Effects of differential pulse frequencies of chicken gonadotrophin-releasing hormone-I (cGnRH-I) on laying hen gonadotrope responses in vitro

MC Soñez[1], CA Soñez[2], MT Mugnaini[2], M Haedo[3], SA Romera[3], DM Lombardo[1], GA Delhon[1]

[1]Histology and Embryology, Faculty of Veterinary Science, University of Buenos Aires, C1427CWO, Buenos Aires, [2]Embryology and Cell Biology, Animal Anatomy Department, Agronomy and Veterinary Faculty, National University of Río Cuarto, and [3]Virology Institute INTA Castelar, Buenos Aires, Argentina

Abstract

The aim of this work was to determine the effects of cGnRH I pulse frequencies on FSH and LH release and the changes in features and number of cultured laying hen FSH-cells and LH-cells in vitro. Primary adenohypophyseal cell cultures taken from laying hens were stimulated by four 5 min pulses using 1 or 10 nM cGnRH, administered with interpulses between pulses at 15, 30 or 60 min. Pulse frequencies and dose dependent effects were examined in six separate experiments including two controls. After the last interpulse time, the supernatants were collected and stored at –70° C until the performance of an indirect enzyme-linked immunosorbent assay (ELISA) using chicken LH and chicken FSH antisera at 1:1000 and 1:2000 dilutions, respectively. Supernatants were coated in duplicate on the inner surface of Immulon 2 plates and later blocked with the optimal solutions. They were incubated with each antiserum and subsequently with isotype-specific peroxidase-labeled anti-rabbit antibodies. Hydrogen peroxide/*o*-phenylenediamine was added as substrate/chromogen and the optical density (OD) was determined at 492 nm. The ABC immunocytochemical method was performed to characterize and re-count the gonadotropes employing anti-chicken FSH and anti-chicken LH as primary antibodies. The number of FSH-LH cells was obtained using stereological analysis and the data were statistically processed. The ODs obtained for each anti-hormone were compared with the control groups and with each other. Significant differences were found in number of aggregated-positive LH cells, which decreased with 1 nM cGnRH-I, 15 *vs.* 30 min pulses, increased with 30 *vs.* 60 min pulses, and also with 10 nM cGnRH-I, 30 *vs.* 60 min pulses. Aggregated positive FSH cells, however, did not show significant differences in percentage at any GnRH dose or pulse frequencies, but did show activity at low pulse frequencies of 15 and 30 min. The results suggest that LH cells varied in percentage in a dose dependent manner at higher pulse frequency (15 min) and were dose independent at low pulse frequency (60 min) and showed inactive features; while FSH cell numbers were unaffected showing features of activity at low pulse frequencies. High and moderate pulse frequencies of cGnRH-I (15-30 min) increased the FSH release in dose independent manner without changes in features or percentage of FSH cells. Low pulse frequency (60 min) of cGnRH-I increased LH release dose independently disminished LH cell percentage and showed changes in cells' features. These results in avian cells showed differences in responses to GnRH pulse frequencies from those reported earlier in mammals.

Key words: cGnRH-I, laying hens, primary gonadotrope cells, pulse frequencies

Chicken gonadotropin-releasing hormone-I (cGnRH-I) is the predominant gonadotropin-releasing hormone in adult birds. It contributes to the differential regulation of luteinizing hormone (LH) and follicle stimulating hormone (FSH) biosynthesis and secretion (Ball and Hahn 1977, Sullivan and Silverman 1993). cGnRH-I is released from the hypothalamus in a pulsatile manner and transported to the anterior pituitary where it binds to high affinity receptors on gonadotrope cells. Chicken pituitary gonadotrope cells are two separate populations that contain either LH or FSH since the early embryonic stages (Proudman et al. 1999, Puebla-Osorio et al. 2002).

It is known that treatment of primary chicken pituitary cells with cGnRH or its analogues promotes LH release (Bonney et al. 1977). Experiments in vivo had indicated that 1– 10 nM cGnRH-I or II promote LH release and premature laying in hens. Such an effect on LH release in vitro also was observed when cultured pituitary cells were incubated for 2 h with cGnRH I or II (Deery 1974, Chou et al. 1985). The release of FSH seems to be regulated partly by cGnRH-I, whereas the underlying mechanism regulating LH release is unclear (Bruggeman et al. 1998). Dannies (1999) reported that LH and FSH do not have the same patterns of stimulated release, nor is the pattern of mobilization of the two kinds of granules after stimulation. The frequency of GnRH pulses has demonstrated differential effects on LH and FSH gene expression and secretion in rats (Kaiser et al. 1997a). High GnRH pulse frequencies (30 min) were associated with LH synthesis and secretion, whereas low GnRH frequencies (2 h) favored FSH synthesis and secretion in mammalian pituitary cells (Besecke et al. 1996, Kaiser et al. 1997a). The differential effects of cGnRH administered in pulse frequencies on gonadotrope cells activity in gonadotropin synthesis and secretion in birds, however, have not been studied exhaustively, and the underlying mechanisms remain unknown.

The aim of the study reported here was to describe FSH and LH release, and changes in features and number of cultured laying hen FSH and LH cells after administration of 1 or 10 nM cGnRH-I at high (15 min), medium (30 min), and low (60 min) pulse frequencies.

Materials and methods

Animals and cell dispersion

Laying hens (Brown & Nick) in their initial laying period (18-week-old birds) were obtained commercially (Nuestra Huella S.A., Buenos Aires). Groups of 12 animals were maintained in cages for 48-72 h with water and food *ad libitum.* Adenohypophyses recovered immediately after decapitation were collected in supplemented culture medium (D-S-BSA, pH 7.4, Dulbecco's modified Eagle's medium, 1% nonessential amino acid solution MEM Aa X 100, 0.26% gentamicine, 1% L-glutamine and 0.1% bovine serum albumin, all from Sigma-Aldrich Inc. (St. Louis, MO). Glands were sectioned in a laminar flow cabinet, rinsed several times using D-S-BSA and treated enzymatically with 0.1% trypsin solution (porcine trypsin 1:250; Sigma) at 37° C in a humidified 5% CO_2-95% air (CO_2 incubator) atmosphere for 1 h. DNase II 0.2% (type V; Sigma) was added for 2 min followed by centrifugation at 400 X g for 10 min. Trypsin enzymatic treatment was inhibited using 0.2% trypsin inhibitor type II-S (Sigma) for 10 min at 37° C. After centrifugation for 10 min at 400 X g, the cell suspension was gently dispersed in Dulbecco's Modified Eagle's Medium supplemented with 20% fetal bovine serum (D-S-SFB) (Bioser Certified, Buenos Aires, Argentina) with additional 10 µl aliquots of DNase and filtered through a 160 µm pore size nylon mesh. Cell viability (> 85%) was determined by the 0.4% trypan blue exclusion method.

Cultures

Two hundred fifty microliters of the high density cell suspension were plated over 13 mm sterile coverslips on 24-well plates. The coverslips previously had been treated with 0.01% poly-L-lysine (Sigma) to improve adhesion. Final plating densities were 1.5-2 x 10^5 cells/well in a total volume of 0.5 ml medium (D-S-SFB 20%). Specimens were placed in the CO_2 incubator at 37° C for 2 h to allow cells to settle and attach. Finally, D-S-SFB 20% was added to a final volume of 750 µl/well. The cultures were maintained at 37° C for 4 days (96 h) under humidified, 95% CO_2-5% air atmosphere.

Experiments

Monolayers of primary adenohypophyseal cell cultures were stimulated by four 5 min cGnRH-I pulses using 1 or 10 nM cGnRH (chicken [Gln[8]] LHRH; Sigma) administered in one of three ways, i.e., with interpulses between pulses of 15 or 30 or 60 min. Pulse frequencies and dose dependent effects were examined in six separate experiments including two controls. The total times for pulses plus interpulses were 1.5, 2.5 and 4.5 h, respectively. Pulse frequencies and dose dependent effects were examined in six independent experiments and eight wells/plate for each dose. Pulse stimulation was achieved by

replacing the medium in each well with serum-free medium containing the cGnRH-I dose for 5 min. Then the medium was discarded and replaced with serum-free medium while maintaining the cultures under humidified 95% CO_2-5% air atmosphere at 37° C for an interpulse time according to each frequency tested. The same procedure was repeated four times to accumulate the effects of four pulses of 1 or 10 nM cGnRH-I. Initial (C_1) and final procedural controls (C_2) were included in each experiment. C_1 was cultured without stimulation treatment and C_2 received serum-free medium without GnRH in the pulse frequency tested in each experiment. After the last interpulse, the supernatants were collected and stored at – 70° C until the enzyme-linked immunosorbent assay (ELISA) test was performed.

Immunocytochemistry (ICC)

Cell monolayers were fixed for 15 min in Bouin's solution after the last interpulse time and processed for ICC using the ABC procedure. The coverslips were rinsed with 0.5 M Tris-buffered saline pH 7.6 (TBS) and three times with 0.12:100 Triton X 100: TBS (v/v). An indirect amplified avidin-biotin system (Vectastain ABC Kit PK-6200, Vector Labs., Burlingame, CA) was performed on cell populations. Nonspecific binding sites were blocked using 1% milk solution and goat normal serum in 20-30 min incubations. Polyclonal anti-chicken FSH and anti-chicken LH, kindly provided by Dr. JA Proudman (United States Department of Agriculture, Agriculture Research Service), were used as primary antibodies and diluted 1:6000 and 1:8000 in TBS, respectively. Incubations were at room temperature in a humidified chamber for 60 min for the primary antibody and 30 min for the secondary antibody (rabbit IgG biotinilated) and for the avidine peroxidase. The antigen-antibody complex was revealed using 0.003% 3,3'-diaminobenzidine tetrahydrochloride solution (DAB) for 10 min. Weak hematoxylin staining was carried out, then the specimens were dehydrated, cleared and mounted in synthetic Canada Balsam on microscope slides. The slides from each pulse treatment were batch processed through the ICC procedure. Control procedures involved omission of the primary antibody or incubation with immunoabsorbed primary antibody (diluted first antibody with 7 μg/ml FSH or LH, 24 h at 4° C). Qualitative analyses were based on the following parameters: signal intensity, nucleus: cytoplasm ratio, intercellular relation, extracellular secretions and morphological features of nuclei. Characteristics of stimulated cellular populations were compared with C_1 and with C_2, to differentiate

stimuli effects vs. pulse method. Granule storage was determined qualitatively by the immunostain intensity and considered as synthesis-secretion rates to classify the cells into low, moderate, or strong immunoreactive or positive cells.

Statistical analysis

The number of anterior pituitary cells containing FSH or LH was obtained by microscopic stereological analysis (VIDAS Kontron system). More than 400 cells were counted per slide. Gonadotrope cell groups were defined according to the following parameters: isolated (I), aggregated (A), immunoreactive (ir) or positive cells. These parameters were used to determine statistically differences among 15 vs. 30 vs. 60 min pulse frequencies. cGnRH-I doses and pulse frequencies were the statistical variables for comparing 1 nM or 10 nM cGnRH-I applied for 15 vs. 30 vs. 60 min. The numbers of gonadotrope cells were expressed as percentages of the total population sample. Relative values data were processed with one way ANOVA using STATA software 5.0. Quantitative data represent the mean ± SD of six separate experiments, two for each pulse frequency and 3-4 replicate slides for each hormone and cGnRH-I dose. A mean percentage of FSH- or LH-containing cells was determined for each pulse frequency from 3-4 replicate slides for each experiment. Kruskal-Wallis, Scheffé or Bonferroni statistical tests were applied to compare mean values among pulse frequencies for each gonadotrope group. Increases in the number of FSH or LH cells was interpreted as a signal of activity in the synthesis and storage of the hormone. A *p* value of < 0.05 was considered significant.

ELISA

The culture media collected after the last interpulse time of each independent experiment were stored at -70° C until performing the ELISA tests to determine the pulse frequency effects on FSH and LH release. Ninety-six well plates (Immulon 2, Dynatech Laboratories, Corston, Bath, UK) were sensitized with supernatants overnight at 4-8° C in a 1:1 ratio in carbonate/bicarbonate buffer (pH 9.6). The plates were washed three times with 0.05% PBS-Tween 80. All washes used the same buffer. The same antibodies employed in ICC were used to recognize the cFSH and cLH released. The dilutions tested were 1:2000 and 1:6000 of anti-cFSH and 1:1000 and 1:4000 of anti-cLH. Positive controls consisted of wells sensitized with pure hormones and incubated with the respective anti-serum. Negative

controls consisted of sensitizing with the pure hormones and incubating with the other antiserum (data not shown). After incubation with the primary antibodies, the plates were washed three times and goat isotype-specific anti-rabbit anti-bodies (KPL; Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD) were added and incubated for 1 h at room temperature. Two different enzyme labels were tested: peroxidase and streptavidine phosphatase (biotinylated antibodies) for 1 h at room temperature.

The substrate/chromogen was H_2O_2/o-phenylenediamine (Sigma). Optical density (OD) was determined at 490 nm with a Dynatech microELISA reader and p-nitrophenyl phosphatase disodium (PPD) (Sigma). The ODs obtained for each antiserum (FSH, LH) were compared with those of the initial controls (C_1) and with each other.
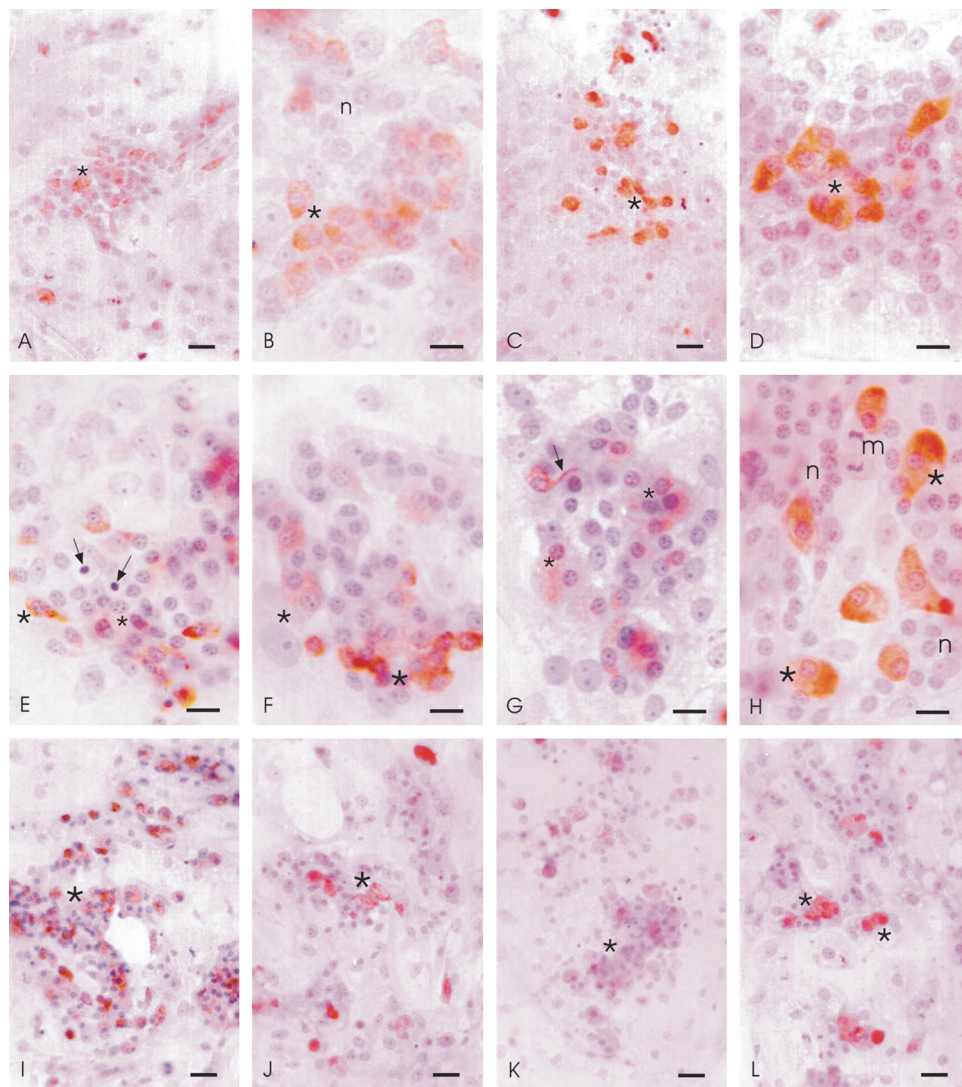

Fig. 1. (A-L) FSH cells and LH cells of control cultures (C1-C2) and also cells stimulated with cGnRH-I using pulse frequencies of 15 min (A-D), 30 min (E-H) and 60 min (I-L). A-B) FSH cells of C2, and from the experiment in which cultures were stimulated with 10 nM cGnRH-I, respectively. C-D) LH cells of C2, and from the experiment in which cultures were stimulated with 1 nM cGnRH-I, respectively. E-G) FSH cells of C2, and from the experiment in which cultures were stimulated with 1 and 10 nM cGnRH-I, respectively. H) LH cells of C1. I) FSH cells of C2. J-L) LH cells of C1 and from the experiment in which cultures were stimulated with 1 and 10 nM cGnRH-I, respectively. Light, moderate and strong immunoreactive cell (∗); n, nucleus of secretory cells; (↑) pyknotic nucleus (E), cytoplasmic process of A (+) cell surrounding a secretory cell type (G); m, mitosis. Scale bars = 20 μm.

Results

The percentage of the initial gonadotrope cells was obtained from C_1 cultures. Before stimulation, FSH and LH cells represented 16.61 and 14.44% of the total population, respectively (Table 1). Most cells in control and stimulated populations in mitosis were non-immunoreactive (Fig. 1H). Secretory cell types were well defined by their small, round nucleus with condensed and dispersed chromatin, slight basophilic nucleoplasm and one or two nucleoli (Fig. 1B,H). Nonsecretory cell types, probably fibroblasts or phagocytes, were characterized by their comparatively larger nuclei containing very dispersed chromatin and clear nucleoplasm. Some of them surrounded a clear space around one or more small LH or FSH-cells or non-ir cells with pyknotic nuclei. Some "C" shaped positive cells with typical cell nuclei were adhering to an LH or FSH-cell or non-ir cell. Control sections were ir-negative. Neither FSH-LH synthesis nor secretion seemed to be altered by pulse simulation procedures in the final controls (C_2), because there were no significant differences in the percentages of gonadotropes between both controls C_1 and C_2, and their features were similar. ELISA provided a sensitive method for detecting hormone release after cGnRH-I treatment in vitro (Table 1).

Cultures stimulated with cGnRH-I at each 15 min pulse frequency

FSH cells treated with 1 or 10 nM cGnRH-I (Fig. 1B) showed granule densities and cytoplasmic volumes similar to those of control cultures (Fig. 1A). Cultures treated with 10 nM, however, contained cells with abundant FSH-immunoreactive inclusions and less condensed chromatin. FSH cells with low to moderate reactivity and nucleus:cytoplasm ratios of 1:3 were predominant in the cultures. Comparison of treated and untreated cultures (Fig. 1C,D) showed that LH cells were inactive, especially those that received 1 nM cGnRH-I (Fig. 1D) (Table 2). These cells contained lower granule densities than the controls and were surrounded by cells containing LH-immunoreactive inclusions. Cultures treated with 10 nM cGnRH-I showed cells with greater cytoplasmic volume than those treated with 1 nM cGnRH-I. The ELISA results show that the release of FSH was increased with both cGnRH-I doses employed at this pulse frequency, while the release of LH was similar to controls (Fig. 4).

Cultures stimulated with cGnRH-I at each 30 min pulse frequency

FSH cells treated with 1 or 10 nM cGnRH-I contained higher granule densities than control populations (Fig. 1E,F,G). Aggregated positive cells [A(+)] with moderate to strong immunoreactivity were the predominant cells with uniform granules distribution and nucleus:cytoplasm ratios of 1:3 to 1:4. Isolated FSH cells (Fig. 1I) also showed strong immunoreactivity. Occasionally, FSH-positive cells with long cytoplasmic processes were observed around secretory cell type (Fig. 1G).

Treated LH cells did not differ from controls except for the greater nucleus:cytoplasm ratio (Fig. 1H). Cultures treated with both cGnRH-I doses contained aggregated LH cells with moderate to strong immunoreactivity, nucleus:cytoplasm ratios of 1:3 to 1:4, and nuclei partially obscured by secretory granules.

ELISA results showed that FSH was released by both doses of cGnRH-I at this pulse frequency, while the release of LH was disminished compared to the control, also with both cGnRH-I doses (Fig.4).

Cultures stimulated with cGnRH-I at each 60 min pulse frequency

A moderate decrease in the cytoplasmic volume of aggregated FSH cells was detected in cultures treated with 1 nM cGnRH-I compared to the treated and control populations (Fig. 1I). No differences in granule densities of FSH cells were observed, however, in any of the populations examined. Cell to cell association was decreased in aggregated LH cells treated with 10 nM cGnRH-I. (Fig. 1L). The nucleus:cytoplasm ratios of treated LH cells were lower than in the initial controls (C_1), but similar to the final controls (C_2) (Fig. 1J,K,L). Most LH cells showed moderate to strong immunoreactivity and uniform distribution of the secretory granules. This lower pulse frequency with both doses of cGnRH-I significantly increased LH release, whereas FSH release was similar to that of the controls. (Fig.4).

Statistical analysis

No significant statistical differences in cell number were detected for isolated immunoreactive,

Table 1. Mean total percentages of gonadotrope cells in initial control populations (C_1)

Gonadotrope cell groups	Fsh-trope cells (%)	Lh-trope cells (%)
Aggregated – [A (+)]	14.09	12.77
Isolated [I (+)]	2.52	1.67
A + I (+)	16.61	14.44

Table 2. Significant statistical differences in number of LH-trope cells after pulse-frequencies with 1 – 10 nM cGnRH-I

Lh-trope cell groups	1 nM cGnRH-I			10 nM cGnRH-I		
	Pulse-frequencies	P Value	Mean ± SD	Pulse-frequencies	P Value	Mean ± SD
A (+)	15 vs 30 min	0.001	4.67 ± 5.68 vs 16.69 ± 4.32			
	30 vs 60 min	0.011	16.69 ± 4.32 vs 7.74 ± 3.24	30 vs 60 min	0.028	14.74 ± 3.39 vs 7.38 ± 3.24

A (+) Aggregated immunoreactive cells

aggregated ir FSH cells treated with either 1 or 10 nM cGnRH-I with all the pulse frequencies tested (Fig. 2). Significant statistical differences were detected for aggregated ir LH cells as shown in Table 2, Fig. 3.

Discussion

We describe here for the first time differential cGnRH-I pulse frequency effects on the number and features of primary laying hen gonadotrope cells and on FSH-LH release in vitro. We hypothesize that their variations in number and changes in morphological features are indirect signs of biosynthesis and secretion of chicken FSH and LH under cGnRH-I regulation in vitro. The doses of cGnRH-I (1-10 nM) that we employed previously have demonstrated effectiveness in LH release in dispersed and perfused fowl pituitary cells (Chou et al. 1985, King et al. 1986). Our primary pituitary cells originated from laying hens, a period in which the higher hypothalamic content of cGnRH-I had been demonstrated in turkeys (Rozenboim et al. 1993). The total times of pulses plus interpulses (1.5, 2.5 and 4.5 h) of stimulation on cultures was minimized to avoid the inhibitory effects of GnRH on protein synthesis that King et al. (1986) reported in chicken pituitary cells.

The total quantity of both gonadotropes that we determined for controls cultures (C_1) before the GnRH stimulation was in agreement with the findings reported in mammals (Kaiser et al. 1997b). The low initial increase in the total number of FSH cells (2%) over LH cells suggests that baseline FSH release was not higher than the LH release. If this is so, it constitutes a difference from rat gonadotropes (Dannies 1999). Alternatively, this finding could have been due to the fact that hens were young and in their initial laying period. Studies of female broiler breeder chickens have demonstrated that the plasma FSH concentrations peaked about 3 weeks before the first oviposition in association with increased pituitary FSH and median eminence cGnRH-I (Bruggeman et al. 1998). The profile of basal LH and FSH secretion from the mammalian pituitary in vitro, in the absence of added secretagogue, resembled that of the peripheral blood levels of each gonadotropin and the control of basal and of GnRH-stimulated FSH and LH release seemed to occur by separate mechanisms (Evans et al. 1999).

In our experimental model, the percentages of LH cells decreased significantly when they received 1 nM of cGnRH-I every 15 min, and increased significantly with both doses with stimulation every 30 vs. 60 min; thus the responses were dose dependent and dose independent, respectively. The increase in nucleus:cytoplasm ratio of LH cells observed with cGnRH-I 10 nM in pulse frequency of 15 min suggests a probable increase in LH synthesis and storage. The qualitative features of LH cells stimulated with a pulse frequency of 60 min and with both doses of GnRH showed that the synthesis, storage and release of the hormone determined by ELISA were relevant. Dose dependent responses of LH releasing chicken GnRH have been demonstrated in dispersed chicken anterior pituitary cells (King et al. 1986).

cGnRH-I showed no effect on the percentage of FSH cells, which was similar with both doses and with all pulse frequencies we tested. Previous studies that showed FSH and LH release from chicken pituitary cells involved continuous stimulation for 2 h with cGnRH-I (Millar et al. 1986). Higher release of LH in vitro and in vivo compared to FSH induced by chicken LH-RH-I has been documented in
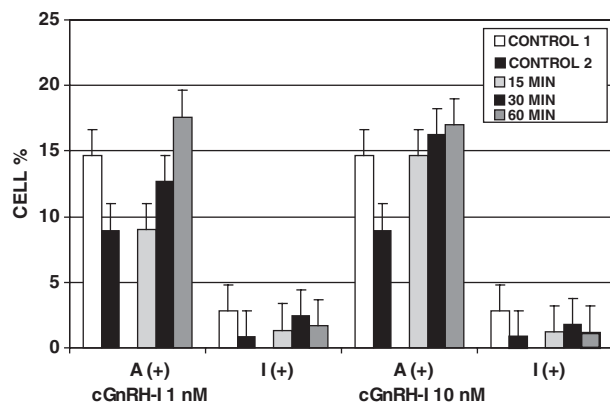
Fig. 2. Mean number of FSH cells after the stimulation with 1 and 10 nM cGnRH-I using 15, 30, and 60 min pulse frequencies. No significant differences were found with any doses or frequencies employed.

Fig. 3. Mean number of LH cells after the stimulation with 1–10 nM cGnRH-I using 15, 30, and 60 min pulse frequencies. *Significant differences applying 1 nM cGnRH-I for A (+) cells 15 vs. 30 min pulse frequencies (p = 0.001), 30 vs. 60 min pulse frequencies (p = 0.011) with 10 nM cGnRH-I for A(+) cells 30 vs. 60 min (p = 0.026).

Japanese quail and chickens (Hattori et al. 1986). A qualitative response of FSH cells to 1 nM cGnRH-I under 15 min pulses was observed, which was indicated by the increase in their granule storage. FSH cell features consisting of extensive cytoplasmic volume and dense storage of granules observed in most culture populations in all the cGnRH-I pulse frequencies tested could indicate that they were active in synthesis and storage of the hormone independent of GnRH pulses. FSH was released differentially with both doses of cGnRH-I at 15 min
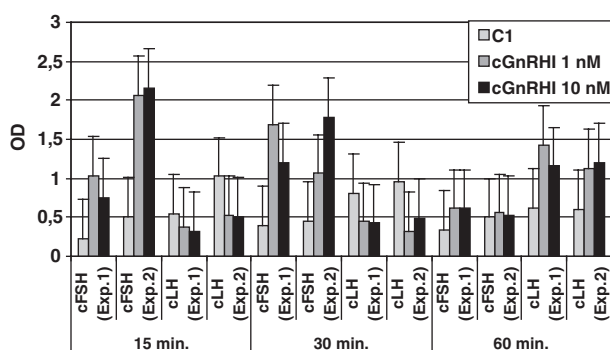

Fig. 4. LH and FSH release toward the medium after the stimulation with 1-10 nM cGnRH-I using 15, 30, 60 min pulse frequencies (Experiments 1 and 2) determined by ELISA (OD measured at 492 nm). The release of FSH increased with the both cGnRH-I doses employed at high 15 min pulse frequency, while the release of LH was similar to controls. At moderate 30 min pulse frequency, FSH was released by both doses of cGnRH-I, while the release of LH was disminished compared to the control, also with both cGnRH-I doses. The low pulse frequency of 60 min of cGnRH-I increased LH release with both doses, whereas FSH release was similar to controls.
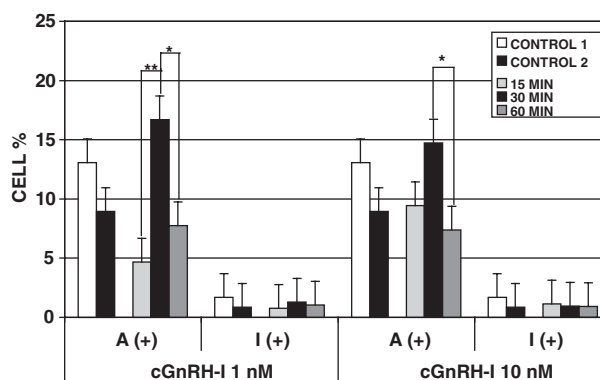
pulse frequencies with respect to the controls. By contrast, in male rat pituitary cells, 5 min pulses of 10 nM GnRH applied every 60 min stimulated FSH mRNAβ, while pulses of GnRH every 30 and 15 min decreased FSH mRNAβ synthesis, and higher GnRH pulse frequencies suppressed FSH mRNAβ by pituitary follistatin production (Besecke et al. 1996, Dalkin 1999). The mechanisms involved in controlling FSH production and secretion have not been well established and several other factors have been implicated as selective regulators of FSH release (Padmanabhan and McNeilly 2001).

The active phagocytes we described surrounding the secretory cell types could be folliculo-stellate cells. We have reported their presence in pituitary of *Columba livia* and *Nothura maculosa* (Soñez et al. 1990, 1997). They probably play a paracrine role in synthesis and secretion of pituitary hormones as in mammals (Besecke et al. 1996, Houben et al. 1990). The long immunoreactive cytoplasmic processes of LH cells suggest that intercellular communication and some paracrine mode interactions were established in these avian culture cells.

Laying hen gonadotrope responses stimulated with the high, medium and low cGnRH-I pulse frequencies that we tested were different from those reported for rat primary and cell line gonadotrope cells (Kaiser 1997a,b), rat and dispersed chicken anterior pituitary cells (King et al. 1986, Besecke et al. 1996). High pulse frequency of 15 min with 1 nM cGnRH-I caused significant changes in the percentage of LH cells, while both doses of cGnRH-I (1-10 nM) in a low pulse frequency (60 min) also changed the percentage of LH cells present. The percentage of FSH cells was not modified by any doses or by

high, medium or low pulse frequencies of cGnRH-I. Cellular activity appeared to change, however, with the low pulse frequencies of 15 min and the release was increased. There are few studies on GnRH regulation in FSH and LH biosynthesis and secretion in birds (Chou et al. 1985, King et al. 1986, Bonney et al. 1997, Proudman et al. 2006). Hattory et al. (1986) reported autonomous FSH production and release, whereas LH was rigidly controlled by GnRH in immature cockerels. Proudman et al. (2006) have examined the two variants of cGnRH (I and II) stimulating release of FSH and LH in chickens and concluded that the mechanism by which independent release of FSH occurs in chickens remains unresolved.

Four cGnRH-I pulses in vitro seemed to induce changes in the percentage of LH cells, and LH synthesis and storage in a cGnRH-I dose dependent and independent manner with high pulse frequencies of 15 min and low pulse frequencies of 60 min, respectively; however, there were no significant changes in the percentage of FSH cells. The 15 min pulse frequency caused FSH release to the medium with both doses, while LH release was determined with 60 min pulse frequency and also with both cGnRH-I doses. Additional work will be required to examine subtle differences that might account for the responses observed. Differential effects of GnRH pulse frequencies on the mechanisms of FSH and LH biosynthesis and secretion in avian gonadotrope cells must be investigated further.

Acknowledgments

References

Ball GF, Hahn TP (1977) GnRH neuronal systems in birds and their relation to the control of seasonal reproduction. In: Parhar IS, Sakuma Y, Eds. *GnRH Neurons. Gene to Behavior*. Brain Shuppan, Japan. pp. 325-342.

Besecke LM, Guendner MJ, Schneyer AL, Bauer-Dantoin AC, Jameson JL, Weiss J (1996) Gonadotropin releasing hormone regulates follicle-stimulating hormone-β gene expression through an activin/follistatin autocrine or paracrine loop. *Endocrinology* 137: 3667-3673.

Bonney RC, Cunningham FJ (1997) Stimulation of luteinizing hormone release from chicken pituitary cells by analogues of luteinizing hormone-releasing hormone. *Gen. Comp. Endocr.* 32: 205-207.

Bruggeman V, D'Hondt E, Berghman L, Onagbesan O, Vanmontfort D, Vandesande F, Decuypere E (1998) The effect of food intake from 2 to 24 weeks of age on LHRH-I content in the median eminence and gonadotrophin levels in pituitary and plasma in female broiler breeder chickens. *Gen. Comp. Endocr.* 112: 200-209.

Chou H, Johnson AL, Williams JB (1985) Luteinizing hormone releasing activity of [Gln^8]-LHRH and [His^5, Trp^7, Tyr^8]-LHRH in the cockerel, in vivo and in vitro. *Life Sci.* 37: 2459-2465.

Dalkin AC, Haisenleder DJ, Gilrain JT, Aylor K, Yasin M, Marshall JC (1999) Gonadotropin-releasing hormone regulation of gonadotropin subunit gene expression in female rats: actions on follicle-stimulating hormone β messenger ribonucleic acid (mRNA) involve differential expression of pituitary activin (β-B) and follistatin mRNAs. *Endocrinology* 140: 903-908.

Dannies PS (1999) Protein hormone storage in secretory granules: mechanisms for concentration and sorting. *Endocrine Rev.* 20: 3-21.

Deery D.J. (1974) Determination by radioimmunoassay of the luteinizing hormone-releasing hormone (LHRH) content of the hypothalamus of the rat and some lower vertebrates. *Gen. Comp. Endocr.* 24: 280-285.

Evans JJ, Janmohamed S (1999) In vitro basal and GnRH-stimulated secretion of gonadotrophins reflects long-lasting modulatory effects, and peripheral levels are not predicted by pituitary responsiveness to GnRH. *Eur. J. Endocrinol.* 141: 512-520.

Hattori A, Ishii S, Wada M (1986) Effects of two kinds of chicken luteinizing hormone-releasing hormone (LH-RH), mammalian LH-RH and its analogs on the release of LH and FSH in Japanese quail and chicken. *Gen. Comp. Endocr.* 64: 446-455.

Houben H, Tilemans D, Denef C (1990) Toward a functional significance of peptides and biogenic amines produced by the anterior pituitary. *J. Endocrinol. Invest.* 13: 855-863.

Kaiser UB, Jakubowiak A, Steinbergger A, Chin WW (1997a) Differential effects of gonadotropin-releasing hormone (GnRH) pulse frequency on gonadotropin subunit and GnRH receptor messenger ribonucleic acid levels in vitro. *Endocrinology* 138: 1224-1231.

Kaiser UB, Conn PM, Chin WW (1997b) Studies of gonadotropin-releasing hormone (GnRH) action using GnRH receptor-expressing pituitary cell lines. *Endocrine Rev.* 18: 46-70.

King JA, Davidson JS, Millar RP (1986) Desensitization to gonadotropin-releasing hormone in perifused chicken anterior pituitary cells. *Endocrinology* 119: 1510-1518.

Millar RP, Milton RC, Follett BK, King JA (1986) Receptor binding and gonadotropin-releasing activity of a novel chicken gonadotropin-releasing hormone ([His^5, Trp^7, Tyr^8] GnRH) and a D-Arg^6 analog. *Endocrinology* 119: 224-231.

Padmanabhan V, McNeilly AS (2001) Is there an FSH-releasing factor? *Reproduction* 121: 21-30.

Perez FM (1993) Cell-to-cell interactions inhibit protein synthesis, GH and LH release in male rat adenohypophyseal cells in vitro. *Endocr. J.* 1: 49-55.

Proudman JA, Vandesande F, Berghman LR (1999) Immunohistochemical evidence that follicle-stimulating hormone and luteinizing hormone reside in separate cells in the chicken pituitary. *Biol. Reprod.* 60: 1324-1328.

Proudman JA, Scanes CG, Johannsen SA, Berghman LR, Camp MJ (2006) Comparison of the ability of the three endogenous GnRHs to stimulate release of follicle-stimulating hormone and luteinizing hormone in chickens. *Domest. Anim. Endocrinol*. 31: 141-53.

Puebla-Osorio N, Proudman JA, Compton AE, Clements KE, Decuypere F, Vandesande F, Berghman LR (2002) FSH-and LH-cells originate as separate populations and at different embryonic stages in the chicken embryo. *Gen. Comp. Endocr.* 127: 242-248.

Rozenboim I, Silsby JL, Tabibzadeh C, Pitts GR, Youngren OM, El Halawani ME (1993) Hypothalamic and posterior pituitary content of vasoactive intestinal peptide and gonadotropin-releasing hormones I and II in the turkey hen. *Biol. Reprod.* 49: 622-626.

Soñez MC, von Lawzewitsch I, Ibañez N, Perez R (1990) Identification of glycoprotein and protein secreting pituitary cells in pigeon (Columba livia) employing human hormones antisera. *Com. Biol.* 9: 85-98.

Soñez MC, von Lawzewitsch I (1997) Ultrastructural identification of pituitary cells in Nothura maculosa (Tinamidae. Temminck, 1815) *Biocell* 21: 103-114.

Sullivan KA, Silverman AJ (1993) The ontogeny of gonadotropin-releasing hormone neurons in the chick. *Neuroendocrinology* 58: 597-608.