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Pulse Frequency-Dependent Gonadotropin Gene Expression by Adenylate Cyclase-Activating Polypeptide 1 in Perifused Mouse Pituitary Gonadotroph LbetaT2 Cells[1]

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ABSTRACT

We examined how pulsatile stimulation with adenylate cyclase-activating polypeptide 1 (ADCYAP1) affected gonadotrophs. In static culture, gonadotropin-releasing hormone (GnRH) stimulated transcription of all the gonadotropin subunits. In contrast, ADCYAP1 increased common alpha-glycoprotein subunit gene (*Cga*) promoter activity but failed to increase luteinizing hormone beta (*Lhb*) and follicle-stimulating hormone beta (*Fshb*) promoters. Messenger RNAs for *Lhb* and *Fshb* were slightly but significantly increased by ADCYAP1 stimulation. The results of cotreatment of the cells with GnRH and ADCYAP1 was not different from the effects of GnRH alone on *Lhb* and *Fshb* transcriptional activities as well as on mRNA expressions. To determine the effect of pulsatile ADCYAP1 stimulation on gonadotropin subunit gene expression, perifused LbetaT2 cells were stimulated either at high frequency (5-min ADCYAP1 pulse every 30 min) or at low frequency (5-min ADCYAP1 pulse every 120 min). High-frequency ADCYAP1 pulses preferentially increased *Lhb* gene expression 2.29-fold ± 0.15-fold, and low frequency pulses resulted in a 1.55-fold ± 0.16-fold increase. *Fshb* gene expression was increased 1.87-fold ± 0.3-fold by high-frequency ADCYAP1 pulses and 4.3-fold ± 0.29-fold by low-frequency pulses. These results were similar to the frequency-specific effects of pulsatile GnRH. Follistatin (*Fst*) gene expression was specifically increased by high-frequency GnRH pulses. High-frequency ADCYAP1 pulses increased *Fst* to a larger extent (4.7-fold ± 0.57-fold) than did low-frequency pulse (2.72-fold ± 1.09-fold). ADCYAP1 receptor gene (*Adcyap1r*) expression was increased significantly following pulsatile GnRH regardless of pulse frequency. Low-frequency ADCYAP1 pulses, however, increased *Adcyap1r* expression (16.49-fold ± 8.41-fold) to a larger extent than high frequency pulses did. In addition, high-frequency ADCYAP1 pulses specifically increased *Gnrhr* (GnRH receptor) expression by 4.38-fold ± 0.81-fold; however, low-frequency pulses did not result in an increase. These results suggest that ADCYAP1, like GnRH, specifically regulates *Lhb* and *Fshb* subunit gene in a pulse frequency-specific manner. This regulation may involve alteration in numbers of GnRH and ADCYAP1 receptors as well as FST expression.

follicle-stimulating hormone, gonadotropin-releasing hormone, luteinizing hormone, neuroendocrinology, pituitary

INTRODUCTION

The pituitary gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), are under the control of the hypothalamic decapeptide gonadotropin-releasing hormone (GnRH). These gonadotropins regulate gonadal development and function in vertebrates. GnRH is released in a pulsatile manner from the hypothalamus into the portal vascular system. It serves as a major regulator of the reproductive system by stimulating the synthesis and release of LH and FSH [1]. The pattern of GnRH release from the hypothalamus varies physiologically over the reproductive cycle [2, 3]. Changes in GnRH pulse frequency have been shown to differently regulate gonadotropin subunit gene expression [4]. A study using rhesus monkeys showed that rapid GnRH pulse frequencies increased the secretion of LH, whereas slower frequencies resulted in a decline in LH secretion but an increase in FSH secretion [5]. This phenomenon also has been confirmed in pituitary cell or gonadotroph cell lines. In these lines, *Lhb* gene expression is maximally stimulated by a GnRH pulse interval of 30 min, whereas *Fshb* gene expression is preferentially stimulated by a slower, 2-h GnRH pulse frequency [6, 7]. Although GnRH undoubtedly is the main regulator of gonadotropin secretion and gene expression, other factors exist that also affect gonadotropin expression.

Adenylate cyclase-activating polypeptide 1 (ADCYAP1; also known as PACAP) was first isolated from an extract of ovine hypothalamus on the basis of its ability to stimulate cAMP in rat pituitary cells [8]. It is present in two amidated forms [8]. This peptide may be involved in gonadotropin synthesis and release, either alone or in cooperation with GnRH. In the male rat, intra-atrial injection of ADCYAP1 increases the plasma level of LH [9]. Evidence that ADCYAP1 can induce gonadotropin release by a direct action at the pituitary was obtained mainly through in vitro studies using anterior pituitary cells or gonadotroph cell lines. A physiological concentration of ADCYAP1 increases LH and FSH as well as free common alpha-glycoprotein subunit (CGA) release in normal gonadotrophs [8, 10–12]. The stimulatory effects of ADCYAP1 on gonadotropin production also are evident in single gonadotroph cell models, such as αT3–1 cells or LβT2 cells [13, 14].

When bound to their respective ligands, receptors for both GnRH and ADCYAP1 initiate a complex network of signaling pathways that includes the phospholipase C (PLC)/protein kinase C (PKC)/calcium and adenylate cyclase/protein kinase A (PKA) pathways. Differences in the two signaling cascades exist, however. The predominant receptor for ADCYAP1,

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ADCYAP1 receptor 1 (ADCYAP1R), mainly couples to GNAS (known as Gs) protein and induces rapid cAMP production, which ultimately activates PKA [8]. The GnRH receptor mainly couples to GNAQ (known as Gq) to activate PLC, resulting in the rapid production of inositol phosphate, which dramatically elevates intracellular calcium elevation and activates PKC [15].

The secretion of GnRH from hypothalamic neurons occurs in a pulsatile fashion. It may be regulated by the recently identified G protein-coupled receptor GPR54 and its ligand, kisspeptin [16]. The temporal pattern of ADCYAP1 secretion and how it is regulated, however, remain poorly understood. In the rat, ADCYAP1 immunoreactivity also has been demonstrated in the nerve terminals within the median eminence with a higher ADCYAP1 concentration in the pituitary stalk [17–19]. This suggested the possibility that ADCYAP1 derived from hypothalamus acts on the anterior pituitary as a hypothalamic factor. *Adcyap1* mRNAs are widely distributed throughout the central nervous system, with the highest concentration found within the diencephalon in the lateral habenucular nucleus, the paraventricular nucleus, and the supraoptic nuclei. Because the GnRH cells have been known to be localized within these region [20], a possibility exists that ADCYAP1 and GnRH are colocalized or coordinately released. In addition, previous observations have demonstrated that ADCYAP1 was detectable in rat pituitary gonadotrophs at proestrus [21]. *Adcyap1* mRNA levels in the anterior pituitary vary across the rat estrous cycle, and *Adcyap1* mRNA levels significantly increase 3 h before the gonadotropin surge and then decline [22]. This evidence also suggested that ADCYAP1 produced within the pituitary gland works as a paracrine/autocrine factor.

Given the pulsatile nature of gonadotropin and GnRH secretion, ADCYAP1 secretion also likely varies across the reproductive cycle; however, a specific pattern of ADCYAP1 release has not yet been determined. GnRH pulse frequency-dependent expression of *Lhb* and *Fshb* has been described in association with follistatin (FST) expression, GnRH receptor concentration, and mitogen-activated protein kinase (MAPK) 3/1 (also known as extracellular signal-regulated kinases 1 and 2) activation [23–26]. Gonadotropin subunit expression also varies with different modes of ADCYAP1 stimulation [12, 27].

It remains unclear whether ADCYAP1 derived from the hypothalamus or paracrine/autocrine ADCYAP1 produced within the pituitary gland has the greatest effect on gonadotropin subunit gene expression. In the present study, we examined the effect of ADCYAP1 pulse frequency on gonadotropin subunit gene expression to distinguish between its effect and those of GnRH and ADCYAP1 using LβT2 cells as a model for the single gonadotroph. Expression levels of gonadotropin subunits, FST, and receptors for ADCYAP1 and GnRH were measured after exposure to low- and high-frequency ADCYAP1 pulses. In the present study, we used 38 amidated forms of ADCYAP1 (also known as PACAP38) based on the previous observation that PACAP38 has higher-affinity binding to rat anterior pituitary cell membranes compared to the 27 amidated forms of ADCYAP1 (also known as PACAP27) [28].

MATERIALS AND METHODS

Cell Culture: Perifusion System

The LβT2 cells (kindly provided by Dr. P.L. Mellon of the University of California, San Diego, CA) were maintained in a monolayer culture in high-glucose Dulbecco modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37°C in a humidified atmosphere of 5% CO_2 in 95% air. The perifusion system used for the present study was designed and validated in our laboratory and has been described previously [25]. Briefly, LβT2 cells were plated in perifusion chambers mounted on glass slides [29] coated with Matrigel (Becton Dickinson and Co. Labware). The cells were then incubated for 24 h in a static culture system in high-glucose DMEM containing 10% heat-inactivated FBS and 1% penicillin-streptomycin at 37°C in a humidified atmosphere of 5% CO_2 in air. The chambers were then mounted in the perifusion system and continuously perifused with high-glucose DMEM containing 1% heat-inactivated FBS and 1% penicillin-streptomycin at a constant flow rate of 0.25 ml/min. During perifusion, the cells were treated with medium alone or with pulsatile ADCYAP1 (PACAP38) or GnRH at varying frequencies (one pulse every 30 min or one pulse every 120 min). In the present study, we used PACAP38 because of its higher binding affinity to anterior pituitary cells compared to that of PACAP27 [30]. Up to nine chambers were run simultaneously. GnRH pulses were delivered by a set of peristaltic pumps controlled by a time controller (Chrontrol XT; Chrontrol Corp.). Cells were perifused for a total of 18 h, and stimulation was performed as indicated.

Reporter Plasmid Construct and Luciferase Assay

The reporter constructs used in these experiments were generated by fusing −797/+5 of the rat *Lhb* gene, −2000/+698 of the rat *Fshb* gene, or −846/0 of the human *Cga* gene cDNA in *pXP2* as described previously [31]. LβT2 cells were cotransfected by electroporation with 2.0 μg of gonadotropin subunit-Luc and pRL-TK (0.1 μg of DNA; Promega), which contained the *Renilla* luciferase under the herpes simplex virus thymidine kinase promoter. The activities of firefly luciferase and *Renilla* luciferase were measured by the Dual Luciferase Reporter Assay System (Promega) with a luminometer (TD-20/20; Promega) according to the manufacturer's protocol. The ratio of the luminescence signals of firefly luciferase to that of *Renilla* luciferase was determined.

RNA Preparation, Reverse Transcription, and Real-Time Quantitative PCR

Total RNA from untreated or treated LβT2 cells was extracted using the commercially available extraction method Trizol-S (Gibco BRL Life Technologies) according to the manufacturer's instructions. To obtain cDNA, 2.0 μg of total RNA were reverse transcribed using an oligo-dT primer (Promega) and prepared using a First Strand cDNA Synthesis Kit (Invitrogen) in reverse transcription (RT) buffer. The preparation was supplemented with 0.01 M dithiothreitol and 1 mM each of dNTP, and 200 U of RNase inhibitor/human placenta ribonuclease inhibitor (Ribonuclease Inhibitor, code 2310; Takara) in a final volume of 25 μl. The reaction was incubated at 37°C for 60 min. Quantification of *Lhb*, *Fshb*, and *Fst* mRNAs, as well as mRNAs for *Adcyap1r1* and *Gnrhr* (GnRH receptor) expression was obtained through real-time quantitative PCR (ABI Prism 7700 Sequence Detector; PerkinElmer Applied Biosystems) using Brilliant SYBR Green QPCR Master Mix (Stratagene). The PCR primers were designed based on the published sequences for *Lhb*, *Fshb* [32], *Fst* [23], *Adcyap1r* [33], and *Gnrhr* [34], whereas the internal reference *Gapdh* primer was purchased from Sigma Chemical Co. Real-time PCR amplification and product detection was performed using an ABI PRISM 7700 Sequence Detection System as recommended by the manufacturer (User Bulletin 2). The simultaneous measurement of mRNA (*Lhb*, *Fshb*, *Fst*, *Adcyap1r,* and *Gnrhr*) and *Gapdh* permitted normalization of the amount of cDNA added per sample. Each assay included a standard-curve sample in duplicate, a no-template control, and the cDNA sample from the treated LβT2 cells in triplicate. For each set of primers, a no-template control and a no-reverse transcriptase control were included. The thermal cycling conditions were as follows: 92°C for 2 min for denaturation, followed by 40 cycles of 92°C for 30 sec, 54°C for 30 sec, and 75°C for 30 sec, followed by 75°C for 5 min for extension. Reaction conditions were programmed on a Power Macintosh 7100 (Apple Computer) linked directly to the ABI PRISM 7700 sequence detector. The crossing threshold was determined using PRISM 7700 software. Postamplification dissociation curves were performed to verify the presence of a single amplification product in the absence of DNA contamination.

Statistical Analysis

All experiments were independently repeated at least three times. Each experiment was performed with triplicate samples (in real-time quantitative PCR, luciferase assays) for each experimental group. Values were expressed as the mean ± SEM. Statistical analysis was performed using the one-way ANOVA, followed by Duncan's multiple-range test. A level of $P < 0.05$ was considered to be statistically significant.

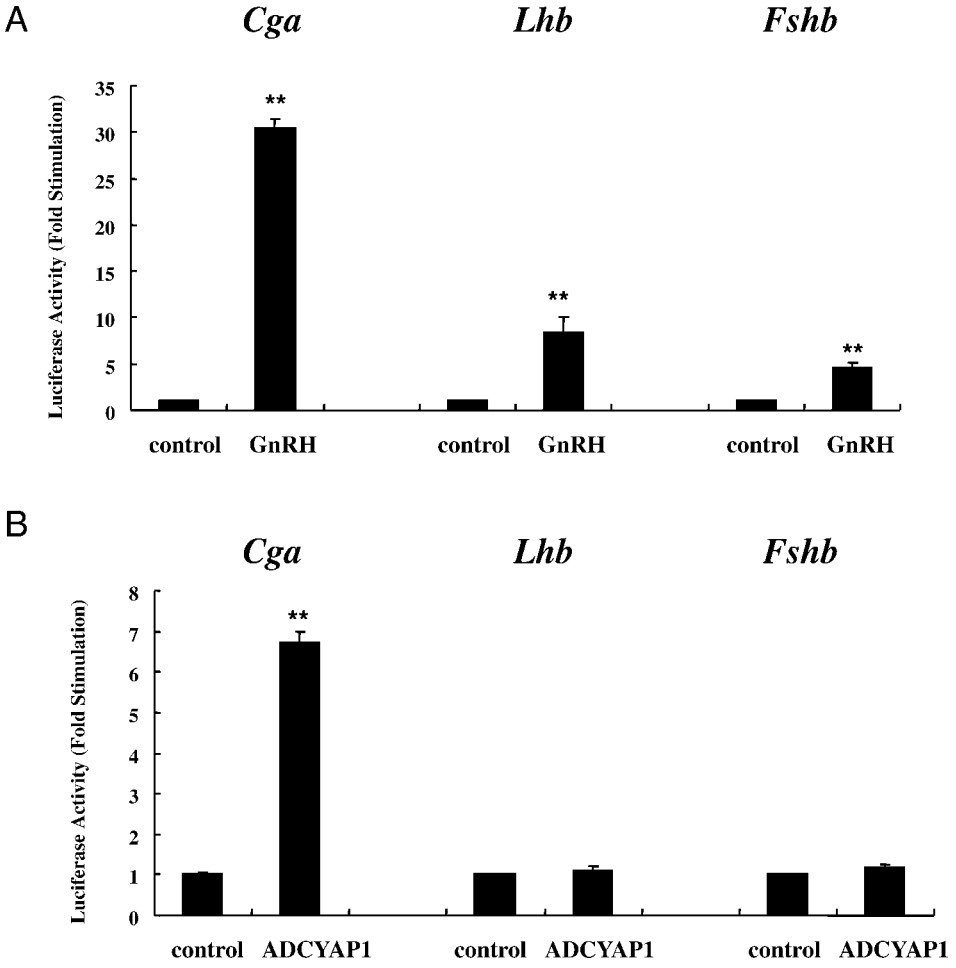

FIG. 1. Effects of GnRH and ADCYAP1 on gonadotropin subunit promoter activities in static culture. LβT2 cells were cotransfected with 0.1 μg of pRL-TK vector and 2.0 μg of luciferase vector linked with either the gonadotropin *Cga*, *Lhb,* or *Fshb* subunit and cultured in six-well plates for 48 h. The cells were then replaced with DMEM containing 1% FBS. Next, 100 nM GnRH (**A**) or 100 nM ADCYAP1 (**B**) was added directly to the culture dish, and the cells were cultured for 6 h and then assayed. The luciferase activity was measured and expressed as the fold-stimulation of the control. Values are expressed as the mean ± SEM of three independent experiments done with triplicate samples. $^{**}P < 0.01$ vs. control.

RESULTS

Effect of ADCYAP1 on Gonadotropin Subunit Transcription

We confirmed that both *Lhb* and *Fshb* gonadotropin beta subunit and *Cga* promoter activities were increased by the addition of 100 nM GnRH. In contrast, 100 nM ADCYAP1 failed to increase *Lhb* and *Fshb* promoter activities. ADCYAP1 increased only *Cga* promoters by 6.7-fold ± 0.28-fold compared to nonstimulated cells (Fig. 1).

Effects of ADCYAP1 on GnRH-Induced Gonadotropin Subunit Transcription and mRNA Expression

ADCYAP1 failed to increase both *Lhb* and *Fshb* promoter activities, as shown in Figure 1. In addition, combined treatment of the cells with GnRH and ADCYAP1 failed to modify the increasing effect of GnRH on *Lhb* and *Fshb* promoters (Fig. 2A). In mRNA determination by real-time PCR, ADCYAP1 increased *Lhb* mRNA expression 1.52-fold ± 0.25-fold and *Fshb* mRNA expression 1.37-fold ± 0.14-fold, respectively. The effects of ADCYAP1 on *Lhb* and *Fshb* mRNA expression were slight but, in both cases, statistically significant. Endogenous gonadotropin beta mRNA expression by GnRH was not affected by cotreatment with 100 nM GnRH and 100 nM ADCYAP1 (Fig. 2B).

Gonadotropin Subunit Gene Expression Following Pulsatile ADCYAP1 Stimulation

Next, pulse frequency-dependent gonadotropin subunit expression, driven by GnRH and ADCYAP1, was examined by quantitative real-time PCR. LβT2 cells were cultured in a perifusion system and stimulated with pulsatile GnRH (10 nM, 5 min/pulse) at a frequency of one pulse every 30 min or one pulse every 2 h for 18 h. These pulse frequencies were chosen based on previous studies indicating that these frequencies were optimal for *Lhb* and *Fshb* gene expression and secretion [4, 5, 26, 35]. Similar to the previous observation, *Lhb* promoter activity was stimulated to a greater extent by high-frequency GnRH pulses, whereas *Fshb* promoter activity was preferentially stimulated at the lower GnRH pulse frequency (Fig. 3A). Interestingly, pulsatile ADCYAP1 stimulation resulted in mRNA expression patterns similar to those produced by GnRH. Treatment of the LβT2 cells for 18 h with high-frequency ADCYAP1 pulses significantly increased *Lhb* gene expression by 2.29-fold ± 0.15-fold. The increase in *Lhb* at low-frequency ADCYAP1 pulse (1.55-fold ± 0.16-fold) was statistically lower compared to that at high-frequency pulses. *Fshb* gene expression was significantly increased (4.3-fold ± 0.29-fold) by ADCYAP1 pulses at a lower frequency, and this increase was statistically significant compared to *Fshb* expression at high-frequency ADCYAP1 pulse (1.87-fold ± 0.3-fold) (Fig. 3B). These results suggest that ADCYAP1, like GnRH, has the ability to stimulate gonadotropin subunit gene expression in a pulse frequency-dependent fashion. Each frequency of ADCYAP1 pulse did not increase transcriptional activities for *Lhb* and *Fshb*, and perifused continuous ADCYAP1 treatment did not down-regulate *Lhb* and *Fshb* mRNA expressions (data not shown).

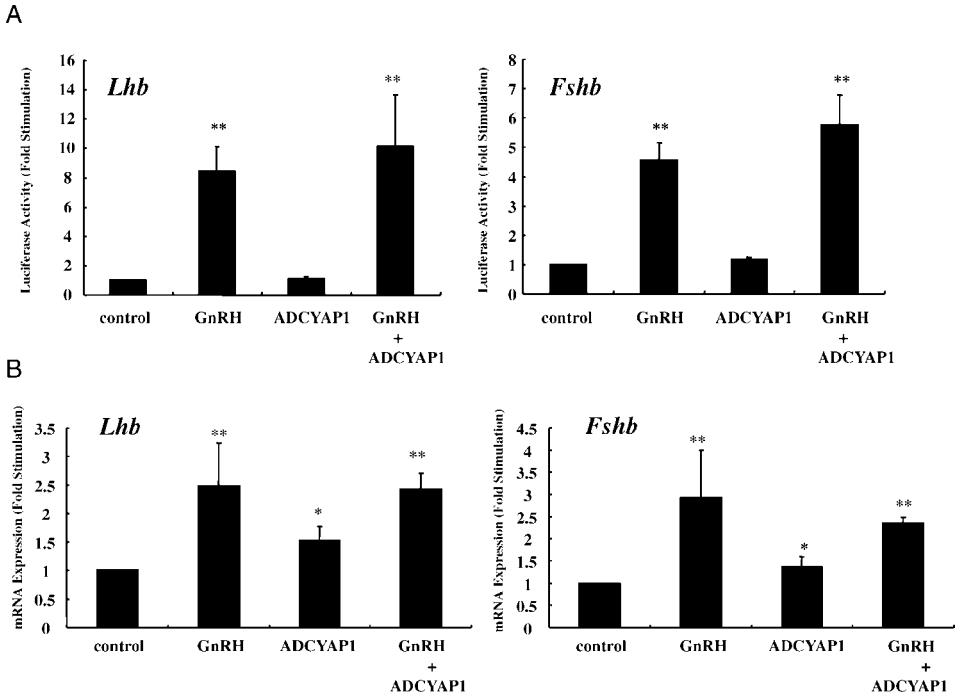

FIG. 2. Effect of ADCYAP1 on GnRH-induced gonadotropin subunit promoter activities and mRNA expressions in static culture. **A**) LβT2 cells were cotransfected with 0.1 μg of pRL-TK vector and 2.0 μg of luciferase vector linked with either the gonadotropin *Lhb* or *Fshb* subunit and cultured in six-well plates for 48 h. The cells were then replaced with DMEM containing 1% FBS. Next, 100 nM GnRH , 100 nM ADCYAP1, and both 100 nM GnRH and 100 nM ADCYAP1 were added directly to the culture dish, and the cells were cultured for 6 h and then assayed. The luciferase activity was measured and expressed as the fold-stimulation of the control. Values are expressed as the mean ± SEM of three independent experiments done with triplicate samples. **B**) The cells were then replaced with DMEM containing 1% FBS. Next, 100 nM GnRH, 100 nM ADCYAP1, and both 100 nM GnRH and 100 nM ADCYAP1 were added directly to the culture dish, and the cells were cultured for 12 h, after which their mRNA was extracted and reverse transcribed. *Lhb* and *Fshb* mRNA levels were measured with quantitative real-time PCR. Samples for each experimental group were run in triplicate, and the results are expressed as the fold-stimulation over the unstimulated group/control. Results (mean ± SEM) of three independent experiments are shown. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control.

Fst *Gene Expression Following Pulsatile ADCYAP1 Stimulation*

The role of FST in the GnRH pulse frequency-dependent induction of gonadotropin subunit genes has been reported previously [24]. As shown in Figure 3, ADCYAP1 also stimulates the specific expression of gonadotropin subunit genes in a frequency-dependent manner. Next, we examined *Fst* gene expression following pulsatile ADCYAP1 stimulation. Consistent with previous reports, pulsatile GnRH stimulation every 30 min increased *Fst* gene expression to 4.8-fold ± 0.32-fold; however, GnRH pulses every 2 h failed to increase *Fst* expression. Pulsatile ADCYAP1 stimulation every 30 min increased *Fst* expression 4.7-fold ± 0.57-fold over the increase following stimulation with a ADCYAP1 pulse every 120 min (Fig. 4).

ADCYAP1 Receptor Expression Following Pulsatile ADCYAP1 Stimulation

ADCYAP1 executes its action via three different heptahelical G protein-linked receptors: the ADCYAP1R1, VIPR1, and VIPR2 receptors. A previous study demonstrated that high-frequency GnRH pulses increased GnRH receptor mRNA expression more than low-frequency GnRH pulses did [35]. Next, we examined the expression of the *Adcyap1r1* following different frequencies of ADCYAP1 pulse stimulation. Perifused GnRH pulsatile stimulation increased *Adcyap1r1* mRNA expression similarly for both GnRH pulse frequencies (9.52-fold ± 4.78-fold and 8.90-fold ± 4.42-fold for low and high frequency, respectively) (Fig. 5A). In contrast, *Adcyap1r1* mRNA expression was affected by pulse frequency. *Adcyap1r1* mRNA expression was increased 16.49-fold ± 8.41-fold by high-frequency ADCYAP1 pulses, whereas the increase was modest (2.48-fold ± 0.28-fold) following low-frequency ADCYAP1 pulses (Fig. 5B). These results suggest that both GnRH and ADCYAP1 induce *Adcyap1r1* expression. In addition, ADCYAP1 induces its own receptor expression with low-frequency pulses.

GnRH Receptor Expression Following Pulsatile ADCYAP1 Stimulation

Low-frequency ADCYAP1 pulses specifically increased *Adcyap1r1* expression more than high-frequency pulses did. We then investigated how GnRH receptor expression was regulated by pulsatile ADCYAP1 stimulation. *Gnrhr* gene expression was significantly higher following stimulation with high-frequency ADCYAP1 pulses (every 30 min; 4.38-fold ± 0.81-fold) than following low-frequency pulses (every 120 min; 0.66-fold ± 0.52-fold) (Fig. 6). These results show that ADCYAP1 regulates GnRH receptor expression in a pulse frequency-dependent manner in LβT2 cells.

DISCUSSION

It is evident that the pituitary gonadotropins, LH and FSH, are mainly under the control of the hypothalamic peptide GnRH. Other factors, such as gonadal steroids and the gonadal peptides inhibin, activin, and FST, also regulate gonadotropins. In addition, increasing evidence supports a role for ADCYAP1 in regulating gonadotropin synthesis and release, both alone

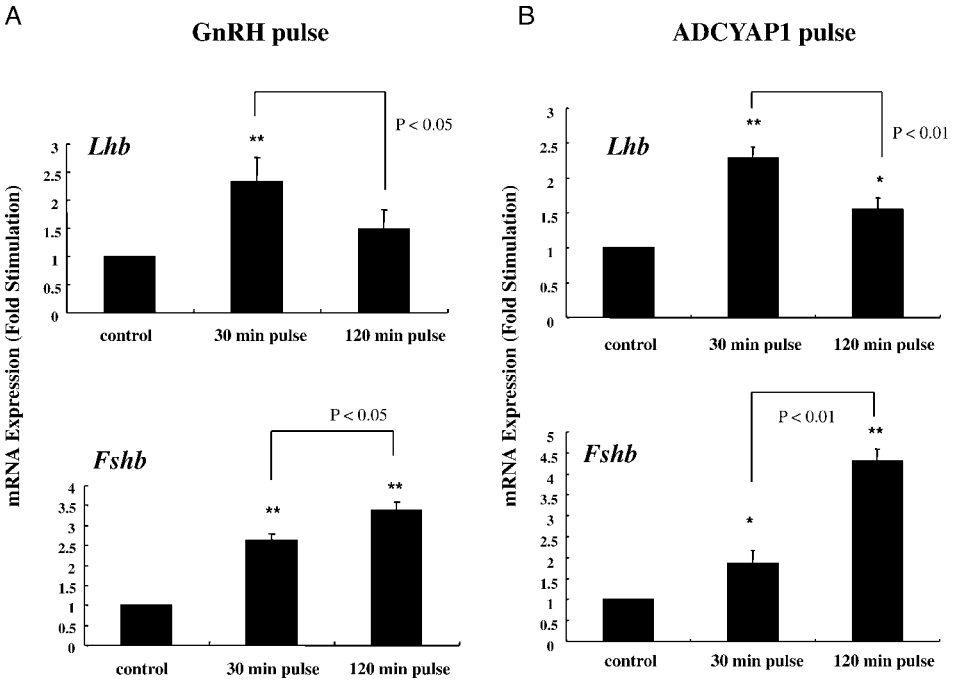

FIG. 3. Gonadotropin subunit gene expression following pulsatile stimulation with either GnRH or ADCYAP1. LβT2 cells were plated in perifusion chambers and perifused with 10 nM GnRH (**A**) or 10 nM ADCYAP1 (**B**) administered at pulse intervals of either 30 or 120 min for 18 h. After the last pulse, cells were harvested and their mRNA extracted and reverse transcribed. *Lhb* and *Fshb* mRNA levels were measured with quantitative real-time PCR. Samples for each experimental group were run in triplicate, and the results are expressed as the fold-stimulation over the unstimulated group/control. Results (mean ± SEM) of three independent experiments are shown. The differences between the 30-min GnRH pulse and the 120-min GnRH pulse in *Lhb* and *Fshb* mRNA expression were statistically significant. The differences between the 30-min ADCYAP1 pulse and the 120-min ADCYAP1 pulse in *Lhb* and *Fshb* mRNA expression also were statistically significant. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control.

and in cooperation with GnRH. For example, the intra-arterial injection of ADCYAP1 into rats results in a dose-dependent increase in the serum LH level [36]. ADCYAP1 also stimulates LH and FSH release in rat monolayer cultures [10, 37] and increases the secretory response to GnRH [10].

Although very little is known about ADCYAP1 in its physiological context, it is believed to be derived from the nerve terminals within the median eminence and delivered into the portal circulation, where it acts as a hypophysiotropic neurohormone [17–19]. In addition, previous reports have suggested that ADCYAP1 produced within the pituitary gland works as a paracrine/autocrine factor [21, 22]. It remains unclear whether ADCYAP1 derived from the hypothalamus or paracrine/autocrine ADCYAP1 produced within the pituitary gland has the greatest effect on gonadotropin subunit gene expression. In the present study, we have shown that ADCYAP1 differently regulated mRNA expression for *Lhb* and *Fshb* in gonadotroph LβT2 cells in a pulse frequency-dependent manner. In addition, the GnRH receptor and the ADCYAP1R1 also were differentially expressed, depending on ADCYAP1 pulse frequency.

The differential regulation of the gonadotropins LH and FSH by hypothalamic GnRH has been well elucidated. In rat and primate models, high-frequency GnRH pulses increased the secretion of LH, whereas slower frequencies resulted in a decline in LH secretion and a rise in FSH secretion [5]. Previous experiments using cultured pituitary cells or gonadotroph cell lines confirmed that *Lhb* gene expression was maximally stimulated by a GnRH pulse interval of 30 min, whereas *Fshb* gene expression was optimally stimulated by a GnRH pulse frequency of 2 h [25, 35]. In the present study, we again confirmed that the specific expression of either the *Lhb* or *Fshb* gene depends on GnRH pulse frequency (Fig. 2). Interestingly, pulsatile administration of ADCYAP1 also specifically increased the mRNA expression for *Lhb* and *Fshb* in a pulse frequency-dependent manner, in which high-frequency ADCYAP1 pulses (every 30 min) increased *Lhb* but lower-frequency pulses (120 min) preferentially increased

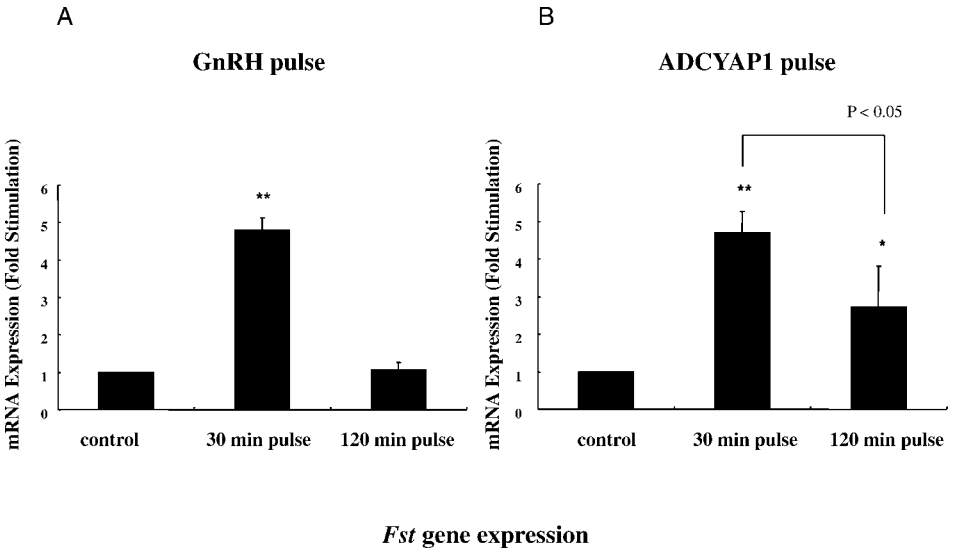

FIG. 4. *Fst* gene expression following pulsatile GnRH and ADCYAP1 stimulation. LβT2 cells were plated in perifusion chambers and perifused with either 10 nM GnRH (**A**) or 10 nM ADCYAP1 (**B**) administered at pulse intervals of 30 or 120 min for 18 h. After the last pulse, cells were harvested and their mRNA extracted and reverse transcribed. *Fst* mRNA levels were measured with quantitative real-time PCR. Samples for each experimental group were run in triplicate, and the results are expressed as the fold-stimulation over the unstimulated group/control. Results (mean ± SEM) of three independent experiments are shown. The difference between the 30-min ADCYAP1 pulse and the 120-min ADCYAP1 pulse was statistically significant ($P < 0.05$). $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control.

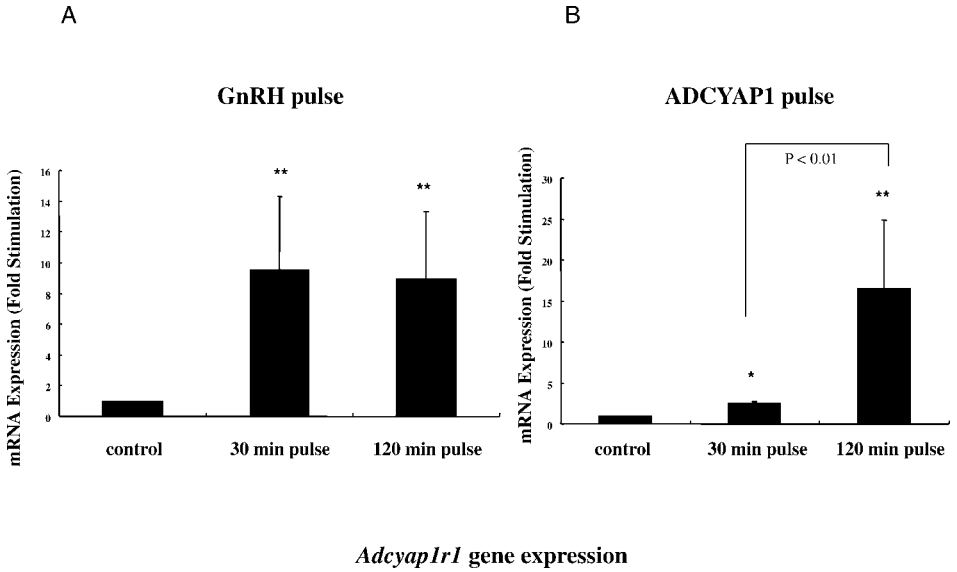

FIG. 5. *Adcyap1r1* gene expression following pulsatile GnRH and PACAP stimulation. LβT2 cells were plated in perifusion chambers and perifused with 10 nM GnRH (**A**) or 10 nM ADCYAP1 (**B**) administered at pulse intervals of 30 or 120 min for 18 h. After the last pulse, cells were harvested and their mRNA extracted and reverse transcribed. *Adcyap1r1* mRNA was measured with quantitative real-time PCR. Samples for each experimental group were run in triplicate, and the results are expressed as the fold-stimulation over the unstimulated group/control. Results (mean ± SEM) of three independent experiments are shown. The difference between the 30-min ADCYAP1 pulse and the 120-min ADCYAP1 pulse was statistically significant ($P < 0.01$). *$P < 0.05$, **$P < 0.01$ vs. control.

Fshb. ADCYAP1 failed to stimulate the transcriptional activity of the *Lhb* and *Fshb* subunits significantly in static culture. Only the *Cga* promoters were increased significantly by ADCYAP1. As determined using real-time PCR, *Lhb* and *Fshb* gene expression levels were only modestly increased compared to the *Cga* level [14]. These results are consistent with a previous observation [12]. In spite of its weak effects on *Lhb* and *Fshb* gene expression in static stimulation, ADCYAP1 effectively regulated *Lhb* and *Fshb* expression when administered in a perifused pulsatile fashion.

The pulsatile nature of GnRH secretion is well established [2, 3]; however, the pattern of ADCYAP1 release (continuous/tonic or pulsatile) has not yet been described. The effect of ADCYAP1 on *Cga*, *Lhb,* and *Fshb* mRNA expression levels depends on the mode of ADCYAP1 delivery [12, 27]. Previous reports demonstrated that pulsatile delivery of ADCYAP1 was a better signal for stimulating gonadotropin release compared with continuous delivery. In addition, in a recent rat study, anterior pituitary *adcyap1* mRNA levels varied across the rat estrous cycle with higher levels on the evening of proestrus [22]. These previous observations do not, however, provide direct evidence supporting the pulsatile release of ADCYAP1. Considering that *Gnrh* mRNA also is significantly increased in the proestrus after ovulation [38], ADCYAP1 might be released from the hypothalamus in a manner similar to that of GnRH.

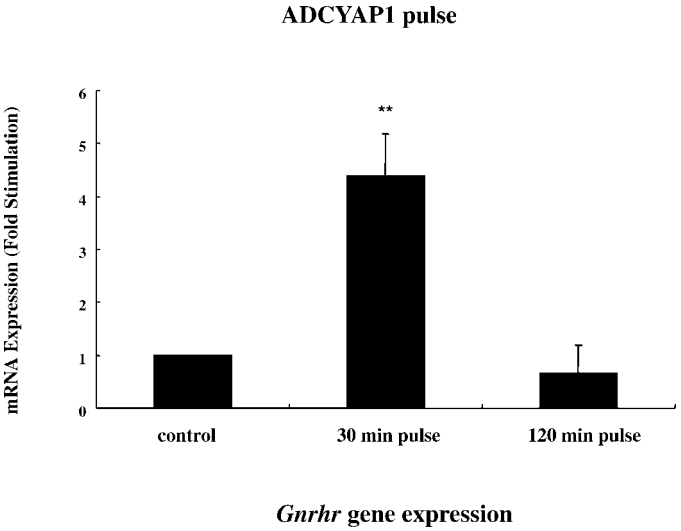

FIG. 6. *Gnrh* receptor gene expression following pulsatile GnRH or ADCYAP1 stimulation. LβT2 cells were plated in perifusion chambers and perifused with 10 nM ADCYAP1 administered at pulse intervals of 30 or 120 min for 18 h. After the last pulse, cells were harvested and their mRNA extracted and reverse transcribed. *Gnrh* receptor mRNA levels were measured with quantitative real-time PCR. Samples for each experimental group were run in triplicate, and the results are expressed as the fold-stimulation over the unstimulated group/control. Results (mean ± SEM) of three independent experiments are shown. **$P < 0.01$ vs. control.

To understand the mechanism behind the differential regulation of *Lhb* and *Fshb* by ADCYAP1, the role of FST should be considered. FST is one of the key regulators of the hypothalamic-pituitary-ovarian axis, and it is produced by both the gonadotroph and folliculostellate cells [39]. Activin, which is produced in various tissues, stimulates the synthesis of FSH by direct action [40, 41]. FST decreases *Fshb* gene expression by binding to and bioneutralizing activin [42]. The role of FST in GnRH pulse frequency-dependent gonadotropin regulation has been described previously [24, 43, 44]. High-frequency ADCYAP1 pulses stimulated *Fst* to a greater extent than low-frequency pulses did. Considering that the high-frequency ADCYAP1 pulses preferentially increased both *Lhb* and *Fst* but slower-frequency pulses decreased *Fshb* more, FST might play a role in the induction of *Lhb* or the reduction of *Fshb* subunits. Obviously, a relationship exists among GnRH, FST, and ADCYAP1. The details of their interaction, however, remain to be clarified.

Changes in the receptor numbers for GnRH and ADCYAP1 also were observed following stimulation with pulsatile GnRH or ADCYAP1. A number of factors affect the expression of the GnRH receptor, most notably GnRH itself. GnRH receptor gene expression, like the gonadotropin subunit genes, is dependent on GnRH pulse frequency [45]. ADCYAP1 also regulates the GnRH receptor by a cAMP-dependent mechanism [46–48]. In the present study, high-frequency ADCYAP1 pulses increased GnRH receptor gene expression to a greater extent than low-frequency pulses did. In this context, the relationship between GnRH receptors and gonadotropin subunit gene expression should be discussed. In the previous reports, it has been shown that when cells were transfected with increasing amounts of *Gnrh* cDNA, *Lhb* luciferase activity was increased in proportion to the number of GnRH receptors. In

contrast, *Fshb* luciferase activity was optimally stimulated when relatively low numbers of GnRH receptors were present, and it was decreased at higher numbers of GnRH receptors [31]. These results suggested the possibility that the numbers of GnRH receptor alter GnRH action, especially on gonadotropin LHB subunit.

Stimulation with either GnRH or ADCYAP1 increased *Adcyap1r1*. In the case of GnRH stimulation, the change was not frequency dependent. In contrast, lower-frequency ADCYAP1 pulses increased *Adcyap1r1* gene expression to a greater extent than high-frequency pulses did. These results imply that a relationship exists between expression of the ADCYAP1R1 and induction of the FSHB subunit.

Bedecarrats and Kaiser [25] showed that when the GnRH receptor was overexpressed, the response of *Fshb* to low-frequency GnRH pulses was eliminated. Therefore, if the cell surface ADCYAP1R1 density mediates ADCYAP1 pulse-dependent differential regulation of gonadotropin subunits, an increase in ADCYAP1R1 may negatively regulate *Lhb* gene expression. The results of this present study suggest that the pulse frequency-specific increases in GnRH and ADCYAP1 receptors in response to ADCYAP1 may be a way in which ADCYAP1 and GnRH regulate gonadotropin expression.

In this present study, we observed ADCYAP1 pulse frequency-dependent expression of the *Lhb* and *Fshb* genes. High-frequency ADCYAP1 pulse preferentially increased *Lhb*, whereas low-frequency ADCYAP1 pulses specifically increased *Fshb*. This pattern was quite similar to that produced by GnRH pulses. FST expression was preferentially increased following high-frequency pulses of either GnRH or ADCYAP1. Low-frequency ADCYAP1 pulses increased *Adcyap1r1* expression, whereas high-frequency pulses increased *Gnrh* receptor expression. It is clear that ADCYAP1 is involved in the differential regulation of gonadotropin subunit gene expression. Further studies are needed to completely clarify the relationships among GnRH, ADCYAP1, their receptors, and gonadotropin subunit expression.

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