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ORIGINAL

Effects of estradiol and progesterone on prolactin transcriptional activity in somatolactotrophic cells

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Abstract. We examined the effects of sex steroids on prolactin promoter activity in rat somatolactotrophic GH3 cells. Both estradiol (E2) and progesterone (P4) were found to inhibit basal prolactin promoter activity, but to potentiate Thyrotropin-releasing hormone (TRH)-induced prolactin promoter activity. P4 had a greater inhibitory effect on basal prolactin promoter activity than E2, and P4 also potentiated TRH-induced prolactin promoter more potently than E2. Combined treatment with E2 and P4 further increased TRH-induced prolactin promoter activity. E2 and P4 also both reduced basal serum response element (SRE) promoter activity, and increased TRH-induced SRE promoter activity. Combination treatment with E2 and P4 reduced basal activity of SRE promoter and increased TRH-induced SRE activity more potently than E2 or P4 alone. In contrast, basal cAMP response element (CRE) promoter activity was not influenced by either E2 or P4, although TRH-induced CRE promoter was potentiated by each of these steroids, and was further increased by E2 and P4 combination treatment. Both E2 and P4 increased TRH-induced extracellular signal-regulated kinase (ERK) phosphorylation; however, intracellular cAMP levels was not influenced by E2 or P4. TRH-induced CRE promoter was inhibited by mitogen-activated protein kinase/ERK kinase (MEK) inhibitor and was increased by overexpression of MEK kinase (MEKK). This study showed that ERK and SRE transcriptional pathways, but not the cAMP/CRE pathway, may be involved in the suppression of basal prolactin promoter activity, whereas both the ERK/SRE and MAP kinase-mediated CRE pathways appear to be involved in the increased transcriptional efficiency of the prolactin promoter induced by TRH stimulation.

Key words: Prolactin, Thyrotropin-releasing hormone (TRH), Estradiol, Progesterone, GH3 cells

THE SEX STEROIDS estradiol (E2) and progesterone (P4) regulate the synthesis and secretion of several pituitary hormones, and play a key role in the regulation of reproductive function. Lactotroph cells, which produce prolactin, are a known target of E2 action. Lactotrophs constitute approximately 15% of the cells of the adenohypophysis; however, this proportion is dependent on age and sex [1]. It has been reported that there is marked hyperplasia of lactotrophs during pregnancy and lactation in humans [2]. Exogenous E2 is known to induce lactotroph hyperplasia [3], and increases prolactin release from rat pituitary cultures by desensitizating lactotrophs to dopamine, a well-known inhibitor of prolactin release [4]. E2 was also reported to stimulate lactotroph proliferation and prolactin gene expression in rats [5].

E2 modulates gene expression through estrogen receptors (ERs), which belong to the nuclear transcription factor superfamily [6]. Ligand-bound ERs form dimers that act on specific estrogen response elements in the promoter regions of estrogen-regulated genes. Three major ER isoforms are known to be expressed in the rat anterior pituitary: ERα, ERβ, and truncated estrogen receptor product-1 (TERP-1) [7], and each isoform is regulated throughout the estrous cycle and by steroids and hypothalamic peptides [8-10].

Although it is well known that dopamine plays a major role in the control of prolactin release [4, 11], prolactin synthesis and secretion are also regulated by several hypothalamic hormones. Thyrotropin-releasing hormone (TRH) is a primary secretagogue [12]. TRH is secreted from the hypothalamus and transported *via* the hypothalamic-hypophysial portal circulation to the pituitary gland, where it stimulates inositol phospholipid metabo-

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lism by activating membrane receptors in prolactin-producing cells and initiating signaling cascades [13].

GH3 cells are a clonal strain of rat pituitary tumor cells, and can synthesize and secrete both prolactin and growth hormone [14]. As GH3 cells have many properties in common with normal lactotrophs, these cells are a valuable model for studying the regulation of prolactin production.

Using GH3 cells, we previously demonstrated that TRH-induced extracellular-signal regulated kinase (ERK) activation is involved in prolactin synthesis but not prolactin secretion [15], and that TRH inhibits DNA synthesis and reduces growth hormone synthesis *via* ERK [16]. We also characterized patterns of prolactin transcriptional activity induced by TRH perifusion [17]. Although all of these studies used cultured GH3 cells, we did not previously examine the effects of sex steroid hormones in these cells. Because sex steroids, including E2 and P4, are present in the pituitary gland, it is important to determine their influence on prolactin synthesis and secretion.

The present study examined the effects of sex steroid hormones on the basal and TRH-induced transcriptional activity of prolactin in GH3 cells.

Materials and Methods

Materials

The following chemicals and reagents were obtained from the indicated sources: Fetal Bovine Serum, Trypsin (GIBCO, Invitrogen, Carlsbad, CA); Dulbecco's modified eagle medium (DMEM), penicillin-streptomycin, TRH, Water Soluble β-Estradiol, Water Soluble Progesterone (Sigma Chemical Co., St. Louis, MO).

Cell culture

GH3 cells were plated in 35-mm tissue culture dishes and incubated 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. After 24 h, the culture medium was changed to high-glucose DMEM containing 1% heat-inactivated FBS and 1% penicillin-streptomycin and incubated without (control) or with the test reagents for the indicated periods of time.

Transfections and luciferase assays

The reporter constructs used were generated by fusing -609/+12 of the prolactin gene to the firefly luciferase cDNA in pGL3, as previously described [16]. GH3 cells were transiently transfected by electroporation [18] with 2.0 μg/dish of reporter construct and 0.1 μg of pRL-TK (Promega), and plated on 35-mm tissue culture dishes. To measure the activity of promoter regions containing a CRE and SRE, cells were transfected with either pCre-Luc (1.0 μg DNA) or pSre-Luc (1.0 μg DNA), which have a CRE enhancer (x 4) or five tandem SRE repeats, respectively, upstream of the TATA boxes of the firefly luciferase gene. For experiments in which E2 and P4 were used, cells were exposed to these steroids 48 h prior to TRH stimulation. When the inhibitor was used, cells were pre-incubated with the inhibitor for 60 min and then stimulated. After stimulation, cells were washed with ice-cold PBS and lysed with PLB (Passive Lysis Buffer, Promega). Cell debris was pelleted by centrifugation at 14,000 x *g* for 10 min at 4ºC, and firefly luciferase and Renilla luciferase activity were measured in the supernatant using the Dual-Luciferase Reporter Assay System and a luminometer (TD-20/20) (Promega) according to the manufacturer's protocol. Luciferase activity was normalized to Renilla luciferase activity to correct for transfection efficiency, and the results were expressed as fold increase compared to the unstimulated control. All experiments were independently performed three times, each in triplicate.

RNA preparation, reverse transcription, and real-time quantitative RT-PCR

Total RNA from treated or untreated GH3 cells was extracted using the Trizol-S extraction method (GIBCO BRL Life Technologies) according to the manufacturer's instructions. To obtain cDNA, 1.0 ug of total RNA was reverse transcribed using an oligo-dT primer (Promega), and a First Strand cDNA Synthesis Kit (Invitrogen) in reverse transcription (RT) buffer. The preparation was supplemented with 0.01 dithiotreitol (DTT), 1 mM dNTPs, and 200 units of RNAse inhibitor/human placenta ribonuclease inhibitor (Ribonuclease Inhibitor, Code No. 2310, Takara, Tokyo, Japan) in a final volume of 10 μL. The reaction was incubated at 37ºC for 60 min. Prolactin mRNA was measured using real-time quantitative PCR (ABI Prism 7000, Perkin Elmer Applied Biosystems, Foster City, CA) following the manufacturer's protocol (User Bulletin No. 2), and utilizing a Universal Probe Library Probe and Fast Start Master Mix (Roche Diagnostics, Mannheim, Germany). Using specific primers for prolactin [16], the simultaneous measurement of mRNA

and GAPDH permitted normalization of the amount of cDNA added per sample. The thermal cycling conditions were: 95ºC for 10 min for denaturation followed by 40 cycles of 95ºC for 15 s and 60ºC for 1 min. The cycle threshold was determined using PRISM 7000 software and post-amplification data were analyzed by the delta-delta CT method using Microsoft Excel.

Western blot analysis

After stimulation, GH3 cells were rinsed with PBS, then lysed on ice with RIPA buffer (PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) containing 0.1 mg/mL phenylmethylsulfonyl fluoride, 30 µg/mL aprotinin, and 1 mM sodium orthovanadate, scraped for 20 sec, and centrifuged at 14,000 x g for 10 min at 4ºC. Protein concentration in the cell lysates was measured using the Bradford method. Ten micrograms of denatured protein/well was separated on a 10% SDS-PAGE gel using a standard protocol. Protein was transferred onto polyvinylidene difluoride membranes (Hybond-P PVDF, Amersham Biosciences, Little Chalfont, UK), which were blocked for 2 h at room temperature in Blotto (TBS with 4.5% milk). Membranes were incubated with phospho-ERK antibody (P-ERK) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) (1:500 dilution) in Blotto overnight at 4ºC, and then washed 3 x 10 min with TBS/1% NP-40. A subsequent incubation with a monoclonal horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was carried out for 1 h at room temperature in Blotto, and the appropriate additional washes were performed. Following chemiluminescence (ECL) detection (Amersham Biosciences, Little Chalfont, UK), membranes were exposed to X-ray film (FujiFilm, Tokyo, Japan). After strip washing (Restore Buffer, Pierce Chemical Co.), membranes were re-probed with ERK antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) (1:1000 dilution) overnight at 4ºC, followed by incubation with HRP-conjugated secondary antibody and continuation of the procedure as described above. Films were analyzed by densitometry using NIH Image, and the intensities of the P-ERK bands were normalized to those of total ERK to correct for protein loading of cellular lysate extracts. Each experiment was repeated at least three times.

Measurement of cyclic-AMP accumulation

GH3 cells were plated on 96-well plates at a density of 10^5 cells/well, cultured for 48 h, and then stimulated with TRH for 1 h. To determine the effects of E2 and P4 on cAMP accumulation, cells were treated with E2 and P4 for 48 h and then intracellular cAMP levels were measured using the cAMP enzyme immunoassay system from Amersham Pharmacia Biotech.

Statistical analysis

All experiments were independently repeated at least three times. Each experiment was performed using triplicate (luciferase assays) or duplicate (Western blots) samples in each experimental group. Values were expressed as means ± SEM. Statistical analysis was performed using one-way ANOVA followed by Duncan's multiple range test. $P < 0.05$ was considered statistically significant.

Results

Effects of E2 on prolactin promoter activity

Basal activity of the prolactin promoter was measured in the presence or absence of E2. Ten nM of E2 slightly inhibited basal prolactin promoter activity. A higher concentration of E2 (1 µM) further inhibited basal prolactin promoter activity (Fig. 1A). TRH increased prolactin promoter activity by up to 2.48±0.5-fold compared to non-stimulated cells in the absence of E2. One µM, but not 10 nM, E2 significantly increased prolactin promoter induction by TRH (Fig. 1B).

Effects of P4 on prolactin promoter activity

Next, the effects of P4 on prolactin promoter activity were determined. Basal prolactin promoter activity was reduced to 0.59±0.18-fold and 0.35±0.01-fold by treatment with 10 nM or 1 µM P4, respectively (Fig. 1C). In contrast, TRH induction of prolactin promoter activity was increased from 3.61±1.0-fold to 5.5±2.49-fold or 7.90±2.90-fold by 10 nM or 1 µM P4, respectively (Fig. 1D).

Effects of combined E2 and P4 treatment on prolactin promoter activity

Both E2 and P4 reduced basal activity of the prolactin promoter to a similar extent, and both increased TRH-induced prolactin transcriptional activity. Thus, we examined the effects of E2 and P4 combination treatment. One µM E2 or P4 reduced basal prolactin promoter activity to 0.57±0.10-fold and 0.34±0.02-fold, respectively. P4 had a greater inhibitory effect on basal prolactin promoter activity than E2 (Fig. 1E).

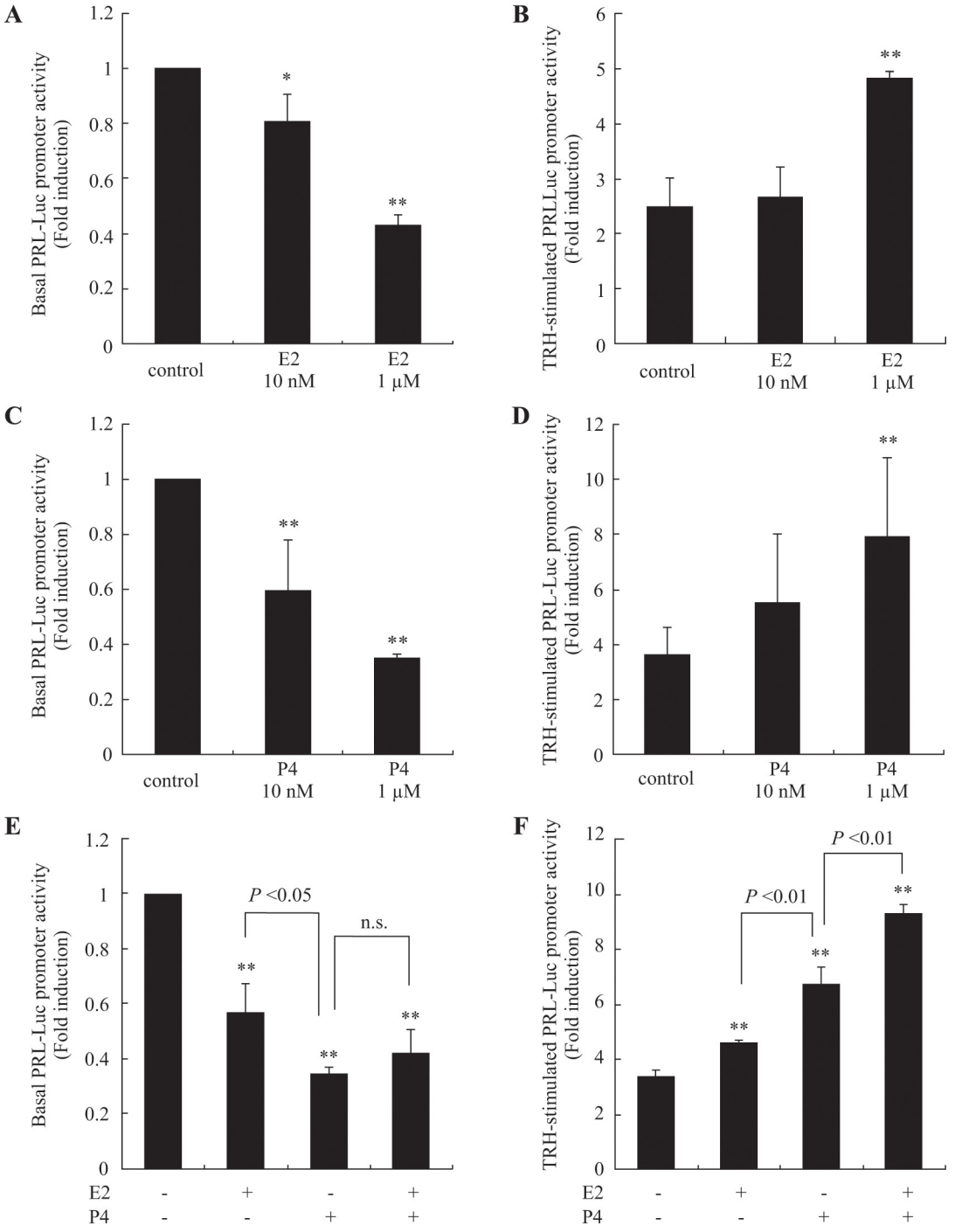

Fig. 1 Effects of E2 and P4 on basal and TRH-stimulated prolactin promoter activity
GH3 cells were co-transfected with 0.1 μg of PRL-TK vector and 2.0 μg of prolactin-luciferase promoter (PRL-Luc). After 48 h of culture with or without (control) the indicated concentrations of E2 (Fig. 1A and 1B), P4 (Fig. 1C and 1D), or combined 1 μM E2 and P4 (Fig. 1E and 1F), cells were treated with (Fig. 1B, 1D, and 1F) or without (Fig. 1A, 1C, and 1E) 100 nM TRH for 6 h. A luciferase assay was performed to measure prolactin promoter activity, which was normalized to PRL-TK activity and expressed as fold activation over unstimulated controls. Values are means ± SEM (three independent experiments were performed using triplicate samples). *$P < 0.05$; ** $P< 0.01$ *vs*. control. The differences between the effects of E2 and P4 on basal and TRH-stimulated prolactin promoter activity were statistically significant (basal activity: $P < 0.05$, TRH-stimulated activity: $P < 0.01$). The difference between the effects of P4 and E2+P4 on TRH-stimulated prolactin promoter activity was statistically significant ($P < 0.01$). n.s.: the difference was not statistically significant.

Combined treatment with E2 and P4 did not further increase the inhibitory effect induced by P4 alone. However, combination treatment with E2 and P4 did further increase TRH-induced prolactin promoter activity compared to E2 or P4 alone (Fig. 1F).

Effects of E2 and P4 on prolactin mRNA expression

We examined the effects of E2 and P4 on prolactin mRNA expression. E2 and P4 did not induce a significant change in basal prolactin mRNA expression (Fig. 2A). In contrast, TRH-induced prolactin mRNA expression was significantly potentiated by both E2 and P4 (Fig. 2B).

Effects of E2 and P4 on SRE promoter activity

The serum response element is a DNA domain in the promoter region that binds to an ERK-mediated transcription factor. We examined the effects of sex steroids on SRE promoter activity. Basal SRE promoter activity was significantly reduced in the presence of 1 μM E2 or P4 (Fig. 3A and C). However, TRH induction of SRE promoter activity was significantly potentiated in the presence of E2 or P4 (Fig. 3B and D). Combination treatment with E2 and P4 had the same inhibitory effects as P4 alone on basal SRE promoter activity; however, TRH-induced SRE promoter activity was increased in the presence of both E2 and P4 compared to E2 or P4 alone (Fig. 3E and F).

Effects of E2 and P4 on CRE promoter activity

Next, we examined whether activity of the cAMP-responsive element (CRE) promoter region is regulated by sex steroid hormones. CRE luciferase activity was slightly increased by 1 μM E2 treatment. This increase in CRE luciferase activity was significant, but limited (Fig. 4A). TRH-induced CRE activity was significantly potentiated by 1 μM E2, going from 5.74±0.80-fold to 7.85±0.5-fold induction (Fig. 4B), whereas basal CRE activity was not affected by the presence of P4. TRH-induced CRE activity was also significantly increased by 1 μM P4 (Fig. 4D). Basal CRE activity was unchanged by combination treatment with E2 and P4 (Fig. 4E), but TRH-induced CRE activity was increased in the presence of E2 and P4 together compared to E2 or P4 alone (Fig. 4F).

Effects of E2 and P4 on TRH-induced ERK phosphorylation

Next, the effects of E2 and P4 on TRH-induced ERK phosphorylation were examined. Pre-treatment with E2 or P4 potentiated TRH stimulation of ERK phosphorylation. Combined treatment with E2 and P4 further increased TRH-induced ERK phosphorylation compared to E2 or P4 alone (Fig. 5).

Effects of TRH on intracellular cAMP accumulation

Because CRE-luciferase activity was stimulated by TRH, we next examined whether TRH increases cAMP and whether sex steroids affect basal cAMP levels in

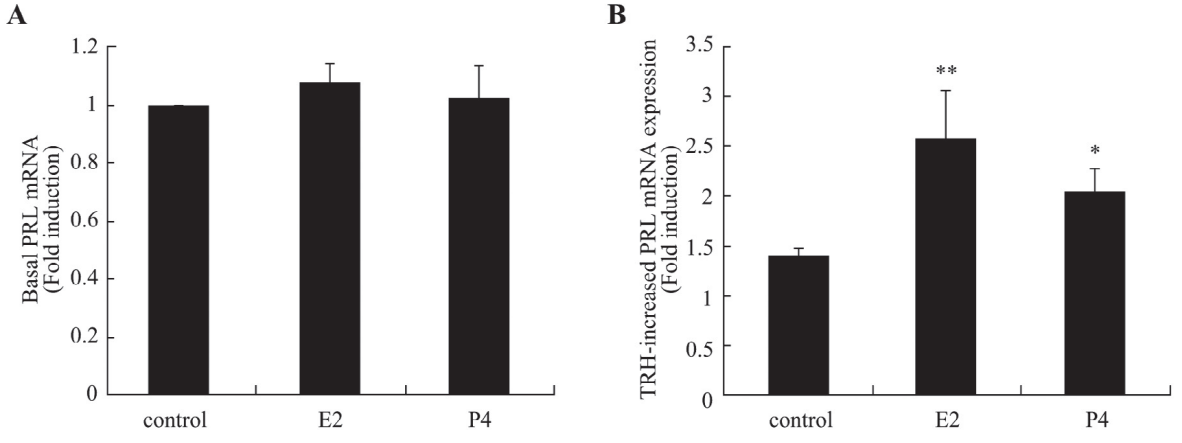

Fig. 2 Effects of E2 and P4 on basal and TRH-stimulated prolactin mRNA expression
GH3 cells were treated with 1 μM E2 and 1 μM P4 for 48 h and then stimulated with (Fig. 2B) or without 100 nM TRH (Fig. 2A) for an additional 24 h. Then, mRNA was extracted and reverse-transcribed. Prolactin mRNA levels were measured by quantitative real-time PCR. Results are expressed as fold stimulation over unstimulated cells and present the means ± SEM of three independent experiments, each performed using triplicate samples. *$P < 0.05$; ** $P < 0.01$ *vs*. control.

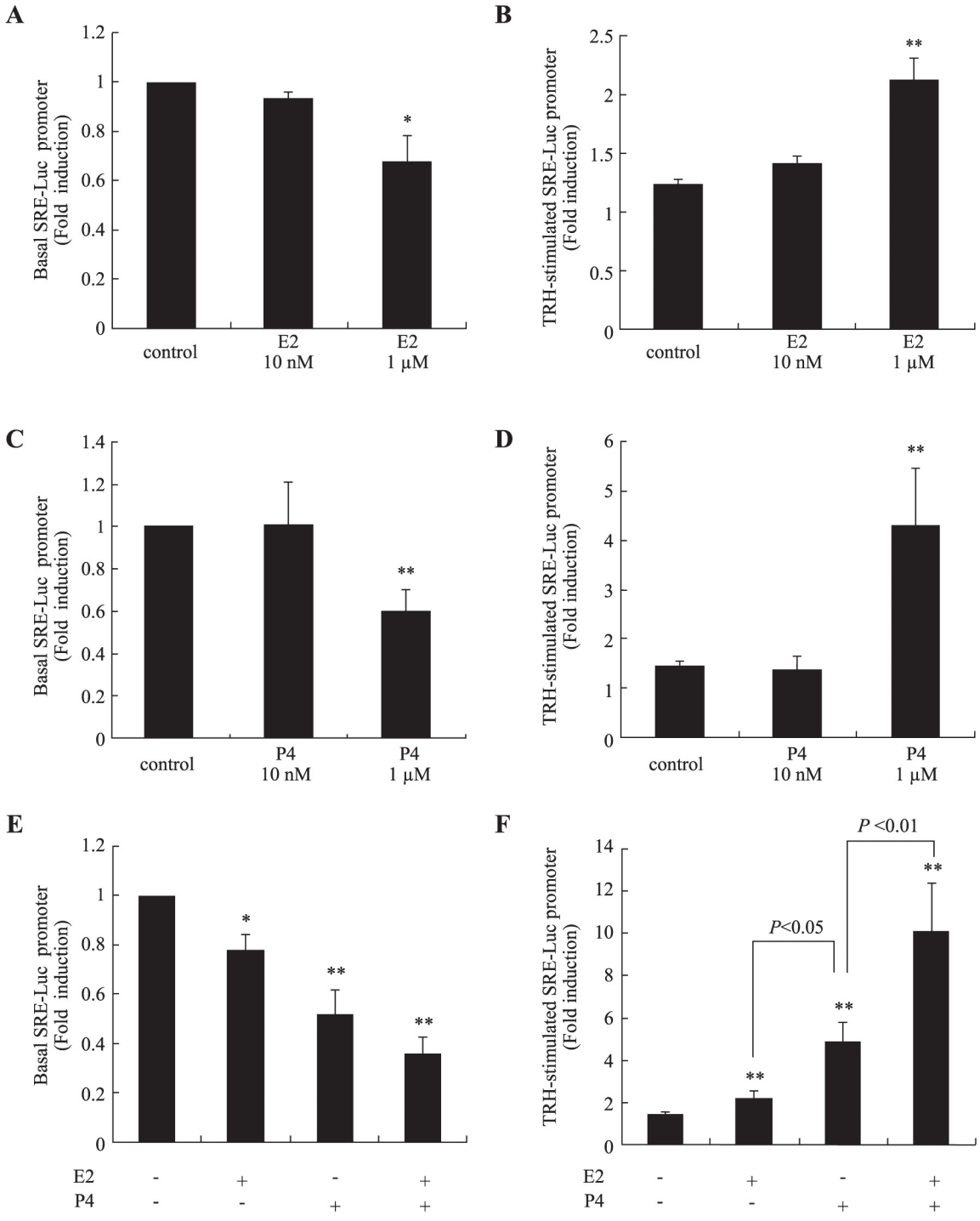

Fig. 3 Effects of E2 and P4 on basal and TRH-stimulated SRE promoter activity

GH3 cells were co-transfected with 0.1 μg of PRL-TK vector and 2.0 μg of SRE-luciferase promoter (SRE-Luc). After 48 h of culture with or without (control) the indicated concentrations of E2 (Fig. 3A and 3B), P4 (Fig. 3C and 3D), or combined 1 μM E2 and P4 (Fig. 3E and 3F), cells were treated with (Fig. 3B, 3D, and 3F) or without (Fig. 3A, 3C, and 3E) 100 nM TRH for 6 h. A luciferase assay was performed to measure prolactin promoter activity, which was normalized to PRL-TK activity and expressed as fold activation over unstimulated controls. Values are means ± SEM (three independent experiments were performed using triplicate samples). *$P < 0.05$; ** $P < 0.01$ *vs*. control. The difference between the effects of E2 and P4 on TRH-stimulated SRE promoter activity was statistically significant ($P < 0.05$). The difference between the effects of P4 and E2+P4 on TRH-stimulated SRE promoter activity was statistically significant ($P < 0.01$).

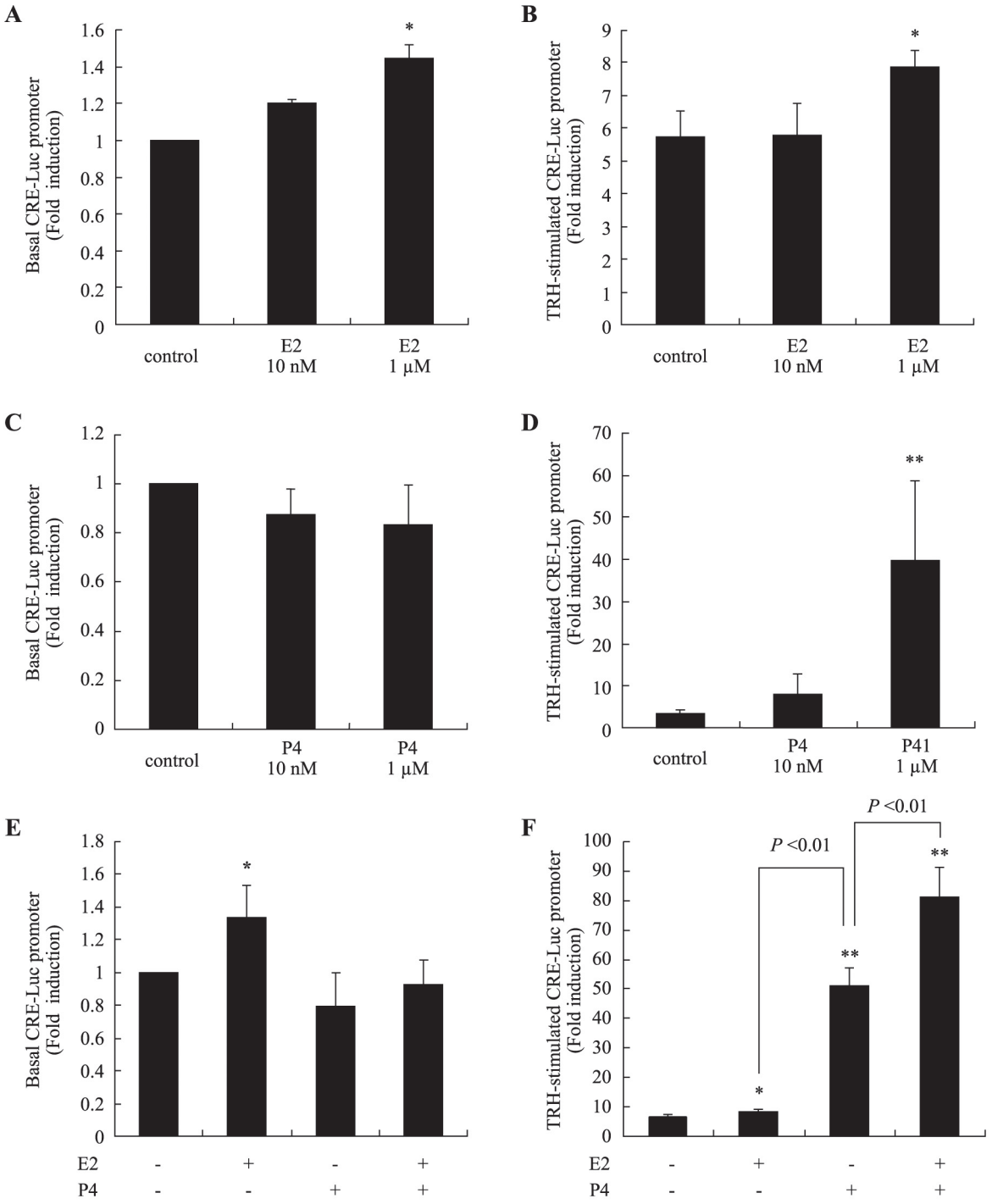

Fig. 4 Effects of E2 and P4 on basal- and TRH-stimulated CRE promoter activity
GH3 cells were co-transfected with 0.1 μg of PRL-TK vector and 2.0 μg of CRE-luciferase promoter (CRE-Luc). After 48 h of culture with or without (control) the indicated concentrations of E2 (Fig. 4A and 4B), P4 (Fig. 4C and 4D), or combined 1 μM E2 and P4 (Fig. 4E and 4F), cells were treated with (Fig. 4B, 4D, and 4F) or without (Fig. 4A, 4C, and 4E) 100 nM TRH for 6 h. A luciferase assay was performed to measure prolactin promoter activity, which was normalized to PRL-TK activity and expressed as fold activation over unstimulated controls. Values are means ± SEM (three independent experiments were performed using triplicate samples). *$P < 0.05$; ** $P < 0.01$ *vs*. control. The difference between the effects of E2 and P4 on TRH-stimulated CRE promoter activity was statistically significant ($P < 0.01$). The difference between the effects of P4 and E2+P4 on TRH-stimulated CRE promoter activity was statistically significant ($P < 0.01$).

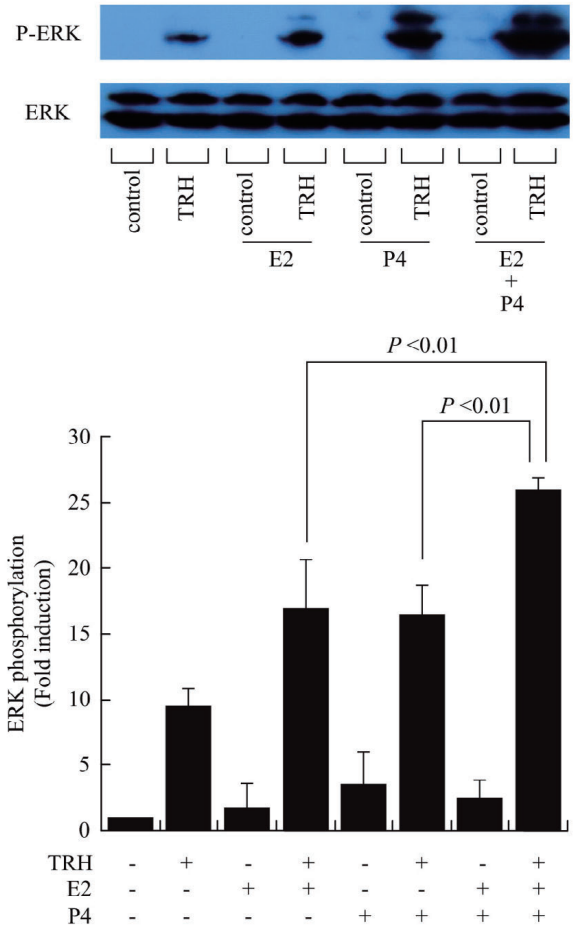

Fig. 5 Effects of E2 and P4 on TRH-induced ERK phosphorylation
After 48 h of culture of GH3 cells with or without (control) E2 and P4, cells were stimulated with 100 nM TRH for 10 min. After cells were harvested, cell lysates (10 µg) were subjected to SDS-PAGE followed by Western blotting and incubation with antibody against phosphorylated ERK (P-ERK) and total ERK (ERK). Values are means ± SEM (three independent experiments were performed using triplicate samples). The differences between ERK phosphorylation by TRH with E2 or P4 alone and ERK phosphorylation by TRH with E2 and P4 combined were statistically significant ($P < 0.01$).

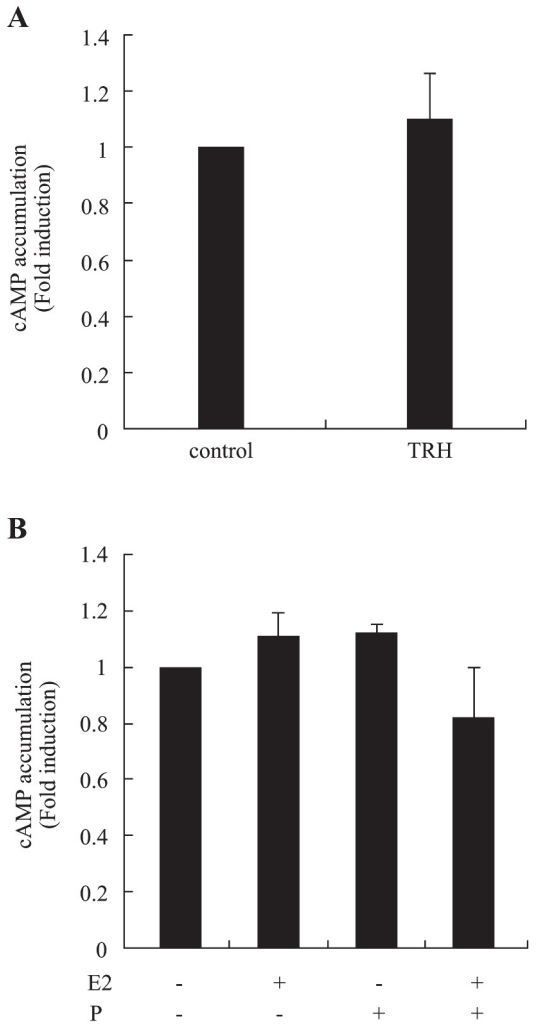

Fig. 6 Effects of TRH, E2, and P4 on cAMP accumulation in GH3 cells
(A) GH3 cells were plated on 96-well plates, incubated for 48 h, and then stimulated with 100 nM TRH for 1 h. (B) Cells were treated with E2 and P4 for 48 h and then intracellular cAMP was measured as described in the Materials and Methods section.

GH3 cells. Treatment of GH3 cells with TRH for 1 h did not increase cAMP accumulation in GH3 cells (Fig. 6A). E2 and P4 treatment also did not influence intracellular cAMP levels in these cells (Fig. 6B).

Possible interaction of the ERK/SRE pathway with CRE promoter activation

To investigate the possibility of crosstalk between the ERK/SRE pathway and CRE promoter activity, the mitogen-activated protein kinase/ERK kinase (MEK) inhibitor U0126 was applied to GH3 cells. After pre-treatment of the cells with U0126, TRH-increased CRE promoter activity was significantly reduced (Fig. 7A). In addition, transfection of cells with pFC-MEK kinase (MEKK), which expresses constitutively active MEKK and activates ERK, strongly increased CRE promoter activity (Fig. 7B).

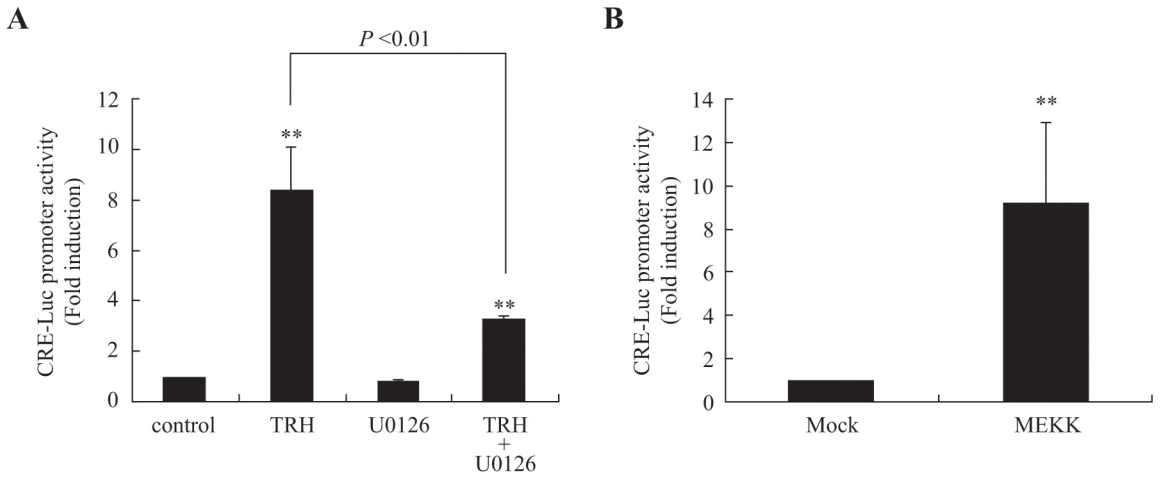

Fig. 7 Effects of a MEK inhibitor on TRH-induced CRE promoter activity and the effects of MEKK overexpression
(A) GH3 cells were co-transfected with 0.1 μg of PRL-TK vector and 2.0 μg of CRE-luciferase promoter (CRE-Luc). After 48 h of culture, cells were pre-incubated with or without (control) the MEK inhibitor U0126 (10 nM) for 60 min and then stimulated with 100 nM TRH for 6 h. (B) GH3 cells were co-transfected with 0.1 μg of PRL-TK vector and 2.0 μg of CRE-luciferase promoter (CRE-Luc) together with 2.0 μg of pFC-MEKK and cultured for 48 h. A luciferase assay was performed to measure CRE promoter activity, which was normalized to PRL-TK activity and expressed as fold activation over unstimulated controls. Values are means ± SEM (three independent experiments were performed using triplicate samples). ** $P< 0.01$ *vs*. control. The difference between CRE promoter activity levels in TRH- and TRH+U0126-stimulated cells was statistically significant ($P < 0.01$).

Discussion

This study investigated the effects of E2 and P4 on basal prolactin promoter activity, and also how these steroids influence TRH-stimulated prolactin promoter activity. Several previous studies have examined the effects of sex steroids on prolactin gene expression and secretion and have found that these steroids exert their effects in association with dopamine. In a study of ovariectomized rats, both E2 and P4 negatively influenced the activity of neuroendocrine dopaminergic neurons and increased prolactin secretion [19]. E2 has also been shown to decrease the number of dopamine receptors in the anterior pituitary [20, 21]. Cloned prolactin-producing GH3 cells also have E2 and P4 receptors [22], and GH3 cell function was shown to be modulated by these steroids. Haug *et al.* demonstrated that E2 stimulates prolactin release, whereas P4 decreases prolactin production as well as inhibiting the stimulatory effect of E2 on GH3 cells [23]. It has also been reported that E2 increases the number of TRH receptors [24, 25].

The actions of E2 and P4 on prolactin promoter activity in the present study were unexpected. We predicted that E2 would positively modulate basal prolactin promoter activity because numerous studies have reported a stimulatory effect of E2 on prolactin synthesis and secretion. However, our studies showed that basal prolactin promoter activity was significantly decreased by both E2 and P4.

Estrogens act by binding to nuclear receptors that interact with specific DNA sequences (estrogen response elements, EREs) present in the promoters of many genes, thereby influencing their expression. Previous studies have shown that the prolactin promoter contains a potential ERE in a region approximately -1500 bp upstream of the rat prolactin gene [26, 27]. The reporter construct used in the present study was generated by fusing -609/+12 of the rat prolactin gene to firefly luciferase cDNA in pGL3. Regardless of whether EREs were present in the construct used for our promoter assay, the fact that basal prolactin promoter activity was reduced by E2 suggests that E2 can modulate prolactin promoters independent of EREs. Although little is known about the effects of progesterone on the prolactin promoter region, P4 also reduced basal prolactin promoter activity in a manner similar to E2. In the present study, basal levels of prolactin mRNA in GH3 cells were not changed by 48 h treatment with E2 or P4 (Fig. 2). This is in contrast with previous studies dem-

onstrating a stimulatory effect of E2 on prolactin; however, in these studies, prolactin protein was measured rather than mRNA. In addition, these studies seemed to indicate that a relatively long time period was needed to observe the stimulatory effect of E2 [23, 24, 28]. It is possible that basal expression of prolactin mRNA may not be influenced directly by basal prolactin transcriptional activity or that the prolactin promoter construct used in this experiment was inhibited by E2 and P4 merely because this promoter region does not contain any EREs. It is also possible that E2 and P4 do not have a substantial effect on basal prolactin expression under physiological conditions. However, these possibilities are only speculative.

Although basal prolactin promoter activity was reduced in the presence of E2 or P4, TRH-induced prolactin promoter activity was increased by these hormones. The effect of P4 was significantly stronger than that of E2, and combination treatment with E2 and P4 further increased the effects of TRH compared to those of E2 or P4 alone. These observations suggest that sex steroids can potentiate the efficacy of TRH in stimulating prolactin promoter activity, and ultimately increase gene and protein expression, although no steroid hormone response element was present in the promoter region used in this study. Similar to the observed effects on promoter activity, TRH-stimulated prolactin mRNA expression was further potentiated by E2 and P4. The inhibitory effects of E2 and P4 on basal prolactin promoters were observed at the lower concentration tested (10 nM) and the effects of these sex steroids on TRH-induced activation of prolactin promoters were observed only at the higher concentration tested (1μM). These results suggest that higher concentrations of these steroids are necessary for potentiation of the effects of TRH. Previous studies have demonstrated that TRH stimulation of prolactin promoter activity is mediated by the Pit-1 binding site within the prolactin promoter [29], and that E2 regulates prolactin gene transcription only if the prolactin promoter is bound by Pit-1 [30]. It has been speculated that a higher concentration of E2 and P4 is required to potentiate Pit-1 related signaling evoked by TRH.

TRH binds to its seven-transmembrane Gq-coupled TRH receptor [31] and activation of this receptor stimulates the activity of phospholipase C, leading to the production of diacylglycerol and inositol 1,4,5-triphosphate, resulting in protein kinase C (PKC) activation and calcium release from intracellular storage sites [32]. ERK activation by TRH is mediated by both PKC-dependent and -independent pathways [33]. It was also reported that TRH has the ability to increase cAMP [34] and can affect prolactin expression *via* the cAMP/protein kinase A (PKA) pathway [35].

We also investigated the effects of E2 and P4 on downstream signal transduction pathways associated with the TRH receptor using SRE and CRE luciferase reporter constructs. The SRE is a DNA domain in the promoter region that binds to an ERK-mediated transcription factor. CRE is known as a transcription factor and the CRE promoter shows activation by cAMP-responsive element-mediated pathways. The effects of sex steroids on basal SRE luciferase activity were similar to their effects on prolactin promoter activity. Basal prolactin promoter activity was reduced by both E2 and P4, with P4 having a stronger inhibitory effect than E2. Similarly, basal SRE luciferase activity was reduced by E2 and P4, and P4 had a greater inhibitory effect. In contrast, basal CRE luciferase activity was not changed by E2 or P4. These results suggest that ERK-mediated pathways play an important role in the effects of E2 and P4 on basal prolactin promoter activity. However, E2 and P4 had similar effects on TRH-induced SRE and CRE luciferase activity. Both E2 and P4 potentiated the effects of TRH on SRE and CRE activity. P4 had a stronger potentiating effect than E2 on TRH-induced SRE and CRE activity and combined treatment with E2 and P4 further increased TRH-induced action. The effects of E2 and P4 on SRE and CRE luciferase activity mirrored these steroids' effects on prolactin promoter activity.

ERK activation was shown to be strongly involved in prolactin synthesis. We previously demonstrated that TRH-induced ERK phosphorylation was completely abolished in the presence of the MEK inhibitor PD098059, and that TRH-induced prolactin protein synthesis was also completely abolished in the presence of U0126. Inhibition of DNA synthesis by TRH was also prevented by a MEK inhibitor [15]. In addition, we also have shown that TRH-stimulated prolactin mRNA expression and prolactin promoter activity were completely abolished in the presence of a MEK inhibitor. Overexpression of MEKK, a factor upstream of ERK, also increased prolactin mRNA expression [16]. Thus, there is extremely strong evidence that ERK activation is involved in control of prolactin expression. This study also showed that there is crosstalk between the ERK and cAMP/PKA path-

ways. A previous study using COS-7 cells transfected with a Gi-coupled receptor showed that cAMP-stimulated ERK pathways were activated *via* the β and γ Gi protein subunits [36]. It was also reported that Rap-1, a Ras homolog, is involved in cAMP-induced activation of the ERK pathways in neurons [37]. In contrast, CRE-binding protein (CREB) has been reported to be phosphorylated by a MAPK-activated protein kinase, probably through $p70^{S6K}$, as well as by PKA [38, 39]. The GH3 cells used in the present study did not increase cAMP accumulation in response to TRH stimulation. In addition, CRE promoter activity stimulated by TRH was significantly reduced in the presence of an ERK inhibitor, and overexpression of MEK increased CRE promoter activity. These observations support the idea that the CRE promoter is activated by an ERK-mediated pathway in GH3 cells. In addition, we found that TRH-induced ERK phosphorylation was enhanced by E2 and P4. Considering these observations, we speculate that ERK pathways activated by TRH stimulation are a major target of sex steroids and ultimately modulate the synthesis of prolactin.

In the present study, basal prolactin promoter activity was decreased similarly by E2 and P4 and TRH-increased prolactin promoter activity was significantly increased by E2 and P4. The mechanisms of these observations remain unknown. It has been demonstrated that during pregnancy and lactation, there is marked hyperplasia of prolactin cells [1, 2]. Increased estrogen levels in pregnancy lead to a progressive increase in cytoplasmic volume and the accumulation of numerous prolactin-containing granules [40]. In cells exposed to high levels of estrogen, it may not be necessary to maintain high levels of prolactin transcriptional activity because prolactin protein is already abundant. In contrast, under conditions when lactation is stimulated by a lactagogue, sensitivity to the lactagogue may be potentiated.

Here we have shown that both E2 and P4 can modulate basal and TRH-induced prolactin promoter activity. E2 and P4 alone reduced the basal activity of the prolactin promoter with a concomitant decrease in SRE-luciferase activity, but not CRE-luciferase activity. In contrast, TRH-induced prolactin promoter activity was potentiated by both E2 and P4. P4 had a greater stimulatory effect than E2, and combined treatment with E2 and P4 further increased TRH-induced prolactin promoter activity. TRH-induced SRE and CRE luciferase activity was modulated by E2 and P4 in a manner similar to the effects on TRH-induced prolactin promoter activity. The fact that TRH did not increase cAMP accumulation in GH3 cells suggests that ERK pathways activated by TRH may be a target of sex steroids, and may mediate the function of these hormones in somatolactotrophic cells.

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Conflict of Interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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