Dependence of Gonadotropin-releasing Hormone-induced Neuronal MAPK Signaling on Epidermal Growth Factor Receptor Transactivation*

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The hypothalamic decapeptide, gonadotropin-releasing hormone (GnRH), utilizes multiple signaling pathways to activate extracellularly regulated mitogen-activated protein kinases (ERK1/2) in normal and immortalized pituitary gonadotrophs and transfected cells expressing the GnRH receptor. In immortalized hypothalamic GnRH neurons (GT1–7 cells), which also express GnRH receptors, GnRH, epidermal growth factor (EGF), and phospholipase C (PMA) caused marked phosphorylation of ERK1/2. This action of GnRH and PMA, but not that of EGF, was primarily dependent on activation of protein kinase C (PKC), and the ERK1/2 responses to all three agents were abolished by the selective EGFR receptor kinase inhibitor, AG1478. Consistent with this, both GnRH and EGF increased tyrosine phosphorylation of the EGFR receptor. GnRH and PMA, but not EGF, caused rapid phosphorylation of the proline-rich tyrosine kinase, Pyk2, at Tyr402. This was reduced by Ca²⁺ chelation and inhibition of PKC, but not by AG1478. GnRH stimulation caused translocation of PKCα and ε to the cell membrane and enhanced the association of Src with PKCα and PKCε, Pyk2, and the EGFR receptor. The Src inhibitor, PP2, the C-terminal Src kinase (Csk), and dominant–negative Pyk2 attenuated ERK1/2 activation by GnRH and PMA but not by EGF. These findings indicate that Src and Pyk2 act upstream of the EGFR receptor to mediate its transactivation, which is essential for GnRH-induced ERK1/2 phosphorylation in hypothalamic GnRH neurons.

The hypothalamic decapeptide, gonadotropin releasing hormone (GnRH), is a primary regulatory factor in the neuroendocrine control of reproduction and is released in an episodic manner from the hypothalamic GnRH neurons. The pulsatile delivery of GnRH to the anterior pituitary gland is essential to maintain the circulating gonadotropin profiles that are necessary for normal reproductive function. In addition to regulating pituitary gonadotropin release, GnRH has extrapituitary actions in neural and nonneural tissues and in several types of tumor cells (1). Immortalized GnRH-producing neurons (GT1–7 neurons) express several G protein-coupled receptors (GPCRs), including those for GnRH and luteinizing hormone/human chorionic gonadotropin (2, 3), as well as α- and β-adrenergic (4), muscarinic (5), and serotonin receptors (6). These cells retain many of the characteristics of the native GnRH neurons, including the ability to maintain pulsatile GnRH release (1, 3). Recent evidence suggests that the autocrine action of GnRH on hypothalamic GnRH neurons is involved in the mechanism of pulsatile GnRH secretion (3).

Agonist activation of specific GPCRs and the resulting dissociation of their cognate G proteins releases α- and βγ-subunits that regulate phospholipase C-β, adenylyl cyclase, and ion channels, which in turn control the intracellular levels of inositol phosphates, Ca²⁺, cAMP, and other second messengers (7, 8). The major signal transduction pathways in cells expressing GnRH receptors are initiated by activation of phospholipase C. The consequent calcium (Ca²⁺) mobilization and activation of protein kinase C (PKC) by GnRH are key elements in the hypothalamic control of gonadotropin secretion from the anterior pituitary gland (1, 3, 5). Activation of PKC and Ca²⁺-mobilization during GnRH receptor stimulation are also responsible for mediating downstream signals leading to activation of extracellularly regulated mitogen-activated protein kinases (ERK1/2 MAPKs) that transmit signals from the cell surface to the nucleus to regulate transcriptional and other processes (7–13). However, the specific PKC isoforms that are involved in GnRH-induced ERK1/2 activation in GT1–7 cells are not known.

Mitogenic signaling by GPCRs can also occur through activation of tyrosine kinases of the Src family, focal adhesion kinases (FAKs), and receptor tyrosine kinases (RTKs). The RTKs involved in GPCR-mediated activation of ERK1/2 MAPKs include the EGFR, platelet-derived growth factor receptor, and insulin-like growth factor receptor (14–16). The GPCRs mediating EGFR transactivation during agonist stimulation include the AT₁ angiotensin receptor (17), the β-adrenoceptor (18), the P2Y2 purinoceptor (19), and receptors for endothelin-1, thrombin, lysophosphatidic acid, and Bradykinin (20, 21). GPCR-mediated transactivation of the EGFR-R initiates the ERK1/2 MAPK cascade through recruitment of adaptor proteins, such as the Shc-Grb2-Sos complex, that activate the small G protein, Ras (14, 22). Depending on the GPCR
agonist and cell type, Ca\(^{2+}\), PKC, G protein \(\beta\gamma\) subunits, and nonreceptor tyrosine kinases including Src and Pyk2, have been implicated in GPCR-induced EGF-R transactivation (14, 22). Endogenous EGF-Rs are expressed in several model systems, including \(\alpha\)T3–1 gonadotrophs, COS-7 cells, and HEK-293 cells, that have been used in studies on GnRH signaling. However, the role of EGF-R transactivation in GnRH-induced ERK activation has been a subject of controversy and is not clearly defined (9, 10, 23). Also, the signaling molecules involved in cross-talk between the neuronal GnRH-R and the EGF-R have not been identified.

Depending upon the cell type, GPCRs mediate both Ras-independent ERK1/2 activation via stimulation of PKC and Ras-dependent ERK activation by receptor and nonreceptor tyrosine kinases (7, 14). GnRH has been found to activate ERK1/2 MAPKs in \(\alpha\)T3–1 gonadotrophs and in COS-7 cells.
(8–10, 12–13) and GH3 cells transfected with the GnRH receptor (11). It also stimulates Jun N-terminal kinase in αT3-1 cells (24) and p38-MAPK in LgrT2 gonadotrophs (25). Activation of these MAPKs by other GPCRs, such as angiotensin II (26, 27), endothelin (28), adrenomedullin (29), and acetylcholine (30), is mediated through the proline-rich protein tyrosine kinase, Pyk2. In general, Pyk2 activation in conjunction with Src kinase appears to be a key element in GPCR-mediated transactivation of the EGF-R (31). However, no information is available on the role of Pyk2 and the nature of its interaction with Src and EGF-R during receptor stimulation by GnRH. The present studies have identified a signaling cascade that mediates GnRH-induced ERK1/2 phosphorylation in immortalized GnRH neurons (GT1–7 cells) and is dependent on receptor-mediated activation of PKC, Src, Pyk2, and the EGF-R.

EXPERIMENTAL PROCEDURES

Materials—GnRH was obtained from Peninsula Laboratories, Inc. (Belmont, CA). EGF was from Invitrogen, and pertussis toxin was from List Biological Laboratories. Protein assay kits were from Pierce. ERK1/2 and anti-phospho-ERK1/2 (Thr202/Tyr204) antibodies were from New England Biolabs, and secondary antibodies conjugated to horseradish peroxidase were from KPL. Antibodies against Src, EGF-R, phospho-EGF-R (Tyr1177), and phosphotyrosine (PY20) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-phospho-Pyk2 (Tyr402) antibody was from BIOSOURCE International. Mouse monoclonal hemagglutinin tag antibody was from Covance (Berkeley, CA). AG1478, Go6983, Ro318220, PP2, BAPTA, PMA, and wortmannin were from Calbiochem, and antibodies against PKC isoforms and Pyk2 were from Transduction Laboratories. LipofectAMINE was from Invitrogen. The Pyk2, dominant negative Pyk2, constitutively active Src, and Csk constructs were provided by Dr. Zvi Naor (University of Tel Aviv). PKC isoform-specific dominant negative and constitutively active constructs tagged with the hemagglutinin epitope were prepared as previously described (32). Western blotting reagents and ECL were obtained from Amersham Biosciences or Pierce.

Cell Culture and Transfections—GT1–7 neurons donated by Dr. Richard Weiner (University of California, San Francisco) were grown in culture medium consisting of 500 ml of Dulbecco’s modified Eagle’s medium containing 0.146 g/liter L-glutamate, 10% heat-inactivated fetal calf serum. DNA transfections were performed with LipofectAMINE according to the manufacturer’s instructions.

Inositol Phosphate Measurements—Cells were labeled for 24 h in inositol-free Dulbecco’s modified Eagle’s medium containing 20 μCi/ml [3H]inositol as previously described (5) and then washed twice with

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**Fig. 3.** GnRH and PMA cause translocation of PKCα and -ε from cytosol to the membrane. **A**, GT1–7 cells express PMA-sensitive and -insensitive PKC isoforms. Cells were treated with PMA (2 μM) overnight (O/N), and cell lysates were analyzed by Western blotting (IB) for various PKC isoforms. **Lower panel**, the ERK1/2 levels in control and PMA-treated cells. **B and C**, serum-starved cells were stimulated with GnRH (200 nM) and PMA (200 nM) for the time periods indicated. After washing with ice-cold PBS, cells were collected and homogenized. The cytosol and membranes were obtained as described under “Experimental Procedures.” Equal amounts of proteins from control and stimulated cells were analyzed by SDS-PAGE and detected for PKC isoforms in cytosol and membranes. As controls, ERK1/2 and Na+/K+ ATPase were probed in cytosol and membranes, respectively. All data are representative of two or three experiments.

**Fig. 4.** The effects of dominant negative PKCα on ERK1/2 activation by GnRH. **A**, expression of native and transfected dominant negative PKCα (dnPKCα) in GT1–7 cells transfected with plasmid DNA (2 and 5 μg) encoding dominant negative PKCα. Cells were washed twice with ice-cold PBS and lysed in Laemmli sample buffer before loading onto 8–16% gradient gels for SDS-PAGE analysis. The expression of PKC constructs was detected using antibody against the hemagglutinin epitope with which these mutant proteins are tagged. Whereas conventional antibody against PKCα detects both native and exogenous dominant negative PKCα proteins, the hemagglutinin antibody detects only the product of transfected dominant negative PKC with no immunoreactivity in the nontransfected (NT) cells. **B**, effects of overexpression of dominant negative PKCα on GnRH-induced ERK1/2 phosphorylation (ERK1/2-P). Serum-starved cells were stimulated with GnRH (200 nM for 5 min) and then washed twice with ice-cold PBS and lysed in Laemmli sample buffer before loading onto 8–16% gradient gels for SDS-PAGE analysis. The quantitated data are shown in the lower panel (n = 4).
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**FIG. 5.** EGF causes marked phosphorylation of ERK1/2 and EGF-R in GT1–7 cells. A, cells were treated with EGF (50 ng/ml) for the time periods indicated, and ERK1/2 phosphorylation was determined as described under “Experimental Procedures.” B, the EGF receptor kinase inhibitor, AG1478 (100 nM) completely inhibits ERK1/2 activation by EGF (50 ng/ml). C, time course effect of EGF (50 ng/ml) on EGF-R phosphorylation (EGF-R-P) at Tyr1173. D, concentration-dependent effects of EGF (4-min stimulation) on EGF-R phosphorylation at Tyr1173. Serum-starved GT1–7 cells treated with EGF were collected in Laemmli sample buffer and analyzed for immunoblotting with anti-phosphotyrosine EGF-R antibody at Tyr1173. E, concentration-dependent inhibitory effect of AG1478 on EGF-induced phosphorylation of the EGF-R at Tyr1173. F, PKC depletion by PMA treatment (2 μM) overnight (ON) abolishes ERK1/2 activation induced by GnRH (200 nM for 5 min) and PMA (100 nM for 5 min) but not by EGF (50 ng/ml). G, lack of effect of PKC inhibitors, Ro318220 (Ro31; 1 μM) and Go6983 (Go69; 1 μM) on EGF-induced ERK1/2 activation. GT1–7 cells were pretreated with inhibitors for 20 min and stimulated with EGF (50 ng/ml) for 5 min.

inositol-free M199 medium and stimulated at 37 °C in the presence of 10 mM LiCl. The reactions were stopped with perchloric acid, inositol phosphates were extracted, and radioactivity was measured by liquid scintillation γ-spectrometry.

**Subcellular Fractionation**—Serum-starved GT1–7 cells were treated with either PMA or GnRH for the times indicated and then washed twice with ice-cold PBS and collected in homogenization buffer containing 25 mM Tris-HCl, pH 7.4, 2 mM EDTA, 10 mM β-mercaptoethanol, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 μM each of aprotinin and leupeptin. After they were kept on ice for 10 min, cells were homogenized with 25 strokes of a Dounce homogenizer. Nuclei and unbroken cells were pelleted by centrifugation at 500 g for 5 min, and the supernatant was centrifuged at 100,000 g for 30 min. The high speed supernatant constituted the cytosolic fraction. The pellet was washed three times and extracted in ice-cold homogenization buffer containing 1% Triton X-100 for 30 min. The Triton-soluble component (membrane fraction) was separated from the insoluble material (cytoskeletal fraction) by centrifugation at 100,000 × g for 20 min.

**Immunoprecipitation**—After treatment with inhibitors and drugs, cells were placed on ice, washed twice with ice-cold PBS, lysed in lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1 mM NaF, 0.25 mM sodium deoxycholate, 1 mM EDTA, 1% Nonidet P-40, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml pepstain, and 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride), and probe-sonicated (Sonifier cell disruptor). Solubilized lysates were clarified by centrifugation at 8000 × g for 10 min, precleared with agarose, and then incubated with specific antibodies and protein A- or G-agarose. The immunoprecipitates were collected, washed four times with lysis buffer, and dissolved in Laemmli buffer. After heating at 95 °C for 5 min, the samples were centrifuged briefly, and the supernatants were analyzed by SDS-PAGE on 8–16% gradient gels.

**Immunoblot Analysis**—Cells grown in six-well plates and at 60–70% confluence were serum-starved for 24 h before treatment at 37 °C with selected agents. The media were then aspirated, and the cells were washed twice with ice-cold PBS and lysed in 100 μl of Laemmli sample buffer. The samples were briefly sonicated, heated at 95 °C for 5 min, and centrifuged for 5 min. The supernatant was electrophoresed on SDS-PAGE (8–16%) gradient gels and transferred to polyvinylidene difluoride membranes. Blots were incubated overnight at 4 °C with primary antibodies and washed three times with TBST before probing with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Blots were then visualized with enhanced chemiluminescence reagent (Amersham Biosciences or Pierce) and quantitated with a laser-scanning densitometer. In some cases, blots were stripped and reprobed with other antibodies.

**RESULTS**

GnRH treatment of GT1–7 cells caused transient stimulation of ERK1/2 that reached a peak at 5 min and declined thereafter toward the basal level over 30 min (Fig. 1A). GnRH-induced ERK1/2 activation was concentration-dependent over the 0.2–200 nM range and was abolished by the GnRH receptor antagonist, [D-pGlu1,D-Phe2,D-Trp(3,6)]GnRH (Fig. 1, B and C). GnRH receptors are primarily coupled to Gα13 proteins, but some of the physiological actions of GnRH are known to occur through activation of G0 or G13 proteins (5, 33). In GT1–7 neurons, nanomolar GnRH concentrations cause marked elevation of inositol phosphate production through Gα1-mediated activation of phospholipase C and also stimulate CAM production. Higher concentrations of GnRH (0.1–1 μM) reduce intracellular CAM levels in a pertussis toxin-sensitive manner, suggesting that GnRH activates a Gi protein in GT1–7 cells (34). Consistent with this, pertussis toxin had a modest inhibitory effect (~30%) on GnRH-induced ERK activation, suggesting partial involvement of G0 protein(s) in MAPK signaling in GT1–7 cells (Fig. 1D).

The roles of PKC and Ca2+ in agonist-stimulated activation of ERK1/2 were evaluated in studies with PKC inhibitors and the Ca2+ chelators BAPTA-2AM and EGTA. GnRH-induced ERK1/2 activation was found to be highly PKC-dependent and was abolished by the PKC inhibitors Ro318220 and Go6983 (Fig. 2A). These inhibitors had no effect on ERK1/2 activation induced by basic fibroblast growth factor (bFGF) (Fig. 2B) or isoproterenol, a β2-adrenoreceptor agonist (data not shown). Consistent with its critical role in GnRH action, depletion of PKC by prolonged PMA treatment (1 μM for 16 h) abolished agonist-induced ERK1/2 activation (Fig. 2C). However, ERK1/2
activation by GnRH was less sensitive to Ca\(^{2+}\) chelation by EGTA and BAPTA (Fig. 2D). Consistent with this, the PKC activator, PMA, was much more effective than the Ca\(^{2+}\) ionophore, ionomycin, in eliciting ERK1/2 activation (Fig. 2E). These findings suggest that GnRH receptor-mediated ERK1/2 activation in GT1–7 cells is predominantly dependent on PKC.

A major role of PKC in GnRH-induced ERK activation has also been reported in other cell types (8–10), but little information is available about the involvement of specific PKC isoforms in this cascade. GT1–7 cells were found to contain several immunoreactive PKC isoforms, including α, δ, ε, and λ. Overnight PMA stimulation (2 μM) caused down-regulation of PKCa, δ, and ε but not PKCd (Fig. 3A). Among the PKC sensitive PKC isoforms, only PKCa and ε were translocated from cytosol to the cell membrane during treatment with GnRH and PMA (Fig. 3B). These effects of GnRH and PMA were specific, since no changes in the levels of ERK1/2 and Na\(^+/K^{-}\)-ATPase were found in cytosol and membranes, respectively (Fig. 3C). The predominant role of PKCa in GnRH-induced ERK activation was confirmed in studies with constitutively active and dominant negative mutants of PKCa. These results showed that GnRH-induced ERK1/2 phosphorylation was attenuated by dominant negative PKCa (dnPKCa; Fig. 4) and was increased with transfection of constitutively active PKCa (data not shown).

It is well established that transactivation of receptor tyrosine kinases such as the EGF-R contributes to GPCR-mediated ERK1/2 activation in certain cell types (14, 22). However, studies on the role of the EGF-R in GnRH action have not given consistent results (9, 10). Thus, whereas Grosse et al. (10) implicated transactivation of the EGF-R in GnRH-induced stimulation of ERK1/2 phosphorylation in T3–1 pituitary gonadotrophs, Benard et al. (9) subsequently reported that the major pathway of ERK1/2 activation was through PKC and activation of Raf-1 and did not involve the EGF-R. Since GT1–7 cells express receptors for both EGF and GnRH, we evaluated the role of the EGF-R in GnRH-induced MAPK signaling. In this cell type, EGF, like GnRH, caused transient activation of ERK1/2 (Fig. 5A). As expected, the selective EGF-R kinase inhibitor, AG1478, blocked the ERK1/2 activation induced by EGF (Fig. 5B). EGF stimulation caused rapid and marked phosphorylation of the EGF-R at Tyr\(^{1173}\) in a time- and concentration-dependent manner (Fig. 5, C and D). Our data suggest a potential role of PKC in GnRH-induced ERK1/2 activation. To determine whether PKC acts upstream or downstream of the EGF-R, we examined the effect of PKC inhibition on EGF-induced ERK1/2 activation. Whereas PKC depletion by prolonged PMA treatment or PKC inhibitors abolished the effects of PMA and GnRH, it had no effect on EGF responses (Fig. 5, E and F). These data indicate that EGF-induced ERK1/2 activation is PKC-independent and that PKC acts upstream of the EGF-R during GnRH signaling in GT1–7 cells.

To examine the involvement of EGF-R in GnRH-induced ERK1/2 activation in GT1–7 cells, cells were pretreated with AG1478 and stimulated with GnRH (200 nM for 5 min). As
shown in Fig. 6A, GnRH-stimulated ERK1/2 phosphorylation was also abolished by AG1478, indicating its absolute dependence on transactivation of the EGF-R. The inhibitory action of AG1478 on GnRH-induced ERK1/2 activation was selective and did not affect bFGF-stimulated ERK1/2 phosphorylation (Fig. 6B). Consistent with the role of EGF-R in GnRH signaling, GnRH also stimulated phosphorylation of the EGF-R as measured with anti-phosphopeptide antibodies that recognize the phosphorylated molecule at Tyr1173 or Tyr1168 (Fig. 6C), the major sites of Src kinase phosphorylation (35) and Grb2 binding (36), respectively. These data demonstrate that transactivation and phosphorylation of the EGF-R are essential for GnRH signaling through ERK1/2 in GT1–7 cells.

Since GnRH-induced ERK1/2 activation is primarily dependent on PKC and GnRH causes PKC activation through generation of diacetyl glycerol (8–10), we investigated the effects of PMA on this cascade. The results revealed that PMA caused marked ERK1/2 activation that was abolished by prior PKC depletion (as shown above in Fig. 5D) and by PKC inhibitors, Ro318220 and Go6983 (Fig. 7, A and B). To determine whether PMA mimics the effects of GnRH with respect to EGF-R transactivation, GT1–7 cells were treated with AG1478 and stimulated with PMA. As shown in Fig. 7C, PMA-induced ERK1/2 activation was extinguished in a dose-dependent manner by the EGF-R kinase inhibitor, AG1478, indicating that GnRH-induced ERK1/2 activation occurs through EGF-R transactivation in a PKC-dependent manner.

Since there is no consensus on the types of intermediate proteins involved during GPCR-induced transactivation of the EGF-R (14, 36, 37), we examined the roles of Src and Pyk2 in GnRH-induced EGF-R phosphorylation and ERK activation. In GT1–7 cells, the highly selective Src kinase inhibitor, PP2, and the Src negative regulatory kinase, Csk, attenuated the activation of ERK1/2 by GnRH (Fig. 8, A and B). Similarly, Src inhibition abolished the effect of PMA on ERK1/2 activation (Fig. 8C). In contrast, Src inhibition and Csk had no effect on EGF-induced ERK1/2 responses (Fig. 8D). These data suggest that Src has a critical role in GnRH-induced activation of the EGF-R and ERK1/2. Since our data show that both PKC and Src act upstream of EGF-R, we examined the interaction between PKC and Src. As shown in Fig. 8E, GnRH stimulation increased the association of PKCα and −ε with Src. The dependence of GnRH-mediated ERK1/2 activation on Pyk2, in ERK1/2 activation by some GPCRs has been shown (26–30). However, nothing is known about the role of Pyk2 in GnRH signaling. GnRH stimulation of GT1–7 cells caused a marked increase in Pyk2 tyrosine phosphorylation at residue 402 that commenced within 1 min and was sustained for up to 30 min (Fig. 9A). Like ERK1/2 activation, the stimulatory effect of GnRH on Pyk2 activation was sensitive to both PKC inhibition and Ca2+ chelation (Fig. 9B). Consistent with the potential involvement of PKC in this cascade, PMA also caused phosphorylation of Pyk2 at Tyr402, and this effect was attenuated by PKC inhibition but not by AG1478 (Fig. 9C). These data suggest that GnRH-induced Pyk2 activation is primarily PKC-dependent in GT1–7 cells.

The dependence of GnRH-mediated ERK1/2 activation on Pyk2 was evaluated in studies with dominant negative Pyk2 mutants (PKM). Overexpression of PKM attenuated the stimulatory effects of GnRH and PMA on ERK1/2 activation, and Pyk2 overexpression enhanced the effect of GnRH on ERK1/2 activation (Fig. 10A). These data show that Pyk2 has an important role in GnRH-induced ERK1/2 activation in GT1–7 cells. Previous studies have shown that, depending on the cell
types, GPCR stimulation leads to interaction of Src with Pyk2 and also that these proteins can cause activation of one another (20, 37). Whether such an interaction occurs following GnRH stimulation is not known. An analysis of the cell lysates immunoprecipitated with anti-Src antibody and immunoblotted with Pyk2 antibody revealed that GnRH increased the association of Src with Pyk2. Furthermore, Src also co-immunoprecipitated with the EGF-R in GT1–7 cells (Fig. 10B). Taken together, these results indicate that ERK1/2 activation by GnRH leads to recruitment of a multicomponent signaling complex that includes PKCα/ε, Src, Pyk2, and the EGF-R.

In contrast, although EGF caused marked activation of ERK1/2, it had no effect on Pyk2 phosphorylation (Fig. 11A). Moreover, Pyk2 activation by GnRH was not prevented by AG1478 (Fig. 11B), indicating that GnRH-induced Pyk2 activation precedes that of the EGF-R transactivation. Whereas PKM decreased ERK1/2 activation by GnRH and PMA, it had no effect on EGF-induced ERK1/2 activation and EGF-R phosphorylation (Fig. 11, C and D). Because both Pyk2 and FAK show high sequence similarity, cellular localization, and signaling characteristics (38, 39), we determined whether GnRH-induced ERK1/2 activation also involves FAK activation. In contrast to its marked effect on Pyk2 activation, GnRH had little effect on FAK phosphorylation (data not shown). These data suggest that GnRH causes selective activation of Pyk2 through its specific receptors in GT1–7 cells.

**DISCUSSION**

The activity of the mammalian pituitary-gonadal axis is dependent on the pulsatile secretion of GnRH from hypothalamic GnRH neurons. Many of the genomic effects of GnRH in its neuroendocrine target cells are believed to be mediated by the activation of MAPKs, which convey GnRH signaling from the cell surface to the nucleus for regulation of genes controlling the functions of GnRH neurons and pituitary gonadotropes (1, 8). However, the roles of intermediate signaling molecules such as Pyk2 and the EGF-R in this pathway have not been clearly defined. Our results show that GnRH causes rapid, marked, and transient phosphorylation of ERK1/2 through transactivation of the EGF-R in GT1–7 cells. The signaling response to GnRH also involves the PKC-dependent phosphorylation of two nonreceptor tyrosine kinases, Src and Pyk2. To date, there has been no indication that this mechanism, with consequent transactivation of the EGF-R,
operative during GPCR-induced MAPK signaling in neuronal cells.

The requirement for growth factor receptor transactivation in GnRH-induced ERK1/2 phosphorylation in other cell types is controversial. Whereas observations in immortalized pituitary gonadotrophs (αT3–1 cells) and COS-7 cells expressing GnRH receptors have suggested that GnRH-induced ERK1/2 activation involved transactivation of the EGF-R, more recent studies found no role of EGF-R transactivation in the phosphorylation of MAPK in αT3–1 cells (9) and HeLa cells expressing GnRH-R (23). Our data show that EGF-Rs are abundantly expressed in GT1–7 cells and, when stimulated, undergo marked autophosphorylation, leading to activation of ERK1/2. More significantly, transactivation of the EGF-R kinase is essential for GnRH-induced ERK1/2 activation in GT1–7 neurons, which is completely prevented by the selective EGF-R kinase inhibitor AG1478. Furthermore, GnRH causes selective phosphorylation of the EGF-R at Tyr1173, a target site for Src action (35) and at Tyr1068, a binding site for the Grb2/Src homology 2 domain (36) (Fig. 6).

Whereas involvement of the EGF-R in GPCR-mediated ERK1/2 activation is well documented (14, 17, 18, 22), the molecular mechanisms responsible for this cascade are not clearly defined. Depending on the cell type, GEF-R transactivation has been reported to be mediated by Gz protein βγ-subunits (40, 41), Ca2+ (26, 30), PKC (10, 37, 42), and hirpin-binding EGF (HB-EGF) released by matrix metalloproteinases (43, 44). However, the latter does not appear to be a universal mechanism for transactivation of the EGF-R by GPCRs (17, 45). Recently, the metabotropic glutamate receptor-5 has been shown to directly interact with the EGF-R, bypassing phospholipase Cβ, PKC, and Ca2+ (46). In the present study, the use of pharmacological inhibitors and constitutively active and dominant negative mutants of relevant signaling molecules has defined PKC, Src, and Pyk2 as critical factors in GnRH-mediated EGF-R transactivation and the consequent increase in ERK1/2 phosphorylation.

Tyrosine kinases implicated in cell signaling include Src family kinases, RTKs such as the EGF-R, the FAK family, Pyk2, and JAK kinases. Pyk2 belongs to the FAK family and is activated by tyrosine phosphorylation in response to several GPCRs (39) as well as by stress stimuli (47) and membrane depolarization (36). Pyk2 has also been implicated in the regulation of ion channels (48), cell adhesion and motility, neurite outgrowth (38, 39), and the induction of long term potentiation in CA1 hippocampal cells (49). Under various experimental conditions, Pyk2 has been shown to participate in the activation of all three major MAPKs: ERK1/2 (26, 48, 50–52), p38 MAPK (28), and Jun N-terminal kinase (27). However, no evidence about the role of Pyk2 in GnRH-induced ERK1/2 activation has been available. Our data have established that GnRH causes marked stimulation of Pyk2 phosphorylation and that overexpression of a kinase-inactive Pyk2 mutant impairs ERK1/2 activation by GnRH and PMA (Figs. 9 and 10). We have also demonstrated that Pyk2 acts upstream of the EGF-R, since EGF failed to activate Pyk2 and dominant negative Pyk2 had no effect on EGF-induced responses. Moreover, GnRH-induced phosphorylation of Pyk2 was not affected by AG1478 (Fig. 11).

The present data also show that GnRH enhances the association of Pyk2 with c-Src (Fig. 10), an interaction that results from binding of the autophosphorylated Tyr402 of Pyk2 to the Src homology 2 domains of c-Src (50–52). Expression of wild type Pyk2 induces phosphorylation of Shc and increases its association with Grb2 (52), a finding consistent with GnRH-induced phosphorylation of the EGF-R at Tyr1173 (Fig. 6). On the other hand, a mutant form of Pyk2 that cannot complex with c-Src behaves as a dominant negative inhibitor of GPCR-stimulated ERK1/2 activation (50). Our studies using both the selective Src inhibitor, PP2, and the C-terminal Src kinase,
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Csk, have demonstrated the essentiality of Src in the activation of ERK1/2 by GnRH and PMA (Fig. 8). In contrast, the lack of effect of Src inhibition on EGF-induced ERK1/2 activation indicates that Src acts upstream of EGF-R in GT1–7 cells. Since Src was essential for GnRH-induced phosphorylation of the EGF-R, these findings indicate that activation of Src/Pyk2 has a critical role in transducing signals from the GnRH-R to EGF-R transactivation in GnRH neuronal cells. These results are consistent with recent studies in fibroblasts from knockout mice showing that Src kinases are critical for activation of Pyk2 and that Src and Pyk2 are indispensable for EGF-R activation by GPCR s (31). In contrast, ERK1/2 activation by GnRH in αT3–1 pituitary gonadotrophs was independent of Pyk2 as well as EGF-R transactivation. Instead, it was primarily mediated through the direct activation of Raf-1 by PKC and to a lesser extent by Ras activation that was dependent on dynamin and Src (9). Similarly, whereas ERK1/2 activation by endothelin-1 in rat mesangial cells involved Pyk2, it was independent of EGF-R activation (28). This is clear that the matrix of signaling molecules utilized during GPCR stimulation is highly variable among different cell types, in which several specific patterns of interactions and phosphorylations are now becoming evident.

Earlier studies on the role of PKC isoforms in GnRH action demonstrated activation of PKCζ and -ε (53) and PKCζ2, -δ, -ε, and -ζ in αT3–1 cells (54) and activation of PKCo and -β in rat pituitary cells (55). However, no information was available about the specific PKC isoform(s) involved in GnRH-induced ERK activation in GT1–7 cells. We found that GT1–7 cells contain PKC isoforms α, δ, ε, and λ and that both GnRH and PMA increased the translocation of PKCo and -ε to the cell membrane and enhanced their association with Src (Figs. 3 and 8). Moreover, GnRH-stimulated phosphorylation of Pyk2 and ERK1/2 in GT1–7 cells is primarily dependent on PKC, since both pharmacological PKC inhibition and PKC depletion by PMA abolished ERK1/2 activation by GnRH and PMA but not by EGF (Figs. 2, 7, and 8). Overexpression of dominant negative PKCo also attenuated ERK1/2 activation by GnRH (Fig. 4), but not by EGF (data not shown). These data suggest that whereas PKCα is an important mediator of GnRH-induced signals, its stimulatory action is upstream of the EGF-R in GT1–7 cells. GPCR-mediated activation of Pyk2 and ERK1/2 is reported to be dependent on both Ca2+ (26, 30, 41, 50) and PKC (19, 37, 39, 51). PKC is also known to cause activation of Src (24) and the EGF-R (10, 24, 44, 45) in several cell types. In fact, PKCo and -δ undergo direct physical and functional interactions with the EGF-R and Pyk2, respectively (56, 57). Thus, PKC can stimulate signaling cascades by targeting a variety of intermediary proteins.

In many cells, the pathways of GPCR- and RTK-mediated ERK1/2 activation converge primarily at the level of the EGF-R (20, 22, 41, 58). Following agonist-induced tyrosine phosphorylation of the EGF-R, the signaling pathways involved in ERK1/2 activation by GPCRs and EGF-Rs appear to be identical (14, 20). This also applies to the action of GnRH in GT1–7 cells. In conclusion, our results show that GnRH causes rapid phosphorylation of ERK1/2 through transactivation of the EGF-R. Such cross-regulation between the GnRH and EGF receptors occurs through the rapid activation of PKC, Src, and Pyk2 by GnRH. Our data support the view that agonist stimulation of neuronal GnRH receptors induces the assembly of a multiprotein signaling complex that includes PKCo/ε, Sro/Pyk2, and the EGF-R. A summary of the manner in which GnRH causes phosphorylation of ERK1/2 in GT1–7 cells is shown in Fig. 12.
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