Activation of Follistatin Promoter by GnRH in L β T2 Gonadotroph Cells

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Abstract. Follistatin (FS) is produced and secreted from gonadotroph cells in pituitary gland as well as granulosa cells in the ovary. In the present study, we found that the FS promoter is activated by GnRH in the gonadotroph cell line, L β T2. Therefore, we examined the signal transduction pathways involved in the mechanism. The activation of the FS promoter by GnRH was inhibited by calphostin C, a protein kinase C inhibitor, and U0126, a MAP kinase kinase (MEK) inhibitor. Phosphorylation by protein kinase C of myristoylated alanine-rich C kinase substrate (MARCKS) in L β T2 cells was observed after 3-min treatment with GnRH and declined after 30 min. The subsequent activation of MAP kinase was also transient, and down-regulation of protein kinase C completely inhibited the MAP kinase activation by GnRH, suggesting that the transient activation of protein kinase C led to the transient activation of MAP kinase. Although phorbol 12-myristate 13-acetate treatment increased phosphorylation of MARCKS and activated MAP kinase, it did not activate the FS promoter. Genistein, a tyrosine kinase inhibitor, completely inhibited the GnRH-induced activation of the FS promoter, while no inhibition of the MAP kinase pathway was observed. These results suggest that the activations of both the protein kinase C and tyrosine kinase pathways are necessary for the activation of the FS promoter in gonadotroph cells.

Key words: Follistatin, GnRH, Gonadotroph cells, MAP kinase, Protein kinase C

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FOLLISTATIN (FS) was first identified from ovarian follicular fluid on the basis of its ability to suppress the secretion of follicle-stimulating hormone (FSH) from pituitary gonadotrophs [1–4]. FS is a monomeric glycoprotein structurally unrelated to inhibin [3]. FS binds to activin with high affinity [5] and inhibits the activity of activin to stimulate FSH secretion [6–8]. In ovary, the granulosa cells are responsible for producing and secreting FS in most species [9–12]. FS expression in granulosa cells is increased through both cyclic AMP

and protein kinase C pathways [13–17]. In cultured rat granulosa cells, FSH and gonadotropin-releasing hormone (GnRH) increased FS protein production through the cyclic AMP and protein kinase C pathways, respectively. The expression of FS is not limited to the ovary, and FS is secreted within the pituitary gland, where it exerts autocrine/paracrine effect [18]. It has been reported that FS expression in gonadotrophs is regulated by GnRH at the steady state mRNA levels [18, 19].

GnRH is a hypothalamic peptide and serves as a critical regulator for the synthesis and secretion of FSH [20, 21]. FSH is composed of two noncovalently linked subunits, α and β . It is well known that the synthesis of the β -subunit (FSH β) is the rate-limiting step in the production of FSH [20]. Slow-frequency GnRH pulses increase FSH β mRNA through activation of the

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FSH β promoter, whereas fast-frequency GnRH pulses decrease FSH β mRNA [22, 23]. It has been reported that fast-frequency, but not slow-frequency, GnRH pulses increase FS mRNA [19]. As an increase in FS synthesis results in the inhibition of the activin-induced activation of FSH synthesis, the GnRH-induced activation of FS synthesis may be involved in the decrease of FSH synthesis in the case of fast-frequency GnRH pulses. However, the intracellular mechanisms by which GnRH activates FS synthesis are unknown at present.

L β T2 cells, derived from a pituitary tumor in a transgenic mouse, are a clonal strain of cultured gonadotroph cell line producing FSH and GnRH receptor [24]. L β T2 cells have proven useful for studies on the molecular mechanisms involved in the GnRH-induced synthesis of FSH. We and other laboratories have reported that GnRH induced the transient activation of the mitogen-activated protein (MAP) kinase pathway in the cells [25, 26]. However, it is still not clear at present why the activation by GnRH of the MAP kinase pathway is transient. Given that L β T2 cells synthesize FS [27], it is of interest to study whether GnRH activates the FS promoter in the cells.

In this study, we placed the luciferase reporter gene downstream of the rat FS promoter and found that the GnRH treatment of L β T2 cells activated the FS promoter. Therefore, we precisely examined the signal transduction pathways from the GnRH receptor stimulation to activation of the FS promoter.

Materials and Methods

Materials

The following chemicals and reagents were obtained from the indicated sources: fetal calf serum (FCS), Hy-Clone (Logan, UT); des-Gly¹⁰, [D-Ala⁶]-LH-RH ethylamide (GnRH) and PD098059, Sigma Chemical Co. (St Louis, MO); recombinant human activin A, R&D Systems Inc. (Minneapolis, MN); antide (*N*-Ac-D-Nal1-D-Cpa2-D-Pal3-Ser4-Lys(Nic)6-Leu7-Ilys8-Pro9-D-Ala10-NH₂), a GnRH antagonist, Bachem (King of Prussia, PA); calphostin C, genistein and herbimycin A, Calbiochem Novabiochem (La Jolla, CA); U0126, Signaling Technology (Beverly, MA); anti-active MAP kinase antibody (rabbit pTEpY), Promega (Madison, WI); anti-MAP kinase antibody, Zymed Laboratories

Inc. (San Francisco, CA); anti-MARCKS antibody, Chemicon International (Temecula, CA); Dulbecco's modified Eagle's medium (DMEM), Nissui Pharmaceutical Co. (Tokyo, Japan); and pFC-MEK1, pFC-MEKK, and pFC-protein kinase A (pFC-PKA) plasmid vectors, Stratagene (La Jolla, CA). Anti-P-MARCKS antibody was developed and purified as previously reported [28].

Culture of L β T2 cells

L β T2 cells were kindly provided by Dr. P.L. Mellon (University of California, San Diego, CA). L β T2 cells were cultured in DMEM containing 10% FCS, 50 U/ml penicillin, and 50 μ g/ml streptomycin and maintained at 37°C in an atmosphere of 95% air-5% CO₂ [26]. The cells in passages 9 to 14 were used in this study. Two or three days before experiments, 2–3 $\times 10^5$ cells and 5–8 $\times 10^5$ cells were plated on 35-mm and 65-mm petri dishes (Nunc, Roskilde, Denmark), respectively. When test reagents were added, L β T2 cells were incubated in DMEM without FCS at 37°C for the indicated times with or without the test reagents. When we examined the effects of some inhibitors, the cells were preincubated with each inhibitor as indicated. We chose the concentrations of antide (100 nM), U0126 (10 μ M), PD098059 (50 μ M), calphostin C (800 nM), genistein (50 μ M) and herbimycin A (50 μ M) as directed by the instructions provided by the manufacturers. The cells were incubated with or without GnRH in the presence or absence of each inhibitor. After incubation for the indicated times, the medium was quickly aspirated off, and the cells were washed once with PBS and used immediately for luciferase assay or frozen in liquid N₂.

Reporter plasmid construct and luciferase assay

Genomic DNA encoding rat FS gene was used to obtain the FS promoter [29]. Polymerase chain reaction (PCR) was carried out to amplify the fragment containing FS promoter based on the published sequences (GenBank accession no. M31586) [30]. Using the PCR primers that consisted of a sense primer containing the *Bgl* II restriction site (5'-ACGCAGATCTCCCGAGCGCTGGAGCAG-3') and an antisense primer containing the *Hind* III restriction site (5'-GGCTAAGCTTGCGATTCAATGGACGTCAG-3'), the FS promoter region (–570 to +157; the transcrip-

tional start site was numbered as +1) was amplified. The fragment of FS promoter region was excised with *Bgl* II and *Hind* III restriction enzymes and inserted into the *Bgl* II and *Hind* III sites of pGL3-basic luciferase reporter vector (Promega Corp.; termed pGL3-FSp). Both strands of the FS promoter region were sequenced with RV primer 3 and GL primer 2 (Promega Corp.), using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit and ABI PRISM 310 sequencer (Perkin-Elmer Corp.). L β T2 cells were cotransfected with pGL3-FSp (1.0 μ g DNA) and pRL-TK (0.1 μ g DNA; Promega Corp.) which contains *Renilla* luciferase under the herpes simplex virus thymidine kinase promoter, using 3 μ l of FuGENE 6 transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN) in 1 ml standard medium. In some experiments, the cells were cotransfected with pFC-MEK1 (2.0 μ g DNA), pFC-MEKK (1.0 μ g DNA), pFC-PKA (1.0 μ g DNA) or pCAGGSneo expression vector (a gift from Prof. J. Miyazaki, Osaka University, Osaka, Japan; mock-transfected cells) in addition to pGL3-FSp and pRL-TK. After incubation of the cells for 36 h, the cells were treated with chemicals for each experiment. The activities of firefly luciferase and *Renilla* luciferase were measured by the Dual Luciferase Reporter Assay System (Promega Corp.) with a luminometer (TD-20/20, Turner designs, Sunnyvale, CA) according to the manufacturer's protocol. The ratio of luminescence signal by firefly luciferase to that by *Renilla* luciferase was determined. We repeated the same experiments three or five times with reproducible results. We combined all the data of independent experiments in figures and for statistical analysis.

Preparation of cell extracts and immunoblot analysis

The procedures for preparation of cell extracts were carried out at 4°C. Frozen cells in 60-mm petri dish were scraped and solubilized in 400 μ l of a homogenization buffer containing 50 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) (pH 7.4), 0.1% Triton X-100, 4 mM ethyleneglycol bis (2-aminoethylether) tetraacetic acid (EGTA), 10 mM EDTA, 15 mM Na₄P₂O₇, 100 mM β -glycerophosphate, 25 mM NaF, 0.1 mM leupeptin, 75 μ M pepstatin A, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na₃VO₄, and 100 nM calyculin A [26, 31]. After sonication, the insoluble materials were removed by centrifugation at 15,000 $\times$ g for 20 min. The protein

concentration was determined by the method of Bradford [32] with bovine serum albumin (BSA) as a standard. Cell extracts were treated with SDS sample buffer [33] and boiled for 2 min. Samples containing the same amount of proteins were subjected to SDS-PAGE in 9 or 10% acrylamide and transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was incubated overnight with the anti-active MAP kinase (1:1,000) or anti-P-MARCKS antibody (1:100). The membrane was washed three times with blocking solution containing 4.5% nonfat dry milk, 100 mM Tris-HCl (pH 7.5), 0.9% NaCl, and 0.1% Tween-20, and then washed more than four times with Tris-buffered saline with Tween-20 (TTBS) containing 100 mM Tris-HCl (pH 7.5), 0.9% NaCl, and 0.1% Tween-20 at room temperature, followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5,000). Immunoreactive proteins were detected using the enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech, Little Chalfont, UK) as directed by the instructions provided by the manufacturer. For reprobing, the membrane was incubated with stripping buffer (62.5 mM Tris-HCl (pH 6.7), 100 mM 2-mercaptoethanol, and 2% SDS) at 50°C for 30 min. The membrane was washed with blocking solution and TTBS as described above and was subjected to immunoblot analysis.

Statistical evaluation

We repeated the same experiments three or five times with reproducible results. We combined all the data of independent experiments. Values were expressed as the mean $\pm$ SE. Statistical analysis was performed using one-way ANOVA plus Duncan's multiple range test. $P < 0.05$ was considered statistically significant.

Results

Activation of FS promoter by GnRH

To measure the activity of FS promoter, we constructed the luciferase reporter gene driven by the fragment (−570 to +157) of FS promoter region (termed pGL3-FSp). The sequencing of the obtained fragment revealed that the nucleotide sequence was 100% identical to that of the reported sequence (GenBank acces-

sion no. M31586). When L β T2 cells were transfected with pGL3-FSp and treated with increasing doses of GnRH, FS promoter activity was significantly increased from 1 nM GnRH after 8 h of treatment (Fig. 1). FS promoter activity was increased to $259.6 \pm 10.1\%$ of control by 10 nM GnRH. The activation of FS promoter by GnRH at 10 nM was higher than that at 1 nM but not different from that at 100 nM or 1,000 nM. Figure 2 shows the time course of FS promoter activation by 10 nM GnRH. FS promoter activity was increased to 202.7 ± 11.6 and $203.0 \pm 23.1\%$ of control at 8 and 12 h, respectively. From these results, we decided to treat L β T2 cells with 10 nM GnRH for 8 h in the following experiments. To confirm that the activation of FS promoter by GnRH was receptor mediated, we examined the effects of antide, a GnRH antagonist. The GnRH-induced FS promoter activation was completely inhibited by addition of 100 nM antide (data not shown). FS promoter activity was not affected by treatment with 40 ng/ml activin A for 8 h.

Involvement of MAP kinase in FS promoter activation

It has been reported that GnRH induced the transient activation of the MAP kinase pathway in L β T2 cells [25, 26]. Therefore, we examined whether or not the

activation of the MAP kinase pathway was necessary for the GnRH-induced activation of the FS promoter. The stimulatory effect of GnRH was completely abolished by U0126, a specific MAP kinase/extracellular signal-regulated kinase activating kinase (MEK) inhibitor (Fig. 3). U0126 alone slightly inhibited the basal FS promoter activity. In addition to U0126, we found that PD098059, another MEK inhibitor, completely inhibited the GnRH-induced FS promoter activation (data not shown). These results suggested that the MAP kinase pathway was involved in the GnRH-induced activation of the FS promoter. Next, we asked whether or not activation of the MAP kinase pathway was sufficient for the activation of the FS promoter. For this purpose, we transfected the cells with pFC-MEK1 or pFC-MEKK to overexpress constitutively active MEK1 or MEK kinase (MEKK), respectively. Overexpression of MEK1 and MEKK resulted in an approximately 2.5- and 4.5-fold activation, respectively, compared with mock-transfected cells (Fig. 4). It has been reported that activation of the cyclic AMP pathway activates the FS promoter in primary cultured rat granulosa cells [13]. However, overexpression of the catalytic subunit of cyclic AMP-dependent protein kinase (cAMP-kinase) had no stimulatory effect on FS promoter activity in L β T2 cells (Fig. 4).

Fig. 1. Activation of the FS promoter by increasing doses of GnRH. L β T2 cells were cotransfected with pGL3-FSp (1.0 μ g) and pRL-TK (0.1 μ g) for 36 h. The cells were then treated without or with GnRH at the indicated doses for 8 h. The activity without GnRH (control) was taken as 100%, and from this value, other values were calculated. Values are the mean $\pm$ SE. ** $P < 0.01$; * $P < 0.05$ (vs. control).

Fig. 2. Time-course effects of GnRH on FS promoter activation. L β T2 cells were cotransfected with pGL3-FSp (1.0 μ g) and pRL-TK (0.1 μ g) for 36 h. The cells were then treated with 10 nM GnRH for the indicated time intervals. The activity with GnRH treatment for 0 h (control) was taken as 100%, and from this value, other values were calculated. Values are the mean $\pm$ SE. * $P < 0.05$ (vs. control).

Fig. 3. Effects of U0126 on FS promoter activation by GnRH. LβT2 cells were cotransfected with pGL3-FSp (1.0 μg) and pRL-TK (0.1 μg) for 36 h. The cells were then treated without (Control) or with either or both of 10 μM U0126 and 10 nM GnRH for 8 h. The activity is expressed as a percentage of the control. Values are the mean ± SE. ** $P < 0.01$; * $P < 0.05$ (vs. control). The difference between GnRH and GnRH plus U0126 was statistically significant ($P < 0.01$).

Fig. 5. Effects of calphostin C on FS promoter activation by GnRH. LβT2 cells were cotransfected with pGL3-FSp (1.0 μg) and pRL-TK (0.1 μg) for 36 h. The cells were then preincubated without or with 800 nM calphostin C (Cal. C) for 60 min, and further treated without (Control) or with either or both of 800 nM calphostin C and 10 nM GnRH for 8 h. The activity is expressed as a percentage of the control. Values are the mean ± SE. ** $P < 0.01$; * $P < 0.05$ (vs. control). The difference between GnRH and GnRH plus calphostin C ($P < 0.01$) was statistically significant.

Fig. 4. Effects of overexpression of MEK1, MEKK and catalytic subunit of cAMP-kinase on FS promoter activation. LβT2 cells were cotransfected with pGL3-FSp (1.0 μg) and pRL-TK (0.1 μg) together with either pCAGGSneo (1.0 μg; MOCK), pFC-MEK1 (2.0 μg; MEK1), pFC-MEKK (1.0 μg; MEKK) or pFC-PKA (1.0 μg; PKA) for 36 h. The value of mock-transfected cells was taken as 100%, and from this value, other values were calculated. Values are the mean ± SE. ** $P < 0.01$; * $P < 0.05$ (vs. MOCK).

Involvement of protein kinase C in FS promoter activation

We next examined the possible involvement of protein kinase C in the GnRH-induced activation of the FS promoter. In Figure 5, GnRH activated the FS promoter to $226.5 \pm 5.8\%$ of control. In the presence of calphostin C, a specific protein kinase C inhibitor, GnRH activated the FS promoter only to $131.2 \pm 1.3\%$ of control.

To further examine the possible involvement of protein kinase C in the activation of FS promoter, protein kinase C was down-regulated by pretreatment with 1 μM phorbol 12-myristate 13-acetate (PMA) for 24 h. We found that down-regulation of protein kinase C abolished the stimulatory effect of GnRH (Fig. 6). It was interesting to note that treatment of the cells with 50 nM PMA for 8 h did not activate the FS promoter. Furthermore, we examined the time course effects of 50 nM PMA until 24 h (*i.e.* 8 h, 16 h, and 24 h). However, no stimulatory effect of PMA on the FS promoter activity was observed (data not shown).

Fig. 6. Effects of down-regulation of protein kinase C on FS promoter activation by GnRH. L β T2 cells were preincubated without or with 1 μ M PMA for 24 h, and further treated without or with 10 nM GnRH or 50 nM PMA for 8 h. The activity without PMA pretreatment and without GnRH and PMA was taken as 100% (control), and from this value, other values were calculated. Values are the mean $\pm$ SE. ** P <0.01 (vs. control). The difference between GnRH without PMA pretreatment and GnRH with PMA pretreatment (P <0.01) was statistically significant.

Activation of protein kinase C and MAP kinase by GnRH and PMA

We next examined whether or not PMA activates protein kinase C and MAP kinase at the comparable levels with GnRH (Fig. 7). Myristoylated alanine-rich C kinase substrate (MARCKS), a major substrate for protein kinase C, is distributed in a variety of cells [34]. Since serines 152 and 156, numbered according to rat MARCKS, are phosphorylated only by protein kinase C, the increase in phosphorylation of these sites has been used as an indicator for protein kinase C activation in intact cells [35]. In a previous study, we produced a specific antibody (anti-P-MARCKS antibody) against MARCKS phosphorylated at these sites [28]. Using this antibody, we first examined whether or not the activation levels of protein kinase C by GnRH and PMA were comparable with each other. When the cells were treated with GnRH or PMA for 30 min, strong phosphorylation of MARCKS was detected by both treatments (Fig. 7A). When the antibody to MARCKS was used for immunoblot analysis, no significant changes in immunoreactivities were observed

Fig. 7. Effects of GnRH and PMA on MARCKS phosphorylation (A) and effects of down-regulation of protein kinase C on MAP kinase activation by GnRH and PMA (B). A, L β T2 cells were treated without (Control) or with 10 nM GnRH or 50 nM PMA for 30 min. The cell extracts (18 μ g) were subjected to SDS-PAGE, and immunoblot analysis was carried out with anti-P-MARCKS antibody at a dilution of 1:100. The position of phosphorylated MARCKS is indicated. B, L β T2 cells were preincubated without or with 1 μ M PMA for 24 h, and further treated without or with 10 nM GnRH or 50 nM PMA for 30 min. The cell extracts (18 μ g) were subjected to SDS-PAGE, and immunoblot analysis was carried out with anti-active MAP kinase antibody at a dilution of 1:1,000. After the antibody was stripped, immunoblot analysis with anti-MAP kinase antibody was carried out at a dilution of 1:1,000. The positions of ERK1 and ERK2 (P-ERK2 and ERK2) are indicated.

(data not shown). We next compared the levels of the activation of MAP kinase by GnRH and PMA. When the anti-MAP kinase antibody was used for immunoblot analysis, we found that both 44-kDa extracellular signal-regulated kinase 1 (ERK1) and 42-kDa ERK2 occurred in L β T2 cells (Fig. 7B). Immunoblot analysis with anti-active MAP kinase antibody showed that both GnRH and PMA treatments for 30 min strongly activated ERK2 (Fig. 7B). Furthermore, down-regulation of protein kinase C completely inhibited the activation of ERK2 by GnRH as well as PMA. When the antibody to MAP kinase was used for immunoblot analysis, no significant changes in immunoreactivities were observed by any treatments (Fig. 7B). These results indicated that PMA as well as GnRH strongly activates protein kinase C and MAP kinase under our assay conditions, and that activation of protein kinase

C is necessary for the activation of MAP kinase by GnRH. In addition, the results of Fig. 6 and Fig. 7A suggested that activation of protein kinase C is necessary but not sufficient for the activation of the FS promoter.

Time course of activation by GnRH of protein kinase C and MAP kinase

We next examined whether or not GnRH treatment activates protein kinase C more rapidly than MAP kinase (Fig. 8). Phosphorylation of MARCKS by 10 nM GnRH became detectable at 3 min, and then reached a maximal peak at 5 min and lasted until 20 min. Inter-

estingly, phosphorylation of MARCKS was declined from 30 min and was not observed in 90 min. In contrast, activation of MAP kinase reached a maximal peak at 10 min and lasted until 30 min. Activation of MAP kinase was declined from 45 min. These results suggested that the activation of protein kinase C by GnRH is transient and precedes the activation of MAP kinase.

Time course of activation of MAP kinase by overexpression of MEK1 and MEKK

In addition, we examined the time course activation of MAP kinase by overexpressing constitutively active MEK1 and MEKK (Fig. 9). Overexpression of MEK1 activated ERK2 after 12 h of transfection. It was interesting that the ERK2 activation lasted until 36 h of transfection. The activation levels of MAP kinase after 24 and 36 h of transfection were not different in other experiments (data not shown). Overexpression of MEKK activated ERK2 after 24 h of transfection and the activation lasted until 36 h of transfection. When the anti-MAP kinase antibody was used for immunoblot analysis, no significant changes in the immunoreactivities were observed. These results indicated that overexpression of MEK1 and MEKK induced the sustained activation of ERK2 for longer than 12 h, which was different from the transient activation by GnRH treatment.

Involvement of tyrosine kinases in the GnRH-induced activation of FS promoter

We found that PMA treatment activates protein ki-

Fig. 8. Time course of MARCKS phosphorylation and MAP kinase activation by GnRH. L β T2 cells were treated with 10 nM GnRH for the indicated time intervals. The cell extracts (20 μ g) were subjected to SDS-PAGE, and immunoblot analysis was done with anti-P-MARCKS antibody (1:100) or anti-active MAP kinase antibody (1:1,000). After the anti-active MAP kinase antibody was stripped, immunoblot analysis with anti-MAP kinase antibody was carried out at a dilution of 1:1,000. The positions of ERK1 and ERK2 (P-ERK2 or ERK2) are indicated.

Fig. 9. Effects of overexpression of MEK1 and MEKK on MAP kinase activation. L β T2 cells were transfected with pFC-MEK1 (2.0 μ g; MEK1) or pFC-MEKK (1.0 μ g; MEKK) for the indicated time intervals. The cell extracts (20 μ g) were subjected to SDS-PAGE, and immunoblot analysis was carried out with anti-active MAP kinase antibody at a dilution of 1:1,000. After the antibody was stripped, immunoblot analysis with anti-MAP kinase antibody was carried out at a dilution of 1:1,000. The positions of ERK1 and ERK2 (P-ERK2 or ERK2) are indicated.

Fig. 10. Effects of genistein on FS promoter activation by GnRH (A) and overexpression of MEK1 (B). A, L β T2 cells were cotransfected with pGL3-FSp (1.0 μ g) and pRL-TK (0.1 μ g) for 36 h. The cells were then treated without (Control) or with either or both of 50 μ M genistein and 10 nM GnRH for 8 h. B, L β T2 cells were cotransfected with pGL3-FSp (1.0 μ g) and pRL-TK (0.1 μ g) with either pCAGGSneo (2.0 μ g; MOCK) or pFC-MEK1 (2.0 μ g; MEK1) in the presence or absence of 50 nM genistein or 10 μ M U0126 for 36 h. The activity is expressed as a percentage of the control. Values are the mean $\pm$ SE. * $P < 0.05$ (vs. mock). The differences between GnRH and GnRH plus genistein and between MEK1 and MEK1 plus U0126 were statistically significant ($P < 0.01$).

nase C and MAP kinase but does not mimic the GnRH effect on the activation of the FS promoter. Therefore, we considered the possibility that GnRH but not PMA might activate some other pathways, which were necessary for the GnRH-induced activation of the FS promoter. As one of the candidates, we considered the possibility that the tyrosine kinase pathway is involved in the GnRH action. Therefore, we examined whether or not genistein, a tyrosine kinase inhibitor, inhibits the effect of GnRH on the FS promoter activity (Fig. 10A). Genistein completely abolished the GnRH-induced activation of the FS promoter. Genistein alone slightly inhibited the basal FS promoter activity. In addition to genistein, 50 μ M herbimycin A, another tyrosine kinase inhibitor, completely inhibited the GnRH-induced FS promoter activation (data not shown). However, genistein did not inhibit the activation of the FS promoter by overexpression of MEK1, suggesting that the tyrosine kinase pathway was not downstream of the MAP kinase pathway (Fig. 10B). As expected, U0126 completely inhibited the FS promoter activation by overexpression of MEK1 (Fig. 10B). We next examined whether genistein inhibits the GnRH-induced activation of MAP kinase (Fig. 11). Time course of

activation of ERK2 by GnRH was not affected by the addition of genistein. These results suggested that the tyrosine kinase pathway was not upstream of the MAP kinase pathway.

Discussion

FS is known to be one of the key regulators of the hypothalamic-pituitary-ovarian axis. FS was first identified from ovarian follicular fluid by its ability to inhibit the activin-induced FSH synthesis. In the ovary, FS is secreted mainly from granulosa cells. It is now known that FS is expressed in the cells in pituitary gland, including gonadotroph cells. It has been reported that FS mRNA in gonadotroph cells was increased by fast-frequency GnRH pulses [19]. However, the molecular mechanisms involved in the reaction have yet to be clarified. The mRNA of the β -subunit of luteinizing hormone (LH β) has been reported to be increased by fast-frequency GnRH pulses through activation of its promoter [27, 36]. Recent experiments using GnRH agonist and GnRH antagonist showed that GnRH plays a key role in GnRH receptor expression, as well as LH

Fig. 11. Effects of genistein on MAP kinase activation by GnRH. L β T2 cells were treated with 10 nM GnRH in the presence or absence of 50 μ M genistein for the indicated time intervals. The cell extracts (20 μ g) were subjected to SDS-PAGE, and immunoblot analysis was carried out with anti-active MAP kinase antibody (A) at a dilution of 1:1,000. After the antibody was stripped, immunoblot analysis with anti-MAP kinase antibody (B) was carried out at a dilution of 1:1,000. The positions of ERK1 and ERK2 (P-ERK2 or ERK2) are indicated.

and FSH expression [37]. In human, the GnRH secretion may be regulated by estrogen and leptin [38]. It has been reported that MAP kinase contributes to the GnRH-induced LH β promoter activation in L β T2 cells [25, 26]. In this study, we placed the luciferase reporter gene downstream of the rat FS promoter and found that the single and chronic treatment of L β T2 cells can activate the FS promoter. To our knowledge, this is the first report on the activation of the FS promoter by GnRH in L β T2 cells. Using this system, we examined the signal transduction pathways for the FS promoter activation. Figure 12 shows the pathways that appear to play roles in the phenomena studied.

Our conclusion is that the activation of both MAP kinase and protein kinase C are necessary for the GnRH-induced activation of the FS promoter for the following reasons: 1) U0126 and PD098059 inhibited the FS promoter activation by GnRH, and 2) calphostin C and down-regulation of protein kinase C inhibited the FS promoter activation by GnRH. We thus examined whether or not activation of protein kinase C is necessary for MAP kinase activation. To detect the activation of protein kinase C, we examined the phosphorylation of MARCKS by protein kinase C with a specific antibody which we developed in a previous study [28]. Interestingly, phosphorylation of MARCKS was rapid and transient. Time-course comparison of MARCKS phosphorylation and MAP kinase activation suggested that the activation of protein kinase C is followed by the activation of MAP kinase. Furthermore, MAP kinase activation by both GnRH and PMA was completely inhibited by down-regulation of protein kinase C. These results suggested that transient activation of

Fig. 12. The pathways that appear to play roles in the GnRH actions in L β T2 gonadotroph cells.

protein kinase C induces transient activation of MAP kinase.

We found that overexpression of constitutively active MEK1 and MEKK activates the FS promoter. These results suggested that the activation of MAP kinase is sufficient for the activation of the FS promoter. As PMA treatment strongly activated MAP kinase, we expected that PMA treatment also activates the FS promoter. However, PMA treatment did not activate the FS promoter at all. These results suggested that the activation of protein kinase C and MAP kinase by PMA treatment is not sufficient for the activation of FS promoter. When we examined the time course activation of MAP kinase, we found that the activation by overexpression of MEK1 and MEKK lasts much longer than that by GnRH treatment. We confirmed that the activation of MAP kinase by PMA treatment is transient

(data not shown). These results suggest that transient activation of MAP kinase by GnRH and PMA is not sufficient for the activation of the FS promoter, while sustained activation of MAP kinase by overexpression of MEK1 and MEKK is sufficient for the activation of the FS promoter.

We hypothesized that pathways other than protein kinase C and MAP kinase are necessary for the GnRH-induced activation of the FS promoter. As one of the candidates, we considered the possibility that the tyrosine kinase pathway is involved in the reaction. As expected, genistein completely inhibited the GnRH-induced activation of the FS promoter. It is interesting to note, however, that genistein did not inhibit the activation of MAP kinase by GnRH, hence it is quite possible that the tyrosine kinase pathway is involved in the

GnRH actions in a MAP kinase-independent manner. The identification of the specific tyrosine kinases involved in the mechanism is important for the understanding of the signal transduction.

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