Transcriptional Down-Regulation of Human Gonadotropin-Releasing Hormone (GnRH) Receptor Gene by GnRH: Role of Protein Kinase C and Activating Protein 1*

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ABSTRACT

Clinical applications of GnRH agonists (GnRHa) are based primarily on the decrease in gonadotropin release after down-regulation of the GnRH receptor (GnRHR) by continuous GnRHa administration. However, the molecular mechanisms underlying the transcriptional regulation of the human GnRHR gene after prolonged GnRHa treatment remain poorly understood. In the present study, GnRHa-mediated regulation of human GnRHR gene transcription was studied by transiently transfecting the mouse gonadotrope-derived (αT3-1) cells with a 2297-bp human GnRHR promoter-luciferase construct (p2300-LucF). A dose- and time-dependent decrease in human GnRHR promoter activity was observed after GnRHa treatment. An average 71% decrease in promoter activity was observed after 24-h treatment with 0.1 μM GnRHa, which was blocked by cotreatment of the GnRH antagonist, antide. This effect was mimicked by phorbol 12-myristate 13-acetate (TPA) administration. In addition, the GnRHa- and TPA-mediated decrease in the human GnRHR promoter activity was reversed by a specific protein kinase C (PKC) inhibitor, GF109203X, or depletion of PKC by TPA pretreatment. These findings indicate that the activation of the PKC pathway is important in regulating the human GnRHR gene expression.

By progressive 5′-deletion studies, we have identified a 248-bp DNA fragment (~1018 to ~771, relative to the translation start site) at the 5′-flanking region of the human GnRHR gene that is responsible for the GnRHa-mediated down-regulation of human GnRHR promoter activity. Analysis of this sequence reveals the existence of two putative activating protein-1 (AP-1) sites with 87% homology to the consensus sequence (5′-TGA^G/C/A^C/-A′-3′), located at −1000 to −994 (5′-TTAGACA-3′, in complementary orientation) and −943 to −937 (5′-TGAATTA-3′). Using competitive gel mobility shift assays, AP-1 binding was observed within this 248-bp region. Site-directed mutagenesis of the putative AP-1-binding site located at −1000 to −994 abolished the GnRHa-induced inhibition. Further competitive GMISA and supershift experiments confirmed the identity of AP-1 binding in this region. By the use of Western blot analysis, a significant increase in c-Jun (100%; P < 0.05) and c-Fos (50%; P < 0.05) protein levels was observed after GnRHa treatment in αT3-1 cells. In addition, our data suggested that a change in AP-1 composition, particularly c-Fos, was important in mediating GnRHa-induced inhibition of human GnRHR gene expression.

We conclude that activation of the PKC pathway by GnRH is important in controlling human GnRHR gene expression. In addition, the putative AP-1-binding site located at −1000 to −994 of the human GnRHR 5′-flanking region has been functionally identified to be involved in mediating this down-regulatory effect. (Endocrinology 141: 3611–3622, 2000)

The hypotalamic decapeptide GnRH plays an important role in reproductive development and function by controlling the secretion and biosynthesis of gonadotropins from anterior pituitary gonadotropes through activation of the GnRH receptor (GnRHR) (1–3). There is evidence that GnRH responsiveness in pituitary gonadotropes is correlated to GnRHR number (4–7). Changes in GnRHR numbers in the pituitary glands of several species have been characterized during different physiological conditions (4, 5, 8–10). Treatment with GnRH results in a biphasic response in the gonadotropes with respect to GnRHR number. Short-term pulsatile treatment results in GnRHR up-regulation, whereas prolonged exposure to high concentrations of GnRH induces GnRHR down-regulation (11–13). Since the isolation of GnRHR complementary DNA, studies have shown that a change in GnRHR messenger RNA (mRNA) levels is one of the mechanisms regulating the expression of pituitary GnRHR. However, contradictory results have been reported. For instance, it has been demonstrated that chronic GnRH agonist treatment in pituitary cultures reduced GnRHR mRNA levels in sheep (14), cows (15), and αT3-1 cells (16). Others have reported no change in GnRHR mRNA levels in αT3-1 cells (17) and in the rat (18). To understand the molecular mechanism involved in transcriptional regulation of GnRHR gene expression, the 5′-flanking region of the GnRHR gene was isolated from...
mouse (19, 20). Progressive 5′-deletions in the mouse GnRH gene promoter revealed that the binding sites for steroidogenic factor-1 (SF-1), activating protein-1 (AP-1), and a novel element referred to as the GnRH-activating sequence appear to be responsible for regulating cell-specific expression of the mouse GnRH gene (21).

Using αT3–1, the homologous up-regulation of mouse GnRH gene (mGnRH) by GnRH was studied (22, 23). A significant increase in luciferase activity was observed after GnRH agonist (GnRHa) treatment for 4 h. Progressive deletion and mutation studies have identified a putative AP-1-binding site that responds to the GnRH-induced stimulation of luciferase activity (22, 23). This GnRHa-induced increase in mGnRH promoter activity was mimicked by phorbol ester treatment. Pretreatment with a specific protein kinase C (PKC) inhibitor blocked the GnRH- and phorbol 12-myristate 13-acetate (TPA)-induced increase luciferase activity, suggesting the involvement of PKC in regulating the expression of the mouse GnRH gene (22, 23). The depletion of PKC by pretreatment with 10 nM TPA for 20 h prevented the increase in mGnRH promoter activity caused by GnRH, and TPA treatment further supported the role of PKC (22).

Clinically, GnRH analogs have proven to be efficacious in treating a wide variety of gonadal hormone-dependent disorders, such as endometriosis, precocious puberty, and polycystic ovarian syndrome (24). In addition, GnRH agonists have been used extensively in assisted reproductive technologies (24, 25). The clinical applications of GnRH agonists are based primarily on the decrease in gonadotropin release as a result of GnRH receptor down-regulation by continuous GnRH agonist administration (26, 27). To date, the molecular mechanisms underlying the transcriptional regulation of the human GnRH gene after continuous GnRH treatment remain poorly understood. As the first step in understanding the possible transcriptional regulation of the human GnRH gene by GnRH, a 2297-bp 5′-flanking region of the human GnRH gene (28) was functionally characterized by luciferase reporter gene assays in a pituitary gonadotrope-derived αT3–1 cell line.

Materials and Methods

Cells and cell culture

Mouse pituitary gonadotrope-derived αT3–1 cell line, provided by Dr. P. L. Mellon (Department of Reproductive Medicine, University of California, San Diego, CA), were maintained in DMEM (with 4.5 mg/ml glucose; Life Technologies, Inc., Burlington, Canada) supplemented with 10% FBS (HyClone Laboratories, Inc., Logan, UT). Cultures were maintained at 37 C in a humidified atmosphere of 5% CO2 in air. Cells were passaged when they reached about 90% confluence by trypsin/EDTA solution (0.05% trypsin/0.53 mm EDTA).

Preparation of human GnRHR promoter-luciferase constructs

A 2.3-kb 5′-flanking region of human GnRHR-luciferase construct (p2300-LucF) was prepared as previously described (28). Progressive 5′-deletion constructs were prepared using various restriction endonucleases or exonuclease III/SI nuclease digestion (Pharmacia Biotech, Piscataway, NJ). The 5′-deleted human GnRHR promoter clones NdeI-HLuc, SacI-HLuc, PstI-HLuc, SpeI-HLuc, and p167-Luc were prepared by digesting p2300-LucF with NdeI, SacI, PstI, SpeI, and HindIII. Deletion clone p577-Luc was prepared by exonuclease III/SI nuclease digestion. The positive clones were identified by restriction mapping and DNA sequence analysis using a T7 DNA sequencing kit (Pharmacia Biotech). Plasmid DNA for transfection studies was prepared using QIAGEN Plasmid Maxi Kits (QIAGEN, Chatsworth, CA) following the manufacturer’s suggested procedure. The concentration and integrity of DNA were determined by measuring absorbance at 260 nm and by agarose gel electrophoresis, respectively. Purified plasmid DNA was then dissolved in 0.1 × TE (1 mM Tris-Cl, pH 7.5, and 0.1 mM EDTA) to a final concentration of 1 μg/μl.

Site-directed mutagenesis

Mutations were introduced by a three-step PCR mutagenesis method as described previously (29), using mutagenic primers mAP-1(−1000) and mAP-1(−943) and universal primers UP-T3F, UP, and T7R (see Table 1 for complete sequence information). Mutagenesis was confirmed by sequence analysis after the mutagenesis reaction.

Transient transfections and reporter assay

Transfections were carried out using the lipofectin procedure as recommended by the manufacturer (Life Technologies, Inc.). To correct for different transfection efficiencies of various luciferase constructs, the RSV-LacZ plasmid was cotransfected into cells with the GnRHR promoter-luciferase construct. Briefly, 5 × 104 cells were seeded into six-well tissue culture plate 24 h before the day of transfection. Five micrograms of the promoter-luciferase construct and 2.5 μg RSV-LacZ were combined with 8 μl lipofectin reagent in 200 μl serum-free medium. Lipofectin and DNA were incubated together for 45 min at room temperature. It was then diluted to 1 ml with serum-free medium and applied to the cells. Incubation of the cells with transfection cocktail was continued for 24 h at 37 C. Then, 1 ml medium containing 20% FBS was added. Cell lysate was prepared 24 h later with 200 μl of the luciferase reporter buffer (Promega Corp., Madison, WI). Cellular lysates were assayed for luciferase activity immediately. Luminescence was measured using the TROPIX OPIOCOMP I Luminometer (Bio/Can Scientific, Mississauga, Canada). β-Galactosidase activity was also measured and used to normalize the transfection efficiency. Promoter activity was calculated as luciferase activity/β-galactosidase activity. A pro-

Table 1. Oligonucleotides used in the current study

<table>
<thead>
<tr>
<th>Oligo name</th>
<th>Sequence (5′ to 3′)</th>
<th>Purpose</th>
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<tbody>
<tr>
<td>UP-T3F</td>
<td>GTGCCCTCTCCGAAAGGCTTCAAGCGAACAAATGACCCCCTCAGCTAAAGG</td>
<td>Site-directed mutagenesis</td>
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<tr>
<td>UP</td>
<td>GTGCCCTCTCTGGAAAGGGTCAAA</td>
<td>Site-directed mutagenesis</td>
</tr>
<tr>
<td>T7R</td>
<td>CGTAAACGCCGACGCACTAGG</td>
<td>Site-directed mutagenesis</td>
</tr>
<tr>
<td>mAP-1(−1000)</td>
<td>TTTAATCCGACGGTCGTTCTGCA</td>
<td>Site-directed mutagenesis</td>
</tr>
<tr>
<td>mAP-1(−943)</td>
<td>CATGGTGGTGGCCTCTGCGCG</td>
<td>Site-directed mutagenesis</td>
</tr>
<tr>
<td>hG-AP-1</td>
<td>TTGGCATTAAGCACAAAATGCA</td>
<td>Probe</td>
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<td>Mutated hG-AP-1a</td>
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<td>Competitor</td>
</tr>
<tr>
<td>Mutated hG-AP-1b</td>
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<td>Mutated AP-1</td>
<td>CGCTTAGATCTAGCAGGAAA</td>
<td>Competitor</td>
</tr>
<tr>
<td>Pre</td>
<td>GATCCCAGAAGGATGTCAGCTACA</td>
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FIG. 1. Time- and dose-dependent regulation of human GnRHR-luciferase activity in αT3–1 cells treated with GnRHa. A, The p2300-LucF transfected αT3–1 cells were transfected with 0.1 μM GnRHa for the indicated times. Treatments were initiated at the same time, and the control and treated cells were harvested at each time point of interest. The RSV-αlacz vector was cotransfected to normalize for varying transfection efficiencies. Luciferase units were calculated as luciferase activity/SD from triplicate assays in four separate experiments. a, P < 0.001 from control; b, P < 0.05 vs. the immediately adjacent group.

Pharmacological treatments

Pharmacological reagents, including a GnRH agonist, d-(Ala6)GnRH (GnRHa), a GnRH antagonist (antide), and TPA, were purchased from Sigma-Aldrich Corp. (Oakville, Ontario, Canada). The PKC inhibitor bisindolylmaleimide I (GF109203X) was obtained from Calbiochem (La Jolla, CA). In time-course experiments, treatments of the transfected cells were initiated at the same time with the indicated agents, and the treated cells were harvested at each time point of interest. In experiments in which the effects of GnRHa, antide, and TPA on GnRHR-Luc activity were studied, the cells were treated with the corresponding drug for 24 h before luciferase and β-galactosidase activities were measured.

Gel mobility shift assay (GMSA)

Oligodeoxynucleotides corresponding to the putative AP-1 element (hG-AP-1) at the human GnRHR 5′-flanking region (−1004 to −988), mutated hG-AP-1a, mutated hG-Ap-1b, and their complements were synthesized by the Oligonucleotide Synthesis Laboratory (University of British Columbia, Vancouver, Canada) and annealed to form an 18-bp double-stranded oligodeoxynucleotides (Table 1). Consensus AP-1, mutated AP-1, and progesterone response element (PRE) oligonucleotide DNA; c-Fos antibody (catalog no. sc-52X), c-Jun/AP-1 antibody (catalog no. sc-45X); GATA-2 antibody (catalog no. sc-276X); CREB response element-binding protein (CREB-1) antibody (catalog no. sc-186X); and progesterone receptor (PR) antibody (catalog no. sc-539X) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Probes for GMSA were end radiolabeled with [32P]ATP by T4 polynucleotide kinase (Life Technologies, Inc.) and separated from unincorporated radio nucleotides by passage over a Sephadex G-50 or G-25 column. Nuclear extracts was prepared from GnRHa-treated αT3–1 cells according to the method described previously (30). Protein concentrations were determined by a modified Bradford assay (Bio-Rad Laboratories, Inc., Hercules, CA). GMSAs were carried out in 20 μl containing 20 mM HEPES (pH 7.5), 20 mM KCl, 20 mM NaCl, 1.5 mM MgCl2, 1 mM EDTA, 10% glycerol, 2 μg poly(dI-dC), 5 μg nuclear proteins, 2 mg/ml BSA, and radiolabeled probe.

For the competition assays, the unlabeled DNA was added simultaneously with the labeled probe. Antibodies used in supershift experiments were added to the nuclear extract at room temperature for 1 h before the addition of labeled probe. The binding mixture was incubated at room temperature for 20 min and separated in 4–8% polyacrylamide gel containing 30% TBE (Tris-borate-EDTA: 0.09 M Tris-borate and 2 mM EDTA, pH 8.0). Before loading of samples, the gel was prerun for 90 min at 100 V at 4 °C. Electrophoresis was carried out at 30 mA at 4 °C. The gel was then dried under vacuum and exposed to x-ray film (X-OMAT AR film, Eastman Kodak Co., Rochester, NY) at −70 °C.
Western blot analysis

For Western blot analysis, GnRHa-treated αT3–1 cells were incubated in 75 ml cell lysis RIPA [containing 1× PBS (pH 7.4), 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 μg/ml phenylmethylsulfonylfluoride, 30 μg/ml aprotinin, and 10 μg/ml leupeptin] for 15 min on ice. The cellular debris was removed by centrifugation, and the protein concentration in the cell lysates was determined using a modified Bradford assay (Bio-Rad Laboratories, Inc.). Aliquots (35 μg) were taken from the total cell lysates and subjected to SDS-PAGE under reducing conditions. The separated proteins were then electrophoretically transferred onto nitrocellular paper (Hybond-C, Amersham Pharmacia Biotech, USA). The membranes were blocked with 5% (wt/vol) nonfat milk in Tris-buffered saline containing 0.05% Tween-20 for at least 1 h before the addition of c-Jun or c-Fos antibodies in a final concentration of 0.2 μg/ml. Monoclonal antibodies against PKC isoforms (anti-PKC mAB kit, catalogue no. S85080) were purchased from Transduction Laboratory, Inc. (BD Biosciences, Mississauga, Canada). The antibodies used for PKC isoforms were in the following dilutions: 1:1000 for PKCα, 1:250 for PKCβ, 1:500 for PKCδ, and 1:1000 for PKCe. All antibody incubation and washing were performed in Tris-buffered saline with 0.05% Tween-20. The Amersham Pharmacia Biotech enhanced chemiluminescence system (ECL) was used for detection. Membranes were visualized by exposure to Kodak X-OMAT film. The radioautograms were then scanned and quantified with Scion Image Released-β software (Scion Corp., Bethesda, MD).

Data analysis

For transfection assay, data are shown as the mean ± sd of triplicate assays in at least three independent experiments. For Western blot analysis, data were obtained from three independent experiments. All data were analyzed by one-way ANOVA followed by Tukey’s multiple comparison test or *t* test, using the computer software PRISM (Version 2, GraphPad Software, Inc., GraphPad Software, Inc., San Diego, CA). Data were considered significantly different from each other at *P* < 0.05.
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Introduction

The role of the pituitary gonadotropes in regulating human GnRHR gene expression is complex and involves multiple signaling pathways. Our preliminary studies showed that a 24-h transfection with 8 μl lipofectin resulted in the highest transfection efficiency. As a result, all transfections hereinafter were performed accordingly. To determine the effects of GnRHa, antide, and TPA on the promoter activity of the human GnRHR 5′-flanking region, p2300-LucF was transiently transfected into αT3–1 cells and treated with the corresponding pharmacological agent before measurement of luciferase activity. A dose- and time-dependent decrease in human GnRHR promoter activity was observed after GnRHa treatment (Fig. 1). A longer posttransfection incubation resulted in increased basal luciferase activity. A significant decrease in luciferase activity (31%; P < 0.001 vs. control) was observed after 6 h of 0.1-μM GnRHa treatment, and maximum inhibition (71%; P < 0.001) was reached after 24 h of 0.1-μM GnRHa treatment (Fig. 1A). In addition, this inhibitory effect was observed when treating the p2300-LucF-transfected cells with a concentration as low as 0.1 nM GnRHa for 24 h (Fig. 1B). To investigate whether the activation of GnRHR is essential for inhibiting the human GnRHR promoter activity, a GnRH antagonist, antide, was used to treat the p2300-LucF-transfected αT3–1 cells for 24 h, alone or in combination with GnRHa. No significant change in human GnRHR promoter activity was observed after antide treatment alone, and the magnitude of inhibition by GnRHa was reduced from 55% to 20% (P < 0.001) and was completely blocked in the presence of 0.1 and 10 μM antide, respectively (Fig. 2). These results suggest that the activation of GnRHR by GnRHa is important and results in activation of an intracellular mechanism that subsequently inhibits human GnRHR gene expression in the pituitary cells. Similar to the results obtained with GnRHa treatment, a dose- and time-dependent inhibition of human GnRHR promoter activity was observed after TPA treatment (Fig. 3). A significant decrease (30%; P < 0.01) in luciferase activity was observed after 6 h of 10 μM TPA treatment, and the degrees of inhibition increased with time in culture (Fig. 3A). The inhibitory effect of GnRHa on GnRHR promoter activity was mimicked by the administration of TPA, suggesting that the PKC pathway is involved in regulating human GnRHR gene expression in pituitary gonadotropes at the transcriptional level.

Results

Effects of GnRHa, antide, and TPA on human GnRHR promoter-luciferase activity

As human pituitary gonadotrope cells were unavailable to us, we used the mouse αT3–1 pituitary tumor cell line, which expresses high levels of GnRHR, as an experimental model for our transient transfection assays. Our preliminary studies showed that a 24-h transfection with 8 μl lipofectin resulted in the highest transfection efficiency. As a result, all transfections hereinafter were performed accordingly. To determine the effects of GnRHa, antide, and TPA on the promoter activity of the human GnRHR 5′-flanking region, p2300-LucF was transiently transfected into αT3–1 cells and treated with the corresponding pharmacological agent before measurement of luciferase activity. A dose- and time-dependent decrease in human GnRHR promoter activity was observed after GnRHa treatment (Fig. 1). A longer posttransfection incubation resulted in increased basal luciferase activity. A significant decrease in luciferase activity (31%; P < 0.001 vs. control) was observed after 6 h of 0.1-μM GnRHa treatment, and maximum inhibition (71%; P < 0.001) was reached after 24 h of 0.1-μM GnRHa treatment (Fig. 1A). In addition, this inhibitory effect was observed when treating the p2300-LucF-transfected cells with a concentration as low as 0.1 nM GnRHa for 24 h (Fig. 1B). To investigate whether the activation of GnRHR is essential for inhibiting the human GnRHR promoter activity, a GnRH antagonist, antide, was used to treat the p2300-LucF-transfected αT3–1 cells for 24 h, alone or in combination with GnRHa. No significant change in human GnRHR promoter activity was observed after antide treatment alone, and the magnitude of inhibition by GnRHa was reduced from 55% to 20% (P < 0.001) and was completely blocked in the presence of 0.1 and 10 μM antide, respectively (Fig. 2). These results suggest that the activation of GnRHR by GnRHa is important and results in activation of an intracellular mechanism that subsequently inhibits human GnRHR gene expression in the pituitary cells. Similar to the results obtained with GnRHa treatment, a dose- and time-dependent inhibition of human GnRHR promoter activity was observed after TPA treatment (Fig. 3). A significant decrease (30%; P < 0.01) in luciferase activity was observed after 6 h of 10 μM TPA treatment, and the degrees of inhibition increased with time in culture (Fig. 3A). The inhibitory effect of GnRHa on GnRHR promoter activity was mimicked by the administration of TPA, suggesting that the PKC pathway is involved in regulating human GnRHR gene expression in pituitary gonadotropes at the transcriptional level.

Involvement of PKC in the inhibitory effect of GnRHa on GnRHR promoter activity

To investigate the possible role of the PKC pathway in regulating human GnRHR gene expression, a highly specific PKC inhibitor (GF109203X) was used to block the PKC-dependent pathway. αT3–1 cells were transiently transfected with p2300-LucF and treated with vehicle, 1 or 5 μM GF109203X, 0.1 μM GnRHa, 10 μM TPA, or 0.1 μM GnRHa or 10 μM TPA plus GF109203X (Fig. 4). Administration of GF109203X alone did not affect GnRHR promoter activity. However, in the presence of 1 μM GF109203X, the inhibitory effects of GnRHa and TPA on human GnRHR promoter activity were reduced from 62.5% to 30% (P < 0.01) and from 57% to 30% (P < 0.05), respectively (Fig. 4, A and B). These inhibitory effects were completely reversed by 5 μM GF109203X. These results indicate the participation of the PKC pathway in the control of human GnRHR gene expression at the transcriptional level after GnRHa treatment. The role of PKC in regulating transcriptional activation of the GnRHR gene was further examined by depleting PKC by treatment with 0.1 μM TPA for 24 h. Using Western blot analysis (Fig. 5A), pretreatment of αT3–1 cells with TPA resulted in significant decreases in PKCα (75%; P < 0.001), PKCβ (60%; P < 0.001), PKCδ (80%; P < 0.001), and PKCe (40%; P < 0.001) levels, with no significant decrease in
basal luciferase activity (Fig. 5B). However, TPA-induced inhibition of GnRHR promoter activity was completely blocked in PKC-depleted cells. Similarly, TPA pretreatment resulted in a significant reduction of GnRH-induced inhibition of GnRHR promoter activity (Fig. 5B). These results indicate that the PKC pathway is important in regulating at least in part human GnRHR gene expression at the transcriptional level.

Identification of the human GnRHR promoter sequence required for GnRH-mediated inhibition

To localize a specific region that mediates the responsiveness to GnRHa down-regulation of GnRHR promoter activity, progressive deletion constructs containing fragments of the human GnRHR 5′-flanking region were fused to a luciferase reporter gene and transfected into αT3–1 cells. All deletion clones were functionally active in αT3–1 cell, with a minimal 7-fold increase in luciferase activity compared with pGL2-Basic, except p167-HLuc (Fig. 6). A deletion in the human GnRHR 5′-flanking sequences up to the PstI site (1018 bases away from the translation start site) did not affect its responsiveness to GnRHa inhibition. However, further deletion to the SpeI site (771 bases away from the translation start site) abolished the GnRHa-induced inhibitory effects on luciferase activity (Fig. 6). These results suggest that this 248-bp DNA fragment located between PstI and SpeI sites in the human GnRHR 5′-flanking region is responsible for the GnRH-mediated down-regulation of human GnRHR gene expression in αT3–1 cells.

Identification of putative AP-1 binding in the 248-bp GnRHR 5′-flanking region

Sequence analysis of this 248-bp DNA fragment reveals the existence of two potential AP-1-binding sites, located at positions –1000 to –994 (5′-TTAGACA-3′; in complementary orientation) and –943 to –937 (5′-TGAATTA-3′), with 87% sequence identity to the AP-1 consensus sequence (the nucleotide different from the consensus AP-1 sequence is underlined). GMSAs were conducted using the 248-bp DNA (–1018 to –771, relative to translation start site) as a probe and nuclear extracts isolated from αT3–1 cells after GnRHa treatment. A deletion in the human GnRHR 5′-flanking sequences up to the PstI site (1018 bases away from the translation start site) did not affect its responsiveness to GnRHa inhibition. However, further deletion to the SpeI site (771 bases away from the translation start site) abolished the GnRHa-induced inhibitory effects on luciferase activity (Fig. 6). These results suggest that this 248-bp DNA fragment located between PstI and SpeI sites in the human GnRHR 5′-flanking region is responsible for the GnRH-mediated down-regulation of human GnRHR gene expression in αT3–1 cells.
polyacrylamide gel (data not shown). As a result, a 10-min incubation time was chosen in this particular study. As shown in Fig. 7, there were three DNA-protein complexes formed with the 248-bp DNA fragments. The specificity of these complexes was confirmed by the addition of both specific (unlabeled 248-bp probe; 21018 to 2771) and nonspecific (unlabeled DNA fragment; 22300 to 22000, relative to translation start site) DNA competitors (Fig. 7, lanes 2 and 3). Furthermore, the competition with increasing concentrations of unlabeled AP-1 consensus element displaced the binding of two sequence-specific protein complexes (Fig. 7, bands 1 and 2), suggesting the presence of AP-1-binding sites within this region.

**AP-1 mediates GnRH-induced transcriptional down-regulation of the GnRHR gene**

To test the role of these AP-1-binding sites in mediating the GnRH inhibitory effect, the two putative AP-1-binding sites were mutated in SHLuc (Fig. 8). The mutated constructs were transiently transfected into αT3–1 cells and treated with 0.1 μM GnRH for 24 h. Site-directed mutation of the putative AP-1-binding site located at −943 to −937 did not alter GnRH-induced inhibition of GnRHR promoter activity. However, mutation of the AP-1-binding site at −1000 to −994 abolished the GnRH-induced inhibition (Fig. 8). These effects were again mimicked by TPA treatment (data not shown).

**The putative AP-1 response element binds c-Fos and c-Jun proteins from αT3–1 cells after GnRH treatment**

To confirm the identity of the AP-1-binding site in the human GnRHR promoter, the GMSA was repeated with a synthetic oligodeoxynucleotide containing the putative AP-1-binding element (hG-AP-1; −1000 to −994) in the presence of the AP-1 consensus element and antibodies against c-Fos and c-Jun. As shown in Fig. 9, two DNA-protein complexes were formed in the presence of excess mutated hG-AP-1a and hG-AP-1b.
were formed (bands 1 and 2) using nuclear extract isolated from GnRHa-treated αT3–1 cells. These bands were eliminated by incubation with increasing competitor DNA fragment containing a consensus AP-1 site or unlabeled probe, but not with a competitor containing mutated AP-1 site, mutated hG-AP-1 site, or nonrelated sequence (PRE; Fig. 9). Furthermore, the addition of anti-c-Jun or anti-c-Fos antibodies supershifted these complexes, supporting the binding of c-Jun and c-Fos to the hG-AP-1 (Fig. 10).

Regulation of AP-1 protein by GnRHa

The confirmation of AP-1 binding in the GnRHR promoter suggests that the regulation of AP-1 production, including c-Jun and/or c-Fos, by GnRH might be the molecular mechanism controlling the expression of this gene. To test this possibility, Western blot analysis was performed using protein isolated from GnRHa-treated αT3–1 cells. A 100% increase ($P < 0.05$) in c-Jun protein was observed after 3 h of 0.1 μM GnRHa treatment and was maintained up to 24 h (Fig. 11A). Similarly, a significant increase in c-Fos levels (50%; $P < 0.05$) was observed after GnRHa treatment (Fig. 11B). The GnRHa-mediated regulation of AP-1 protein production was further examined by gel mobility assay using hG-AP-1. Interestingly, differential AP-1 binding was observed. Only one DNA-protein complex was formed with the nuclear extract isolated from non-GnRHa-treated αT3–1 cells, whereas two DNA-protein complexes were formed with the nuclear extract isolated from GnRHa-treated αT3–1 cells (Fig. 12A). The increase in DNA-protein complex intensity implied that more AP-1-like protein was available after GnRHa treatment. In addition, the DNA-protein complex formed with nuclear extract isolated from non-GnRHa-treated cells was supershifted only by anti-c-Jun, not by anti-c-Fos, antibodies (Fig. 12B). Similar to those shown in Fig. 10, the DNA-protein complexes formed by nuclear extract isolated from GnRHa-treated αT3–1 cells were supershifted by both c-Jun and c-Fos antibodies (Fig. 12C). These results suggest that a different composition of AP-1 complex, particularly with c-Fos, may bind to the hG-AP-1 site after GnRHa treatment.

Discussion

It has been shown that GnRH can down-regulate GnRHR number (31, 32) and mRNA levels in the pituitary cells of rats (9), sheep (10, 14), and cows (15). Studies of transcriptional regulation of GnRHR gene expression in gonadotroph cells have been reported since the isolation of the 5′-flanking region of the mouse (19, 20) and human (33, 34). It has been shown that the preferential expression of mouse and human GnRHR in gonadotropes was controlled by the existence of several transcriptional factors, including SF-1 and its binding sites in the mouse GnRHR 5′-flanking region (28, 35). Although the 5′-flanking regions of the human GnRHR gene (33, 34) have been isolated, the molecular mechanisms controlling the transcriptional regulation of the GnRHR gene expression in the human remains unclear. In the present study 2297 bp of the human GnRHR gene 5′-flanking region were used to examine the transcriptional regulation of the human GnRHR gene by GnRHa and TPA in αT3–1 cells. As no human pituitary gonadotrope cells were available, we employed mouse gonadotrope-derived αT3–1 cells as an experimental model in the present study. Our results suggest that one potential mechanism for down-regulation of the human GnRHR is by decreasing its gene expression, as continuous administration of GnRH resulted in the reduction of luciferase activity. Prolonged incubation (24 h) of the transfected αT3–1 cells with a GnRH agonist inhibits human GnRHR promoter activity in a dose-dependent manner. In addition, our results indicate that activation of GnRHR by GnRH is essential for the regulation of GnRHR gene activity, as no reduction in luciferase activity was observed.
We did not observe any increase in human GnRHR promoter responsiveness of the transfected cells to GnRH treatment (23). While the basal luciferase activity was increased by prolonged transfection, it reduced the responsiveness of the transfected cells to GnRH treatment (22). Although the basal luciferase activity after 4 h of treatment (23) was observed after 6 h of treatment (22), it is possible that a species-specific regulator(s) required to control the increase in expression of human GnRHR gene is not present in αT3–1 cells; or 4) a different regulatory mechanism was used in controlling the human gene vs. the rodent gene.

It is well known that agonist occupancy of GnRHR results in a Gq/11-mediated activation of phospholipase Cβ (PLCβ), with consequent generation of diacylglycerol and inositol phosphates, which, in turn, activates PKC and elevates cytosolic Ca²⁺, respectively (36, 37). We have shown in the present study that activation of the PKC pathway is involved in controlling human GnRHR gene expression at the transcriptional level. The inhibition of human GnRHR promoter activity by GnRHa can be mimicked by TPA administration. Our data from the experiments using the specific PKC inhibitor, GF109203X, further confirmed that GnRH acts through a PKC-dependent pathway in regulating transcription of the human GnRHR gene. The role of PKC in regulating transcriptional activation of the GnRHR gene was further demonstrated by depleting PKC by pretreatment with 0.1 μM TPA. In agreement with previous studies (38), we observed a significant decrease in PKCa, -β, -δ, and -ε isoforms after PKC depletion. In addition, depletion of endogenous PKC eliminated the TPA-induced inhibition of GnRHR promoter activity, further supporting the role of PKC. However, the GnRHa-induced inhibition of GnRHR promoter activity cannot be abolished completely after depletion of PKC by TPA pretreatment, suggesting that an additional mechanism might be involved. It has been recently shown that the desensitizing effect of GnRH remained unchanged despite the presence of PKC inhibitor or down-regulation of PKC by TPA pretreatment in αT3–1 cells (38). Apart from PLCβ, phospholipase A2 (PLA) and phospholipase D (PLD) are also activated by GnRH, which resulted in the production of arachidonic acid and phosphatidic acid (PA), respectively (38–41). PA can be converted to diacylglycerol, by a specific PA phosphohydrolase, which activates Ca²⁺-independent PKC isoforms such as novel PKC (42). In addition, arachidonic, oleic, linoleic, and linolenic acids (derived from PA via PLA) were found to be capable of supporting the activation of specific PKC isoforms (43). Recent studies have demonstrated that GnRHa activates and regulates various PKC isoforms in αT3–1 cells (38, 44–46). Of particular interest, GnRHa activated PKCδ (46), which is insensitive to either GnRH or TPA down-regulation (38). Perhaps the stimulation of this TPA-insensitive PKC isoform after GnRHa activation is also involved in regulating the expression of the human GnRHR gene.

The mechanism(s) underlying GnRHR homologous desensitization remains unclear and might be different from other G protein-coupled receptors (47) due to the lack of C-terminal tail and parts of the third intracellular loop (48). As GnRH exposure to gonadotropes resulted in a decrease in GnRHR number and mRNA levels, modification of GnRHR synthesis has been proposed as a mechanism of GnRHR homologous desensitization. Thus, a
decrease in GnRHR promoter activity may be a mechanism of GnRHR desensitization. This is the first demonstration of GnRH down-regulation of the human GnRHR promoter activity. Using deletion, mutation, and gel mobility shift studies, a putative AP-1-binding site located between −1000 and −994 was identified as being responsible for this inhibitory effect. Interestingly, AP-1 has been shown to be important in regulating gonadotrope-specific expression of mouse GnRHR and to mediate the GnRH-induced increase in mGnRHR promoter activity, as mutation of the AP-1 site resulted in a significant loss of promoter activity as well as GnRHR responsiveness (21–23). However, we did not observe any significant loss of promoter activity after mutating the AP-1 site located at −1000 to −994, suggesting that this AP-1 site may not regulate basal human GnRHR gene expression. Nevertheless, a recent study demonstrated that the inhibition of rat GnRHR gene expression at the transcriptional level by phorbol ester may be mediated by AP-1, as deletion of a putative AP-1-binding site abolished this TPA inhibition (50). Similarly, overexpression of c-Jun and c-Fos in rat pituitary cells significantly reduced gonadotropin α-subunit promoter activity (51). These studies support the potential role of AP-1 in down-regulation of gene expression.

Activation of GnRHR in primary cultures of rat pituitary gonadotropes and αT3–1 cells by GnRH caused an increase in mRNA levels of c-Jun, c-Fos and JunB (52). The GnRHa-induced expression of these genes was mimicked by activation of PKC by phorbol ester. In addition, depletion of cellular PKC by prior treatment of TPA reduced GnRHa- and TPA-induced expression of these genes, further supporting the role of PKC in mediating the GnRH stimulatory effect (52). In agreement with these studies, a significant increase

in c-Fos and c-Jun protein levels was observed after GnRHa treatment in the present study by Western blot analysis. Using nuclear extracts from αT3–1 cells, with or without GnRHa treatment, a differential binding of AP-1 was observed in h-G-AP-1. Our data suggested that the possible mechanism of GnRHa-mediated inhibition in human GnRHR promoter activity may be the result of a change in AP-1 complex composition, as there was no retarded migration of the AP-1 complex caused by c-Fos antibody in non-GnRHa-treated nuclear extract. Furthermore, a second DNA-protein complex, which can be retarded by both c-Jun and c-Fos antibodies, was only observed in GnRHa-treated nuclear extract. These results indicate that c-Fos may play an important role in mediating GnRHa-induced inhibition of human GnRHR gene expression. Several studies have demonstrated the negative transcriptional regulatory action of c-Fos (53, 54). A dose-dependent decrease in rat GnRHR promoter activity (53) and clusterin promoter activity (54) was observed after cotransfection of increasing c-Fos expression vector. Furthermore, this inhibitory effect was reversed in the presence of mutant c-Fos expression vector (53, 54). It is worth noting that AP-1 is a family of nuclear factors composed of either homodimeric Jun or heterodimeric Fos-Jun complexes that interact with the AP-1-binding site to regulate gene expression at the transcriptional level. The Jun family includes c-Jun, JunB, and JunD, whereas Fos gene family members include c-Fos, FosB, Fra-1, and Fra-2. The differential expression among these gene products could certainly affect the composition of AP-1 and their actions (55). In the present study we only studied the possible role of c-Jun and c-Fos in mediating GnRHa-induced down-regulation of GnRHR gene expression. Studies of the involvement of other AP-1 proteins are currently underway.

In summary, we demonstrated a decrease in human GnRHR promoter activity after GnRHa treatment in αT3–1 cells. Activation of GnRHR and the PKC pathway is important for transcriptional down-regulation of the human GnRHR gene. In addition, a putative AP-1-binding site in the human GnRHR 5′-flanking region (−1000 to −994) has been functionally identified to be involved in the molecular mechanism of this down-regulation.

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