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A mechanism for the differential regulation of gonadotropin subunit gene expression by gonadotropin-releasing hormone

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ABSTRACT **The hypothalamic hormone gonadotropin-releasing hormone (GnRH) is released in a pulsatile fashion, with its frequency varying throughout the reproductive cycle. Varying pulse frequencies and amplitudes differentially regulate the biosynthesis and secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) by pituitary gonadotropes. The mechanism by which this occurs remains a major question in reproductive physiology. Previous studies have been limited by the lack of available cell lines that express the LH and FSH subunit genes and respond to GnRH. We have overcome this limitation by transfecting the rat pituitary GH_3 cell line with rat GnRH receptor (GnRHR) cDNA driven by a heterologous promoter. These cells, when cotransfected with regulatory regions of the common α, LHβ, or FSHβ subunit gene fused to a luciferase reporter gene, respond to GnRH with an increase in luciferase activity. Using this model, we demonstrate that different cell surface densities of the GnRHR result in the differential regulation of LH and FSH subunit gene expression by GnRH. This suggests that the differential regulation of gonadotropin subunit gene expression by GnRH observed *in vivo* in rats may, in turn, be mediated by varying gonadotrope cell surface GnRHR concentrations. This provides a physiologic mechanism by which a single ligand can act through a single receptor to regulate differentially the production of two hormones in the same cell.**

The hypothalamic decapeptide gonadotropin-releasing hormone (GnRH) plays a critical role in reproductive development and function by regulating the biosynthesis and secretion of the pituitary gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH). GnRH is released into the hypophysial portal circulation and transported to the anterior pituitary, where it binds to specific, high-affinity cell surface receptors on gonadotropes, the pituitary cell type that produces gonadotropins. LH and FSH are heterodimers, each composed of a common α subunit associated noncovalently with a unique β subunit (1).

The stimulation of gonadotropin secretion by GnRH is dependent on the pulsatile nature of GnRH delivery to the anterior pituitary. Administration of exogenous GnRH in a continuous fashion results in the down-regulation of LH and FSH secretion, whereas pulsatile GnRH stimulates LH and FSH secretion, in several species, including primates and rat (2–4). Furthermore, the frequency and amplitude of GnRH pulses secreted by the hypothalamus, which vary during different phases of the menstrual or estrous cycle, regulate differentially LH and FSH secretion (5–8). Similarly, the levels of gonadotropin subunit gene expression in the rat pituitary vary by 2- to 4-fold, depending on the GnRH pulse frequency and amplitude (8–11). For example, one pulse of GnRH per hour maintains average levels of gonadotropin subunit mRNA levels and secretion. Increasing the frequency of GnRH pulses increases LHβ gene expression and the secretion of LH. Lower frequencies result in a decline in LHβ gene expression and LH secretion but a rise in FSHβ gene expression and FSH secretion. The common α-subunit gene expression is less stringently regulated by GnRH pulse frequency, but it is produced in excess and, therefore, is not a major determinant of the rate of FSH or LH biosynthesis.

cDNAs encoding the GnRH receptor (GnRHR) have recently been isolated from several species (12–16). The deduced amino acid sequence reveals a protein with seven putative transmembrane domains, characteristic of the family of guanine nucleotide-binding protein (G protein)-coupled receptors (17). The GnRHR is coupled to pertussis toxin-insensitive G proteins of the $G_{q/11}$ family (18). The cellular responses to GnRHR activation include inositol phospholipid turnover, which leads to calcium mobilization and protein kinase C activation (19, 20).

The mechanism by which GnRH is able to regulate differentially LH and FSH biosynthesis and secretion is unknown. Previous studies have been limited by the lack of available cell lines that express the LH and FSH subunit genes and respond to GnRH. We have taken advantage of the availability of the GnRHR cDNA to facilitate studies of the mechanisms of action of GnRH. GH_3 cells are a well-characterized pituitary cell line generated from a rat pituitary adenoma that express prolactin (PRL) and growth hormone in a regulated fashion (21). These cells express thyrotropin-releasing hormone (TRH) receptors endogenously and respond to TRH with an increase in PRL gene expression, biosynthesis, and secretion (22). Like the GnRHR, the TRH receptor is a member of the family of G protein-coupled receptors and is coupled to pertussis toxin-insensitive G proteins of the $G_{q/11}$ family (23). Furthermore, like GnRH, TRH activates its receptor to stimulate inositol phospholipid turnover, leading to calcium mobilization and protein kinase C activation (24). Thus, the effects of TRH and GnRH appear to be mediated through similar intracellular signal transduction pathways.

We hypothesized that GH_3 cells may present a useful model for the study of the mechanisms of GnRH action. We have therefore transfected the GH_3 cell line with the rat GnRHR cDNA driven by the cytomegalovirus promoter. These cells bind GnRH specifically and with high affinity and are able to respond to a GnRH agonist with an increase in PRL promoter activity, mRNA levels, and secretion. In addition, when co-

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Abbreviations: GnRH, gonadotropin-releasing hormone; GnRHR, GnRH receptor; GnRHAg, des-Gly^{10}, [D-Ala^6]-GnRH ethylamide (GnRH agonist); FSH, follicle-stimulating hormone; LH, luteinizing hormone; G protein, guanine nucleotide-binding protein; PRL, prolactin; TRH, thyrotropin-releasing hormone.

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transfected with the regulatory region of the human gonadotropin α-subunit gene fused to a luciferase reporter gene (αLUC), these cells respond to either TRH or GnRH with an increase in luciferase activity (25–27). Moreover, GH_3 cells transfected with the gonadotropin subunit genes have been used for the study of the regulation of gonadotropin secretion; the regulation of LH and FSH secretion appears to be similar in these cells to primary pituitary cells, providing affirmation of their value as a model of gonadotrope function (28). We therefore used these cells as a model for the study of the regulation of gonadotropin subunit gene activity by GnRH.

MATERIALS AND METHODS

Materials. The GnRH agonist des-Gly^{10}, [D-Ala^6]-GnRH ethylamide (GnRHAg) and TRH were purchased from Sigma. ^{125}I-labeled buserelin {des-Gly^{10}, [[^{125}I]iodoTyr^5, *tert*-butyl-D-Ser^6]-GnRH ethylamide} was obtained from Hoechst–Roussel.

Reporter Genes and Expression Vectors. An expression vector encoding the rat GnRHR was prepared by subcloning the rat GnRHR cDNA sequence into pcDNA1 (Invitrogen), an expression vector containing a cytomegalovirus promoter for high levels of expression (25). The reporter constructs used had the regulatory regions of the human α (29) (−846/0; i.e., base pairs −846 to 0 relative to the transcriptional start site), rat LHβ (30) (−791/+5), and rat FSHβ (31) (−2000/+1709) genes fused to the luciferase gene in the pXP2 vector (32) (αLUC, LHβLUC, and FSHβLUC, respectively). The αLUC plasmid was kindly provided by J. Larry Jameson. The LHβ construct was prepared by PCR amplification of the −791/+5 region, which was then subcloned into the *Bam*HI/*Hin*dIII polylinker restriction sites in pXP2. The FSHβ construct was prepared by subcloning the *Hin*dIII/*Bal* I genomic fragment (−2000/+1709) into pXP2. An expression vector expressing β-galactosidase driven by the Rous sarcoma virus promoter (RSV-βGAL) was used as an internal standard and control (33).

Cell Culture and Transfection. GH_3 cells were maintained in monolayer culture in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) fetal bovine serum at 37°C in humidified 5% CO_2/95% air. Cells were transfected by electroporation. In each experiment, ≈5 × 10^6 GH_3 cells were suspended in 0.4 ml of Dulbecco's phosphate-buffered saline (PBS) plus 5 mM glucose, containing the DNA to be transfected. The cells received a single electrical pulse of 240 V from a total capacitance of 1000 μF, using an Invitrogen electroporator II apparatus. After electroporation, cells were plated in serum-containing medium. Twenty-four hours after transfection, medium was replaced. Cells were treated with hormones as indicated for various intervals and harvested 48 h after electroporation. Cells were harvested and lysed in 125 mM Tris·HCl, pH 7.6/0.5% (vol/vol) Triton X-100 buffer. Luciferase and β-galactosidase assays were performed as described (33, 34). Luciferase activities were expressed relative to levels of β-galactosidase activity.

GnRH Binding Assay. The GnRH analog binding assay was conducted using a range of concentrations of ^{125}I-labeled buserelin in DMEM/0.1% bovine serum albumin prepared as reported (35). One hundred microliters of a cell suspension (1–2 × 10^6 cells) was added to each tube, and the assay was allowed to come to equilibrium (2–3 h at 4°C) in a final volume of 600 μl. Binding was terminated by layering each sample over 2 ml of DMEM/0.3 M sucrose at 4°C and centrifuging at 2000 × *g* for 10 min at 4°C. The supernatant was aspirated and discarded. The cell pellet was resuspended in 1 ml of PBS, and radioactivity was determined in a Beckman 5500 γ counter. Nonspecific binding of ^{125}I-labeled buserelin was determined using cells transfected with pcDNA1 vector DNA.

Statistical Analysis. Data were analyzed by Student's *t* test for independent samples when appropriate. A value of $P < 0.05$ was considered statistically significant. The errors in the ratios were calculated by standard methods of propagation of errors in computation (36).

RESULTS

Expression of Gonadotropin Subunit Gene Reporter Constructs in GH_3 Cells and Regulation by GnRH. We transiently transfected GH_3 cells with reporter constructs in which regulatory regions of the human α, rat LHβ, and rat FSHβ genes were fused to the luciferase gene (αLUC, LHβLUC, and FSHβLUC, respectively). When transfected into GH_3 cells, expression levels were significantly higher than those of the promoterless luciferase vector (pXP2) (32) alone—250-fold higher for αLUC, 5-fold higher for LHβLUC, and 12-fold higher for FSHβLUC. Cotransfection of the GnRHR cDNA resulted in the additional specific stimulation of luciferase activity by GnRHAg of 10-fold for αLUC, 8-fold for LHβLUC, and 4-fold for FSHβLUC. TRH, in turn, was also able to stimulate the expression of the gonadotropin subunit gene reporter constructs in these transfected cells, although to

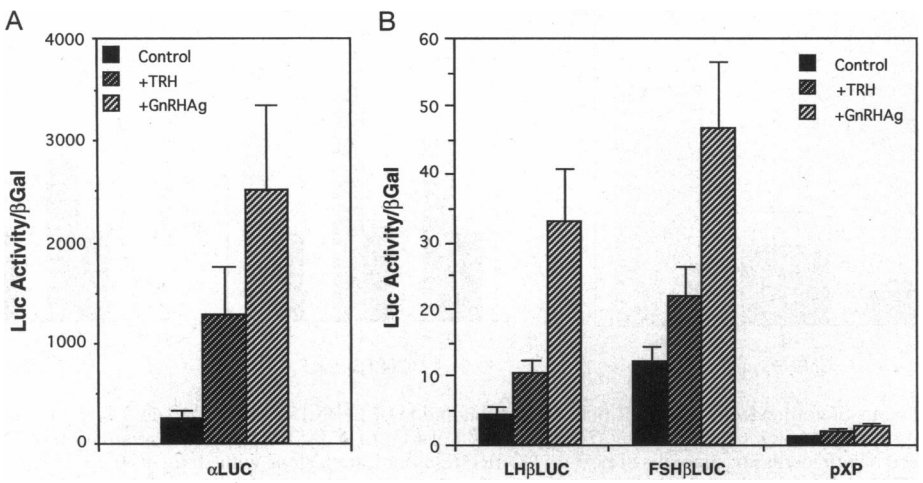

FIG. 1. Basal and TRH- and GnRH-stimulated expression of αLUC (*A*) and LHβLUC, FSHβLUC, and pXP2 (*B*) in GH_3 cells. Cells were cotransfected with 20 μg of αLUC, LHβLUC, FSHβLUC, or pXP2, 20 μg of pcDNA1-GnRHR, and 9 μg of RSV-βGAL. Cells were treated with 100 nM GnRHAg, 1 μM TRH, or vehicle for 6 h prior to harvesting. All experiments were repeated at least three times. Each bar represents the mean ± SEM for nine samples from three independent experiments.

a lesser degree than GnRHAg (5-fold for αLUC, 2-fold for LHβLUC, and 2-fold for FSHβLUC) (Fig. 1).

Differential Influence of GnRHR Density on the Regulation of Gonadotropin Subunit Gene Expression by GnRH. The magnitude of stimulation of gonadotropin subunit promoter activity in response to GnRH in these cells is influenced by the number of GnRHRs expressed. When these cells were cotransfected with LHβLUC and progressively increasing amounts of GnRHR cDNA, the degree of stimulation of luciferase activity by GnRH was increased in proportion to the number of GnRHRs—i.e., the more receptor cDNA transfected, the greater the fold increase in luciferase activity in response to GnRH stimulation (Fig. 2*A*). The same was true for αLUC activity (Fig. 2*B*). In contrast, FSHβLUC activity was optimally stimulated when relatively low numbers of GnRHRs were expressed; interestingly, at higher numbers of cell surface GnRHRs, the magnitude of stimulation of FSHβLUC by GnRH was decreased (Fig. 2*C*). We have confirmed that when GH_3 cells are transiently transfected with progressively increasing amounts of GnRHR cDNA, the average number of GnRHR expressed on the cell surface, as determined by a GnRH binding assay, also increases (Fig. 2*D*). Furthermore, these effects are specific to the GnRH response and are not a nonspecific effect of the transfection: the total amount of DNA transfected was kept constant in all experiments, and the stimulation of the subunit genes by TRH was unaffected by the number of GnRHRs (Figs. 2 *E* and *F*).

As a result of these differences in the regulation of LHβ and FSHβ subunit gene expression by GnRH at different densities of GnRHR, the ratio of LHβ to FSHβ subunit gene expression varies, depending on the cell surface concentration of GnRHR (Fig. 3). At low concentrations of GnRHR expression, the degree of stimulation of LHβ and FSHβ promoter activities by GnRH is approximately equivalent, whereas at higher concentrations of GnRHR, LHβ is stimulated by GnRH to a much greater degree than FSHβ. This change in the ratio of LHβ to FSHβ stimulation by GnRH is similar to that observed *in vivo* under conditions of varying GnRH pulse frequencies (8).

DISCUSSION

GH_3 cells appear to be a useful model for the study of the regulation of expression of the gonadotropin subunit genes by GnRH. The exogenous α, LHβ, and FSHβ subunit genes are expressed, albeit at low levels, in this cell line. Furthermore, the activities of these reporters are stimulated in response to GnRH to a similar extent compared to the responses of primary pituitary gonadotropes to GnRH. In addition, desensitization of the response to continuous GnRH does occur in these cells (25). Admittedly, the kinetics of desensitization may be different in GH_3 cells than in primary pituitary cells, as has been shown for responsiveness of PRL secretion to TRH (37). Nonetheless, the heterologous GH_3 cell system that we have used has an advantage over primary pituitary cells, in that the expression of the GnRHR is driven by a cytomegalovirus promoter, which is not regulated by GnRH. The concentration of GnRHRs can therefore be externally manipulated and is independent of the mode of administration of GnRH. In this way, we can study the effects of GnRHR concentrations on gonadotropin subunit gene expression independent of GnRH

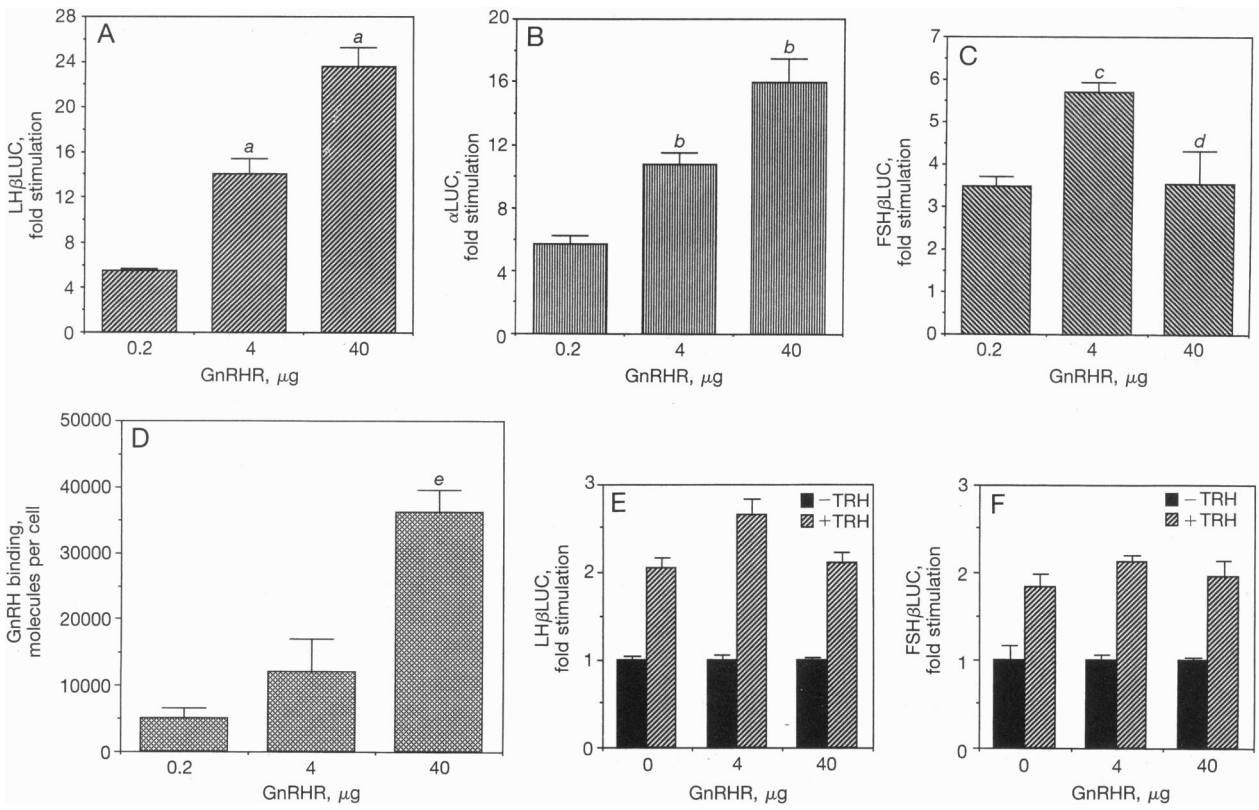

FIG. 2. Effects of amounts of cotransfected GnRHR plasmid on stimulation of LHβLUC by GnRH (*A*), stimulation of αLUC by GnRH (*B*), stimulation of FSHβLUC by GnRH (*C*), GnRH binding (*D*), stimulation of LHβLUC by TRH (*E*), and stimulation of FSHβLUC by TRH (*F*). GH_3 cells were cotransfected with increasing amounts of pcDNA1-GnRHR, as indicated, along with 20 μg of αLUC, LHβLUC, or FSHβLUC and 12 μg of RSV-βGAL. pcDNA1 vector DNA was added as needed so that the total amount of DNA in each transfection was constant. Cells were then treated with 100 nM GnRHAg, 1 μM TRH, or vehicle for 6 h prior to harvesting. Each experiment was repeated at least three times, in duplicate or triplicate. Each value represents the mean ± SEM for four to nine samples. *a*, $P < 0.025$ vs. preceding GnRHR amount; *b*, $P < 0.05$ vs. preceding GnRHR amount; *c*, $P < 0.01$ vs. 0.2 μg of GnRHR; *d*, $P < 0.01$ vs. 4 μg of GnRHR. Each value of GnRH analog (buserelin) binding activity in *D* represents the mean ± SEM for five or six samples, from two independent experiments. *e*, $P < 0.005$ vs. 4 μg of GnRHR.

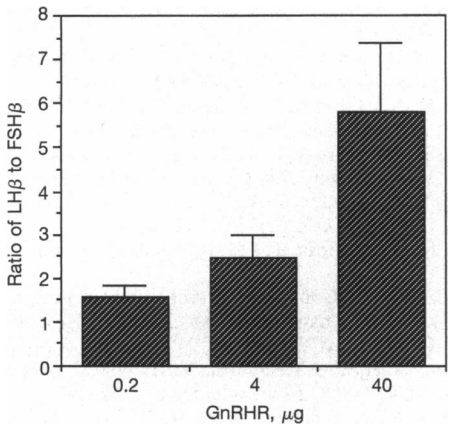

FIG. 3. Effect of the amount of GnRHR cDNA transfected on the ratio of the degree of stimulation of LHβLUC activity by GnRH to the degree of stimulation of FSHβLUC activity by GnRH. As the number of GnRHRs expressed increases, the degree of stimulation of LHβ also increases, relative to FSHβ.

pulse frequency. Furthermore, GH_3 cells do not express the gonadal peptides inhibin, activin, and follistatin. These peptides are produced not only in the gonads but also in the pituitary gland and regulate FSH biosynthesis and secretion with relatively little effect on LH (38–40). These peptides have been postulated to play a role in the differential regulation of LH and FSH. Our experiments suggest that GnRH is capable of differentially regulating LH and FSH independent of these gonadal peptides.

The expression of the α and LHβ subunit genes is optimally stimulated at relatively high cell surface densities of GnRHRs, whereas FSHβ gene expression is optimally stimulated at lower cellular concentrations of the receptor. These data suggest that the mechanisms by which GnRH regulates α and LHβ gene expression may be distinct from those by which FSHβ gene expression is regulated. Furthermore, the signal transduction pathways activated by GnRH may be different at low vs. high GnRHR numbers. At low concentrations of the GnRHR, stimulation with GnRH results in the stimulation of all three subunit genes. This may occur through a single signal transduction pathway, resulting in the activation of the same transcription factor(s), which binds to the same or similar cis-acting DNA elements in all three genes. At high concentrations of the GnRHR, the α and LHβ subunit genes are further stimulated, which could be attributed to greater activation of the same stimulatory pathway. In contrast, the FSHβ gene is relatively inhibited, suggesting that a second signal transduction pathway has been activated, which results in the specific inhibition of the FSHβ gene, with no effects on the α and LHβ genes (Fig. 4).

The details of the different intracellular signaling pathways activated by GnRH at low vs. high GnRHR numbers remain to be elucidated. Cross-talk between G proteins has been reported: a single G protein may regulate more than one effector, and, conversely, more than one G protein may regulate the same effector. For example, the activation of parathyroid hormone and thyroid-stimulating hormone receptors results primarily in an increase in intracellular cAMP levels but has also been shown to cause stimulation of phospholipase C (41–43). One could hypothesize that at low GnRHR numbers, activation of the receptor stimulates effectors coupled to $G_{q/11}$, whereas at higher GnRHR numbers, another G protein is activated as well, resulting in the activation of a second signaling pathway. Alternatively, differential activation of two signal transduction pathways may occur via a single G protein. The βγ subunits of a G protein can mediate signals as well as the α subunit (44, 45). This confers to any G protein the potential for dual signaling. This has been best described for the G_i protein; the α subunit inhibits adenylyl cyclase activity, whereas at higher concentrations the βγ subunits can stimulate the activity of some adenylyl cyclase subtypes (46, 47). Similarly, G protein βγ subunits have been shown to be capable of stimulating the β2 isoform of phospholipase C (48). Thus, ligand binding to one receptor can stimulate one effector pathway through G_α and a second pathway through $G_{\beta\gamma}$. The GnRHR may couple to different G proteins at low vs. high cellular receptor numbers or, alternatively, different signaling pathways may be activated by the α and βγ subunits of a single G protein.

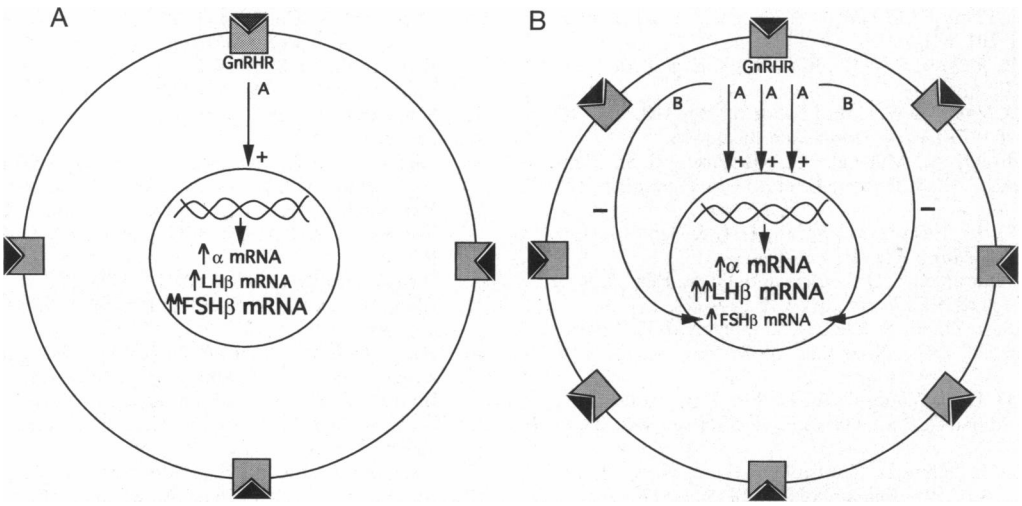

FIG. 4. Model of the mechanism of differential regulation of the gonadotropin subunit genes by GnRH at low GnRHR concentrations (*A*) and high GnRHR concentrations (*B*). (*A*) At low cell surface GnRHR concentrations, when GnRH binds to its receptors, a signal transduction pathway, pathway A, is activated, resulting in the stimulation of the expression of all three of the gonadotropin subunit genes, α, LHβ, and FSHβ. (*B*) At higher cell surface GnRHR concentrations, when GnRH binds to the now greater receptor numbers, signal transduction pathway A is activated to an even greater extent, resulting in the greater stimulation of the α and LHβ subunit genes. In addition, a second signal transduction pathway, pathway B, is now also activated. Activation of pathway B results in the specific inhibition of the expression of the FSHβ gene, with no effects on the α and LHβ genes. The net effect is that α and LHβ gene expression is maximally stimulated at relatively high GnRHR concentrations, whereas FSHβ gene expression is optimally stimulated at lower cell surface GnRHR concentrations.

Observations have been made *in vivo* in rats that cell surface GnRHR numbers are regulated by varying GnRH pulse frequencies (49–51). The highest concentrations of cell surface GnRHRs, as reflected by GnRH binding activity, occur at a GnRH pulse frequency of every 30 min, which has also been shown to stimulate preferentially LH biosynthesis and secretion. Lower cell surface GnRHR numbers occur at a GnRH pulse frequency of every 2 h, which has been shown to stimulate preferentially FSH biosynthesis and secretion. GnRHR concentration in primary rat pituitary cells is 2- to 3-fold higher when the GnRH pulse frequency is every 30 min, compared to that at a frequency of every 2 h. This magnitude of change in GnRHR numbers is similar to that seen when 4 μg vs. 40 μg of GnRHR cDNA is transfected into GH_3 cells, the concentrations at which the maximal differences in LHβ and FSHβ gene expression were observed. Similar effects of varying GnRH pulse amplitudes have also been observed (9). We therefore speculate that varying GnRH pulse frequencies and amplitudes may regulate differentially LH and FSH subunit gene expression *in vivo* in rats by regulating pituitary GnRHR concentrations and hence determining the intracellular signaling pathways activated by GnRH. However, this hypothesis remains to be tested in primary pituitary cells.

In summary, we have demonstrated that different cell surface concentrations of GnRHR result in differential degrees of stimulation of LH and FSH subunit gene expression by GnRH. This suggests that the differential regulation of gonadotropin subunit gene expression by GnRH observed *in vivo* in rats may, in turn, be mediated by varying gonadotrope cell surface GnRHR concentrations. This provides a physiologic mechanism whereby a single ligand can act through a single receptor to regulate differentially two different hormones in the same cell.

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