Gonadotropin Subunit and Gonadotropin-Releasing Hormone Receptor Gene Expression Are Regulated by Alterations in the Frequency of Calcium Pulsatile Signals*

D. J. HAISENLEDER, M. YASIN, AND J. C. MARSHALL

Division of Endocrinology, Department of Medicine, University of Virginia Health Sciences Center, Charlottesville, Virginia 22908

ABSTRACT

Previously, we have shown that intermittent calcium (Ca\(^{2+}\)) stimulates increase \(\alpha\), LH\(b\), and FSH\(b\) messenger RNAs (mRNAs), and only LH\(b\) mRNA was increased by continuous Ca\(^{2+}\). As gonadotropin subunit and GnRH receptor (GnRH-R) mRNAs are differentially regulated by alterations in GnRH pulse interval, we aimed to determine whether changes in the frequency of Ca\(^{2+}\) signals play a role in this effect. Cultured adult female rat pituitary cells in perfusion were given pulses of the Ca\(^{2+}\) channel activator BayK 8644 (10 \(\mu\)M), with 10 mM KCl in the injectate), at intervals of 16, 60, or 180 min for 24 h (vehicle pulses or 100 pM GnRH to controls). Pulsatile Ca\(^{2+}\) influx stimulated a rise in all mRNAs examined (\(P < 0.05\) vs. vehicle controls); however, optimal pulse intervals differed. \(\alpha\) and LH\(b\) mRNAs were maximally stimulated by 16- or 60-min pulses (57% and 74% increases, respectively), with 180-min pulses being less effective. In contrast, FSH\(b\) and GnRH-R mRNAs were selectively stimulated by 180-min pulses (51% and 41% increases, respectively). Pulsatile GnRH produced similar increases in GnRH-R and subunit mRNAs (53–78% vs. controls). These results reveal that alterations in the frequency of Ca\(^{2+}\) signals can regulate gonadotrope gene expression in a differential manner, producing effects similar to previous findings for GnRH. Thus, intermittent increases in intracellular Ca\(^{2+}\) may be an important step in the transmission of GnRH pulse signals from the plasma membrane to the gene. (Endocrinology 138: 5227–5230, 1997)

We and other investigators have used both \textit{in vivo} and \textit{in vitro} models to show that alterations in GnRH pulse frequency can regulate the expression of various gonadotrope genes [including the gonadotropin subunits, GnRH receptor (GnRH-R) and follistatin] in a differential manner (1–3). More specifically in rats, \(\alpha\), LH\(b\), and follistatin messenger RNAs (mRNAs) are maximally stimulated by faster (15- to 30-min) pulse intervals (1, 2), whereas FSH\(b\) and GnRH-R are increased by slower (120- to 240-min) pulse intervals (1, 3). Other results have shown that the effects of GnRH pulse frequency on gonadotropin subunit mRNA expression involve increased gene transcription (4). However, the site(s) in the intracellular signal transduction pathway that plays the key role in frequency modulation remains to be determined.

Calcium has been implicated as a major component in the mechanism of action of GnRH. Activation of the GnRH-R stimulates a transient increase in intracellular Ca\(^{2+}\) that results from inositol 1,4,5-trisphosphate-induced Ca\(^{2+}\) release from intracellular storage pools and Ca\(^{2+}\) influx from voltage-sensitive (L-type) channels (5). Recently, we have shown that alterations in intracellular Ca\(^{2+}\) play a critical role in GnRH regulation of gonadotropin subunit genes (6). Specifically, administration of the Ca\(^{2+}\) channel blocker, verapamil, inhibits the GnRH-induced rise in gonadotropin subunit mRNAs. Also, pulsatile increases in intracellular Ca\(^{2+}\) via pulses of the L-type channel activator BayK 8644 are more effective in stimulating gonadotropin subunit mRNA expression than a continuous elevation in Ca\(^{2+}\) (via treatment with the Ca\(^{2+}\) ionophore A23187).

This study was conducted to investigate whether a critical site in GnRH frequency modulation is at the level of the plasma membrane receptor or at a downstream intracellular location(s). The strategy was to bypass the receptor and determine whether intermittent increases in intracellular Ca\(^{2+}\) (at various intervals) could reproduce the stimulatory effect of GnRH pulse patterns on gonadotropin subunit and GnRH-R mRNA expression. The measurement of GnRH-R mRNA was included because this receptor plays a critical role in gonadotrope physiology, and the expression of this gene has been shown to be regulated by pulsatile GnRH in female rats \textit{in vivo} in a frequency-dependent manner [i.e. maximal increases seen with 240-min pulses, whereas faster (8-min) pulses are ineffective (3)].

Materials and Methods

\textbf{In vitro perfusion system}

Adult random cyclic female rat pituitaries were collected (using procedures approved by the University of Virginia animal research committee), dissociated, and plated for 48 h (7–8 \(\times\) 10\(^6\) cells/well) to allow attachment to plastic coverslips (22 mm) that were coated with Matrigel...
(Collaborative Biomedical Products, Waltham, MA). The in vitro procedure and culture medium constituents have been previously described (6). To allow LHβ mRNA expression in response to pulsatile GnRHI testosterone [T; at a concentration present on proestrus (7, 8); 0.5 ng/ml] was added during the last 24 h of plating and during perfusion. After plating, the coverslips were inserted into custom-made chambers and allowed to equilibrate for 1 h before initiating treatment. The perfusion flow rate was 200 μl/min, and 100-μl pulses were administered over a 10-sec duration via Autosyringe pumps (Auto-Syringe, Inc., Hooksett, NH).

**Experimental protocol**

Chambers received pulses of the Ca²⁺ channel activator, BayK 8644 plus potassium chloride [BK KCl; peak chamber concentrations, 10 μM (BK) and 10 mM (KCl)] at intervals of 16, 60, or 180 min for 24 h. This treatment paradigm was selected due to previous data showing that BK is more effective in stimulating pituitary secretion or mRNA responses in the presence of a threshold depolarization concentration of K in the medium (6, 9). Control groups received either vehicle pulses (0.2% ethanol/medium) or pulses of GnRH (peak chamber concentration, 100 pm) every 60 min. This GnRH treatment protocol was selected, as previous experiments in our laboratory had shown that it will stimulate both gonadotropin subunit and GnRH-R mRNAs expression. The complete study was conducted over 2 separate experiments (12 chambers/experiment). All treatment groups were represented in each experiment. Data from both experiments are combined and shown in the figures and table [total n per treatment group = 5, with the exception of GnRH (n = 4); Exp 1: controls, n = 3, GnRH, n = 2; BK + KCl (16 min), n = 3; BK + KCl (60 min), n = 2; BK + KCl (180 min), n = 2; Exp 2: controls, n = 2; GnRH, n = 2; BK + KCl (16 min), n = 2; BK + KCl (60 min), n = 3; BK + KCl (180 min), n = 3].

**Measurements**

To assess secretory responses over the experimental duration, perifusate samples (10-min fractions) were collected from each chamber after 3 and 21 h of treatment. LH and FSH were measured by RIA using reagents provided by the NIDDK. The RIA standards were NIDDK RP-3 (for LH) and RP-2 (for FSH).

After completion of each experiment, the cells were recovered, total RNA was extracted with phenol, and α, LHβ, FSHβ, and GnRH-R mRNAs were determined by dot blot hybridization, as previously described (3, 4). mRNA concentrations were expressed as femtomoles of complementary DNA bound per 100 μg pituitary DNA and are presented as the percent increase vs. vehicle-pulsed controls.

**Statistical analysis**

The data were analyzed by one-way ANOVA, with differences between treatment groups determined by Duncan’s multiple range test.

**Results**

Figure 1 shows the effects of vehicle, GnRH, and BK + KCl pulses on gonadotrope mRNA concentrations. Pulsatile Ca²⁺ signals stimulated a significant increase in gonadotropin subunit and GnRH-R mRNAs; however, differences were observed in the optimal pulse interval. α and LHβ mRNAs were stimulated by 16- and 60-min pulses, with maximal increases seen after the more rapid (16-min) pulses (57% increase for α and 74% increase for LHβ; P < 0.05 vs. vehicle controls). In contrast, significant increases in FSHβ and GnRH-R mRNAs were only seen after slower (180-min) Ca²⁺ pulses (FSHβ, 51% increase vs. controls; GnRH-R, 41% increase; P < 0.05). Significant differences between BK + KCl pulse interval groups were not seen for gonadotropin subunit or GnRH-R mRNAs. GnRH pulses also stimulated a rise in each mRNA examined (53–78% vs. vehicle controls; P < 0.05). With the exception of GnRH-R, the magnitude of responses to pulsatile Ca²⁺ influx were similar to that seen for GnRH.

Figure 2 shows the LH secretory pattern to pulsatile BK + KCl. As we and other investigators have shown previously (6, 10), LH secretory activity in perifused pituitary cells decreased over the 24-h experimental duration; however, responsiveness to BK + KCl or GnRH was maintained. Pulse intervals of 60 or 180 min produced a pulsatile secretory pattern that was present throughout the perfusion time course. Of interest, the pattern of the LH secretory response in chambers that received 60-min pulses of BK + KCl was similar to that seen for 60-min pulses of GnRH. A clear pulsatile LH release pattern was not seen in the 16-min interval group, which may reflect the 10-min duration over which perifusate fractions were collected. As presented in Table 1, BK + KCl pulses stimulated significant increases in LH and FSH secretion (P < 0.05 vs. vehicle controls). Although secretory responses were maintained over the experimental duration, LH and FSH release vs. that in vehicle controls decreased after 21 h of shorter (16- and 60-min) interval pulses (P < 0.05 vs. 3 h point in the same treatment group). As suggested in Fig. 2, the magnitude of LH release responses to Ca²⁺ pulses after 3 h of treatment were significantly greater than that seen for GnRH (P < 0.05). Also, LH secretory responses to BK + KCl or GnRH were greater (5- to 10-fold increases vs. controls) than those of FSH (2- to 3-fold increases).
The present investigation provides the first evidence that alterations in the Ca\(^{2+}\) signal pattern, as would occur in response to GnRH pulse stimulation, can differentially regulate gonadotrope gene expression. More specifically, \(\alpha\) and LH\(\beta\) mRNAs were stimulated by faster (16- to 60-min) pulse intervals, and FSH\(\beta\) and GnRH-R were increased by the slower (180-min) interval. Although we are not certain that the magnitude and duration of intracellular Ca\(^{2+}\) responses to pulses of BK\(+\)KCl are the same as those seen in response to GnRH, this treatment does result in a pulsatile LH secretory pattern similar in profile to that observed after GnRH pulse stimuli. As GnRH pulses produce similar mRNA responses, the results are probably of physiological significance and suggest that transient increases in intracellular Ca\(^{2+}\) may play a critical role in transmitting frequency modulatory information from the plasma membrane to the nucleus.

A recent study by Kaiser et al. (11) used GH\(3\) cells cotransfected with plasmids containing gonadotropin subunit promoter/reporter gene constructs and various concentrations of GnRH-R expression vector. The results showed that maximal ratios of \(\alpha\) and LH\(\beta\) to FSH\(\beta\) promoter responses to GnRH were seen in cells expressing higher levels of GnRH-R. The hypothesis developed from these findings was that \(\alpha\) and LH\(\beta\) are maximally stimulated by faster interval GnRH pulses via the previously described frequency-dependent rise in intracellular Ca\(^{2+}\) (12). According to this hypothesis, the increase in cell surface receptors would enhance the GnRH signal transduction pathway for \(\alpha\) and LH\(\beta\), but in-

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**TABLE 1.** LH and FSH secretory responses *in vitro* to pulsatile BayK 8644 plus KCl (BK\(+\)KCl; 16, 60, or 180 min) or GnRH (60-min interval) after 3 or 21 h of treatment

<table>
<thead>
<tr>
<th></th>
<th>LH (3 h)</th>
<th>LH (21 h)</th>
<th>FSH (3 h)</th>
<th>FSH (21 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100 ± 6</td>
<td>100 ± 7</td>
<td>100 ± 3</td>
<td>100 ± 5</td>
</tr>
<tr>
<td>GnRH</td>
<td>600 ± 56</td>
<td>488 ± 63</td>
<td>245 ± 13</td>
<td>283 ± 60</td>
</tr>
<tr>
<td>BK + KCl (16 min)</td>
<td>1033 ± 75</td>
<td>570 ± 67</td>
<td>330 ± 41</td>
<td>152 ± 42*</td>
</tr>
<tr>
<td>BK + KCl (60 min)</td>
<td>980 ± 148</td>
<td>525 ± 60</td>
<td>298 ± 57</td>
<td>166 ± 25</td>
</tr>
<tr>
<td>BK + KCl (180 min)</td>
<td>953 ± 140</td>
<td>830 ± 94</td>
<td>300 ± 46</td>
<td>208 ± 40</td>
</tr>
</tbody>
</table>

Results are presented as the percent increase vs. vehicle-pulsed control values (peak values from each chamber vs. mean value for controls). The mean ± SEM are shown. Control perifusate levels were: LH, 3.3 ± 0.2 ng/ml/100 μg pituitary DNA (3 h) and 2.2 ± 0.2 (21 h); FSH, 4.6 ± 0.3 (3 h) and 3.9 ± 0.2 (21 h).

*Values in all treatment groups are significantly increased \(P < 0.05\) vs. control values for the same time point with the exception of BK + KCl (16 min), FSH (21 h).

**Discussion**

The present investigation provides the first evidence that alterations in the Ca\(^{2+}\) signal pattern, as would occur in response to GnRH pulse stimulation, can differentially regulate gonadotrope gene expression. More specifically, \(\alpha\) and LH\(\beta\) mRNAs were stimulated by faster (16- to 60-min) pulse intervals, and FSH\(\beta\) and GnRH-R were increased by the slower (180-min) interval. Although we are not certain that the magnitude and duration of intracellular Ca\(^{2+}\) responses to pulses of BK\(+\)KCl are the same as those seen in response to GnRH, this treatment does result in a pulsatile LH secretory pattern similar in profile to that observed after GnRH pulse stimuli. As GnRH pulses produce similar mRNA responses, the results are probably of physiological significance and suggest that transient increases in intracellular Ca\(^{2+}\) may play a critical role in transmitting frequency modulatory information from the plasma membrane to the nucleus. In a previous report, we found that 60-min pulses of BK\(+\)KCl stimulated a significant increase in FSH\(\beta\) mRNA levels (6). In the present study, FSH\(\beta\) mRNA levels were also increased (28%) after 60-min pulses, but statistical significance was not obtained. Thus, similar to previous *in vivo* findings for responses to pulsatile GnRH (1), more rapid pulse intervals may be effective to some degree; however, FSH\(\beta\) mRNA is optimally stimulated by the slower BK\(+\)KCl pulse interval.

A recent study by Kaiser et al. (11) used GH\(3\) cells cotransfected with plasmids containing gonadotropin subunit promoter/reporter gene constructs and various concentrations of GnRH-R expression vector. The results showed that maximal ratios of \(\alpha\) and LH\(\beta\) to FSH\(\beta\) promoter responses to GnRH were seen in cells expressing higher levels of GnRH-R. The hypothesis developed from these findings was that \(\alpha\) and LH\(\beta\) are maximally stimulated by faster interval GnRH pulses via the previously described frequency-dependent rise in GnRH-R numbers (12). According to this hypothesis, the increase in cell surface receptors would enhance the GnRH signal transduction pathway for \(\alpha\) and LH\(\beta\), but in-
hhibit a pathway specific to FSHβ. The present data do not support the view that differential regulation of gonadotropin subunit genes is mediated solely by the number of GnRH-R. We were able to reproduce frequency-induced alterations in gonadotropin gene expression using a model in which the stimulus bypassed the GnRH receptor. Although our data clearly show that frequency modulation can occur by actions downstream from the plasma membrane, it does not exclude a role at the receptor level. Indeed, recent information relating to GnRH-induced effects on G protein expression, cross-talk between G protein-regulated systems, and rapid/acute desensitization to GnRH stimulation support a role for frequency modulation at the receptor level (13).

The mechanism(s) involved in the Ca²⁺ frequency effect on gonadotrope gene expression remains to be determined. However, alterations in intracellular Ca²⁺ can potentially influence various signal transduction pathways. Ca²⁺ plays a critical role in the activation of calmodulin kinase pathways and has been shown to affect cAMP signaling in the pituitary (5, 14). Ca²⁺ is also an important regulator of the protein kinase C pathway and is required for the activation of specific protein kinase C isoforms (i.e. α and β II) that have been identified within gonadotrope cells (15). Data in gonadotrope-derived α T-3 cells reveal that an increase in intracellular Ca²⁺ stimulates a rise in α and β II protein kinases C mRNAs (16). Recent unpublished studies (in collaboration with Dr. Margaret Shupnik, University of Virginia) have shown that BK stimulates an increase in mitogen-activated protein kinase activity in α T-3 cells, an intracellular pathway that may mediate the α-subunit transcriptional response to GnRH (17). Thus, Ca²⁺ can influence several signal transduction pathways that have been shown to regulate gonadotrope gene expression. This provides the potential for Ca²⁺ to play a pivotal role in gonadotrope responses to alterations in GnRH pulse patterns, perhaps via regulatory actions on cross-talk between signal transduction pathways.

Previous in vivo studies from our laboratory have shown that administering pulsatile GnRH to ovarioctomized, GnRH-deficient female rats does not stimulate a rise in LHβ mRNA levels unless the animals are treated with T (8). Other data reveal that the optimal serum concentration of T (0.5 ng/ml) was similar to the level observed on proestrus (7, 8), which may suggest that T plays an important role in the rapid rise in LHβ mRNA levels seen during the GnRH-induced LH surge (1). The results of recent in vitro studies have also shown that pulsatile GnRH does not increase LHβ mRNA expression in female rat pituitary cells when T is not added to the culture medium (6, 18, 19). However, the present findings reveal that pretreating the cells with a female physiological dose of T (0.5 ng/ml) allows for a LHβ mRNA response to GnRH, demonstrating that T is acting at the pituitary level. Although the mechanism(s) for this effect of T remain to be determined, it is interesting to note that T is not required for pulsatile BK to stimulate an increase in LHβ mRNA (6). This could suggest that a critical site of T action is at the plasma membrane. In support of this, a recent study found that T modulates intracellular Ca²⁺ responses to GnRH in male rat gonadotrope cells (20). If the critical site for T action is at the plasma membrane, further investigations will be needed to determine how this effect is LHβ specific.

Based on the present findings, we conclude that intermittent increases in intracellular Ca²⁺ may be an important step in the transmission of GnRH pulse signals from the plasma membrane to the gene. Also, as Ca²⁺ pulses can generally reproduce the effect of pulsatile GnRH on gonadotropin subunit and GnRH-R mRNA expression, it is likely that frequency modulation occurs at a site(s) downstream from the plasma membrane receptor.

Acknowledgments

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References