Signaling Responses to Pulsatile Gonadotropin-releasing Hormone in LβT2 Gonadotrope Cells*

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Rie Tsutsumi†§, Devendra Mistry†¶**, and Nicholas J. G. Webster†‡¶**‖

From the †Medical Research Service, Veterans Affairs San Diego Healthcare System, San Diego, California 92161, the **Department of Medicine and the †Biomedical Sciences and ‡Interfaces Graduate Programs, University of California, San Diego, La Jolla, California 92093, and †Tokushima University, Tokushima 770-8503, Japan

The hypothalamic neuropeptide gonadotropin-releasing hormone (GnRH) is secreted in a pulsatile fashion by hypothalamic neurons, and alterations in pulse frequency and amplitude differentially regulate gonadotropin synthesis and release. In this study, we investigated the kinetics of Gs and Gq signaling in response to continuous or pulsatile GnRH using fluorescence resonance energy transfer reporters in live mouse LβT2 gonadotrope cells. cAMP and protein kinase A-dependent reporters showed a rapid but transient increase in fluorescence resonance energy transfer signal with increasing doses of constant GnRH, and in contrast diacylglycerol (DAG) and calcium reporters showed a rapid and sustained signal.

Multiple pulses of GnRH caused multiple pulses of cAMP and protein kinase A activation without desensitization, but the DAG and calcium reporters were rapidly desensitized resulting in inhibition of calcium and DAG responses. At the transcriptional level, both a CAMDependent cAMP-response element reporter and a DAG/calium-dependent AP-1 reporter showed a pulse frequency-dependent increase in luciferase activity. However, constant GnRH stimulation gave very little cAMP-response element activation but very strong AP-1 activation. Based on these data, we propose that both the GnRH-R-Gs and Gq pathways are responsive to pulses of GnRH, but only the Gq pathway is responsive to constant GnRH. Furthermore, the Gq pathway is subject to desensitization with multiple GnRH pulses, but the Gs pathway is not.

The hypothalamic hormone gonadotropin-releasing hormone (GnRH) is the central regulator of the mammalian reproduction system. It acts in the anterior pituitary via a specific GnRH receptor (GnRH-R) on the plasma membrane of gonadotrope cells where it triggers the synthesis and secretion of LH and FSH, which in turn regulate production of gonadal steroids and reproduction (1, 2). Physiologically, GnRH is secreted in a pulsatile fashion by hypothalamic neurons (2). Gonadotrope responsiveness is modulated by both the GnRH concentration and by the frequency or pattern of its administration. During the female reproductive cycle, estrogen increases the GnRH pulse frequency and amplitude during the pre-ovulatory phase resulting in the LH surge and ovulation. Progestrone then slows and diminishes the hypothalamic GnRH pulses resulting in a preferential increase in FSH to stimulate the next round of follicle development (3). How the gonadotrope responds to the different pulse frequencies and amplitude to differentially produce LH or FSH is poorly understood.

All of GnRH effects are mediated by the GnRH-R, which is a member of the G-protein-coupled receptor family. In primary pituitary cultures, G-GH3, and LβT2 cells, the GnRH-R couples to Gs and Gq/11 but not Gi. In αT3-1 pituitary precursor cells as well as CHO-K1 and COS-7 cells, the receptor seems to couple exclusively to Gq/11 (4, 5). Coupling to Gi and G12 has also been reported (6). Several reports have also shown increases in second messengers such as cAMP, inositol 1,4,5-trisphosphate, Ca2+, DAG, and PKC with GnRH treatment (7–9). All of these studies, however, have used acute tonic treatment rather than pulsatile stimulation.

In this study, we have investigated the kinetics of Gi and Gq/11 signaling in response to GnRH pulses of varying frequency and amplitude in LβT2-immortalized gonadotrope cells as a model for how gonadotropes decode GnRH pulses. The dynamics of the cAMP-PKA and DAG-Ca2+ responses were monitored in live cells using fluorescence resonance energy transfer (FRET) reporters over >4 h. The reporters showed a rapid but transient increase in FRET signal with increasing doses of GnRH. In the context of multiple pulses, a strong FRET signal was observed with every pulse with no desensitization for the cAMP pathway, but the DAG-Ca2+ pathway was rapidly desensitized. At the level of transcriptional activation, increasing the pulse frequency caused a strong activation of both CRE-dependent and AP-1-dependent reporters; however, the response to constant GnRH treatment was dramatically different. The AP-1 reporter responded to constant GnRH similar to a high frequency pulse, but the CRE reporter responded like slow GnRH pulses.
**EXPERIMENTAL PROCEDURES**

**Materials**—GnRH was purchased from Sigma. Anti-phosphorylated CREB (Ser-133) was purchased from Cell Signaling Technology (Beverly, MA). Rabbit polyclonal anti-CREB-1 and horseradish peroxidase-linked anti-rabbit antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). The FRET plasmid plasmid AKAR2 was from Dr. Roger Y. Tsien (University of California, San Diego); plasmids AKAR3 and indicator of cAMP using Epac were from Dr. Jin Zhang (The Johns Hopkins University); plasmid DAGR was from Dr. Alexandra Newton (University of California, San Diego), and GCaMP2 was from Dr. Junichi Nakai (National Institute for Physiological Sciences, Okazaki, Japan). DMEM and fetal bovine serum were purchased from Invitrogen. CRE and AP-1 reporter plasmids were purchased from Stratagene (La Jolla, CA). All other reagents were purchased from either Sigma or Fisher.

**Cell Culture**—LβT2 cells were maintained in monolayer cultures in DMEM supplemented with 10% fetal bovine serum in a humidified 10% CO2 atmosphere at 37 °C. For FRET assay, cells were plated onto sterilized glass coverslips in 35-mm dishes coated with poly-lysine and grown to 50–90% confluency in DMEM with 10% fetal bovine serum. Cells were then transfected with the FRET reporter plasmids with FuGENE-HD transfection reagent (Roche Applied Science) or electroporated using a Microporator at 1300 V, 20-ms pulse width, and 2 pulses (BTX/Harvard Apparatus, Holliston, MA) and allowed to grow for 24–48 h before imaging.

**cAMP Immunoassay**—LβT2 cells were plated in serum-free media with 0.1% bovine serum albumin for 16 h. GnRH (1–100 nm) was added for 5 min and then removed and incubated in DMEM with 0.1% bovine serum albumin for the indicated times. The cells were rapidly washed with ice-cold phosphate-buffered saline, and the intracellular cAMP content was determined using the cAMP direct Biotrak EIA kit (GE Healthcare).

**Fluorescence Imaging**—Cells on coverslips were washed twice with Hanks’ balanced salt solution buffer with 25 mM HEPES and 1% glucose and were maintained in the dark at 37 °C. Coverslips were mounted in a temperature-controlled perfusion cell on a Zeiss Axiovert microscope with a 40×/1.3 NA oil-immersion objective lens. Typically, 3–8 fluorescent cells were analyzed in a single field. The intensity within selected cell regions of interest was measured in both cyto direct (excitation 440 and emission 480 nm) and FRET (excitation 440 and emission 535 nm) channels. Because yellow fluorescent protein is more photobleachable, the intensity within regions of interest was also measured in yellow direct channel (excitation 495 and emission 535 nm) to monitor photobleaching. Prolonged illumination was avoided to prevent photobleaching during the measurement. Exposure time was 30–240 ms, and images were taken every 10 s and processed using the SimplePCI software. The ratios of cyan-to-yellow emission for ICUE or yellow-to-cyan emissions for AKAR3, AKAR2, and DAGR were calculated at different time points and normalized by dividing all ratios by the emission ratio just 5 min before stimulation.

**PKA Kinase Assay**—Samples were prepared as for the cAMP immunoassay. PKA kinase activity was determined by PepTag assay for nonradioactive detection of cAMP-dependent protein kinase assay kit as recommended by the manufacturer (Promega, Madison, WI).

**CRE and AP-1 Reporter Assays**—LβT2 cells were maintained in 10-cm diameter dishes in DMEM-supplemented cell lysates with 10% fetal bovine serum at 37 °C with 10% CO2. On the day before the transfection experiment, LβT2 cells (3 × 105 cells per well) were plated in 12-well plates (BD Biosciences). Each well was transfected with 500 ng of CRE or AP-1 reporter plasmid and 50 ng of tk-lacZ. The following day, the cells were switched to serum-free DMEM supplemented with 0.1% bovine serum albumin. After incubation for 24 h, the cells were treated with 1, 10, or 100 nm GnRH for 5 min every 30, 60, or 120 min for 6 h. Cell lysates were assayed directly for luciferase (Luciferin, Sigma) and β-galactosidase (Galacto-Light Plus, Tropix, Bedford, MA) activity according to the manufacturer’s instructions in a 96-well plate using a Veritas microplate luminometer (Turner Biosystems, Sunnyvale, CA).

**Western Blotting**—LβT2 cells were grown to confluence in 6-well plates, washed once with phosphate-buffered saline, and incubated in serum-free medium overnight. Cells were stimulated with agonists for various times at 37 °C. Thereafter, cells were washed with ice-cold phosphate-buffered saline, lysed on ice in SDS sample buffer (50 mM Tris, 5% glycerol, 2% SDS, 0.005% bromophenol blue, 84 mM dithiothreitol, 100 mM sodium fluoride, 10 mM sodium pyrophosphate, and 2 mM sodium orthovanadate, pH 6.8), boiled for 5 min to denature proteins, and sonicated for 5 min to shear the chromosomal DNA. Equal volumes (30–40 µl) of these lysates were separated by SDS-PAGE on 10% gels and electrophoresed to polyvinylidene difluoride membranes (Immobilon-P, Millipore, Bedford, MA). The membranes were blocked with 5% nonfat dried milk in TBS/Tween (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20). Blots were incubated with primary antibodies in blocking buffer for 60 min at room temperature and then incubated with horseradish peroxidase-linked secondary antibodies followed by chemiluminescent detection. For the phospho-specific antibodies, the polyvinylidene difluoride membranes were immediately stripped by placing the membrane in stripping solution (10 mM Tris, 0.5% Nonidet P-40, 2% sodium dodecyl sulfate, 50% v/v methanol) for 10 min at room temperature and then incubated with horseradish peroxidase-linked secondary antibodies followed by chemiluminescent detection. For the phospho-specific antibodies, the polyvinylidene difluoride membranes were immediately stripped by placing the membrane in stripping buffer (0.5 mM NaCl and 0.5 mM acetic acid) for 10 min at room temperature. The membrane was then washed once for 10 min in TBS/Tween, re-blocked, and blotted with antibodies to the unphosphorylated form of the enzyme to control for equal protein loading.

**Statistics and Mathematical Modeling**—Statistics were analyzed by analysis of variance with Tukey post-tests, and FRET responses were fit to bell-shaped or exponential curves using Prism 4 (GraphPad Software, La Jolla, CA).

**Bell-shaped curves** were fit to Equation 1,

\[
y = y_{\text{max}} + \frac{(y_{\text{min}} - y_{\text{max}})}{1 + 10^{(\log t_{1/2}) - \log x + n+1}} + \frac{(y_{\text{min}} - y_{\text{max}})}{1 + 10^{(\log t_{1/2}) + n+2}}
\]

(Eq. 1)

where \(y_{\text{max}}\) is the peak of the curve; \(y_{\text{min}}\) and \(y_{\text{end}}\) are the initial and final plateaus; \(x\) is the time; \(t_{1/2}\) and \(t_{1/2}\) are the midpoints of
Pulsatile GnRH Induces $G_{s}$ and $G_{q/11}$ Signaling

FIGURE 1. GnRH pulse treatment induces pulses of cAMP generation. A, LβT2 cells were incubated in serum-free media at 37 °C for 16 h. GnRH (1, 10, or 100 nM) was then added for 5 min and then washed away, and the cells were incubated for additional times as indicated. Intracellular cAMP was determined on cell lysates by enzyme immunoassay. Data are from three separate experiments done in triplicate wells. Results are means ± S.D. Asterisks indicate significance versus untreated $p < 0.05$. B, cells were transfected with the cAMP-dependent ICUE reporter and cAMP levels monitored by FRET. Graphs show normalized FRET from cells stimulated with a single 5-min pulse of 1 nM (blue), 10 nM (green), or 100 nM (red) GnRH. Black bar indicates the period of GnRH treatment. C, graphs show normalized FRET from transfected cells stimulated with constant 1 nM (blue), 10 nM (green), or 100 nM (red) GnRH. After pulse treatment, cells were washed out with Hanks’ media with 25 mM HEPES and 1% glucose at 37 °C. Black bar indicates the period of GnRH treatment. D–F, cAMP levels in transfected cells were monitored by FRET in response to multiple GnRH pulses over a 4-h perfusion. Graphs show normalized emission ratio (cyan/yellow) from cells stimulated with GnRH at 1 nM (blue), 10 nM (green), or 100 nM (red) GnRH for 5 min every 120 min (D), every 60 min (E), or every 30 min (F). Arrows indicate GnRH pulses. Data are the mean of three to four independent assays. $G$, mean normalized FRET signal for pulses of 1, 10, and 100 nM GnRH (n = 14). H, area under the curve of mean FRET signal. *** indicates $p < 0.001$ versus 1 nM GnRH; ### indicates $p < 0.001$ versus 10 nM GnRH. I, inhibition of phosphodiesterases augments FRET signal but does not change the kinetics. Cells were transfected with the cAMP-dependent ICUE3 reporter and cAMP levels monitored by FRET. Graphs show normalized FRET from cells stimulated with 10 nM GnRH for 5 min then stimulation with 10 nM GnRH for 5 min in the presence of 50 μM isobutylmethylxanthine.

RESULTS

Pulsatile GnRH Stimulation Induces Intracellular Pulses of cAMP—We initially measured the cAMP response to a single pulse of GnRH. LβT2 cells were starved for 16 h and then stimulated with increasing doses of GnRH (0.1, 1, 10, and 100 nM) for 5 min. The GnRH was removed; the cells were then harvested at different times, and cAMP was measured by radioimmunoassay. GnRH caused a dose- and time-dependent increase in cAMP (Fig. 1A). The peak GnRH effect was observed at 10 min, and cAMP levels returned to basal levels by 60 min. No increases in cAMP were observed at the lowest dose of 0.1 nM, but a significant increase was observed at 1 nM GnRH at 10 min. Higher amplitude GnRH pulses allowed increases in cAMP to be observed earlier, and the elevations in cAMP were maintained longer. To measure the cAMP response to GnRH in real time, we used an Epac-based FRET reporter ICUE3 (10). This

Equation 2:

$$y_{inc} = (y_{max} - y_{min})(1 - e^{-x/k}) + y_{min}$$

Equation 3:

$$y_{dec} = Se^{-k_1(x-x_0)} + y_{min}$$

Equation 4:

$$y_{dec} = S_1e^{-k_{off}(x-x_0)} + S_2e^{-k_{on}(x-x_0)} + y_{min}$$
reporter contains the cAMP-binding domain from Epac (amino acids 1–881) fused between enhanced cyan fluorescent protein (N-terminal) and the citrine variant of the yellow fluorescent protein (YFP, C-terminal). Binding of cAMP reorients the enhanced cyan fluorescent protein and citrine domains causing a change in FRET. LbT2 cells were transfected with ICUE and then imaged over a period of 30 min using a temperature- and pH-controlled perfusion cell mounted on the fluorescence microscope. Cells were given increasing doses of constant GnRH (1, 10 and 100 nM) or a 5-min pulse of GnRH at the same concentrations. Constant GnRH treatment induced a dose-dependent transient increase in FRET that was maximal at 5 min and had returned to basal levels by 15 min (Fig. 1B). A similar response was obtained with a single pulse of GnRH (Fig. 1C). The dose response and time course are consistent with the measurement of cAMP levels by radioimmunoassay (Fig. 1A). Interestingly, with constant GnRH treatment the elevation of cAMP was only transient despite the continued presence of GnRH and, furthermore, did not respond to a second GnRH pulse (data not shown). Cells transfected with ICUE were then imaged using multiple pulses of GnRH over 4 h. Five-minute pulses of 1, 10, or 100 nM GnRH were administered at intervals of 30, 60, or 120 min. A strong transient FRET signal was observed with each pulse irrespective of the pulse frequency, and the amplitude of the FRET signal did not diminish with subsequent pulses indicating that this pathway does not desensitize (Fig. 1, D–F). The FRET signals for all the pulses were averaged and modeled mathematically to derive kinetic parameters for the response. The data were fit to bell-shaped curves, which were significantly different for each dose of GnRH (p < 0.0001). All GnRH doses gave maximal responses at 3 min despite the GnRH pulse continuing for 5 min (Fig. 1G). The higher doses of GnRH resulted in a faster activation of FRET (1 nM GnRH, half-life $t_{1/2(on)} = 116$ and $t_{1/2(off)} = 288$ s, slope factors $n_{H(on)} = 0.011$ and $n_{H(off)} = 0.0056$; 10 nM GnRH, $t_{1/2(on)} = 69$ and $t_{1/2(off)} = 315$ s, $n_{H(on)} = 0.021$ and $n_{H(off)} = 0.0067$; 100 nM GnRH, $t_{1/2(on)} = 85$ and $t_{1/2(off)} = 199$ s, $n_{H(on)} = 0.012$ and $n_{H(off)} = 0.0027$, respectively). Integration of the area under the FRET curve showed a significant dose-dependent increase in FRET (Fig. 1H). To investigate the origin of the rapid extinction of the cAMP signal, we tested whether phosphodiesterase inhibition would alter the response of the ICUE reporter. Cells expressing the ICUE reporter were imaged and stimulated initially with a pulse of GnRH and then with a pulse of GnRH in the presence of isobutylmethylxanthine to block cAMP hydrolysis (Fig. 1I). Phosphodiesterase inhibition caused an augmentation in the response but did not alter the kinetics, suggesting that phosphodiesterase activation is not responsible for the deactivation.

**Other G<sub>s</sub>-coupled Receptors Do Not Show the Same Pulse Sensitivity**—To test whether other G<sub>s</sub>-coupled receptors show the same kinetics, ICUE-expressing cells were imaged and stimulated with continuous GnRH, isoproterenol, or pituitary adenylate cyclase-activating polypeptide (PACAP) to activate GnRH and β-adrenergic or PAC1-R receptors, respectively, over 30 min. As a control, we stimulated cells with forskolin to activate adenylate cyclase directly. As before, GnRH caused a transient increase in FRET that was maximal at 3 min and then decreased to basal over 20 min (Fig. 2A). In contrast, PACAP, isoproterenol, and forskolin gave a sustained FRET signal over 30 min (Fig. 2A). We modeled the change in FRET mathematically. The GnRH response fit a bell-shaped curve ($t_{1/2(on)} = 56$ and $t_{1/2(off)} = 231$ s, $n_{H(on)} = 0.011$ and $n_{H(off)} = 0.0025$) similar to our earlier analysis. The isoproterenol data fit to a one-phase exponential increase ($t_{1/2} = 1.5$ min, $k_{on} = 0.0062$), but the forskolin and PACAP data fit better to bell-shaped curves ($t_{1/2(on)} = 82$ and $t_{1/2(off)} = 842$ s, $n_{H(on)} = 0.0054$ and $n_{H(off)} = 0.0011$, p < 0.0001; PACAP, $t_{1/2(on)} = 129$ and $t_{1/2(off)} = 4933$ s, $n_{H(on)} = 0.0079$ and $n_{H(off)} = 1 \times 10^{-4}$, p < 0.0001). The on-rates were similar for all agonists and likely reflect the diffusion-limited mixing of the medium containing the agonist in the chamber. With a flow rate of 0.05 ml/min and a chamber volume of 100 μl, the expected $t_{1/2(on)}$ would be ~1 min. We also tested the FRET response to a 5-min pulse of the same agonists (Fig. 2B). All agonists gave transient increases in FRET that decayed with different off-rates. GnRH gave the expected pulse signal with a maximum at 3 min that fit to a bell-shaped curve ($t_{1/2(on)} = 49$ and $t_{1/2(off)} = 311$ s, $n_{H(on)} = 0.0089$ and $n_{H(off)} = 0.0095$), but the off-rate ($n_{H(off)} = 0.0095$ versus 0.0025 for constant GnRH) was significantly faster. As with the constant GnRH treatment, the FRET signal decreases after 3 min despite the continued presence of GnRH. The responses to isoproterenol and forskolin gave FRET signals that increased exponentially to a maximum at 5 min then decreased exponentially when the agonist was removed (isoproterenol, $t_{1/2(on)} = 1.6$ min and $k_{on} = 0.007$, $t_{1/2(off)} = 0.96$ min and $k_{off} = 0.012$; forskolin, $t_{1/2(on)} = 0.96$ min and $k_{on} = 0.012$, $t_{1/2(off)} = 0.61$ min and $k_{off} = 0.019$). The on- and off-rates are again consistent with the diffusion-limited mixing and suggest that these targets rapidly activate and deactivate as the agonist is applied and then removed. It also implies that activation of β-adrenergic signaling is only determined by the availability of ligand, unlike GnRH. The FRET response to a 5-min pulse of PACAP is more complicated (Fig. 2B). The increase in FRET is consistent with the data from constant PACAP treatment, but the decrease in FRET signal upon removal of PACAP followed a two-phase exponential decay ($t_{1/2(on)} = 1.5$ min and $k_{on} = 0.0078$, $t_{1/2(off)} = 0.64$ min and $k_{off} = 0.018$, $t_{1/2(off)} = 27$ min and $k_{off} = 0.00043$) with a rapid partial loss of signal followed by a much slower loss of the remaining signal.

**Pulsatile GnRH Stimulation Induces Intracellular Pulses of PKA**—We then investigated signaling downstream of cAMP by measuring protein kinase A activation. Initially, cells were given a single 5-min pulse of 100 nM GnRH and harvested at various times, and PKA enzyme activity was measured in cell extracts. A single pulse of GnRH caused a transient increase in PKA activity that was maximal by 15 min and returned to basal by 90 min (Fig. 3A). We then performed FRET using a novel protein kinase A activity reporter AKAR3 (11). This reporter contains an N-terminal enhanced cyan fluorescent protein followed by the phosphoamino acid binding domain from FHA1, a PKA substrate peptide sequence, and a circularly permuted variant of Venus YFP. Phosphorylation of the PKA target sequence allows the FHA1 domain to bind intramolecularly, and changes the protein conformation and increasing FRET. Cells were stimulated with increasing concentrations of constant GnRH (1, 10, and 100 nM) or a single 5-min pulse of GnRH at the same
Concentrations. Constant GnRH treatment induced a dose-dependent transient increase in FRET that was maximal at 5 min and had returned to basal levels by 15 min (Fig. 3B). A similar response was obtained with a single pulse of GnRH (Fig. 3C). As before, the FRET signal with tonic GnRH rapidly returned to basal levels despite the continued presence of GnRH. Cells transfected with AKAR3 were imaged over 4 h, and 5-min pulses of GnRH were administered at intervals of 30, 60, or 120 min as before (Fig. 3D–F). A strong transient FRET signal was observed with each pulse, and the amplitude of the FRET signal did not diminish with subsequent pulses in agreement with the cAMP reporter ICUE (Fig. 1, D–F). The FRET signals for all the pulses were averaged and modeled mathematically to derive kinetic parameters for the response. The data were fit to bell-shaped curves. The on-rate $k_{on}$ and off-rate $k_{off}$ are the on- and off-rates, respectively, and $t_{1/2}$ is the half-life of the response.

FIGURE 2. Other Gs-coupled receptors do not show the same pattern of cAMP generation. Cells were transfected with the cAMP-dependent ICUE reporter and cAMP levels monitored by FRET. A, stimulation of FRET by perfusion of constant 10 nM GnRH, 10 μM isoproterenol, 20 nM PACAP, and 10 μM forskolin for 20 min. B, stimulation of FRET by perfusion of a 5-min pulse of 10 nM GnRH, 10 μM isoproterenol, 20 nM PACAP, and 10 μM forskolin. Graphs show normalized FRET (mean ± S.E.) from ICUE-transfected cells. Curves were fit to exponential increases, decreases, or bell-shaped curves. $k_{on}$ and $k_{off}$ are the on- and off-rates, respectively, and $t_{1/2}$ is the half-life of the response.
Pulsatile GnRH Induces \( G_s \) and \( G_{q/11} \) Signaling

GnRH pulse treatment induces pulses of PKA activity. A. L8T2 cells were incubated in serum-free media at 37 °C for 16 h. GnRH (1, 10, or 100 nM) was added for 5 min and then washed away, and cells were incubated for further times as indicated. PKA kinase activity was determined on cell extracts. Asterisks indicate significant difference from vehicle-treated control, \( p < 0.05 \). Data are expressed as fold basal activity (mean ± S.D.) from three samples. B, cells were transfected with the PKA-dependent AKAR3 reporter, and PKA activity was monitored by FRET. Graphs show normalized FRET from cells perfused with constant 1 nM (blue), 10 nM (green), or 100 nM (red) GnRH. Black bar indicates the period of GnRH treatment. C, graphs show normalized FRET from cells perfused with a single 5-min pulse of 1 nM (blue), 10 nM (green), or 100 nM (red) GnRH. After pulse treatment, cells were washed out with Hanks’ with 25 mM HEPES and 1% glucose at 37 °C. D-F, PKA activity in transfected cells was monitored by FRET in response to multiple GnRH pulses over a 4-h perfusion. Graphs show normalized FRET from cells stimulated with GnRH at 1 nM (blue), 10 nM (green), or 100 nM (red) GnRH for 5 min every 120 min (D), every 60 min (E), or every 30 min (F). Arrows indicate GnRH pulses. Data are the mean of three to four independent assays. G, mean normalized FRET signal for pulses of 1, 10, and 100 nM GnRH (\( n = 14 \)). H, area under the curve of mean FRET signal. * and *** indicate \( p < 0.05 \) or 0.001 versus 1 nM GnRH; ### indicates \( p < 0.001 \) versus 10 nM GnRH.

Shaped curves, which were significantly different for each GnRH dose \( (p < 0.001) \). All GnRH doses gave maximal responses at 3 min despite the GnRH pulse continuing for 5 min (Fig. 3G). The FRET signal showed similar kinetics with the three GnRH doses (1 nm GnRH, half-life \( t_{1/2(on)} = 116 \) and \( t_{1/2(off)} = 220 \) s, slope factors \( n_{H(on)} = 0.008 \) and \( n_{H(off)} = 0.0055 \); 10 nM GnRH, \( t_{1/2(on)} = 78 \) and \( t_{1/2(off)} = 220 \) s, \( n_{H(on)} = 0.012 \) and \( n_{H(off)} = 0.0046 \); 100 nM GnRH, \( t_{1/2(on)} = 87 \) and \( t_{1/2(off)} = 218 \) s, \( n_{H(on)} = 0.0099 \) and \( n_{H(off)} = 0.0027 \) respectively). Integration of the area under the FRET curve again showed a significant dose-dependent increase in FRET (Fig. 3H). These results indicated that activation of PKA is tightly coupled to cAMP second messenger generation.

Pulsatile GnRH StimulationInduces Phosphorylation of CREB—We then tested a second protein kinase A activity reporter, AKAR2, that shows rapid activation but slower deactivation kinetics as the phosphorylated site is less accessible to phosphatases (12). The AKAR2 reporter is similar to AKAR3 but contains a citrine YFP instead of the Venus domain. Constant GnRH (1, 10, and 100 nM) produced a dose-dependent increase in FRET (Fig. 4A). The response was rapid and sustained over 30 min. A single pulse of GnRH at the same concentrations caused the same rapid transient rise in FRET, but the signal decreased more slowly and did not return to basal within 30 min (Fig. 4B). The response to multiple pulses of GnRH was also noticeably different from AKAR3. At a pulse interval of 120 min, a rapid rise in FRET and a slow decay were observed (Fig. 4C). The maximal signal was dependent on the GnRH dose, as was observed for the constant stimulation, but the signal decayed at a rate that was independent of GnRH dose (\( t_{1/2} \sim 45 \) min). At 1 nM GnRH, the FRET signal had returned to basal by 90 min and by 120 min at 10 nM GnRH, but it did not reach basal before the next pulse at 100 nM GnRH. At higher pulse frequencies, this sawtooth pattern became compressed (Fig. 4D), and at the highest frequency with a pulse interval of 30 min, the stimulation approached constant stimulation (Fig. 4E). Interestingly, the multiple submaximal pulses did not cause a stepwise increase in signal suggesting that the pulses are not additive.

The finding of very different responses with the FRET reporters raised the question of the response of endogenous PKA targets. Many transcriptional responses to cAMP are mediated by PKA phosphorylation of the CREB at Ser-133 (14). Therefore, we examined the kinetics of CREB phosphorylation following a single 5-min pulse of GnRH. Cells were stimulated with increasing doses of GnRH (0, 0.1, 1, 10, and 100 nM) for 5 min and then washed and harvested at different times. CREB Ser-133 phosphorylation was measured by immunoblotting. CREB was rapidly phosphorylated at Ser-133 upon 5 min of GnRH pulse stimulation even at the lowest 0.1 nM dose of GnRH (Fig. 4F). At 1 nM GnRH, the rate of CREB dephosphorylation \( (t_{1/2} \sim 15 \) min) was intermediate between the two A-kinase activity reporters (Fig. 4G). At higher GnRH doses, the dephosphorylation was similar to the slow AKAR2 reporter \( (t_{1/2} \sim 60 \) min). Thus, we observed a rapid dose-independent phosphorylation of CREB with a slower GnRH dose-dependent dephosphorylation.

Pulsatile GnRH StimulationInduces Intracellular Pulses of DAG—The GnRH-R also signals via the \( G_{q/11} \) family of G-proteins, so we measured the DAG response to a single pulse of GnRH. To measure the response in real time, we used a PKC-\( \beta III \)-based FRET reporter DAGR (13). This reporter contains...
the DAG-binding domain from PKC-βII between cyan fluorescent protein and YFP. The FRET signal with this reporter is weaker than with the previous reporters as the conformational change is caused by binding of the PKC-βII C1 domain to the plasma membrane rather than an intramolecular conformational change. LβT2 cells were transfected with DAGR and then imaged over a period of 30 min. Cells were given increasing doses of constant GnRH (1, 10, and 100 nM) or a 5-min pulse of GnRH at the same concentrations. Constant GnRH treatment induced a dose-dependent increase in FRET that was maximal at 5 min and was maintained for the 30-min perfusion (Fig. 5A). The response to a single pulse of GnRH was transient, reaching a maximum at 5 min and then returning rapidly to basal levels. This shows not only that the DAGR reporter responds quickly to changes in DAG, but also that binding of the DAG reporter to DAG does not protect the DAG from being metabolized when GnRH is removed (Fig. 5B). Cells transfected with DAGR were then imaged using multiple pulses of GnRH over 4 h. Five-minute pulses of 10 nM GnRH were administered at intervals of 30, 60, or 120 min. A strong transient FRET signal was observed with the first pulse, but the amplitude of the FRET signal diminished with each subsequent pulse indicating that this pathway desensitizes (Fig. 5, C–E). Desensitization was evident by 60 min after the first pulse, and no further pulses were observed after 120–180 min.

**Pulsatile GnRH Stimulation Induces Pulses of Intracellular Calcium**—We also measured the calcium response in real time using a calmodulin-M13-based fluorescence reporter GCaMP2 (14). This reporter consists of a nonfluorescent split circularly permuted EGFP protein that folds to form a fluorescent protein in the presence of calcium. We and others have previously shown calcium increases, both acute spike and extended plateau phase, in LβT2 cells using calcium dyes, but these dyes are not suitable for the extended perfusion studies to measure the response to multiple pulses. Cells were transfected with GCaMP2 and then imaged over a period of 30 min. Cells were given increasing doses of constant GnRH (1, 10, and 100 nM). Constant GnRH treatment induced a dose-dependent increase in EGFP fluorescence that was maximal at 5 min and was maintained for the 30-min perfusion (Fig. 6A). The perfusion system is not rapid enough to see the acute spike-phase of calcium release, so the GCaMP2 signal likely reflects the plateau-phase calcium increase. We do not see a calcium increase at the lowest GnRH concentration, but both 10 and 100 nM GnRH give robust calcium increases. Cells transfected with GCaMP2 were then imaged using multiple pulses of GnRH over 4 h. Five-minute pulses of 100 nM GnRH were administered at intervals of 30 or 60 min. A strong transient EGFP signal was observed with the first pulse, but the amplitude of the EGFP signal diminished rapidly with each subsequent pulse (Fig. 6, B and C). Desensitization was faster than for the DAG response, being evident by 30 min after the first pulse and complete by 60 min. The multiple pulses were repeated at 10 nM GnRH. At the lower GnRH concentration, only a single
pulse of calcium was observed. As Gq/11 is subject to proteasomal degradation, we measured the calcium response in the presence of MG-132 to inhibit the proteosome. Cells were imaged with pulses of 10 nM GnRH at 30-min intervals in the presence of MG-132 (Fig. 6F). Multiple calcium increases are now observed, but the pulses are still subject to desensitization,
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**FIGURE 7. Response of CRE- and AP-1-dependent transcription to GnRH pulses.** Cells were transfected with the Cre-luciferase reporter plasmid (A) or AP-1-luciferase reporter plasmid (B) and then stimulated with multiple pulses of vehicle (white), 1 nM (blue), 10 nM (green), or 100 nM (red) GnRH at 30-, 60-, or 120-min intervals for 6 h. Parallel wells received either a single GnRH pulse or tonic GnRH. Asterisks indicate significant differences between GnRH treatment and vehicle-treated control, $p < 0.05$.

**DISCUSSION**

Even though pulsatility of GnRH is recognized as a major determinant for differential gonadotropin subunit gene expression and gonadotropin secretion, very little is known about the kinetics of the signaling circuits governing GnRH action in the pituitary. Most studies of GnRH signaling use maximal continual treatment and biochemical end points such as changes in second messengers or phosphorylation of downstream targets. Although some limited temporal information can be gleaned by taking multiple time points, this approach completely ignores the very dynamic changes in signaling following pulsatile GnRH stimulation. To address this issue, we have investigated GnRH signaling in real time in immortalized gonadotropes. Of the multiple signaling pathways activated downstream of the GnRH receptor, we studied activation of the $G_s$-cAMP-PKA and $G_q/11$-DAG-calcium signaling pathways using fluorescence reporters. To our knowledge, this is the first study to document the dynamics of these responses to pulsatile GnRH in live gonadotrope cells over an extended period. It is important to note that our imaging system allows us to see the effects of pulsatile GnRH at physiological concentrations.

We observed distinct differences in the response of the two pathways to continual GnRH. Stimulation of the $G_s$-cAMP-PKA pathway only resulted in a transient increase in cAMP and PKA activation that had returned to basal levels within 10 min, but the $G_q/11$-DAG-calcium pathway maintained the elevated second messenger levels for the entire period of perfusion (Fig. 8A). The signaling responses to multiple pulses of GnRH were also distinct. Multiple pulses of GnRH caused corresponding pulses of cAMP and PKA activation that did not diminish over the 4-h perfusion. In contrast, both DAG and calcium activation rapidly desensitized within a few pulses (Fig. 8B).

We were intrigued by the transience of the cAMP response. Inhibition of phosphodiesterases enhanced the cAMP increase but did alter the kinetics, suggesting that the transience was not due to induction of phosphodiesterase activity. This transience was only observed with GnRH stimulation as PACAP and isoproterenol gave the expected tonic increase in cAMP levels. The kinetics of the FRET response to a pulse of isoproterenol suggesting that loss of $G_q/11$ is only partially responsible for the desensitization.

**Pulsatile GnRH Stimulation Induces Pulse-dependent CRE and AP-1-dependent Transcription—**Having observed differences in the signaling response to multiple GnRH pulses, we then tested whether these differences would cause different transcriptional responses. CREB binds to the CRE that is found in many promoters. Therefore, we measured CRE-dependent transcriptional activation to assess the transcriptional response to pulsatile GnRH. CRE-luciferase was transfected into LBT2 cells that were stimulated with 5-min pulses of GnRH (0, 1, 10, and 100 nM) every 30, 60, or 120 min over 6 h, a single GnRH pulse given at the start, or constant GnRH. At 10 and 100 nM GnRH, we observed a pulse frequency-dependent increase in luciferase activity (Fig. 7A). Activation of CRE-dependent transcription was greatest at the 30-min inter-pulse interval. At the lowest dose of GnRH, only the 30-min pulse interval caused a significant increase in luciferase activity. Interestingly, the response to constant GnRH was identical to the response to a single pulse in agreement with our signaling data.

Calcium-DAG signaling activates PKC to induce transcription via TPA/AP-1-response elements. So we measured AP-1-dependent transcriptional activation. AP-1-luciferase was transfected into LBT2 cells, which were stimulated with pulses of GnRH as before. At 10 and 100 nM GnRH, we observed a pulse frequency-dependent increase in luciferase activity (Fig. 7B). Activation of AP-1-dependent transcription was greatest at the 30-min inter-pulse interval. At the lowest dose of GnRH, we did not observe a significant increase in luciferase activity nor did we observe significant increases with a single pulse of GnRH or the lowest pulse frequency of 120 min at any concentration. Interestingly and unlike the CRE reporter, the response to constant GnRH was as great as the highest pulse frequency, which agrees with the FRET signaling data.
suggested that mixing of the agonist in the imaging chamber is
the limiting factor in the response with on- and off-rates similar
to direct forskolin stimulation of adenylate cyclase. The kinetics
of the PACAP response were unexpected, but it probably
reflects that PACAP has unusual binding properties as it asso-
ciates nonspecifically with membranes through an α-helical
domain (15). The very rapid extinction of the cAMP response
when an agonist is removed is consistent with the measured in
vitro GTPase activity (4 min\(^{-1}\)) of G\(_{\alpha}\) (16).

We have previously shown that the cAMP response in the
LβT2 gonadotrope cell line is blocked by small peptides that
uncouple G\(_{\alpha}\), so we believe the observed cAMP signaling is
mediated via G\(_{\alpha}\) (17). The observed transient kinetics for elevation
of cAMP is consistent with a model in which the G\(_{\alpha}\) protein
functionally re-engages only the unliganded GnRH-R. Studies
on other receptors are consistent with this model. G\(_{\alpha}\) has been
shown to pre-couple to the unliganded prostacyclin receptor, a
predominantly G\(_{\alpha}\)-coupled receptor, but not with α\(_{2A}\)adren-
ergic receptors (18). Another study showed an increase in FRET
between the α\(_{2A}\)-adrenergic receptor and G\(_{\gamma}\) upon ligand stim-
ulation with a decrease in FRET between G\(_{\alpha}\) and G\(_{\gamma}\) consistent
with activation and release of G\(_{\alpha}\) (19).

The desensitization of the G\(_{\gamma}\) signal that we observed in
LβT2 cells has also been observed in αT3-1 cells. Pretreatment
of αT3-1 cells with 100 nm GnRH for 1 or 2 h completely elimi-
nated the subsequent inositol 1,4,5-trisphosphate response to
increasing concentrations of GnRH (20). The GnRH-R only
couples to G\(_{\alpha}\) or G\(_{\gamma}\) in αT3-1 cells, so the observed desensitization
must be the result of G\(_{\alpha}\) signaling rather than G\(_{\alpha}\) signaling (4).
We initially thought that this desensitization was related to the
known proteolytic degradation of G\(_{\alpha}\) (21, 22); however, the
desensitization is only partially prevented by MG-132, so other
mechanisms must also be involved. Two independent reports
have noted that the GnRH-R is not phosphorylated following
GnRH stimulation and does not bind β-arrestins and G-pro-
tein-coupled receptor kinases (20, 23), so classical G-protein-
coupled receptor desensitization can be eliminated. A number
of other negative feedback loops have been demonstrated for
G\(_{\alpha}\) signaling, including the induction of repressors of G\(_{\alpha}\) signaling,
including the RGS family of proteins or the phos-
phorylation and inhibition of phospholipase β1. For example,
the inhibition of a standing outward K\(^+\) current by G\(_{\gamma}\) signaling
in HEK293 cells is relieved by endogenous RGS proteins, and an
RGS-insensitive G\(_{\alpha}\) protein impairs this recovery (24). Alterna-
tively, phospholipase Cβ1 and GDP-bound G\(_{\gamma}\) are stably asso-
ciated in unstimulated PC12 and HEK293 cells (25). Activation
and GTP loading of G\(_{\gamma}\) increase the affinity of binding by 2
orders of magnitude and activate PLCβ1 but do not change the
association or localization. Interestingly, PKCα phosphoryla-
tion of PLCβ1 on serine 887 inhibits enzyme activity, but it is
not known if it alters complex assembly with G\(_{\gamma}\) (26).

A recent publication reported the rapid translocation of
NFAT-EFP to the nucleus in response to multiple pulses of
GnRH in real time in transfected HeLa and LβT2 cells (27).
Although NFAT activation is thought to be dependent on the
calcium activation of calcineurin, and indeed complete removal
of calcium did inhibit the translocation, Armstrong \textit{et al.} (27)
did not observe desensitization of NFAT translocation. NFAT
can also be activated by cAMP signaling in cardiomyocytes and
osteoclasts, however, so the observed NFAT translocation may
reflect cAMP rather than calcium signaling (28, 29). The
observed desensitization of G\(_{\alpha}\) signaling is consistent with a
published mathematical model for pulsatile LH secretion (30).
The original model predicted pulses of LH secretion that did
not desensitize but, allowing for calcium channel desensitiza-
tion and receptor internalization, produced a model in which
LH release steadily declined with each pulse (30). Our results
would argue that receptor internalization is not occurring as
cAMP/PKA responses do not desensitize, and moreover, we
would argue that the mechanism of negative feedback is at the
level of G\(_{\alpha}\)-PLC as both DAG and calcium responses
desensitize.

Many studies have shown the role of the phosphorylation
rate for the initiation of signaling, but the importance of the
dephosphorylation rate on the subsequent signal profile is
underscored by comparing the two PKA-dependent reporters.
One reporter has a very rapid dephosphorylation rate (t\(_{1/2}\) ~ 1
min) resulting in a signal that matches the acute cAMP
response, whereas the other has a slower dephosphorylation
rate (t\(_{1/2}\) ~ 45 min). This slower rate results in a sawtooth pat-
tern of signaling that approximates to a constant signal at
higher pulse frequencies. Dephosphorylation of kinase sub-
strates depends on many factors, including the strength of the
initial signal, the amount and location of the relevant phospha-
tase activity, the intramolecular accessibility of the phosphor-
ylated residue, and the sequestration of the phosphorylated res-
ides by binding proteins. All these factors will modulate the
final response and determine the pattern of the propagated sig-
nal. For example, CREB dephosphorylation was very rapid fol-
lowing a pulse of 1 nm GnRH but slowed significantly with
pulses of GnRH at 10 and 100 nm. This suggests that CREB
phosphorylation will follow each GnRH pulse at low pulse
amplitudes but will convert to a constant activation at higher
pulse frequencies and amplitudes.

The significance of these signal responses for downstream
transcriptional events is exemplified by the induction of the
CRE- and AP-1-dependent reporters. Both CRE- and AP-1-de-
dependent transcriptions are sensitive to GnRH pulse frequency
with maximal induction seen at a 30-min pulse interval, but the
response to continual GnRH is distinct. For the CRE-depen-
dent reporter, tonic stimulation with GnRH gives the same
response as a single GnRH pulse as would be expected from the
FRET data. In contrast, the AP-1-dependent reporter showed a
strong response to continual GnRH. Notably, the transcrip-
tional response to low dose GnRH (1 nm) is only observed with
cAMP-dependent transcription at the highest GnRH pulse fre-
quency. These findings would suggest that the response of
purely cAMP-dependent genes should be sensitive to pulse fre-
quency and much greater than tonic GnRH. We analyzed a
previous microarray dataset of pulse-regulated genes to identify
any whose profile correlated with our CRE reporter (31). Using
Pearson correlation (p > 0.9), the expression profiles of 23
genes correlated with the CRE reporter. Among these genes,
two are particularly noteworthy. The Ngfi-A-binding protein
Nab1 has been implicated in pulse sensing of the LHβ pro-
moter, and the MAD homolog Smad7 has been implicated in
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$Fsh\beta$ promoter activity (31, 32). We performed a similar analysis using the profile of the AP-1 reporter gene. Again using Pearson correlation ($p > 0.9$), 353 genes correlated with the AP-1 reporter. Among these genes, a number are known to, or have the potential to, regulate the gonadotropin genes, including $Egr2$, $Crem$, $Ets2$, $Inh\beta A$, and $Egr1$ as well as the AP-1 subunits c-Jun, JunB, Fra1, c-Fos, and FosB (31, 33). Most gene promoters are targets for multiple signaling pathways, so the final regulation may represent more than these two inputs, and it remains to be determined whether these genes are targets for DAG/Ca$^{2+}$ and cAMP signaling.

Pulsatile GnRH differentially regulates LH and FSH subunit genes, with faster frequencies favoring $Fsh\beta$ transcription and slower frequencies favoring $Fsh\beta$. There is evidence that some of these transcriptional effects may be mediated by the $G_s$ and $G_{q/11}$ pathways. cAMP increases GnRH stimulation of a rat $Lhb$-luciferase transgene in mouse pituitaries and the $Lhb$ promoter in LBT2 cells (34). $Egr1$ is essential for GnRH induction of $Lhb$ and female fertility in vivo (35), and the $Egr1$ gene expression requires several distinct serum-response elements/Erts elements and a cAMP-response element and is induced downstream of ERK (36). We have shown previously that both $G_s$ and $G_{q/11}$ signals contribute to ERK activation in LBT2 cells (17). There is also a connection between $G_s$ and $G_{q/11}$ signaling and $Fsh\beta$ expression. CREB serves to integrate signals for basal- and GnRH-stimulated transcription of the rat $Fsh\beta$ gene (37), and the GnRH-responsive element contains a partial CRE site that binds CREB. Others have shown that GnRH induces binding of the AP-1 complex to the $Fsh\beta$ promoter, and mutation of the AP-1 site reduces GnRH induction (33).

We conclude that the $G_s$-cAMP-PKA and $G_{q/11}$-DAG-calcium pathways downstream of the GnRH-R are very sensitive to GnRH pulse frequency and amplitude and may underlie some of the known differential effects of GnRH on the pituitary gonadotrope. Further studies will be needed to unravel how the distinct patterns of second messenger generation observed here transduce signals to regulate gonadotropin expression and secretion.

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