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Basal and gonadotrophin-releasing hormone-induced biosynthesis and release of luteinizing hormone: effect of calcium deprivation

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Summary

The present study examines the basal and gonadotrophin-releasing hormone (GnRH)-stimulated biosynthesis and release of luteinizing hormone (LH) by pituitary cells in primary culture, and the effect of extracellular calcium deprivation on these events. Pituitaries from ovariectomized adult rats were enzymatically dispersed and cultured for 96 h. The cells were then incubated for 5 h (Expts. 1 and 3) or for different time intervals between 0 and 5 h (Expt. 2), in medium containing [^{14}C]leucine ([^{14}C]leu) and [^{3}H]glucosamine ([^{3}H]gln), with or without GnRH. Total immunoreactive LH (iLH) was measured in the medium and the cell extract by radioimmunoassay. LH translation (as estimated by [^{14}C]leu incorporation into LH; [^{14}C]LH) and LH glycosylation (as estimated by [^{3}H]gln incorporation into LH; [^{3}H]LH) were measured by immunoprecipitation with specific LHβ antiserum in both medium and cell extract. Treating the cells with GnRH caused both time- and dose-dependent increases of iLH in the medium as well as in total (cells plus medium) content, with an approximate ED_{50} of 0.7 nM. GnRH also stimulated LH biosynthesis by increasing both LH polypeptide chain synthesis and LH glycosylation. The effect of GnRH on LH glycosylation was detected earlier than that on translation, the [^{3}H]LH rates of production and release being higher than those of [^{14}C]LH. These findings suggest that GnRH-induced translation and glycosylation of LH are independently regulated. Removal of extracellular calcium resulted in the loss of cellular responsiveness to GnRH, preventing not only the stimulatory effects of GnRH on total and released iLH but also the GnRH-induced incorporation of both [^{14}C]leu and [^{3}H]gln into newly synthesized LH. These observations suggest that GnRH-stimulated LH glycosylation and LH translation involve calcium-dependent mechanisms. Neither the uptake of radiolabeled precursors nor their incorporation into total protein were affected by GnRH or Ca^{2+}-deficient (no added calcium) medium. The results also suggest that the release of newly synthesized LH is regulated differently from previously synthesized stored hormone.

Introduction

Luteinizing hormone (LH) is a glycoprotein hormone which consists of two noncovalently associated subunits, designed α and β, containing two complex oligosaccharides attached to the α subunit and one such oligosaccharide attached to the β subunit (Pierce and Parsons, 1981). In general, the secretory process of glycoproteins from its site of synthesis on bound polysomes of the rough endoplasmic reticulum (RER) via the Golgi complex to the point of discharge at the cell surface comprises sequential events of assembly and maturation (Hirschberg and Snider, 1987). Thus, the biosynthetic pathway for LH is thought to involve the translation of separate messenger RNAs for the α and β subunits (Godine et al., 1982; Chin et al., 1983), a co-translational attachment of the N-linked high mannose oligosaccharide units to specific asparagine residues, and the formation of the complex oligosaccharides by a post-translational glycosylation in which some of the mannose residues are trimmed, followed by sequential addition of the peripheral sugars in the Golgi.

The regulation of the LH secretory process is principally under the hypothalamic decapeptide gonadotro-

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phin-releasing hormone (GnRH) control. It is known that GnRH stimulates the translation (Starzec et al., 1986), glycosylation (Liu et al., 1976) and release of LH by the anterior pituitary gland. Although the mechanisms involved in the GnRH actions are not completely understood, numerous studies have demonstrated the importance of calcium as an intracellular mediator of GnRH-induced LH release (Conn et al., 1987), a finding which is consistent with the concept of stimulus-secretion coupling originally proposed by Douglas (1968). Other actions of the releasing hormone such as down-regulation of receptors, self-priming effect and desensitization do not have this marked dependency on calcium (Pickering and Fink, 1979; Smith and Conn, 1983; Braden and Conn, 1990).

GnRH raises intracellular calcium by a dual mechanism (Limor et al., 1987), influx across the cell membrane and mobilization from intracellular pools. Some observations emphasize the importance of extracellular calcium in maintaining LH release in response to GnRH (Bates and Conn, 1984), but less is known about the possible calcium-dependent mechanisms which may control LH synthesis (Liu and Jackson, 1985a; Ramey et al., 1987b).

The present study examines the basal and GnRH-stimulated apoprotein biosynthesis, glycosylation and release of LH from pituitary cells in primary culture by measuring incorporation of [^{14}C]leucine (translation) and [^{3}H]glucosamine (glycosylation) into immunoprecipitable LH. This work also presents further evidence regarding the calcium requirements for GnRH-induced LH synthesis.

Materials and methods

Media

Several media were utilized for this study. Pituitary cell dispersion was performed in supplemented Spinner minimum essential medium (S-SMEM) which consisted of calcium- and magnesium-free SMEM containing 0.25% bovine serum albumin (BSA), 13 mM Hepes, 50 μg/ml streptomycin, and 50 U/ml penicillin. Four days culture was performed in supplemented Medium 199 (S-M199) which consisted of Medium 199 with Earle's salts plus 0.25% BSA, 25 mM Hepes, 100 μg/ml streptomycin, 100 U/ml penicillin, 50 μg/ml nystatin, 8% horse serum and 2% fetal bovine serum. Both sera were pretreated with dextran-coated charcoal to remove endogenous steroids. For short-term incubations Dulbecco's modified Eagle's medium (DEM), or its variants, supplemented with 0.25% BSA was used. In experiments with labeled precursors, cells were incubated in low glucose (1000 mg glucose/l) leucine-free DEM (leu-free DEM) containing 5 μCi/ml L-[^{14}C(U)]leucine ([^{14}C]leu; 333.0 mCi/mmol) and 30 μCi/ml D-[1,6-^{3}H(N)]glucosamine ([^{3}H]gln; 47.6 Ci/mmol). In studies where extracellular calcium levels were reduced, media were prepared by simply omitting $CaCl_2$ from leu-free DEM (Ca^{2+}-deficient medium), or adding 0.1 mM EGTA to Ca^{2+}-deficient medium (Ca^{2+}-free medium). All media were Millipore-filtered through 0.22 μm filters before use, the final pH being 7.4.

The culture media, antibiotics and chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA), and the sera from Grand Island Biological Co. (Grand Island, NY, USA). The radiolabeled compounds were obtained from New England Nuclear (Boston, MA, USA).

Dispersion and culture of rat anterior pituitary cells

Adult female rats (200–250 g body weight) from our colony of albino rats were ovariectomized 30 days before being used. Animals were housed in a controlled environment (21 ± 1°C; 14 h light : 10 h darkness) with food and water supplied ad libitum. The animals were killed by decapitation. Dispersed pituitary cells were prepared as described previously (Pérez and Apfelbaum, 1992). Briefly, anterior pituitary glands were minced and treated for 20 min with 0.25% trypsin (1 : 250 porcine pancreatic trypsin; Difco, Detroit, MI, USA) and 100 μg deoxyribonuclease (DNase; bovine pancreatic deoxyribonuclease type DN-100; Sigma) in S-SMEM. Tissue blocks were then incubated with 1 mg soybean trypsin inhibitor (type I-S; Sigma) plus 100 μg DNase for another 3 min. The final dispersion of the pituitary cells was accomplished by means of repeated gentle uptake and expulsion with a flame-polished Pasteur pipette. After dissociation, the cells were resuspended in S-M199 and plated in Falcon tissue culture dishes (1.5 ml containing 3×10^6 cells per 35 mm dish) and cultured at 37°C under a humidified atmosphere of 95% air : 5% CO_2. Additional aliquot of 1 ml fresh culture medium was added to each dish 48 h later. After 72 h plated, the medium was removed and replaced by fresh S-M199 and cells cultured for another 24 h. Following the 96 h culture period, the culture medium was discarded and the cells were washed twice (during 45 min each) with 2 ml/dish of DEM medium, to remove serum and nonadhered cells. In experiments which required media other than DEM cells were washed in the particular medium prior to use in short-term incubations.

Short-term incubations of cultured cells

After 96 h of culture, short-term incubations were carried out at 37°C under a humidified atmosphere of 95% air : 5% CO_2. The purpose of the first set of experiments was to evaluate the pituitary cell culture system as a suitable experimental model to study the basal biosynthesis and release of LH and the effect of GnRH on these events. The ability of the cultured

pituitary cells to release LH in response to increasing doses of GnRH was analyzed. Following the removal of the growth medium and washing twice with DEM, cells were incubated in 1.5 ml of DEM for 5 h in the absence (control) or presence of different concentrations (10 pM to 1 μM) of GnRH (Bachem, Torrance, CA, USA). The time-course of [^{14}C]leu and [^{3}H]gln incorporation into LH and total protein was also studied. The cultured cells were washed twice in leu-free DEM and incubated for different time periods (0–5 h) in 1.5 ml of the same medium containing 5 μCi/ml [^{14}C]leu plus 30 μCi/ml [^{3}H]gln with or without 10 nM GnRH.

The objective of the second set of experiments was to establish the effect of extracellular calcium deprivation on basal and GnRH-stimulated LH synthesis and release. Before experiments, cells were washed twice with leu-free DEM containing 1.8 mM calcium (normal Ca^{2+} medium) or Ca^{2+}-deficient medium. The pituitary cells were then incubated for 5 h with the radioactive precursors in 1.5 ml of the appropriate test medium (normal Ca^{2+}, Ca^{2+}-deficient or Ca^{2+}-free medium) in the absence or presence of 10 nM GnRH.

Incubations were terminated by adding 1 ml phosphate buffered saline (PBS) pH 7 and chilling the dishes on ice. The media were removed, centrifuged at $2500 \times g$ for 15 min at 4°C and kept frozen at −20°C until assayed. In incubations with radiolabeled precursors, the media were dialyzed twice against PBS pH 7.5 containing unlabeled precursors (0.2% leucine and glucosamine) and twice against PBS, during a total time period of 24 h at 4°C. Cells were washed once with 2 ml PBS and lysed by incubation in 1.5 ml of ice-cold 50 mM Na_2CO_3 in 0.1 N NaOH for 10 min. The neutralized cell lysates were then frozen and thawed 3 times and treated with Triton X-100 (1% final concentration). Aliquots of each cell extract were taken to determine total cellular radioactivity (uptake) and incorporation of radiolabeled precursors into trichloroacetic acid (TCA)-precipitable protein. The samples were then centrifuged at $35{,}000 \times g$ for 30 min. Supernatants were decanted and stored at −20°C.

Uptake and incorporation of radioactive precursors into total protein

The uptake of labeled precursors was determined by measuring total radioactivity in the cell extracts. Aliquots of the cell extracts were added to scintillation vials and solubilized in Soluene 350 (United Technologies Packard, Downers Grove, IL, USA) for 24 h at room temperature. 10 ml of counting solution were added, and the samples were counted in a Beckman LS 7000 liquid scintillation counter.

To determine incorporation of labeled precursors into total protein, duplicate aliquots of the medium and the cell extract plus 50 μl 0.5% BSA-PBS were precipitated with 10% ice-cold TCA. The precipitates were washed twice with the TCA solution, solubilized with 1 N NaOH, and counted for radioactivity after neutralization with glacial acetic acid.

Total precursor uptake represents the sum of cellular uptake and TCA-precipitable protein in medium.

Immunoprecipitation of radiolabeled LH

[^{14}C]Leucine-labeled LH ([^{14}C]LH) and [^{3}H]glucosamine-labeled LH ([^{3}H]LH) were measured by immunoprecipitation with highly specific LHβ antiserum in both medium and cell extract. All immunoprecipitations were done in 50 mM EDTA-PBS (pH 7.5), containing 0.5% Triton X-100 and 0.5% Nonidet P40. Nonspecifically bound radioactivity was reduced by pre-treating the samples (four replicates each) of cell extract and dialyzed medium with 10 μl of undiluted normal rabbit serum (NRS) and incubating at 4°C for 4 h. The NRS was precipitated with sheep anti-rabbit gamma globulin (ARGG) by incubation overnight at 4°C followed by centrifugation ($2500 \times g$, 30 min, 4°C). The supernatant fraction was used for specific LH immunoprecipitation with LHβ antiserum. Two of the aliquots of the adsorbed samples were incubated with 50 μl LHβ antiserum (1 : 10) and the other two with an equivalent volume of 1 : 10 dilution NRS for 8 h, followed by ARGG overnight at 4°C. The final precipitates were washed twice with detergent buffer and once with PBS, solubilized in 1 ml of 0.2 M acetic acid and centrifuged ($2500 \times g$, 30 min, 4°C). The supernatants were then transferred to scintillation vials and counted in 10 ml scintillation cocktail. The difference between radioactivity precipitated in the presence of antiserum minus that precipitated in the presence of NRS represents the radioactivity specifically incorporated into LH. The means of the ratios of radioactivity precipitated with antiserum vs. that precipitated with NRS, for medium and cell extract samples, respectively, in control groups ranged from 1.0 and 4.0 at 1 h to 2.2 and 5.9 at 5 h for [^{14}C]LH, and from 1.7 and 3.7 to 3.5 and 4.6 for [^{3}H]LH. In the presence of GnRH the ratios ranged from 1.6 and 4.4 at 1 h to 4.0 and 6.6 at 5 h for [^{14}C]LH, and from 2.4 and 3.9 to 6.6 and 5.1 for [^{3}H]LH.

The LHβ antiserum used in this study was prepared and characterized in our laboratory. Antibodies to ovine LHβ were generated by immunization of rabbits with 100 μg ovine LHβ DNW 9-119-3 antigen (kindly provided by Dr. D.N. Ward), using a procedure similar to that of Vaitukaitis et al. (1971). In order to eliminate the cross-reactivity with thyroid-stimulating hormone (TSH) and follicle-stimulating hormone (FSH), anti-LHβ (A-LHβ) serum was adsorbed (Liu et al., 1976) with 200 μg ovine TSH (oTSH-12) per ml serum. After adsorption with oTSH specificity of the antiserum was tested by two procedures. In the first, we compared the

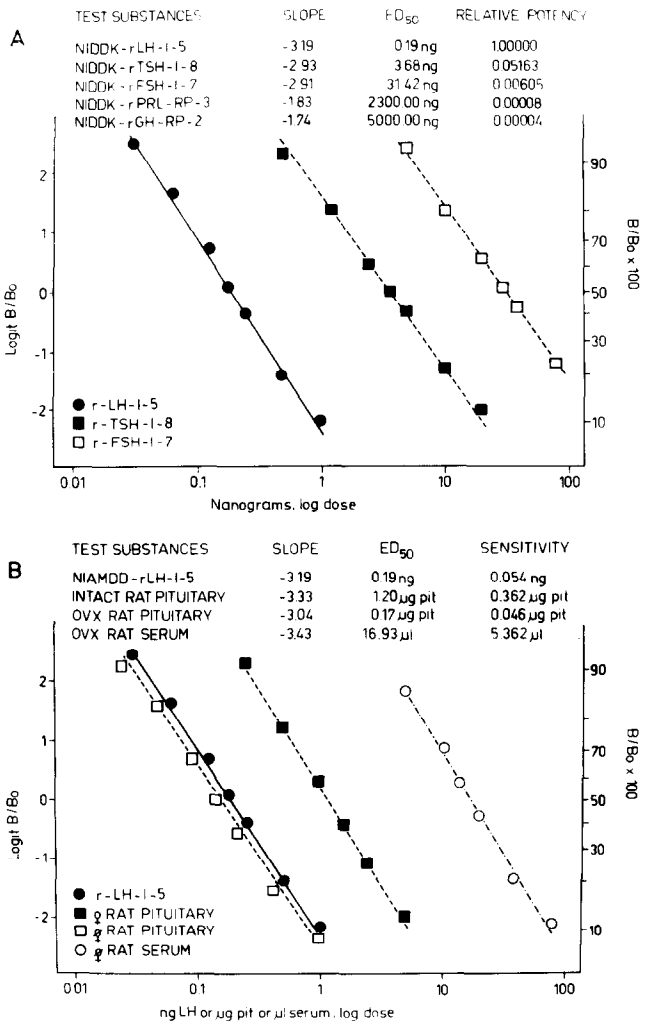

Fig. 1. Characterization of anti-LHβ serum. Dose–response curves generated for different test substances in a system consisting of [^{125}I]LH and anti-LHβ to determine specificity (*A*) and parallelism (*B*). For details see text.

ability of TSH, FSH, LH, prolactin (PRL) and growth hormone (GH) to compete the radioiodinated LH for binding to antibody at an initial dilution of 1 : 100,000. Dose–response curves were generated for rLH-I-5, rFSH-I-7, rTSH-I-8, rPRL-RP-3 and rGH-RP-2 in a system consisting of [^{125}I]LH and A-LHβ serum. Fig. 1*A* summarizes the relative potencies for the antigens tested. In addition, dose–response curves generated for purified rLH-I-5, serum from ovariectomized rats and pituitary extract from both ovariectomized and intact rats, were done to determine parallelism. The least-squares regression line of each dose–response curve was computed and the slopes of those lines were all equal (Fig. 1*B*). In the second procedure we compared the binding of radiolabeled individual LH subunits as well as other highly purified rat anterior pituitary hormones by the antiserum, under the conditions of the immunoprecipitation assay. Approximately 10,000 cpm of the radiolabeled hormones were reacted with A-LHβ at an initial dilution of 1 : 10 in the absence of unlabeled hormones to determine cross-reactivity. The antiserum showed high specificity for [^{125}I]rLH-I-5 (85.7%) and [^{125}I]rLHβ-AFP-7310B (99.4%), minimal cross-reactivity with [^{125}I]rLHα-AFP-7264B (1.3%), [^{125}I]rFSH-I-7 ($< 0.5\%$) and [^{125}I]rTSH-I-8 (4.6%), and no detectable cross-reactivity with [^{125}I]rGH-I-5 or [^{125}I]rPRL-I-5.

The quantity of antiserum needed to precipitate all of the LH present in the sample was determined adding a trace amount of [^{125}I]LH (10,000 cpm) to representative medium and cell extract samples or to control blanks and immunoprecipitating with varying concentrations of A-LHβ. The amount of antiserum finally used was that wich precipitated 100% of the [^{125}I]LH precipitated in the control blanks.

Furthermore, molecular species immunoprecipitated by the A-LHβ serum were identified using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Representative samples of cell extracts were immunoprecipitated with A-LHβ, NRS or A-LHβ in the presence of an excess of highly purified unlabeled rLH-RP-2 (20 μg), using the protocol previously described. In addition a sample of cell extract to which a trace amount of [^{125}I]LH was added, was also precipitated with the antibody. Reduced immune complexes were then mixed with carbonic anhydrase (29,000), soybean trypsin inhibitor (20,000) and lysozyme (14,000), which served as internal molecular weight markers. Samples were electrophoresed in SDS-polyacrylamide slabs gel (1.5 mm thick) with a 4.5% stacking gel (3 cm) and a 15% resolving gel (8 cm). After electrophoresis, the gels were stained with Coomassie blue, destained and each line cut into 2 mm slices. Each gel slice was dissolved in H_2O_2, and its radioactivity quantified by counting in a beta or gamma counter, as applicable. The molecular weights of proteins in each gel slice were determined from a regression line plotted from the positions of the molecular weight markers on the gel. Electrophoresis of radiolabeled pituitary cell culture samples labeled with [^{3}H]gln and [^{14}C]leu for 5 h showed two major peaks of specific incorporation. These peaks, migrating at approximately 22,000 and 17,000 molecular weight, correspond well with the positions of [^{125}I]rLHα and [^{125}I]rLHβ, respectively, running in neighboring lines on the gel (Fig. 2). Since the A-LHβ does not cross-react with the α subunit, these data indicate that the antibody precipitated an α-β subunit complex by recognition of determinants on the β subunit and suggest that the LHα activity found is derived from the LH dimer. Moreover, the identity of the specifically labeled protein appearing on the gels was confirmed to be LH by demonstrating that the addition of an excess of unlabeled LH before immunoprecipitation reduced the radioactivity in both peaks to background levels (Fig. 2). Taken

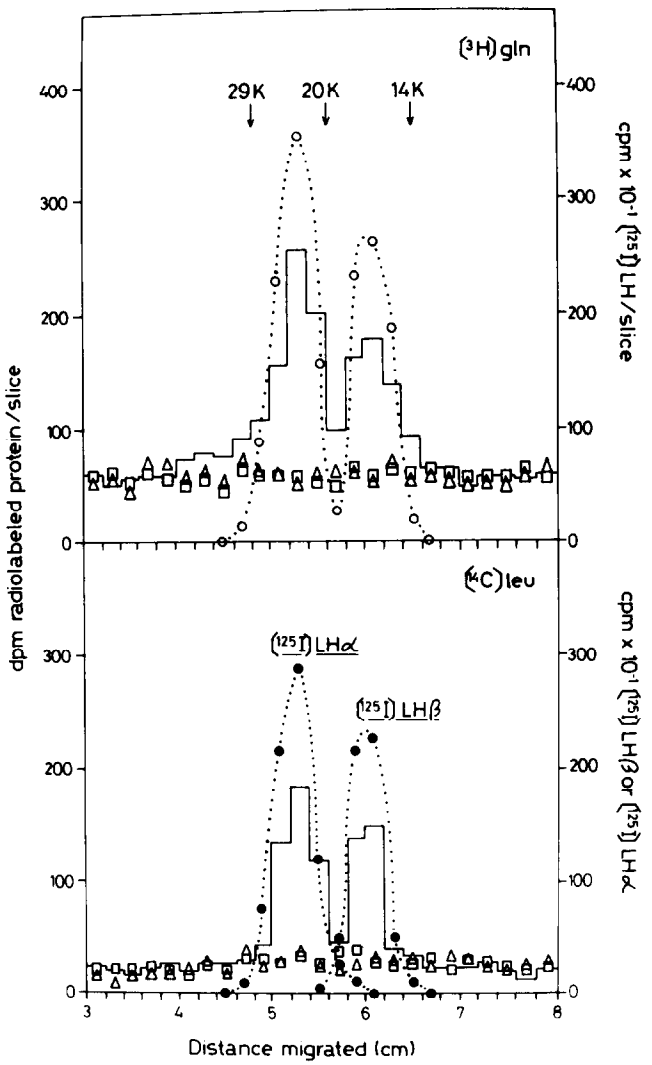

Fig. 2. SDS-PAGE of [^{3}H]glucosamine ([^{3}H]gln; upper panel) and [^{14}C]leucine ([^{14}C]leu; lower panel) from a representative cell extract sample labeled for 5 h in the presence of 10 nM GnRH. Radiolabeled protein was immunoprecipitated with A-LHβ (filled line), NRS (squares), A-LHβ in the presence of excess rLH (triangles) or A-LHβ in the presence of trace amount of [^{125}I]rLH (open circles). The positions of [^{125}I]rLHα and [^{125}I]rLHβ (filled circles) on the gel are also shown.

together, this and the previous experiments demonstrate that, in addition to LHβ subunit, intact LH was specifically precipitated by A-LHβ antiserum.

LH radioimmunoassay

Immunoreactive LH (iLH) was measured in the medium and the cell extract at two dose levels by a double-antibody radioimmunoassay, with reagents supplied by the National Hormone and Pituitary Program, NIDDK, NIH. The amount of LH was calculated in terms of NIDDK rat standard rLH-RP-2. The intra-assay coefficient of variation (c.v.) for the LH assay was 5.1%. The corresponding interassay c.v. was 9.8%. The results were expressed as μg LH per 3×10^6 cells.

Statistics

The data were analyzed by analysis of variance and Duncan's multiple range test. A level of $P < 0.05$ was considered statistically significant. All results are expressed as the mean $\pm$ SEM.

Results

Basal and GnRH-stimulated release and synthesis of LH

iLH. Responsiveness of pituitary cell cultures incubated for 5 h in the presence of different doses of GnRH is shown in Fig. 3. As expected, GnRH at all doses (10 pM to 1 μM) significantly ($P < 0.01$) enhanced iLH release in a dose-related manner, with a maximum response, to 870% of control value, obtained at 0.1 μM GnRH. The ED_{50} value of GnRH action was calculated to be 0.69 nM. Total (cells plus medium) iLH also increased proportionally to the dose of GnRH (Fig. 3), with a minimum effective dose of 1 nM (115% of control; $P < 0.05$) and a maximum response to 126% of control ($P < 0.01$) obtained at 1 μM. On the basis of these findings, a dose of 10 nM GnRH was selected to test responsiveness of pituitary cells as a function of the incubation time.

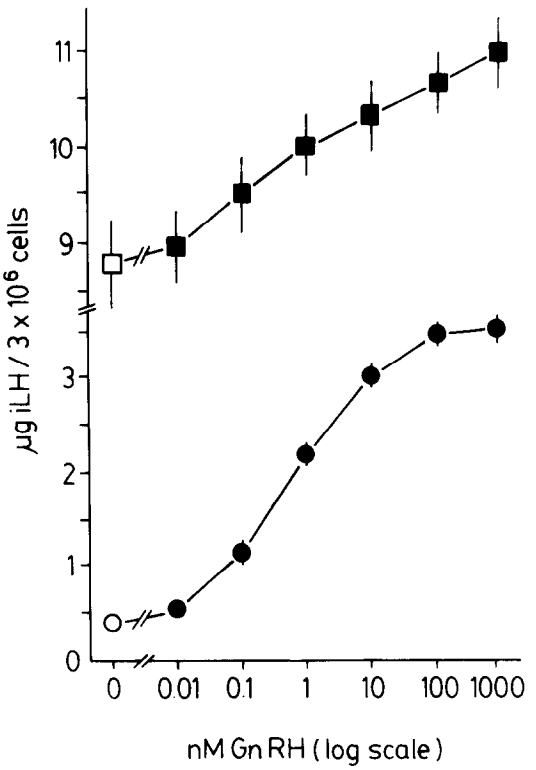

Fig. 3. Release and total system (cells plus medium) content of LH from cultured rat pituitary cells in response to increasing doses of GnRH. Amount of LH released into the medium (circles) and present in the total system (squares) after 5 h incubation in the presence (filled symbols) or absence (open symbols) of GnRH. Each point is the mean value of five cultures and the vertical lines indicate $\pm$ SEM.

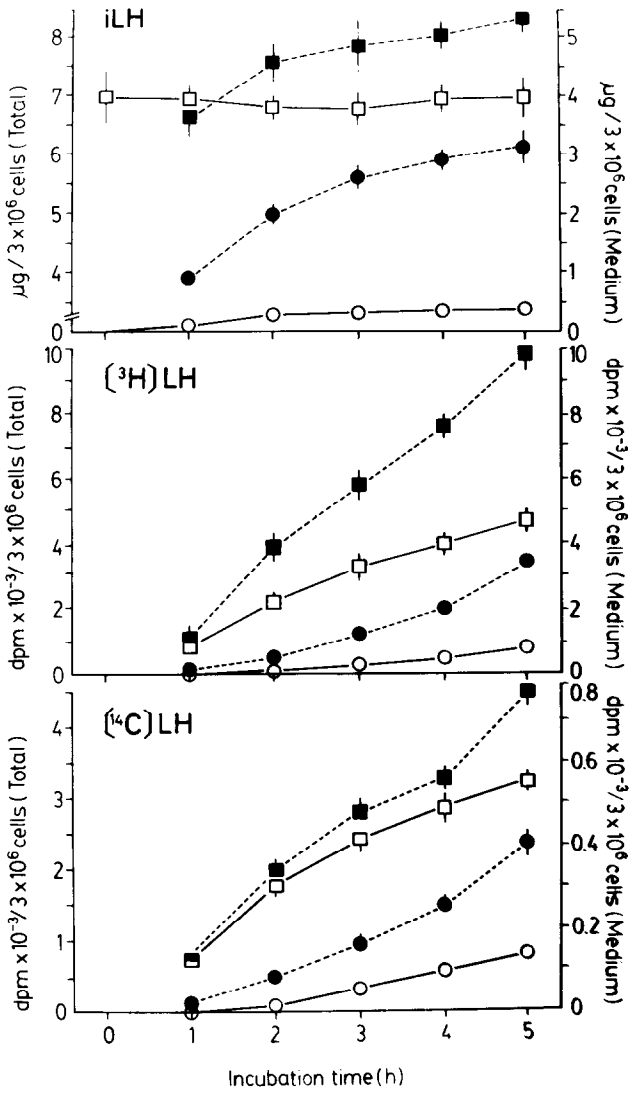

Fig. 4. Time-course of LH released and synthesized from rat anterior pituitary cell cultures. Cells, cultured for 96 h, were incubated in the presence (filled symbols) or absence (open symbols) of 10 nM GnRH for various time periods as described under Materials and methods. Amount of total immunoassayable LH (iLH; upper panel), [^{3}H]glucosamine-labeled LH ([^{3}H]LH; middle panel) and [^{14}C]leucine-labeled LH ([^{14}C]LH; lower panel) in the medium (circles) and total system (squares). Each point is the mean value of five cultures and the vertical lines indicate ± SEM.

As previously observed (Apfelbaum, 1983), basal release of iLH occurred in two phases (Fig. 4), with a higher rate between 0 and 2 h (approximately 95.5 ng/3 × 10^6 cells/h), and a lower rate between 2 and 5 h (approx. 53.7 ng/3 × 10^6 cells/h). There were no significant changes in total iLH content from 0 to 5 h. Addition of 10 nM GnRH into the incubation medium was followed by time-related increases in medium and total content of iLH (Fig. 4). At all times, GnRH greatly stimulated ($P < 0.01$) the basal release of iLH with maximum increases between 0 and 2 h. Total iLH rose linearly with time from 2 to 5 h, being significantly higher than controls at 4 h ($P < 0.05$) and 5 h ($P < 0.01$).

Radiolabeled LH. Comparable patterns of basal release and total (cells plus medium) content of [^{14}C]LH and [^{3}H]LH as a function of the incubation time were observed (Fig. 4). The amount of total radiolabeled LH showed a linear rise up to 3 h, with consecutive increases ($P < 0.01$) of 2.3- (at 2 h) and 3.2-fold (at 3 h) for [^{14}C]LH and 2.5- (at 2 h) and 3.8-fold (at 3 h) for [^{3}H]LH, over the corresponding values found at 1 h. The rate of [^{14}C]leu and [^{3}H]gln incorporation into LH declined slightly between 3 and 5 h. In the medium, the minimum time required to detect [^{3}H]LH was 1 h, whereas [^{14}C]LH was undetectable at 1 h and only trace amounts were measured at 2 h; from this time on a progressive increase in radiolabeled LH release into the medium occurred. Treating the cells with 10 nM GnRH caused a rise in total synthesis and release of [^{14}C]LH and [^{3}H]LH (Fig. 4). This stimulatory effect was more pronounced on glycosylation than on translation of the newly synthesized LH, as established by the mean increases in either the medium ([^{3}H]LH vs. [^{14}C]LH: 401% vs. 274% of control) or total (187% vs. 125%) radiolabeled LH. In addition, GnRH was already effective in inducing a significant increase (176% of control; $P < 0.01$) in total [^{3}H]LH (glycosylation) at 2 h, but longer GnRH exposure was required to obtain a significant increase (4 h: 120% of control; $P < 0.05$) in total [^{14}C]LH (translation). The rate of [^{14}C]leu and [^{3}H]gln incorporation into LH elicited by GnRH was significantly ($P < 0.01$) higher than controls, as established by comparing the slopes of the curves (between 2 and 5 h) of total and released [^{14}C]LH and [^{3}H]LH obtained by regression analysis. The slope values in the absence vs. the presence of 10 nM GnRH, respectively, for total and released radiolabeled LH were 0.503 vs. 0.815 and 0.042 vs. 0.105 for [^{14}C]LH, 0.819 vs. 1.969 and 0.247 vs. 0.926 for [^{3}H]LH.

Calcium-dependent release and synthesis of LH induced by GnRH

To determine if GnRH-induced release and synthesis of LH were dependent on extracellular calcium, pituitary cell cultures were incubated in normal Ca^{2+} (1.8 mM calcium), Ca^{2+}-deficient (no calcium) or Ca^{2+}-free (plus 0.1 mM EGTA) medium. Although it has not been quantified, it is assumed that the Ca^{2+}-deficient medium used is not entirely devoid of calcium. Therefore, the inclusion of 0.1 mM EGTA was done to chelate any remaining calcium.

As shown in Fig. 5, basal release of iLH was not affected by incubating the cells for 5 h in Ca^{2+}-deficient medium, but it was increased in Ca^{2+}-free medium (143% of that in normal calcium medium: $P < 0.01$). Neither Ca^{2+}-deficient nor Ca^{2+}-free medium modified total (cells plus medium) content of iLH. GnRH

(10 nM) added to the normal Ca^{2+} medium stimulated the release (746% of control; $P < 0.01$) and total content (120% of control; $P < 0.01$) of iLH (Fig. 5). Incubating the cells in Ca^{2+}-deficient or Ca^{2+}-free medium dramatically reduced the ability of GnRH to increase the release of iLH (18% of that in normal Ca^{2+} medium), and completely blocked the stimulatory effect of GnRH on total iLH.

Basal levels of medium and total (cells plus medium) [^{3}H]LH and [^{14}C]LH (Fig. 5) were slightly but not significantly diminished by Ca^{2+}-deficient medium, but markedly reduced ($P < 0.01$) by Ca^{2+}-free medium (medium, total: 60%, 42% and 40%, 18% of that in normal Ca^{2+} medium for [^{3}H]LH and [^{14}C]LH, respectively). In normal Ca^{2+} medium, 10 nM GnRH significantly ($P < 0.01$) increased [^{3}H]LH in the medium (451% of control) and in the total system (glycosylation) (210% of control) (Fig. 5). In Ca^{2+}-deficient medium, the GnRH-induced increases in medium and total [^{3}H]LH were significantly inhibited (25% and 48% of those in normal medium; $P < 0.01$) and completely abolished in Ca^{2+}-free medium. As observed for LH glycosylation, under normal calcium conditions GnRH stimulated the incorporation of [^{14}C]leu into immunoprecipitable LH released into the medium (304% of control) as well as total (translation) (132% of control) [^{14}C]LH, while calcium reduction completely prevented any response of the pituitary cells to GnRH (Fig. 5).

Specific activity of intracellular, released and total radiolabeled LH

Table 1 shows the effects of GnRH on newly synthesized LH in the cells, medium and total system when expressed as specific activity (SA; disintegrations per min/μg iLH). At all times studied, GnRH stimulated the total (cells plus medium) SA of [^{3}H]LH (160% of control; $P < 0.01$), whereas the total SA of [^{14}C]LH was slightly but not significantly increased by GnRH. In contrast, the SA of [^{14}C]LH and [^{3}H]LH released in response to GnRH was drastically reduced (30% and 42% of control, respectively; $P < 0.01$). This reduction in SA of released radiolabeled LH in the presence of GnRH was associated with increased ($P < 0.01$) SA of intracellular labeled LH for both [^{14}C]LH (144% of control) and [^{3}H]LH (190%). Lowering extracellular calcium did not alter the SA of radiolabeled LH released under basal conditions (Ca^{2+}-deficient vs. normal Ca^{2+} medium: $P > 0.05$), but it did prevent the reduction in SA of labeled hormone released in response to GnRH (Table 1). In addition, the increased SA of intracellular and total labeled LH in response to GnRH was blocked by incubation of cells in Ca^{2+}-deficient medium, with no effect on basal levels. In the presence of 0.1 mM EGTA, the SA of intracellular, released and total [^{14}C]LH and [^{3}H]LH was markedly diminished ($P < 0.01$) under both basal and GnRH-stimulated conditions (Table 1).

TABLE 1

SPECIFIC ACTIVITY OF RADIOLABELED LH RELEASED, REMAINING IN THE CELLS AND SYNTHESIZED BY RAT PITUITARY CELLS INCUBATED IN THE PRESENCE OR ABSENCE OF GnRH

Specific activity (SA; disintegrations per min/μg iLH) of the radiolabeled LH ([^{14}C]leucine-labeled LH: [^{14}C]LH; [^{3}H]glucosamine-labeled LH: [^{3}H]LH) released (Medium), remaining in the cells (Cells) and containing in the total system (cells plus medium: Total) by rat pituitary cells incubated in the presence (GnRH) or absence (Control) of 10 nM GnRH. Cells were incubated in medium containing 1.8 mM calcium (normal Ca^{2+}), no calcium (Ca^{2+}-deficient) or no calcium plus 0.1 mM EGTA (Ca^{2+}-free) for different time periods (Expt. 2) or for 5 h (Expt. 3). N.D.: not detected. Figures are means ± SEM of five cultures.

Incubation medium	Incubation time (h)	SA (dpm/μg)											
		[^{14}C]LH						[^{3}H]LH					
		Medium		Cells		Total		Medium		Cells		Total	
		Control	GnRH	Control	GnRH	Control	GnRH	Control	GnRH	Control	GnRH	Control	GnRH
Expt. 2													
Normal Ca^{2+}	1	N.D.	18 ± 3	113 ± 9	136 ± 11	111 ± 9	120 ± 10	349 ± 42	138 ± 24	123 ± 9	183 ± 22	127 ± 9	178 ± 22
Normal Ca^{2+}	2	N.D.	44 ± 10	261 ± 22	343 ± 18	254 ± 21	262 ± 15	725 ± 109	271 ± 41	314 ± 30	612 ± 78	326 ± 30	517 ± 57
Normal Ca^{2+}	3	243 ± 35	66 ± 9	364 ± 49	507 ± 42	359 ± 48	367 ± 34	1,154 ± 151	508 ± 66	459 ± 61	894 ± 97	484 ± 62	768 ± 81
Normal Ca^{2+}	4	303 ± 38	89 ± 4	421 ± 53	634 ± 56	415 ± 51	434 ± 27	1,686 ± 253	716 ± 51	529 ± 34	1,085 ± 32	583 ± 41	949 ± 32
Normal Ca^{2+}	5	398 ± 28	132 ± 7	475 ± 38	791 ± 31	466 ± 37	538 ± 30	2,361 ± 160	1,152 ± 115	584 ± 39	1,218 ± 67	673 ± 43	1,181 ± 47
Expt. 3													
Normal Ca^{2+}	5	342 ± 23	122 ± 11	397 ± 16	686 ± 48	404 ± 26	510 ± 31	2,082 ± 81	1,259 ± 92	589 ± 75	1,106 ± 50	660 ± 75	1,153 ± 62
Ca^{2+}-deficient	5	307 ± 26	286 ± 31	369 ± 35	404 ± 31	379 ± 33	385 ± 27	1,894 ± 124	1,795 ± 93	537 ± 79	596 ± 50	619 ± 88	690 ± 51
Ca^{2+}-free	5	89 ± 19	85 ± 7	77 ± 8	73 ± 7	78 ± 8	73 ± 7	857 ± 98	817 ± 107	250 ± 33	251 ± 21	301 ± 36	290 ± 20

Uptake and incorporation of radioactive precursors into total protein

In normal Ca^{2+} medium, basal cellular uptake and incorporation of labeled precursors into total TCA-precipitable proteins increased proportionally to the incubation time (Table 2).

Neither GnRH nor Ca^{2+}-deficient medium significantly altered either pituitary cell uptake of [^{14}C]leu and [^{3}H]gln or the amounts of [^{14}C]leu- and [^{3}H]gln-labeled protein in the total system (Table 2). However, cells incubated during 5 h in medium containing 0.1 mM EGTA greatly reduced ($P < 0.01$) the uptake and incorporation of labeled precursors into total protein in both basal and GnRH-stimulated conditions (Table 2). These results suggest that the effects of EGTA on basal and GnRH-induced incorporation of radiolabeled precursors into LH and total protein may be related to its effects on cellular uptake of [^{14}C]leu and [^{3}H]gln.

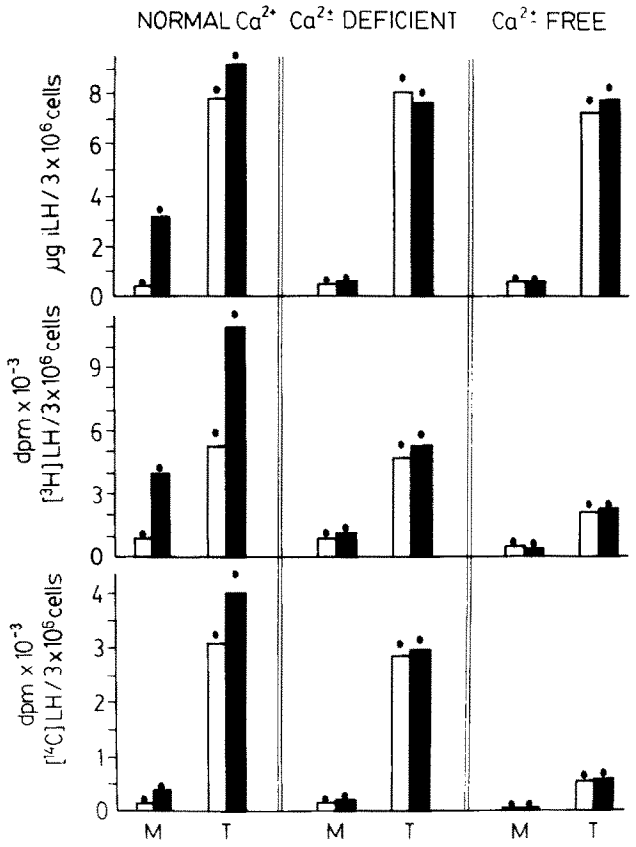

Fig. 5. Extracellular calcium dependence of GnRH-induced release and synthesis of LH. Cultured pituitary cells were incubated for 5 h in medium containing 1.8 mM calcium (normal Ca^{2+}; left), no calcium (Ca^{2+}-deficient; middle) or no calcium plus 0.1 mM EGTA (Ca^{2+}-free; right), in the presence (filled bars) or absence (open bars) of 10 nM GnRH as described under Materials and methods. Amount of total immunoassayable LH (iLH; upper panel), [^{3}H]glucosamine-labeled LH ([^{3}H]LH; middle panel) and [^{14}C]leucine-labeled LH ([^{14}C]LH; lower panel) in the medium (M) and total system (cells plus medium; T). The height of the bars gives the mean value of five cultures and the dot represents ± SEM.

TABLE 2

UPTAKE AND INCORPORATION INTO TOTAL PROTEIN OF RADIOLABELED PRECURSORS BY CULTURED RAT PITUITARY CELLS

Disintegrations per minute (dpm) of [^{14}C]leucine ([^{14}C]leu) and [^{3}H]glucosamine ([^{3}H]gln) taken up and incorporated into total protein (cells plus medium) by rat pituitary cells incubated in medium containing 1.8 mM calcium (normal Ca^{2+}), no calcium (Ca^{2+}-deficient) or no calcium plus 0.1 mM EGTA (Ca^{2+}-free) for different time periods (Expt. 2) or for 5 h (Expt. 3). Figures are means ± SEM of five cultures.

Incubation medium	Incubation time (h)	dpm × 10^{-3}/3 × 10^{6} cells			
		Uptake		Total protein	
		[^{14}C]leu	[^{3}H]gln	[^{14}C]leu	[^{3}H]gln
Expt. 2					
Normal Ca^{2+}	1	182 ± 11	204 ± 14	60 ± 3	52 ± 3
Normal Ca^{2+}	2	434 ± 25	530 ± 38	160 ± 9	116 ± 7
Normal Ca^{2+}	3	586 ± 39	625 ± 36	216 ± 18	156 ± 12
Normal Ca^{2+}	4	727 ± 43	1,006 ± 70	241 ± 16	215 ± 15
Normal Ca^{2+}	5	1,010 ± 78	1,359 ± 81	355 ± 30	332 ± 27
Expt. 3					
Normal Ca^{2+}	5	1,246 ± 89	1,397 ± 93	375 ± 25	317 ± 19
Ca^{2+}-deficient	5	1,193 ± 95	1,225 ± 88	344 ± 25	285 ± 21
Ca^{2+}-free	5	600 ± 4	899 ± 62	143 ± 8	112 ± 6
Normal Ca^{2+} plus 10 nM GnRH	5	1,380 ± 110	1,532 ± 123	413 ± 31	355 ± 30

Discussion

The present study provides evidence for the stimulatory effect of GnRH on LH translation and glycosylation (as estimated by [^{14}C]leucine and [^{3}H]glucosamine incorporation, respectively, into immunoprecipitable LH), and the involvement of calcium ion on these events, using an ovariectomized rat pituitary cell culture system.

Considerable evidence has demonstrated the ability of GnRH to stimulate iLH release. In this study, when cultured cells were stimulated with GnRH, enhanced release of iLH was both time- and dose-dependent, with maximum increase occurring at 2 h, and the ED_{50} being about 0.7 nM. In addition, increased release of iLH induced by GnRH was accompanied by increased total combined iLH content of cells plus medium. In previous studies using incubation of hemipituitary glands from ovariectomized (Apfelbaum and Taleisnik, 1976) and intact (Apfelbaum, 1981) rats, we have consistently shown that GnRH increases total content of iLH, suggesting stimulation of LH synthesis. The present experiments further support our previous data. Accordingly, using a more sensitive and precise methodology such as incorporation of labeled precur-

sors into LH, it was demonstrated that in addition to its well-known effect on LH release, GnRH also stimulated LH biosynthesis by increasing both LH polypeptide chain synthesis and glycosylation. Similar results have been reported by Ramey et al. (1987a). However, results of Khar et al. (1978) and Starzek et al. (1986) suggest that the major effect of GnRH on LH biosynthesis is to stimulate peptide chain synthesis, with a lesser effect on glycosylation. In contrast, other investigators support the idea that GnRH stimulates the glycosylation of LH but fail to demonstrate enhanced synthesis of the polypeptide portion of LH (Azhar et al., 1978; Liu and Jackson, 1985b; Vogel et al., 1986). Although the reasons for the discrepancies are not clear, differences in many variables, including endocrine status of animal donors, experimental design, cell culture procedures, LH antisera, immunoprecipitation techniques, etc., may be factors that contribute to the variability in results between laboratories. The observations made in the present study regarding the stimulatory effect of GnRH on the synthesis of the polypeptide chains of LH after short-term incubations, merit consideration. Although these results correspond qualitatively to those obtained in cultured cells from intact female rats (Khar et al., 1978; Khar and Jutisz, 1980) and intact female rats primed with estradiol (Ramey et al., 1987a, b), present data show expressed effects of GnRH using pituitary cells from ovariectomized rats in the absence of estrogenic priming.

Even though GnRH accelerates both synthesis of the polypeptide chains and glycosylation of LH, the onset and magnitude of the responses were different. The effect of GnRH on LH glycosylation was detected earlier than that on translation, and the rates of production and release of labeled LH were higher for [^{3}H]LH than for [^{14}C]LH. These findings suggest that GnRH-induced translation and glycosylation of LH are independently regulated. Some previous data may support this suggestion. Counis et al. (1987) have demonstrated that in the presence of GnRH, the rate of synthesis of the polypeptide chains of LH subunits significantly increased, regardless of whether glycosylation was blocked. Conversely, GnRH-stimulated LH glycosylation can occur, albeit at a reduced rate, under conditions in which synthesis of the polypeptide chains was totally blocked (Liu and Jackson, 1978).

Previous studies have shown an absolute requirement for extracellular calcium for GnRH stimulation or maintenance of LH release (Bourne and Baldwin, 1980; Bates and Conn, 1984; Chang et al., 1988). In the present study it is also demonstrated that removal of extracellular calcium resulted in the loss of cellular responsiveness to GnRH, preventing not only the stimulatory effects of GnRH on total and released iLH but also the GnRH-induced incorporation of both [^{14}C]leucine and [^{3}H]glucosamine into newly synthesized LH. These observations as well as those by other investigators (Liu and Jackson, 1985a; Ramey et al., 1987b) suggest that GnRH-stimulated LH glycosylation and LH apoprotein synthesis involve calcium-dependent mechanisms similar to GnRH-stimulated LH release. Incubation in Ca^{2+}-deficient (no added calcium) medium inhibited the LH response to 10 nM GnRH, while basal secretion was unaffected. Neither the uptake of radiolabeled precursors nor its incorporation into total protein were significantly altered by GnRH or calcium omission. However, further reduction of calcium by addition of 0.1 mM EGTA to Ca^{2+}-deficient medium (Ca^{2+}-free medium) not only completely abolished GnRH-induced release of LH, but also depressed uptake of precursors, and translation and glycosylation of LH and total proteins under both basal and GnRH-stimulated conditions. The difference may reflect the effects of prolonged exposure to EGTA, with complete removal of calcium from the extracellular environment as well as depletion of intracellular calcium stores to levels below those required to maintain the secretory process.

GnRH stimulated not only the biosynthesis of LH but also enhanced the release of the newly synthesized hormone. However, the specific activity (SA) of radiolabeled LH released into the medium in response to GnRH was markedly decreased compared to that in controls. This indicates that a great excess of unlabeled stored vs. labeled newly synthesized hormone has been released during GnRH stimulation. These observations are consistent with previous reports on other endocrine cells (Walker and Farquhar, 1980; Halban, 1982; Morin et al., 1984) showing that newly synthesized hormone is released preferentially under nonstimulated conditions, whereas the release of stored hormone predominates under stimulated conditions. However, when the release of unlabeled hormone by GnRH was blocked by incubation of cells in Ca^{2+}-deficient medium, the SA of radiolabeled hormone was not reduced. These data are consistent with the coexistence of constitutive and regulated secretory pathways in gonadotrophs, as proposed by Kelly (1985) for specialized cells in protein secretion. Thus, under basal conditions the constitutive component may account for the preferential release of newly synthesized LH, which apparently did not require an external stimulus which triggers exocytosis by altering the level of a cytoplasmic second messenger such as calcium. Conversely, the release of previously synthesized stored hormone by the regulated pathway required the GnRH stimulation, was dependent on extracellular calcium and occurred at a rate much higher than the synthetic rate.

In summary, the results of the present study demonstrate that in addition to the well-known effect on LH release, GnRH stimulates LH biosynthesis by pituitary cells from ovariectomized rats, as determined by incor-

poration of both [^{14}C]leucine (translation) and [^{3}H]glucosamine (glycosylation) into immunoprecipitable LH. GnRH-stimulated LH glycosylation and LH translation, like GnRH-stimulated LH release are mediated by calcium-dependent mechanisms. The release of newly synthesized and that of pre-existing stored hormone appear to be differentially regulated.

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