Regulation of the mitogen-activated protein kinase (MAPK) family by gonadotropin-releasing hormone (GnRH) in the gonadotrope cell line LβT2 was investigated. Treatment with gonadotropin-releasing hormone agonist (GnRHa) activates extracellular signal-regulated kinase (ERK) and c-Jun NH2-terminal kinase (JNK). Activation of ERK by GnRHa occurred within 5 min and declined thereafter, whereas activation of JNK by GnRHa occurred with a different time frame, i.e. it was detectable at 5 min, reached a plateau at 30 min, and declined thereafter. GnRHa-induced ERK activation was dependent on protein kinase C or extracellular and intracellular Ca2⁺, whereas GnRHa-induced JNK activation was not dependent on protein kinase C or on extracellular or intracellular Ca2⁺. To determine whether a mitogen-activated protein kinase family cascade regulates rat luteinizing hormone β (LHβ) promoter activity, we transfected the rat LHβ promoter construct into LβT2 cells. GnRH activated the rat LHβ promoter activity in a time-dependent manner. Neither treatment with a mitogen-activated protein kinase/ERK kinase (MEK) inhibitor, PD98059, nor cotransfection with a catalytically inactive form of a mitogen-activated protein kinase construct inhibited the induction of the rat LHβ promoter by GnRH. Furthermore, cotransfection with a dominant negative Ets had no effect on the response of the rat LHβ promoter to GnRH. On the other hand, cotransfection with either dominant negative JNK or dominant negative c-Jun significantly inhibited the induction of the rat LHβ promoter by GnRH. In addition, GnRH did not induce either the rat LHβ promoter activity in LβT2 cells transfected stably with dominant negative c-Jun. These results suggest that GnRHa differentially activates ERK and JNK, and a JNK cascade is necessary to elicit the rat LHβ promoter activity in a c-Jun-dependent mechanism in LβT2 cells.

GnRH, a hypothalamic decapeptide, serves as a key regulator of the reproductive system. GnRH acts on anterior pituitary gonadotropes to stimulate the synthesis and release of the pituitary gonadotropins LH and FSH. The gonadotropins are subunit hormones, each containing noncovalently linked α- and β-subunits (1, 2). Within a species, the α-subunits are identical, while the β-subunits differ and confer the physiological specificity of the heterodimeric hormone. Each β-subunit as well as the common α-subunit is encoded by different genes on separate chromosomes. When GnRH binds to its seven-transmembrane receptor (3), it induces interaction of the receptor with the heterotrimeric Gα protein, which leads to activation of phospholipase C and formation of inositol 1,4,5-triphosphate and diacylglycerol, leading to elevation of intracellular Ca2⁺ and activation of protein kinase C (PKC) (4–6).

Intracellular transmission of extracellular signals is mediated in large part by several groups of sequentially activated protein kinases, which are collectively known as the mitogen-activated protein kinase (MAPK) cascades. In growth factor signaling, the key elucidated MAPK cascade is the extracellular signal-regulated kinase (ERK). Recent evidence indicates that many G protein-coupled receptors can activate the ERK cascade (7–11). The signals transmitted through the ERK cascade lead to activation of a set of regulatory molecules that eventually initiate cellular responses such as growth and differentiation (12–14). Recently, it has been shown that GnRHa is capable of activating ERK in pituitary organ culture (15) and the αT3–1 gonadotrope cell line (16, 17). However, the ERK cascade is not the only link between membrane receptors and their intracellular targets, and in the past few years several other ERK-like cascades have been identified (18). One of the most studied of these cascades is the Jun NH2-terminal kinase (JNK; also known as stress-activated protein kinase (SAPK); Refs. 18 and 19) cascade, which is known to be activated in response to cellular stresses such as apoptosis (18, 20). ERK, JNK, and p38 (21) constitute the MAPK family. Recent data suggest that GnRHa is capable of activating JNK (22) and p38 (23) in the αT3–1 gonadotrope cell line. It was reported that GnRH induction and basal control of the α-subunit gene seem to occur through the PKC/ERK pathway, protein kinase; JNK, c-Jun NH₂-terminal protein kinase; SAPK, stress-activated protein kinase; iMAPK, a catalytically inactive form of MAPK, dnJun, dominant negative c-Jun; MBP, myelin basic protein; GST, glutathione S-transferase; PAG, polyacrylamide gel electrophoresis; PTX, pertussis toxin; PKC, protein kinase C; FMA, phorbol 12-myristate 13-acetate; BAPTA-AM, 1,2-bis(o-aminophenoxy)ethane-N,N',N''-tetraacetic acid-acetoxyethyl ester; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; LH, luteinizing hormone; FSH, follicle-stimulating hormone; CMV, cytomegalovirus; HA, hemagglutinin.

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while induction of the LHβ gene is dependent on cardiac influx in the αT3–1 gonadotrope cell line, suggesting the differential stimulation of transcription of rat LH subunit genes by GnRH (24). However, the αT3–1 gonadotrope cell line does not express the LHβ gene. Mellon and co-workers (25), using targeted oncogenesis in transgenic mice, have recently generated an immortal gonadotrope cell line (LβT2). The cells of this line express the mRNA of GnRH receptor and of both the α and β-subunits of LH (26, 27).

Taken together, these facts led us to examine whether GnRH stimulates the activity of ERK and/or JNK, and whether the respective cascades play a role in the transcriptional activation of the rat LHβ gene in LβT2 cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—Porobil 12-myrystate 13-acetate (PMA) and myelin basic protein were purchased from Sigma, Bisdionolylmaleimide (GF 109203X) was purchased from Calbiochem (Laufelfingen, Switzerland). GnRH was obtained from Peninsula Laboratories (Belmont, CA). GnRH agonist, [α-Leu5,α-Pro2,NHε-εleuloprolide], was a gift from Takeda Chemical Industries (Japan). ECL Western blotting detection reagents were obtained from Amersham Pharmacia Biotech. [γ-32P]ATP (3000 Ci/ mmol, 30 mCi) from NEN Life Science Products. Erk1 rabbit polyclonal anti-ERK antibody, anti-Myc antibody, and anti-HA antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). PD98059 and the SAPK/JNK assay kit, including NH2-terminal c-Jun fusion protein bound to glutathione-Sepharose beads and a phosphospecific c-Jun antibody (Ser63), were obtained from New England Biolabs (Beverly, MA).

**Cell Cultures**—LβT2 cells (26) were generously provided by P. Mellon (La Jolla, CA). Cells were cultured at 37 °C in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum in a water-saturated atmosphere of 95% O2 and 5% CO2.

**Construction of Expression Plasmids**—The wild type −156 to +7 LHβ promoter construct, cloned upstream of luciferase in PLK5/Sb-Luc vector, was a kind gift from Dr. Y. Sadovsky (Washington University School of Medicine, St. Louis, MO) (28). The plasmid pLNCX-β-gal (K−M) (29) was a kind gift from Dr. A. Gutierrez-Hartmann (University of Colorado Health Science Center, Denver, CO). Plasmid encoding the dominant negative form of Ets-2 (30) was a kind gift from Dr. K. E. Boulukos (Center de Biochimie, Faculté des Sciences, Nice, France). pAPr-etα2, encoding the consensus DNA-binding domain of Ets-2, and pETα2, a kind gift from Dr. M. O. Retik (Ohio State University, Columbus, OH) (31). The plasmids encoding the dominant negative c-Jun (dnJun), pHcC-Jun (S63A, S73A) (32, 33), and TAT-67 (34) were kind gifts from Dr. D. Melora (University of California, San Diego, CA). The plasmids encoding the dominant negative SAPK/JNK (pcDL-SRαSAPK-VFP) and the wild type SAPK/JNK (pcDL-SRα-wt-SAPK) were kind gifts from Dr. E. Nishida (Kyoto University, Kyoto, Japan). Myc-tagged p42MAPK expression plasmid (pEXV-Erk2-tag) was a kind gift from Dr. K. E. Boulukos (Center de Biochimie, Faculté des Sciences, Nice, France). pAPr-etsZ, encoding the consensus DNA-binding domain of SAPK/JNK, or dominant negative c-Jun (dnJun), pLHCc-Jun (S63A, S73A) (32, 33), and TAM-67 (34) were used in the subsequent luciferase and β-galactosidase assays. The plasmid pLNCX-β-gal was used as described previously (40). Briefly, the luciferase assay mixture contained 100 mM potassium phosphate, pH 7.8, and 10 mM dithiothreitol. Vigorous vortexing was used to enhance cell lysis. Unlysed cells and insoluble material were pelleted at 10,000 rpm for 10 min at 4 °C. The supernatant volume was measured, and aliquots of the supernatant were used in the subsequent luciferase and β-galactosidase assays.

**Promoter Assay**—Cells were transfected with rat −156 to +7 LHβ-luciferase construct and CMV-β-galactosidase plasmid (to normalize for cell viability and transfection efficiency) in combination with the indicated plasmids using LipofectAMINE Plus. At 48 h after transfection, serum-deprived cells were incubated with 1 μM GnRHa for 30 min, and cell lysates were immunoprecipitated with anti-HA antibody. The expressed HA-tagged wild type SAPK/JNK or dominant negative SAPK/JNK was eluted with 1% SDS, and the JNK activity was measured as described above.

**β-Galactosidase Assay**—Luciferase assay mixture contained 100 mM potassium phosphate, pH 7.8, 1 mM dithiothreitol, 3.7 mM MgSO4, 530 μM ATP, and 470 μM luciferin plus 20 μl of cell extract in a final volume of 100 μl. Luciferin was added just before measuring light units, which were measured in duplicate during the first 40 s of the reaction at 25 °C in a luminometer (41). The β-galactosidase was assayed as described previously (40). The β-galactosidase buffer contained 60 mM sodium phosphate, pH 7.5, 1 mM MgCl2, 0.80 mg/ml O-nitrophenyl-β-δ-galactopyranoside, and 40 μM β-mercaptoethanol. A standard curve for 100 micromolar to 2 millimolar of β-galactosidase was made with each assay. A 30-μl aliquot of cell extract was incubated with assay buffer until color developed (30–120 min), and the reaction was then stopped by adding Na2CO3 to a final concentration of 625 mM. Absorbance was then read at 405 nm. Luciferase light units were normalized relative to the activity of β-galactosidase. The control value was set at 1 and the data expressed as -fold stimulation relative to control. Data are expressed as the mean ± S.E.

**Statistics**—Statistical analysis was performed by Student’s t test, and p < 0.01 was considered significant. Data are expressed as the mean ± S.E.

**RESULTS**

**Activation of ERK and JNK by GnRH**—To evaluate whether GnRH is activated by GnRH in LβT2 cells, cultured cells were
exposed to GnRHa for the indicated times (Fig. 1A). Cell lysates were immunoprecipitated with anti-ERK antibody and examined for ERK activity by assaying the incorporation of $^{32}$P into MBP. The GnRHa-dependent increase in ERK activity reached a plateau from 5 min through 10 min and rapidly declined thereafter. We next examined the effect of GnRH on the activation of JNK, which is a member of the MAP kinase family. Cultured cells were exposed to GnRHa for the indicated times and cell lysates were incubated with GST-c-Jun fusion protein/GSH-Sepharose beads, followed by SDS-PAGE and Western blot analysis using anti-phospho(Ser$^{63}$) c-Jun antibody, as described under "Experimental Procedures." After the reactions were stopped with Laemmli sample buffer, samples were subjected to SDS-PAGE and autoradiography. Autoradiograms of phosphorylated GST-c-Jun are shown.

**Effect of Pertussis Toxin on GnRH-induced ERK and JNK Activation**—We compared the mechanisms of ERK and JNK activation induced by GnRH. It has been shown that the receptor for GnRH (3–6) is a member of the superfamily of G protein-coupled receptors. To determine what type of G protein is coupled to the GnRH receptor, we pretreated L$\beta$T2 cells with 100 ng/ml pertussis toxin (PTX) for 4 h in order to inactivate $G_i$ and $G_o$ proteins, and then treated the cells with 1 $\mu$M GnRHa for 5 min (Fig. 2A) or 30 min (Fig. 2B, upper panel). PTX clearly caused a decrease in GnRHa-induced ERK activation (Fig. 2A, lane 7). PTX did not have a detectable effect on GnRHa-induced JNK (Fig. 2B, upper panel, lane 7) activation. Thus, although PTX-sensitive G proteins are partly involved in the effect of GnRHa on ERK activity, as previously reported (42), PTX-sensitive G proteins are not involved in the effect of GnRHa on JNK activity, as was also previously reported (22).

**Role of PKC in Activation of ERK and JNK**—Many G protein-linked receptors can mediate stimulation of ERK activity via the phospholipase C-dependent activation of PKC (43–46). Activation of ERK (16, 17) or JNK (22) by GnRH requires PKC in $\alpha$T3–1 cells. Therefore, the role of PKC in GnRH-induced ERK (Fig. 2A) or JNK (Fig. 2B) activation in L$\beta$T2 cells was examined. Exposure of L$\beta$T2 cells to PMA caused stimulation of ERK activity (Fig. 2A, lane 1). However, the ability of PMA to induce the activation of ERK does not necessarily mean that the PKC pathway is involved in GnRH-induced ERK activation, as has been shown in the case of norepinephrine-induced ERK activation in both adipocytes (47) and GT-1 GnRH neuronal cell lines (10). Whether PKC is indeed involved in GnRH signaling was determined using PKC depletion. Pretreatment with 1 $\mu$M PMA for 16 h to deplete most PKC isoforms completely abolished the GnRHa-induced ERK activation (Fig. 2A, lane 6). On the other hand, treatment with 1 $\mu$M PMA for 5 min (Fig. 2B, upper panel, lane 1) or for 30 min (Fig. 2B, lower panel, lane 5) did not induce JNK activation. Moreover, neither pretreatment with 1 $\mu$M PMA for 16 h (Fig. 2B, upper panel, lane 6) nor pretreatment with the selective PKC inhibitor GF109230X at 10 $\mu$M (Fig. 2B, lower panel, lane 4) had any effect on the GnRHa-induced JNK activation. These results suggest that activation of ERK by GnRH was mediated by PKC, whereas activation of JNK by GnRH was not mediated by PKC.

**Role of Extracellular and Intracellular Ca$^{2+}$ in ERK and JNK Activation**—It has been reported that elevated Ca$^{2+}$ is necessary for GnRH-induced ERK activation in $\alpha$T3–1 cells (16, 17). We therefore evaluated the role of extracellular and intracellular Ca$^{2+}$ in the GnRHa-induced ERK (Fig. 3A) and JNK (Fig. 3B) activation in L$\beta$T2 cells. Elimination of extracellular Ca$^{2+}$ by treatment with 3 mM EGTA for 1 min or with 1 $\mu$M nifedipine for 10 min completely attenuated GnRHa-induced ERK activation (Fig. 3A, lanes 3 and 5), indicating that Ca$^{2+}$ influx is required for GnRHa-induced ERK activation. Moreover, treatment with either 50 $\mu$M 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid-acetoxymethyl ester (BAPTA-AM) for 20 min to eliminate intracellular Ca$^{2+}$ (Fig. 3A, lane 6) or 3 mM EGTA for 15 min to eliminate extracellular and intracellular Ca$^{2+}$ (49) (Fig. 3A, lane 4) clearly attenuated GnRHa-induced ERK activation, indicating that intracellular Ca$^{2+}$ is also required for GnRHa-induced ERK activation. On the other hand, elimination of extracellular Ca$^{2+}$ by either treatment with 3 mM EGTA for 1 min or with 1 $\mu$M nifedipine for 10 min or elimination of intracellular Ca$^{2+}$ by treatment with 50 $\mu$M BAPTA-AM had no effect on GnRHa-induced JNK activation (Fig. 3B). These results suggest that GnRHa-induced ERK acti-
involved in the GnRH-induced LH (Fig. 5C). These results suggest that the ERK cascade is not completely abolished the GnRHa-induced ERK activation (Fig. 5B), whereas GnRH-induced JNK activation was independent of extracellular and intracellular Ca$^{2+}$ in L$\beta$T2 cells.

**Stimulation of LHβ Promoter Activity by GnRH**—We sought to determine whether the ERK and/or JNK cascades are involved in the regulation of LHβ synthesis induced by GnRH. A rat LHβ promoter (~156 to +7 bp)-luciferase reporter construct was transiently transfected into L$\beta$T2 cells. As shown in Fig. 4, addition of 100 nM GnRH enhanced the luciferase activity in a time-dependent fashion. To examine whether the stimulation of the LHβ promoter by GnRH is the result of activation of the ERK cascade, either PD98059, an inhibitor of MEK, or an inactive form of MAPK (iMAPK), was used. PD98059 is a highly conserved DNA-binding domain at the carboxyl terminus, and this latter domain defines the Ets family of transcription factors since it lacks homology to other DNA-binding motifs (59, 60). To examine the functional role of Ets transcription factors in GnRH-induced LHβ promoter activation, we first examined whether Ets transcription factors are the nuclear acceptors for GnRH signaling. Members of the Ets transcription factor family contain a transactivation domain, and inhibits both Ets-1- and A-ERK

**FIG. 3.** Role of Ca$^{2+}$ in the activation of both ERK (A) and JNK (B) by GnRH. A

**FIG. 4.** Stimulation of the rat LHβ promoter activity by GnRH. L$\beta$T2 cells were transiently cotransfected with 0.4 μg of the rat LHβ (~156 to +7)-luciferase construct and 0.04 μg of an internal control, pCMVβgal. After transfection, cells were treated with 100 nM GnRH for the indicated times prior to harvesting. Luciferase activity was normalized relative to β-galactosidase activity, and the basal activity was set at 1.0. Data are expressed as the mean-fold activation ± S.E. of six transfections. The activities at 4, 12, and 24 h were significantly different from that of the control (**, p < 0.01).

no effect (Fig. 6A), suggesting that the JNK cascade is involved in the GnRH-induced LHβ promoter activation.

**Role of PKC and Ca$^{2+}$ in GnRH-induced LHβ Promoter Activation**—Since PKC and Ca$^{2+}$ are not involved in GnRH-induced JNK activation (Figs. 2 and 3), we next examined the effect of PKC and Ca$^{2+}$ on GnRH-induced LHβ promoter activation. Pretreatment with 10 μM GF 109203X for 10 min, 3 mM EGTA for 15 min, 1 μM nifedipine for 10 min, or 50 μM BAPTA-AM for 20 min had no effect on GnRH-induced LHβ promoter activation (Fig. 7). Thus, neither PKC nor Ca$^{2+}$ is involved in GnRH-induced LHβ promoter activation, just as they are not involved in GnRH-induced JNK activation.

**A c-Jun Transcription Factor Is a Nuclear Acceptor of the JNK Signaling Cascade**—It has been demonstrated that JNK phosphorylates c-Jun and ATF-2 at the putative regulatory amino-terminal serine residues and thereby increases their transcriptional activities (18, 19). Moreover, JNK has been reported to activate Elk-1, resulting in an increase in c-fos gene expression (54). The Ets domain transcription factor Elk-1 is a substrate for three distinct classes of MAP kinase family members (55–58). In addition, Ets family binding sites have been identified in the rat LHβ promoter between −156 and +7. Therefore, we first examined whether Ets transcription factors are the nuclear acceptors for GnRH signaling. Members of the Ets transcription factor family contain a transactivation domain at the amino terminus and a highly conserved DNA-binding domain at the carboxyl terminus, and this latter domain defines the Ets family of transcription factors since it lacks homology to other DNA-binding motifs (59, 60). To examine the functional role of Ets transcription factors in GnRH-induced activation of the LHβ promoter, the effect of an expression plasmid that encodes a dominant negative Ets construct (pAPr-etsZ) was examined. The pAPr-etsZ construct encodes the highly conserved DNA-binding domain of c-Ets-2 protein devoid of the transactivation domain, and inhibits both Ets-1-
and Ets-2-mediated responses (31) since Ets-1 and Ets-2 recognize the same DNA sequence motif (31, 59). Cotransfection with pAPr-etsZ at doses up to 2.4 μg had no effect on GnRH-induced transcriptional stimulation (Fig. 8A). We also examined the effect of an expression plasmid that encoded a truncated Ets-2 with dominant-negative activity (pRK5-ets-2Δ1–328). Cotransfection with pRK5-ets-2Δ1–328 also had no effect on GnRH-induced transcriptional stimulation (Fig. 8B). These results suggest that the nuclear acceptor for the stimulation of LH promoter activity by GnRH is not a member of the Ets transcription factor family. Since the LHβ promoter does not contain a consensus ATF/CREB site, we next examined whether c-Jun is involved as a nuclear acceptor of a JNK signaling cascade, dnJun, was used to inhibit the phosphorylation of c-Jun. The dnJun mutant cannot be phosphorylated at the Ser63 and Ser73 by alanine, and the mutant consequently blocks the enhanced transactivation promoted by JNK-dependent phosphorylation of these sites (61, 62). Thus, dnJun blocks c-Jun phosphorylation-dependent events of the JNK cascade (32, 61, 62). Cotransfection with a dnJun expression vector significantly attenuated the GnRH-induced LHβ promoter activation (Fig. 9A). In addition, cotransfection of TAM-67, a well-characterized transdominant negative inhibitor of AP-1 owing to a deleted residues 2–122 (34), significantly attenuated the GnRH-induced LHβ promoter activation in a dose-dependent manner (Fig. 9B). We further examined whether the LHβ promoter was activated by GnRH in clonal lines of LβT2 cells, which stably expressed a dominant negative inhibitor (61, 62) of the JNK cascade, dnJun (dnJun-LβT2). GnRH-induced ERK activation was not attenuated in dnJun-LβT2 cells, suggesting that there is no cross-talk between the ERK and JNK cascades (Fig. 9C). Expression of dnJun significantly attenuated the GnRH-induced LHβ promoter activation (Fig. 9D). These results suggest that c-Jun is involved in the GnRH-induced LHβ transcriptional activation.

**DISCUSSION**

Both the biosynthesis and the secretion of the gonadotropins are under the regulation of GnRH. Previous studies indicated that the GnRH receptor couples the G proteins of the Gq/11 family with phosphoinositide turnover and a resultant increase in intracellular calcium concentration and PKC activation, to stimulate secretion of LH and FSH (4–6). However, the molecular mechanisms by which GnRH mediates its transcriptional effects remain largely unknown. It was reported that GnRH-induced activation and basal control of the α-subunit gene seem to occur through the PKC/ERK pathway, while induction of the LHβ gene is dependent on calcium influx using αT3–1 cells, which do not express the LHβ gene (24). The β-subunits confer the physiological specificity of the heterodimeric hormone. Thus, a systematic approach to identifying mechanisms of hormonal regulation of gonadotropin subunit gene expression has been hampered by the lack of an available cell line that expresses the α, LHβ, and FSHβ genes in a regulated manner. An immortal gonadotrope cell line (LβT2) which expresses mRNA for GnRH receptor and for both the α- and β-subunits of LH, has recently been generated (26, 27). Therefore, we examined the mechanism by which GnRH induces the biosynthesis of LHβ using LβT2 cells in this study. As in αT3–1 cells (17, 22), GnRH caused both ERK and JNK activation in LβT2 cells. Although it was reported that activation of ERK by GnRH contributes to stimulation of the α-subunit promoter (16, 24, 63), the role of JNK activation by GnRH has not been clarified hitherto. We present here the first evidence that a JNK cascade is necessary to elicit LHβ promoter activity in a c-Jun-dependent mechanism.
Activation of ERK is induced by phosphorylation of both threonine and tyrosine residues of the enzyme as a result of successive stimulation of Ras, ERK kinase kinase which may be Raf-1, MEK kinase, or an alternative kinase, and MEK (12–14). Protein kinase Ca activates Raf-1 by direct phosphorylation (64). Distinct pathways of G_{i} and G_{q}–mediated ERK activation have been reported (65). The activation of G_{i}–coupled receptors, such as oxytocin (8), prostaglandin F_{2α} (11), and endothelin-1 (11), and prolactin-releasing peptide (40), appears to be PTX-sensitive and PKC-independent. However, in the case of receptors coupled to G_{q}, such as bombesin (43) and thyrotropin-releasing hormone (7), activation is thought to be secondary to stimulation of phosphatidylinositol 4,5-bisphosphate-phospholipase C, leading to production of inositol phosphate and diacylglycerol, with subsequent PKC-mediated stimulation of ERK. In the present study, pretreatment with PTX detectably blocked the GnRHa-induced ERK activation (Fig. 2) and apparent down-regulation of PKC by prolonged incubation with PMA attenuated the stimulation of ERK activity by GnRHa (Fig. 2), suggesting the involvement of both G_{i} and G_{q} protein in GnRHa-induced ERK activation.

One important downstream biochemical event that occurs after ligand binding to many growth-promoting receptors is the activation of members of the MAP kinase family, including ERK and JNK (21). The ERK cascade is strongly activated by growth and differentiation factors, and sustained activation is thought to be an important signal for promoting cell proliferation and differentiation (12–14). The JNK cascade is also activated by cellular stresses (18, 19). These observations suggest the existence of parallel cascades leading to activation of either ERK or JNK. Is the mechanism of GnRHa-induced ERK activation different from that of GnRHa-induced JNK activation? In most cases (7, 10), PKC and Ca^{2+} have been shown to stimulate ERK activity. However, in endothelin-1-stimulated Rat-1 cells, JNK but not ERK activation was inhibited by chelation of Ca^{2+} and by down-regulation of PKC (67). Similarly, in cardiac myocytes, activation of JNK by angiotensin II was strongly suppressed by down-regulation of PKC or by chelation of intracellular Ca^{2+} (68). On the other hand, in GN4 rat liver epithelial cells, angiotensin II activated JNK in a Ca^{2+}-dependent, PKC-independent manner (69). In the present study, GnRHa-induced JNK but not ERK activation was independent of both extracellular Ca^{2+} and intracellular Ca^{2+} (Fig. 3). Moreover, GnRHa-induced ERK activation was PKC-dependent, whereas JNK activation was PKC-independent (Fig. 2). The time course of JNK activation (Fig. 1B) in response to GnRHa was slower than that of ERK activation (Fig. 1A). Thus, the regulation of the JNK activation by GnRHa appeared to be different from that of the ERK activation.

Ca^{2+} is a critical mediator of the induction of gonadotropin secretion by GnRH (5, 70, 71). Studies have shown that calcium ionophores and calcium channel antagonists can stimulate gonadotropin secretion. The stimulatory actions of GnRH on LH and FSH secretion can be inhibited by calcium channel antagonists and culturing the secretory cells in calcium-free medium. Elimination of extracellular Ca^{2+} by treatment with 3 mM EGTA for 1 min or 1 μM nifedipine for 10 min or elimination of intracellular Ca^{2+} by treatment with 50 μM BAPTA-AM for 20 min did not abolish the GnRHa-induced activation of JNK (Fig. 3B). These results suggest that GnRHa-induced activation of JNK is independent of extracellular and intracellular Ca^{2+} and does not seem to involve gonadotropin secretion.
Although little is known regarding the role of the activation of the MAP kinase family in the biosynthesis of hormones, we recently showed that both ERK and JNK are necessary to elicit rat prolactin promoter activity by prolactin-releasing peptide in an Ets-dependent mechanism (40). GnRH-induced activation of the LHβ promoter was not attenuated by either pretreatment with MEK inhibitor PD98059 or by cotransfection with an iMAPK construct (Fig. 5A). On the other hand, GnRH-induced activation of the LHβ promoter was attenuated by cotransfection with a dominant negative SAPK/JNK construct (Fig. 6A). Moreover, the lack of involvement of PKC and Ca²⁺ in GnRHa-induced LHβ promoter activation (Fig. 7) is similar to that in GnRHa-induced JNK activation (Figs. 2 and 3). Thus, although GnRH induces the activation of both ERK and JNK, the JNK cascade seems to be required for the GnRH-induced LHβ promoter activation, as in the case of tumor necrosis factor-α production in mast cells (72). In addition, since it was reported that stimulation of JNK by GnRH was mediated by c-Src in αT3–1 cells (22), we examined the effect of the Src-selective tyrosine kinase inhibitor herbimycin A on GnRHa-induced JNK activation and LHβ promoter activation. Pretreatment with 5 μM herbimycin A for 18 h had no effect on GnRHa-induced JNK activation or LHβ promoter activation (data not shown), suggesting that the mechanism of JNK acti-
tion might be different in other cell lines. The possible mechanisms that cause activation of the JNK cascade in LβT2 cells are under investigation.

No transcription factors that bind to a GnRH-responsive region of the LHβ promoter have been identified yet. JNKs phosphorylate two sites of the NH2-terminal transactivating domain of c-Jun (Ser63 and Ser73), ATF-2/CREB, and Elk-1, thereby increasing their transcriptional activities (18, 19, 54). Although Ets family binding sites have been identified in the rat LHβ promoter (17, 44), GnRH-induced LHβ transcriptional activation might be different in other cell lines. The possible mechanisms that cause activation of the JNK cascade in LβT2 cells are under investigation.

Fig. 9. dnJun inhibits GnRH activation of the rat LHβ promoter. LβT2 cells were transiently cotransfected with 0.4 μg of the rat LHβ (–156 to +7)-luciferase construct and 0.04 μg of an internal control, pCMVβgal, with or without 1.2 μg of pLHCX or pLHCdnJun (A) or 0.4, 0.8, or 1.2 μg of TAM-67 (B), as indicated. In D, LβT2 cells or dnJun-expressing LβT2 (dnJun-LβT2) cells were transiently cotransfected with 0.4 μg of the rat LHβ (–156 to +7)-luciferase construct and 0.04 μg of an internal control. After transfection, cells were treated with 100 nM GnRH for 24 h prior to harvesting. Luciferase activity was normalized relative to β-galactosidase activity, and the basal activity was set at 1.0. Data are expressed as the mean ± fold activation ± S.E. of six transfections. ** indicates p < 0.01 as compared with the respective control. In C, LβT2 and dnJun-LβT2 cells were treated with (lanes 2 and 4, respectively) or without 1 μM GnRH for 5 min (lanes 1 and 3, respectively). Lysates of cells were assayed for ERK activity as described in the legend for Fig. 1A. Autoradiograms of 32P-labeled MBP are shown.
cellular responses to GnRH, such as effects on long term maintenance of the gonadotrope phenotype.

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Activation of the Luteinizing Hormone β Promoter by Gonadotropin-releasing Hormone Requires c-Jun NH₂-terminal Protein Kinase

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