersed into single cells by controlled enzyme treatment (18), yielding about 2×10^6 cells/hypothalamus. Anterior pituitary tissue was also subjected to enzymatic dispersion as described previously (19). Hypothalamic cells (1.5×10^7) or pituitary cells (2×10^7) were incubated for 30 min in a small volume (400 μ l) with preswollen Cytodex-2 beads (Pharmacia, Piscataway, NJ) to allow attachment of cells, then additional culture medium was added. The culture medium [DMEM/Ham's F-12 medium (1:1 vol/vol) with L-glutamate, high glucose (4.5 mg/ml), and 10% heat-inactivated FBS; Life Technologies, Inc.] was changed every 2–3 days.

Immortalized GnRH neurons and pituitary gonadotrophs

GT1-7 GnRH neuronal cells (provided by Dr. Richard Weiner, University of California, San Francisco, CA) and the α T3-1 pituitary gona-

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dotroph cell line (provided by Dr. Pamela Mellon, University of California, San Diego, CA) were grown in the same culture medium employed for primary cultures. When cells reached confluence, they were dispersed by trypsinization, washed twice in culture medium, and cultured on Cytodex-2 beads for perfusion studies or in six-well plates for other studies. The culture medium was changed at 48-h intervals.

Cell perfusion procedure

Before each perfusion, the cell-bead mixture was collected by sedimentation, resuspended in DMEM/F12 (1:1) containing 0.1% BSA and 20 μM bacitracin (Life Technologies, Inc.), and gassed with 95% O_2 -5% CO_2 . After placement in multiple microchamber modules (Cellex Biosciences, Inc., Minneapolis, MN) with an internal volume of 0.5 ml, the bead-attached cells were perfused with medium at a flow rate of 0.15 ml/min at 37 C. Fractions were collected at 5-min intervals and stored at -20 C before RIA. GnRH was measured using [^{125}I]GnRH (Amersham Pharmacia Biotech, Chicago, IL), GnRH standards (Peninsula Laboratories, Inc., Belmont, CA), and primary antibody (donated by Dr. V. D. Ramirez, University of Illinois, Urbana, IL). The intra- and interassay coefficients of variation at 80% binding in standard samples (15 pg/ml) were 12% and 14%, respectively. The sensitivity of the assay, defined as twice the SD at the zero dose, was 0.2 pg/tube. There was no detectable cross-reactivity of the GnRH antibody with the GnRH agonist and antagonist analogs employed in this study (18, 19). LH concentrations in perfusion medium were measured by double antibody RIA using kits obtained from the NIDDK, and results were expressed as nanograms per ml rat LH RP-3 standard. The sensitivity of the assay was 2.5 ng/ml, and the intra- and interassay coefficients of variation were 5% and 8%, respectively (n = 6) (20).

Static cell culture

Hypothalamic or pituitary cells (2×10^6) were cultured in six-well plates and maintained for 3–4 days for measurements of hormone release. Immortalized GT1-7 or $\alpha\text{T}3$ -1 cells (10^6) were cultured in six-well plates, and GnRH and LH release was measured after 48 h of incubation. Hormone release was measured after 1-h incubation at 37 C in DMEM/F12 medium containing 1% BSA and 20 μM bacitracin, pH 7.4. During long term culture of pituitary cells (2×10^6 /well) for 20 days, sampling was performed by removing 1 ml medium and replacing an equal volume of fresh medium in a total volume of 2 ml. Hormone levels were measured at 24-h intervals, and at each time point the released hormone was expressed as the cumulative production.

Intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) measurements

For analysis of $[\text{Ca}^{2+}]_i$, pituitary cells were plated on 25-mm coverslips coated with poly-L-lysine and cultured for 48 h. The coverslips were then washed twice, loaded with 2 μM indo-1/AM (Molecular Probes, Inc., Eugene, OR) for 60 min at 37 C, and mounted on the stage of an inverted Diaphot microscope attached to a dual emission photometry system (Nikon, Garden City, NY). Cells were excited with a 360-nm light beam, and the emission was measured at 405 and 485 nm. After the background subtraction, the ratio of these two intensities was further converted to $[\text{Ca}^{2+}]_i$ using the standard curve constructed by the addition of known concentrations of Ca^{2+} to 10 μM indo-1. Data collection, aided by the FASTINCA program (University of Cincinnati Medical Center, Cincinnati, OH), was performed in 360-msec intervals.

In addition to photometry, Ca^{2+} imaging was also performed where indicated. Coverslips with cells were loaded with 2 μM fura-2/AM (Molecular Probes, Inc.) for 60 min at 37 C, washed, and mounted on a stage of Axiovert 135 microscope (Carl Zeiss, Oberkochen, Germany) attached to the Attofluor Digital Fluorescence Microscopy System (Atto Instruments, Rockville, MD). Cells were illuminated alternatively by 340- and 380-nm beams, and the emission was observed at 505 nm. Changes in $[\text{Ca}^{2+}]_i$ are presented as the ratio of these two fluorescence intensities (F_{340}/F_{380}), and the estimate of the actual concentration was obtained using the Grynkiewicz equation (21). Data were obtained from two to seven gonadotrophs simultaneously at a sampling rate of one point per sec.

RNA isolation and RT-PCR

Total RNA was isolated from $\alpha\text{T}3$ cells, cultured pituitary cells, GT1-7 cells, and fetal hypothalamic cells by the method of Chomczynski and Sacchi (22) and checked for purity. RNA samples were subjected to RT-PCR to determine their content of GnRH mRNA. Four to 6 μg total RNA from each sample were reverse transcribed into complementary DNA in a 20- μl reaction mixture containing an oligo(deoxythymidine) primer using SuperScript II RNase H⁻ reverse transcriptase (Life Technologies, Inc.). Aliquots (2.5 μl) of the RT reaction mixture were subjected to PCR amplification for 35 cycles using the ELONGase Amplification System (Life Technologies, Inc.) in a final volume of 50 μl containing 1.5 mM MgCl_2 , 0.2 μM of each primer, and 0.2 mM of each deoxy-NTP. The PCR conditions for each cycle were denaturation at 94 C for 30 sec, annealing at 60 C for 25 sec, and extension at 72 C for 30 sec. Specific primers, based on the mouse pro-GnRH sequence (23), corresponded to amino acids -9 to -3 for the sense primer (5'-ACT-GTGTGTTTGAAGGCTGC-3') and amino acids 57–51 for the antisense

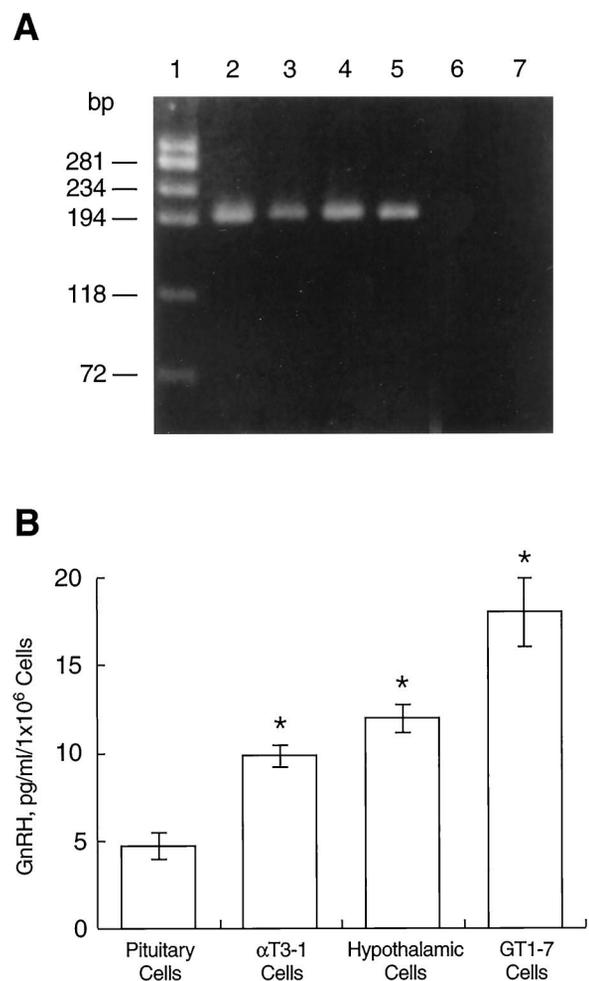


FIG. 1. Production of GnRH by pituitary cells. A, RNA samples isolated from $\alpha\text{T}3$ -1, primary pituitary, GT1-7, and fetal hypothalamic cells (lanes 2–5, respectively) were subjected to RT-PCR using GnRH-specific primers as described in *Materials and Methods*. Shown is an ethidium bromide-stained agarose gel run with the PCR products. Lane 1 contains $\phi\text{X} 174$ DNA/*Hae*III digest markers. Reactions without RT (lane 6) and without RNA sample (lane 7) served as controls. GnRH transcripts of 198 bp, as predicted by the GnRH primers used, were detected in all samples. B, Release of GnRH by normal and immortalized pituitary and hypothalamic cells. Data are the mean \pm SE of six replicates for each cell type. Asterisks indicate significant differences in GnRH release compared to the cultured pituitary cells.

primer (5'-TTCCAGAGCTCCTCGCAGATC-3'). The negative amino acid numbers are within the sequence of the signal peptide (23).

The primer sequences were between the signal peptide and the GnRH-associated peptide, and spanned an intron composed of about 1300 bp. Samples without reverse transcriptase were used as controls for each cell type to exclude the possibility of contamination by genomic DNA, which would yield a 1500-bp segment together with the 198-bp fragment derived from GnRH mRNA transcripts. However, no 1500-bp product was observed under these conditions. Also, a RNA sample extracted from the optic nerve gave no 198-bp DNA product after amplification, and thus served as a negative control. The PCR products were analyzed by agarose gel electrophoresis (4% NuSieve 3:1, FMC BioProducts, Rockland, ME), and the DNA fragments were gel-purified using a QIAEX II Gel Extraction Kit (QIAGEN, Valencia, CA). DNA sequencing was performed using the Thermo Sequence radiolabeled terminator cycle sequencing kit from Amersham Pharmacia Biotech (Arlington Heights, IL).

Immunocytochemistry of cultured pituitary cells and hypothalamic neurons

Immunostaining of LH and GnRH was performed by avidin-biotin-peroxidase and alkaline phosphatase methods. The cultures were washed with 0.01 M PBS, fixed in Bouin's fluid for 30 min, washed, dehydrated, and kept dry at -70°C . For single immunostaining the fixed cultures were rehydrated, treated with 3% H_2O_2 , rinsed, blocked by incubation in 10% normal goat serum in PBS, washed, and incubated overnight at 4°C with rabbit anti-LH serum (1:50,000; NIDDK) or rabbit anti-GnRH serum (1:1,000; provided by Dr. V. D. Ramirez, University of Illinois, Urbana, IL). On day 2, the slides were rinsed and incubated with goat antirabbit IgG-biotin conjugate, followed by avidin-biotin-peroxidase or alkaline phosphatase complex. Specific staining for LH

(blue) was visualized with a Vector blue alkaline phosphatase substrate kit III, and GnRH (brown) was visualized with a diaminobenzidine substrate kit for peroxidase (Vector Laboratories, Inc., Burlingame, CA). Antibody specificity was determined by treating cells with LH or GnRH antiserum preadsorbed with homologous peptides.

For double immunostaining, identified pituitary gonadotrophs were repeatedly washed in PBS, incubated with rabbit IgG for 5 h at room temperature, blocked in 10% normal goat serum, washed, and processed for second immunostaining. There was no formation of positive immunoproductions when the second primary antibodies were preadsorbed with related hormones or omitted, confirming the specificity of the alkaline phosphatase reaction in the double immunostaining.

Data analysis

GnRH, LH, and $\text{LH}\alpha$ pulses were identified, and their parameters were determined by a computer-based algorithm cluster analysis (24). Individual point SDs were calculated using a power function variance model from the experimental duplicates. A 2×2 cluster configuration and a t statistic of 2 for upstroke and downstroke were used to maintain false positive and false negative error rates below 10%. The statistical significance of the pulse parameters and hormone levels was tested by one-way ANOVA. Duncan's multiple range test with critical ranges level of 0.01 was used as a *post-hoc* test.

Results

Expression of GnRH in anterior pituitary cells

RT-PCR analysis of total RNA from anterior pituitary cells, using gene-specific primers based on the mouse pro-GnRH sequence, gave the expected fragment size of 198 bp for

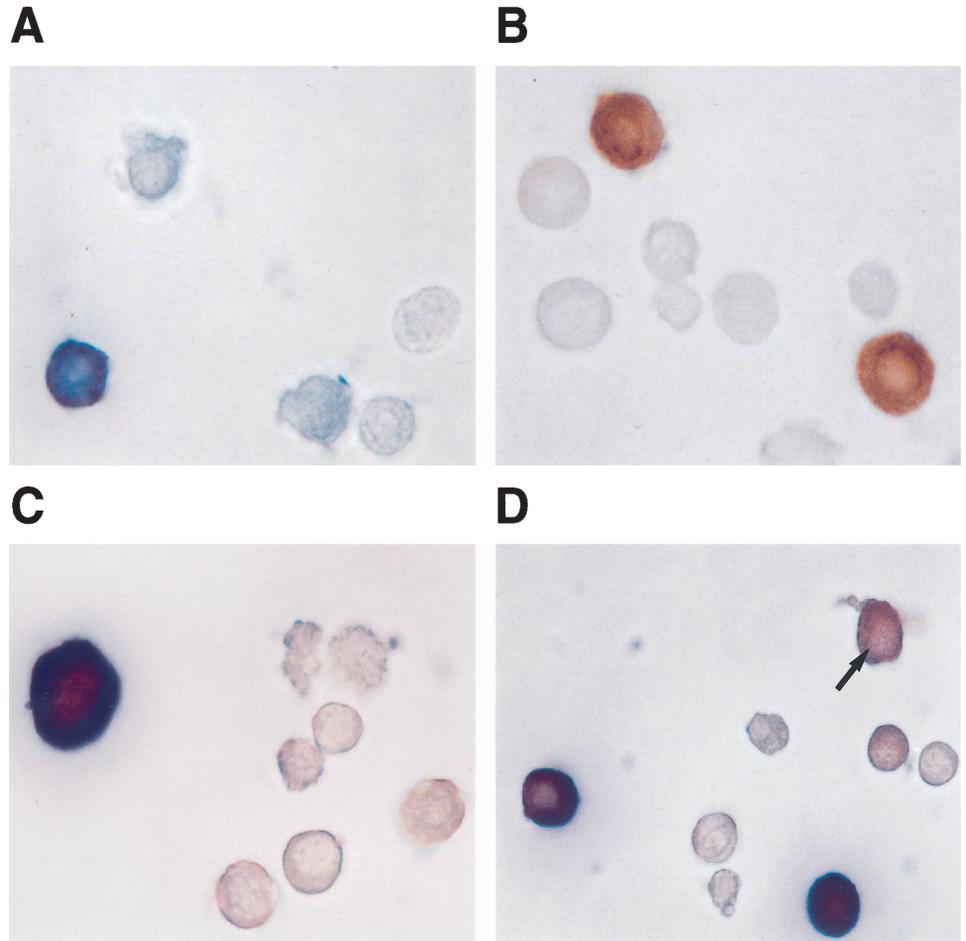


FIG. 2. A and B show positive immunostaining of pituitary cells for LH (blue; $\times 1000$) and GnRH (brown; $\times 1000$) respectively. C and D show double immunostaining for LH (blue) and GnRH (brown) in identified pituitary gonadotrophs. In D, the arrow indicates an unidentified GnRH-positive pituitary cell without LH immunostaining.

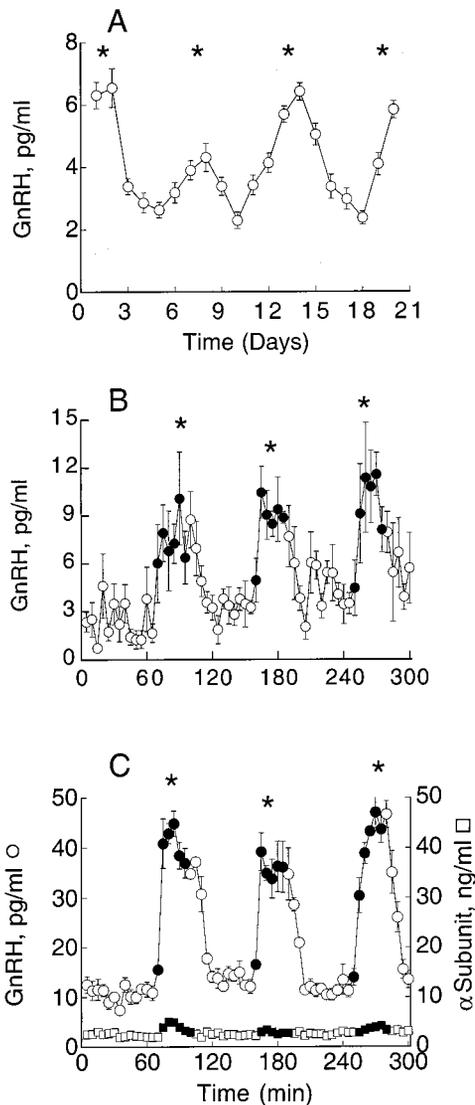


FIG. 3. Basal and GnRH agonist-induced GnRH release. A, GnRH release from cultured pituitary cells. The data are the mean \pm SE of six replicates for each time point. Asterisks indicate significant differences from the lowest level of GnRH over the time course. B, Basal (open circles) and GnRH agonist-induced GnRH release (closed circles) in perfused pituitary cells. Each point is the mean \pm SE of data from four experiments. Asterisks indicate significant ($P > 0.001$) increases in GnRH release. C, Basal and GnRH agonist-induced release of GnRH (open and closed circles) and LH α -subunit (open and closed squares) from perfused α T3-1 cells (mean \pm SE of data from three experiments). Asterisks indicate significant ($P < 0.006$) increases in GnRH release.

GnRH. As shown in Fig. 1A, a single product corresponding to the size predicted by the GnRH primers was observed in both α T3-1 gonadotrophs (lane 2) and cultured anterior pituitary cells (lane 3), similar to that observed in positive control cells (GT1-7 neurons, lane 4; cultured hypothalamic cells, lane 5). No such products were obtained in the absence of reverse transcribed mRNA (lane 6), indicating that the RNA preparation was free of genomic DNA contamination. The DNA sequence analysis of the gel-purified 198-bp fragments from each cell type was identical with the known

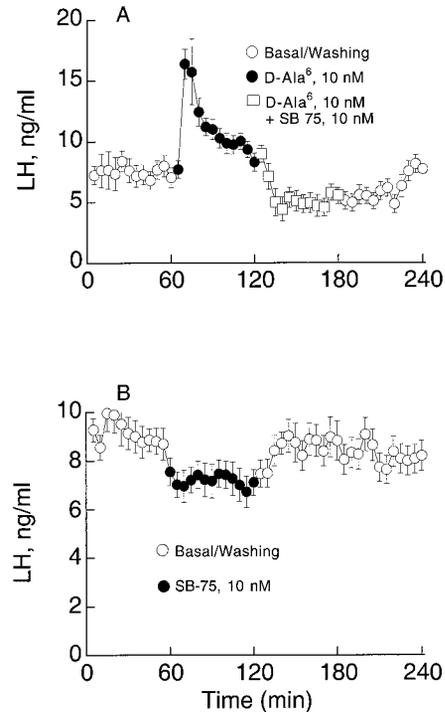


FIG. 4. Effects of GnRH agonist and antagonist analogs on LH release from perfused pituitary cells. A, Basal LH release (open circles) increased ($P < 0.03$; mean \pm SE of data from four experiments) sharply after the addition of 10 nM [D-Ala⁶]Ag (closed circles). Subsequent application of the GnRH receptor antagonist, SB-75 (10 nM; squares) reduced the LH increase to below the basal level ($P < 0.001$; mean \pm SE of data from four experiments). B, Effect of GnRH receptor antagonist (SB-75; 10 nM) on basal LH release.

nucleotide sequence of the GnRH gene (23), thus confirming the authenticity of the amplified DNA fragments. Consistent with the presence of its transcripts, immunoreactive GnRH peptide was released from both cultured pituitary cells and α T3-1 gonadotrophs. The highest amount of GnRH released per 1×10^6 cells during 1-h incubation in DMEM/F12 medium containing 1% BSA and 20 μ g bacitracin at 37 C was in GT1-7 cells, followed by hypothalamic cells, α T3-1 cells, and anterior pituitary cells (by ANOVA, $P < 0.01$; Fig. 1B).

Immunostaining with specific LH antiserum revealed that LH was present in $9 \pm 0.3\%$ of 5772 pituitary cells counted in four experiments. The blue reaction product of the LH immunocytochemical precipitate was distributed throughout the cytoplasm and was absent from the nucleus (Fig. 2A). Single immunostaining with a specific GnRH antiserum revealed that GnRH was present in $11 \pm 0.6\%$ of 4141 pituitary cells counted in three experiments (Fig. 2B). On double immunostaining with both LH and GnRH antisera, 360 of 378 LH-containing cells (95%) also exhibited brown staining for GnRH (Fig. 2, C and D). Also, some GnRH-positive cells were not positively stained for LH, as indicated by the arrow in Fig. 2D. No immunostaining for GnRH (brown) was detectable when the cells were incubated in normal goat serum or with GnRH-preadsorbed primary antibody for GnRH and subsequently treated with the Vector blue alkaline phosphatase.

It is conceivable that hypothalamic GnRH carried in the hypothalamo-hypophyseal portal circulation to the anterior

pituitary gland and internalized after binding to its receptors and stimulating gonadotropin secretion could be the source of the GnRH measured in pituitary extracts and newly cultured pituitary cells. To exclude the contribution of internalized GnRH to the pituitary GnRH pool, primary cultures of pituitary cells were maintained for 20 days to allow ample time for degradation of any internalized peptide and to facilitate the valid measurement of pituitary-derived GnRH. During culture with 50% medium change every day, GnRH measurements revealed fluctuations in GnRH release throughout the duration of culture, with significant increases on days 1–2, 7–8, 12–15, and 19–20 compared with low levels measured on days 4–5, 10, and 17–18 (by ANOVA, $P < 0.01$; Fig. 3A). In dynamic studies, GnRH was also released from pituitary cells (Fig. 3B) and α T3–1 gonadotrophs (Fig. 3C) perfused with DMEM/F12 supplemented with 1% BSA and 20 μ M bacitracin, pH 7.4, at 37 C, using flow rate of 0.15 ml/min. These results indicate that the synthesis and release of GnRH are characteristic features not only of hypothalamic

neurons, but also of anterior pituitary cells. The production of GnRH by mouse α T3–1 gonadotrophs raises the possibility that GnRH is cosecreted with gonadotropins in primary cultures of pituitary cells.

Effects of GnRH agonist analogs on GnRH and LH release

To study the cosecretion of GnRH with gonadotropins, perfused pituitary cells were stimulated with the GnRH agonist, des-Gly¹⁰-[D-Ala⁶]GnRH N-ethylamide ([D-Ala⁶]Ag), which does not cross-react with the antibody employed for RIA of GnRH (19). As expected, perfused pituitary cells responded to a 30-min pulse of 10 nM [D-Ala⁶]Ag with a rapid spike of LH release, followed by a sustained plateau release. The mean level of LH released during the spike and plateau phase (11.0 ± 0.5 pg/ml; $n = 3$) was significantly higher than the basal LH level (7.5 ± 0.3 ; $P < 0.03$; $n = 3$; Fig. 4A). Perfused pituitary cells also responded to successive applications of 10 nM [D-Ala⁶]Ag with a significant (7.5 ± 0.5 vs.

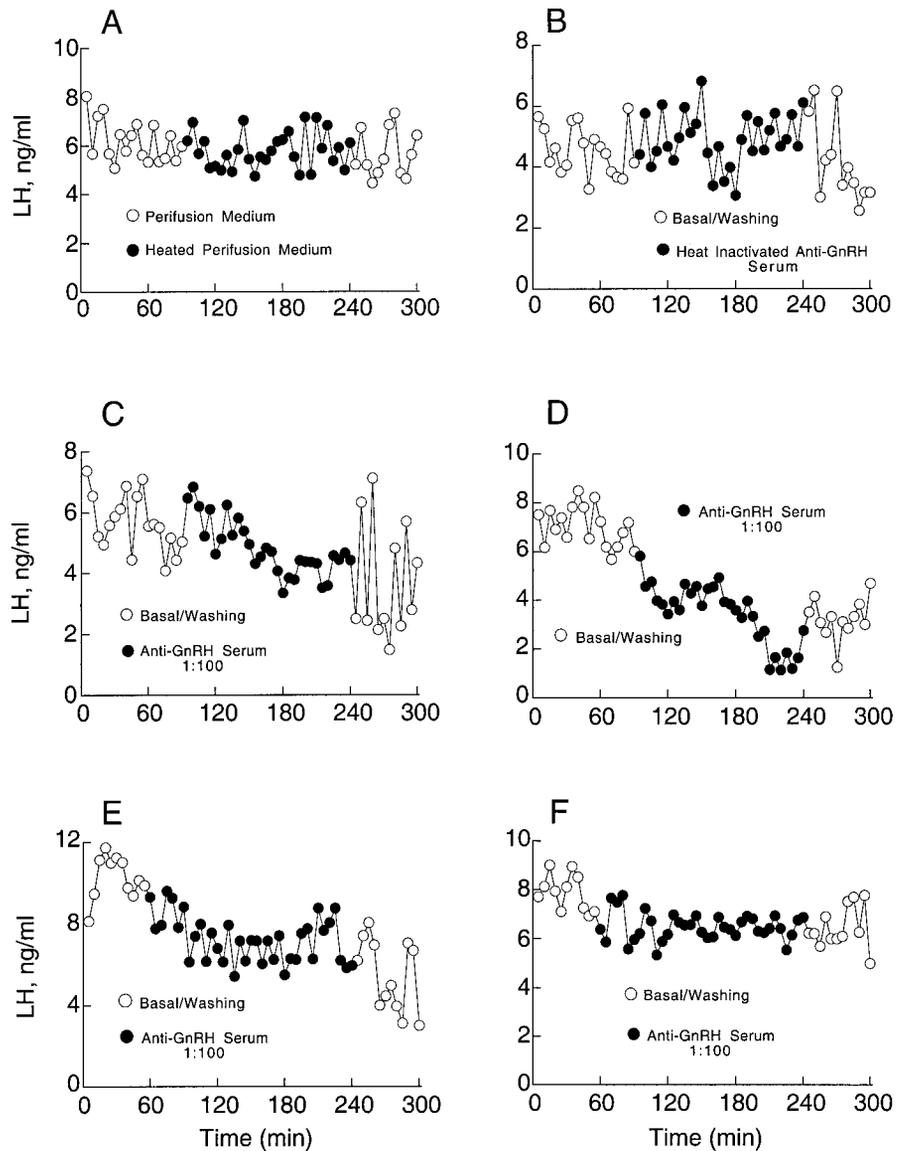
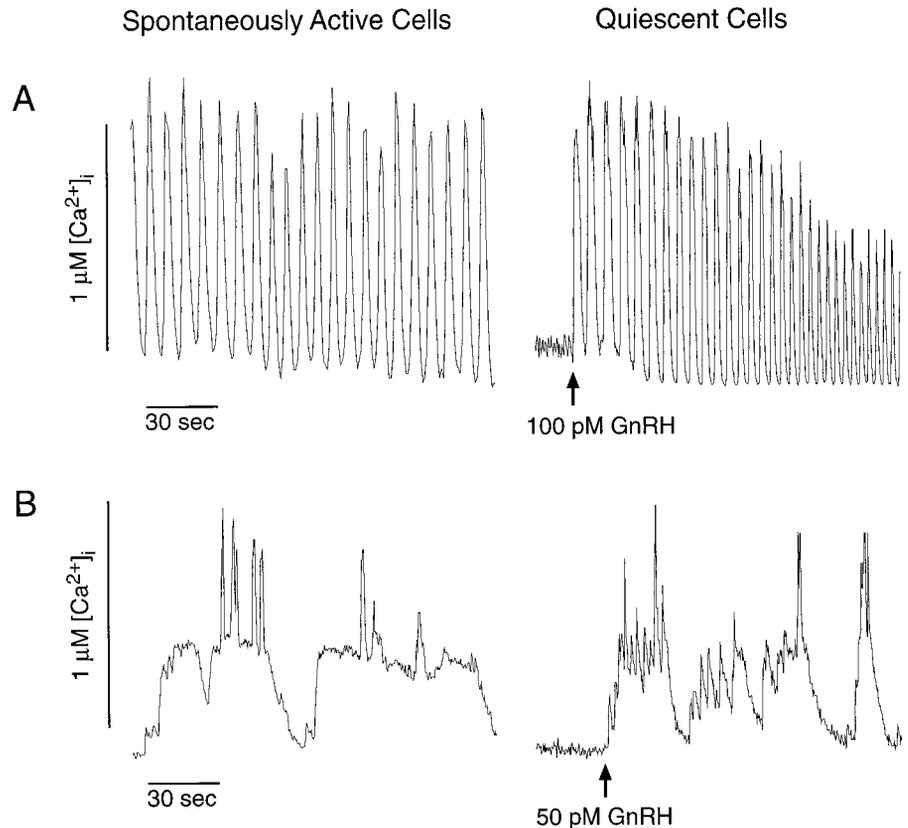


FIG. 5. Effects of anti-GnRH serum on LH release from perfused pituitary cells. A, LH release before (*open circles*) and during (*closed circles*) perfusion with heat-treated medium. B, LH release before (*open circles*) and during (*closed circles*) perfusion with heat-treated medium containing 1% GnRH antiserum. C–F show the changes in LH release during perfusion with medium containing 1% anti-GnRH serum (*closed circles*). Traces are representative results from four experiments.

FIG. 6. Spontaneous and GnRH-induced $[Ca^{2+}]_i$ responses in pituitary gonadotrophs. A, Threshold baseline $[Ca^{2+}]_i$ oscillations in spontaneously active cells (left panel) and GnRH-induced threshold baseline $[Ca^{2+}]_i$ oscillations in quiescent pituitary cells (right panel). B, Subthreshold $[Ca^{2+}]_i$ responses in spontaneously active cells (left panel) and GnRH-induced subthreshold $[Ca^{2+}]_i$ responses in quiescent pituitary cells (right panel). Indo-1 was used as the Ca^{2+} indicator. In this and the following figures, results are representative of at least 10 individual experiments.



2.4 ± 0.2 pg/ml; $P < 0.001$; $n = 4$) increase in GnRH release (Fig. 3B). Also, pituitary cells responded to successive application of 10 nM [D-Ala⁶]Ag with a significant increase in LH release (15.3 ± 2.8 vs. 7.2 ± 1.8 ng/ml; $P < 0.001$; $n = 3$).

Mouse $\alpha T3-1$ gonadotrophs also responded to agonist stimulation with a significant (30.6 ± 2.0 vs. 10.7 ± 0.2 pg/ml; $P < 0.006$; $n = 3$) increase in GnRH release (Fig. 3C) in a manner comparable to that observed in hypothalamic cells (19, 25, 26). This was an unexpected result, as these cells do not exhibit regulated secretion of LH α -subunit in response to GnRH stimulation (27). As shown in Fig. 3C, LH α release was almost completely unaffected by [D-Ala⁶]Ag treatment, in contrast to the severalfold increase in GnRH release. Taken together, these results suggest that a fraction of native gonadotrophs express both gonadotropins and GnRH, and release them in response to activation of GnRH receptors. The results further indicate that the exocytotic pathway in $\alpha T3-1$ gonadotrophs is functional, but that only GnRH is secreted in a regulated manner, whereas LH α -subunit is secreted in a constitutive manner.

Effects of GnRH antagonist analogs and anti-GnRH serum on LH release

To study the physiological relevance of locally produced GnRH on gonadotroph function, the effect of a potent GnRH receptor antagonist ([Ac-D-Nal(2)¹, D-Phe(pCl)², D-Pal(3)³, D-Cit⁶, D-Ala¹⁰]GnRH (SB-75; provided by Dr. A. V. Schally, V.A. Hospital, New Orleans, LA) was studied during sustained agonist stimulation. As shown in Fig. 4A, this antagonist not only abolished the [D-Ala⁶]Ag-induced plateau LH

response, but also caused a prompt decrease in LH release to below the basal level (9.0 ± 0.2 vs. 7.1 ± 0.1 ; $P < 0.01$; $n = 4$). The GnRH receptor antagonist also significantly decreased LH release in unstimulated pituitary cells, an effect that was reversed during the subsequent washing period (Fig. 4B). The effect of GnRH antagonist treatment on basal LH secretion was clearly observed when cells were perfused at flow rate of 0.15 ml/min. These results suggest that locally produced GnRH is sufficient to activate its receptors in pituitary gonadotrophs and contributes to the basal rate of gonadotropin secretion.

The role of pituitary-derived GnRH in the control of basal LH release was also assessed in perfusion studies using anti-GnRH serum. The ED₅₀ of the anti-GnRH serum in RIA at a dilution of 1:50,000 was 11.0 pg/ml. A 1:100 dilution of the anti-GnRH serum was used to neutralize GnRH released from cultured pituitary cells. In control perfusions the mean LH level was 6.2 ± 0.2 ng/ml and did not change when the cells were exposed to heat-treated (100 C) medium (5.8 ± 0.13 ng/ml; Fig. 5A). Heat-inactivated anti-GnRH serum caused a small (4.9 ± 0.16 vs. 4.5 ± 0.18 ng/ml), but insignificant, rise in LH secretion (Fig. 5B). Perfusion of pituitary cells with untreated anti-GnRH serum caused a significant decrease in LH release (7.4 ± 0.2 vs. 5.6 ± 0.15 ng/ml; $P < 0.04$; $n = 4$), as shown by the individual traces in Fig. 5, C and D, and E, and F. The similar inhibitory effects of GnRH antagonist and GnRH antiserum treatment suggest that locally produced GnRH participates in the maintenance of basal LH release in perfused pituitary cells.

Antagonist blockade of $[Ca^{2+}]_i$ oscillations

An analysis of data from 15 independent experiments in cultures from ovariectomized animals revealed that about 20% of gonadotrophs (169 from 856 identified cells) exhibited spontaneous $[Ca^{2+}]_i$ fluctuations at room temperature, whereas in the residual cells the baseline $[Ca^{2+}]_i$ remained constant (quiescent cells). In these experiments, baseline $[Ca^{2+}]_i$ was recorded for several minutes before stimulation with GnRH, which was used to identify gonadotrophs. The majority of spontaneously active cells exhibited small amplitude (50–100 nM) and extracellular Ca^{2+} -dependent $[Ca^{2+}]_i$ fluctuations. However, a fraction of gonadotrophs (34 cells from 856 identified gonadotrophs) showed high amplitude $[Ca^{2+}]_i$ oscillations. These were consistently observed in 2–5% of cells and, in contrast to smaller and influx-dependent $[Ca^{2+}]_i$ fluctuations (28), the presence of extracellular Ca^{2+} was not required for their initiation. These results indicate that high amplitude $[Ca^{2+}]_i$ oscillations occur randomly in a fraction of gonadotrophs and that endogenous Ca^{2+} release is responsible for their initiation.

In further experiments, we compared spontaneous high amplitude $[Ca^{2+}]_i$ oscillations with those induced by GnRH. Some cells (26 of 77) exhibited relatively regular baseline

$[Ca^{2+}]_i$ oscillations (Fig. 6A, *left panel*), typical of those observed after stimulation of quiescent gonadotrophs with 10–100 pM GnRH (*right panel*). The average frequency of these oscillations was 6 ± 0.9 spikes/min, comparable to that observed in quiescent gonadotrophs stimulated with 100 pM GnRH (7 ± 0.8 spikes/min; $n = 34$). In residual cells, oscillations were irregular (Fig. 6B, *left panel*) and resembled the subthreshold type of $[Ca^{2+}]_i$ signaling observed in GnRH-stimulated gonadotrophs (*right panel*).

Addition of intermediate concentrations of GnRH to spontaneously active cells increased the frequency of $[Ca^{2+}]_i$ oscillations in a manner comparable to that observed in gonadotrophs exposed to low and subsequently to higher GnRH concentrations (29). The effects of 100 pM GnRH on a spontaneously active cell are illustrated in Fig. 7A, *left panel*. The increase in spike frequency was also observed in two additional cells stimulated with 1 nM GnRH. In parallel to this, the frequency of $[Ca^{2+}]_i$ oscillations increased from 7 ± 0.9 to 11 ± 0.6 spikes/min in 34 gonadotrophs initially stimulated with 100 pM and subsequently with 1 nM GnRH (Fig. 7A, *right panel*).

Also, spontaneously oscillating cells exposed to high (10 nM) GnRH concentrations showed the typical nonoscillatory

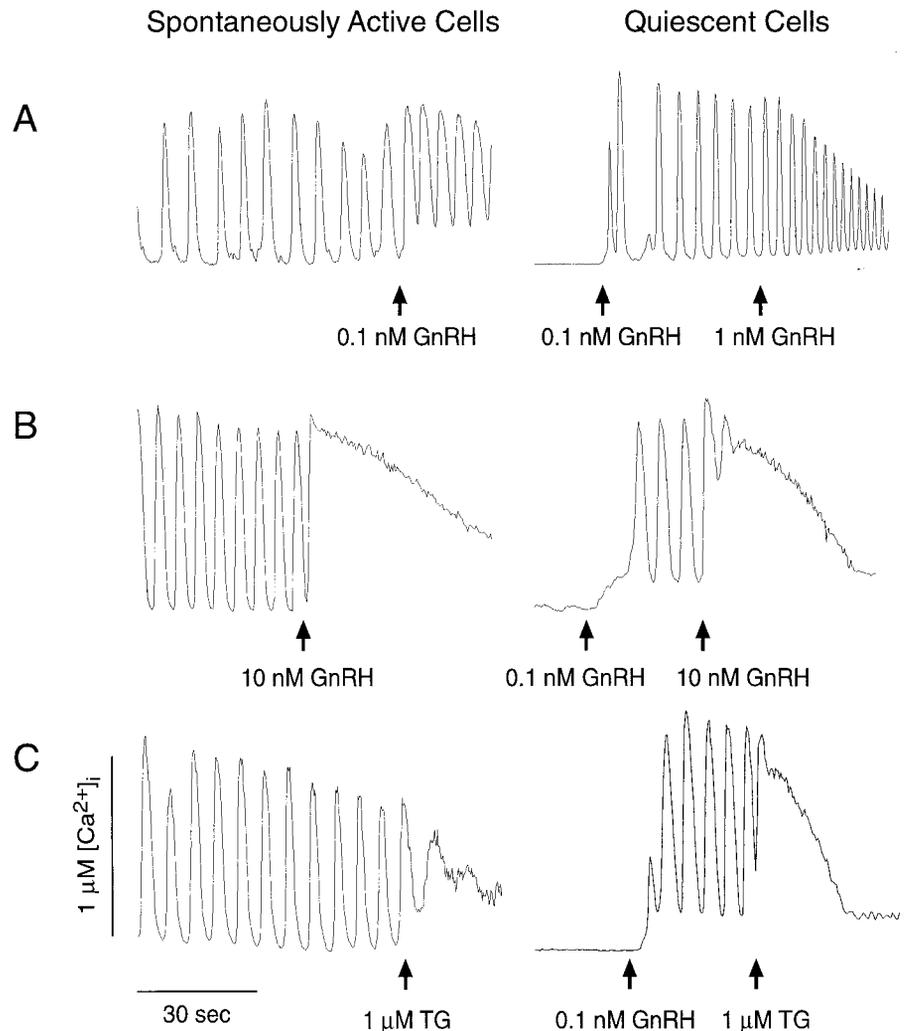


FIG. 7. Comparison of spontaneous and GnRH-induced $[Ca^{2+}]_i$ oscillations in pituitary gonadotrophs. A, GnRH-induced increase in $[Ca^{2+}]_i$ spiking frequency in spontaneously active (*left panel*) and agonist-activated gonadotrophs (*right panel*). B, GnRH-induced transition from the oscillatory to nonoscillatory mode in spontaneously active (*left panel*) and agonist-activated (*right panel*) gonadotrophs. C, Effects of thapsigargin (TG) on baseline $[Ca^{2+}]_i$ oscillations in spontaneously active (*left panel*) and agonist-activated (*right panel*) gonadotrophs. Indo-1 was used as the Ca^{2+} indicator.

prolonged response (Fig. 7B; $n = 3$), again comparable to that observed in gonadotrophs stimulated with low and subsequently with high GnRH concentrations (Fig. 7B, right panel; $n = 25$). Finally, both spontaneous (Fig. 7C, left panel; $n = 2$) and GnRH-induced $[Ca^{2+}]_i$ oscillations (right panel; $n = 27$) were abolished by the addition of thapsigargin, a blocker of endoplasmic reticulum Ca^{2+} -adenosine triphosphatase. These results show that spontaneous $[Ca^{2+}]_i$ oscillations are very similar to those induced by GnRH and suggest that they are initiated by the locally produced neuropeptide.

To test this hypothesis, cultured pituitary cells were treated with the potent GnRH receptor antagonist, [N-Ac-D-Nal (2)¹,D-pCl-Phe²,D-Trp³,D-hArg (Et)⁶,D-Ala¹⁰]GnRH (detirelix; 0.5 μ g/ml). In quiescent cells, detirelix did not affect basal $[Ca^{2+}]_i$ (Fig. 8A). However, in spontaneously active cells with basal $[Ca^{2+}]_i$ oscillations of $6 \pm 0.6 \text{ min}^{-1}$ ($n = 25$; from 10 recordings) the antagonist consistently abolished the $[Ca^{2+}]_i$ oscillations (Fig. 8, B–D). Conversion of the nonoscillatory $[Ca^{2+}]_i$ responses to 100 nM GnRH (Fig. 7B) into the oscillatory pattern ($8 \pm 0.8 \text{ min}^{-1}$; $n = 25$; $P < 0.05$; from 10 recordings) typically elicited by low concentrations of GnRH was usually observed after antagonist blockade of GnRH receptors (Fig. 8, right side of tracings A–D). These results indicate that the apparently spontaneous $[Ca^{2+}]_i$ oscillations are, in fact, elicited by locally produced GnRH. As LH secretion from gonadotrophs is controlled by $[Ca^{2+}]_i$ (30), the occurrence of such oscillations provides an explanation

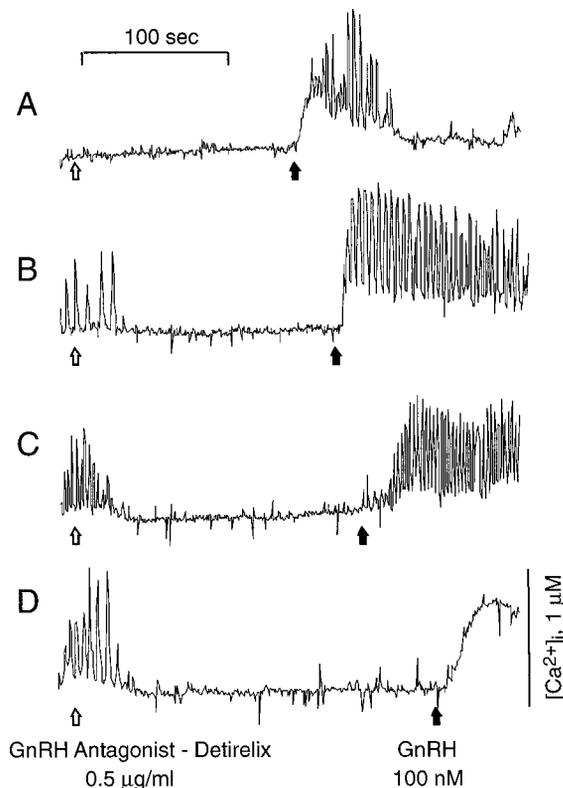


FIG. 8. Inhibition of spontaneous $[Ca^{2+}]_i$ oscillations in single gonadotrophs by a GnRH antagonist. Spontaneous Ca^{2+} oscillations were abolished by the addition of detirelix (0.5 μ g/ml) and were restored by the addition of 100 nM GnRH. Arrows indicate the application times of detirelix and GnRH. Fura-2 was used as the Ca^{2+} indicator.

for the inhibitory effects of GnRH antagonists on basal LH release (Fig. 4).

Discussion

After proteolytic cleavage of the GnRH decapeptide from the pro-GnRH-GAP precursor within the hypothalamic GnRH neurons (31), the neuropeptide product is released as discrete pulses into the hypothalamo-pituitary portal system to regulate gonadotropin secretion (32). Consistent with the primacy of the hypothalamic pulse generator in pituitary regulation, temporal and functional links among hypothalamic multiunit electrical activity, GnRH release, and pituitary gonadotropin secretion have been clearly demonstrated *in vivo* (33). However, several reports describing the production of GnRH at extrahypothalamic sites have raised the question of whether GnRH has a physiological role in peripheral tissues, including the pituitary gland. GnRH immunostaining in long term cultures of dispersed pituitary cells has revealed the presence of GnRH in pituitary gonadotrophs (11–13). Also, expression of GnRH mRNA in pituitary cells (14, 15) and enzymatic processing and release of GnRH from the pituitary gland (16, 17) have been reported. These findings are consistent with the possibility that in addition to the hypothalamic GnRH system, an autonomous GnRH system is operative at the pituitary level.

The present study has addressed the potential regulatory actions of locally formed GnRH at the pituitary level by several approaches. In accord with earlier reports (14, 15), we confirmed the presence of specific GnRH transcripts in pituitary cells and $\alpha T3-1$ gonadotrophs as well as positive immunostaining in anterior pituitary cells. Also, readily measurable release of the GnRH peptide was observed in short and long term static cultures of pituitary cells as well as immortalized gonadotrophs and perifused pituitary cells. When perifusion studies were performed at a slow flow rate (0.15 ml/min), GnRH release was detected under basal conditions as well as during GnRH agonist stimulation. The latter finding demonstrates that GnRH is cosecreted with LH and suggests that the locally produced peptide and its receptors might be involved in an autoregulatory feedback mechanism.

Supportive evidence for the participation of locally produced GnRH in the control of gonadotroph function was provided by the inhibitory action of the potent GnRH receptor antagonist, SB-75, on basal LH release. In earlier studies on pituitary cells perifused with medium at 1 ml/min, basal LH release was very low and was not affected by removal of extracellular Ca^{2+} and abolition of spontaneous electrical activity (28). However, the basal LH release observed when cells were perifused at flow rate of 0.15 ml/min was significantly higher and was consistently reduced after the addition of a GnRH receptor antagonist. When the role of ligand-independent activity of GnRH receptors in basal LH release was tested by the addition of anti-GnRH serum, a significant decrease in secretion was observed. These findings suggest that a low level of GnRH receptor activation by endogenous GnRH contributes to the basal rate of LH release *in vitro*.

The central role of $[Ca^{2+}]_i$ in the control of gonadotropin

secretion by GnRH has been well established (34). In pituitary gonadotrophs, low picomolar concentrations of GnRH induce irregular, low amplitude $[Ca^{2+}]_i$ transients that are classified as subthreshold responses. At higher concentrations, GnRH promotes high amplitude baseline $[Ca^{2+}]_i$ oscillations at a frequency (ranging from 2–25/min) that is dependent on the agonist concentration (29). Such responses to agonist stimulation and the ability of each Ca^{2+} spike to promote exocytosis (30) are consistent with the concept that gonadotropin secretion is frequency coded by the intracellular calcium signal. Also, only high amplitude $[Ca^{2+}]_i$ spikes are able to trigger exocytosis (35). Thus, the partial dependence of basal LH release on locally produced GnRH implies that some gonadotrophs must exhibit baseline $[Ca^{2+}]_i$ spiking in the absence of exogenous GnRH. In accord with this, spontaneous $[Ca^{2+}]_i$ oscillations very similar to those elicited by subthreshold and low threshold concentrations of GnRH were observed in a small proportion of gonadotrophs. Furthermore, the ability of GnRH receptor blockade to abolish such oscillations demonstrates that GnRH receptors participate in their generation.

These findings indicate that endogenously produced GnRH activates the GnRH receptors expressed in pituitary gonadotrophs, leading to subthreshold and low frequency baseline $[Ca^{2+}]_i$ oscillations that elicit a sufficient degree of exocytosis to contribute to the basal level of LH secretion. This local effect of pituitary GnRH could serve to maintain an optimal degree of sensitivity of the gonadotroph population to periodic stimulation by hypothalamic GnRH. This would essentially correspond to an intrinsic form of the well known priming effect of exogenous GnRH, which enhances secretory responses to subsequent GnRH stimulation (36–38).

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