

Roles of Src and Epidermal Growth Factor Receptor Transactivation in Transient and Sustained ERK1/2 Responses to Gonadotropin-releasing Hormone Receptor Activation*

Received for publication, December 18, 2002, and in revised form, February 26, 2003
Published, JBC Papers in Press, March 17, 2003, DOI 10.1074/jbc.M212932200

Bukhtiar H. Shah‡, M. Parvaiz Farshori‡, Anokhi Jambusaria, and Kevin J. Catt§

From the Endocrinology and Reproduction Research Branch, NICHD, National Institutes of Health, Bethesda, Maryland 20892-4510

The duration as well as the magnitude of mitogen-activated protein kinase activation has been proposed to regulate gene expression and other specific intracellular responses in individual cell types. Activation of ERK1/2 by the hypothalamic neuropeptide gonadotropin-releasing hormone (GnRH) is relatively sustained in α T3-1 pituitary gonadotropes and HEK293 cells but is transient in immortalized GT1-7 neurons. Each of these cell types expresses the epidermal growth factor receptor (EGFR) and responds to EGF stimulation with significant but transient ERK1/2 phosphorylation. However, GnRH-induced ERK1/2 phosphorylation caused by EGFR transactivation was confined to GT1-7 cells and was attenuated by EGFR kinase inhibition. Neither EGF nor GnRH receptor activation caused translocation of phospho-ERK1/2 into the nucleus in GT1-7 cells. In contrast, agonist stimulation of GnRH receptors expressed in HEK293 cells caused sustained phosphorylation and nuclear translocation of ERK1/2 by a protein kinase C-dependent but EGFR-independent pathway. GnRH-induced activation of ERK1/2 was attenuated by the selective Src kinase inhibitor PP2 and the negative regulatory C-terminal Src kinase in GT1-7 cells but not in HEK293 cells. In GT1-7 cells, GnRH stimulated phosphorylation and nuclear translocation of the ERK1/2-dependent protein, p90^{RSK-1} (RSK-1). These results indicate that the duration of ERK1/2 activation depends on the signaling pathways utilized by GnRH in specific target cells. Whereas activation of the G_q/protein kinase C pathway in HEK293 cells causes sustained phosphorylation and translocation of ERK1/2 to the nucleus, transactivation of the EGFR by GnRH in GT1-7 cells elicits transient ERK1/2 signals without nuclear accumulation. These findings suggest that transactivation of the tightly regulated EGFR can account for the transient ERK1/2 responses that are elicited by stimulation of certain G protein-coupled receptors.

G protein-coupled receptors (GPCRs)¹ activate mitogen-activated protein (MAP) kinase signaling cascades by a wide vari-

ety of mechanisms. These include the generation of second messengers (Ca²⁺, PKC, and cAMP), G protein subunit coupling to novel effectors, and activation of receptor tyrosine kinases (RTKs), such as EGFR, platelet-derived growth factor receptor, and insulin-like growth factor receptor (1, 2). MAP kinases are a family of serine/threonine protein kinases that are activated by a wide spectrum of stimuli, ranging from mitogens and growth factors to cellular stress and neurotoxic factors. The extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) are closely related members of the MAP kinase family that are predominantly activated by growth factors and GPCRs and play an important role in cell survival, motility, and secretion (3).

Although the physiological roles of MAP kinase activation are diverse, the convergence of signals emanating from GPCRs and RTKs often leads to the activation of mitogenic pathways associated with cell proliferation. The choice between proliferation and differentiation is primarily determined by the duration and strength of ERK1/2 activation, and transient *versus* sustained activation of ERK1/2 can elicit different types of responses. Sustained activation of ERK1/2 (*e.g.* by fibroblast growth factor or nerve growth factor) usually leads to differentiation (4, 5), whereas transient activation (*e.g.* by EGF) often leads to proliferation (6, 7). In general, sustained ERK1/2 activation is associated with the translocation of ERK1/2 from the cytoplasm to the nucleus (7–9). Therefore, transient activation has different consequences for gene expression than sustained activation, because nuclear accumulation of active ERK1/2 results in qualitative differences in the activation of transcription factors (6, 10, 11).

We and others have shown that EGFR transactivation by GPCR agonists, such as angiotensin II, lysophosphatidic acid, bradykinin, thrombin, and endothelin-1, has a major role in ERK1/2 activation (12–14). Depending on the cell type, this process may be inhibited by pertussis toxin, sequestration of free G protein $\beta\gamma$ subunits, Ca²⁺ chelators, and inhibition of PKC, metalloproteinases, and Src (1, 2, 12–15). Whereas GPCR-mediated ERK1/2 activation can be either transient or sustained, EGF-induced responses are usually of short duration (4–6, 14). This suggests that GPCRs that stimulate ERK1/2 phosphorylation through EGFR transactivation tend to mimic the signaling characteristics of EGF-induced receptor activation (16–18). The aberrant expression or dysfunction of RTKs can produce a variety of diseases and developmental disorders (19, 20), and their activities are tightly regulated in

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ These authors contributed equally to this work.

§ To whom correspondence should be addressed: ERRB, NICHD, Bldg. 49, Rm. 6A36, NIH, Bethesda, MD 20892-4510. Tel.: 301-496-2136; Fax: 301-480-8010; E-mail: catt@helix.nih.gov.

¹ The abbreviations used are: GPCR, G protein-coupled receptor; Csk, C-terminal Src kinase; EGF, epidermal growth factor; EGFR, EGF receptor; HB-EGF, heparin-binding EGF; ERK, extracellular signal-regulated kinase; GnRH, gonadotropin-releasing hormone; GnRH-R,

GnRH receptor; MAP, mitogen-activated protein; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; RSK, p90 ribosomal S6 kinase; MEK, MAP kinase; RTK, receptor tyrosine kinase; PBS, phosphate-buffered saline; MEK, MAP kinase/ERK kinase.

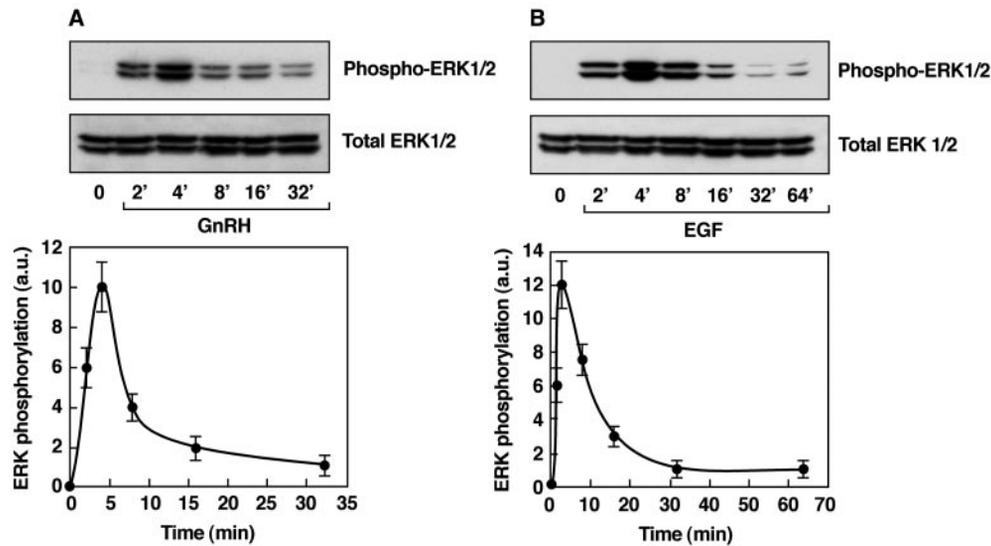


FIG. 1. **Transient phosphorylation responses of ERK1/2 to GnRH and EGF in GT1-7 cells.** A and B, time course of the effects of GnRH and EGF on ERK1/2 phosphorylation (phospho-ERK1/2) at Thr²⁰²/Tyr²⁰⁴ in GT1-7 cells. The cells were stimulated with GnRH (A, 100 nM) and EGF (B, 50 ng/ml) for the time periods indicated, washed with ice-cold PBS, and lysed in Laemmli sample buffer. The cell lysates were analyzed by SDS-PAGE and immunoblotting for phosphorylation of ERK1/2 using phospho-specific (Thr²⁰²/Tyr²⁰⁴) antibodies. The blots were stripped and reprobed with ERK1/2 antibody. The lower panels show total ERK1/2. The data were quantitated, and the means \pm S.E. as arbitrary units (a.u.) are shown in the lower panels ($n = 3-4$).

the course of promoting normal physiological responses. Several mechanisms exist for the attenuation and termination of RTK and ERK1/2 activation (19, 21), and the balance between stimulatory and inhibitory responses ultimately determines the strength and duration of the signals that originate at the cell surface in response to GPCR or RTK stimulation.

The mammalian GnRHR lacks a C-terminal cytoplasmic domain and accordingly is slowly internalized and desensitized (22, 23). For this reason, GnRH-mediated signaling tends to be relatively sustained in pituitary gonadotropes and other cell types expressing endogenous GnRHRs, as well as in receptor-transfected HEK293 cells (10, 24, 25). However, we recently observed that GnRH-induced ERK1/2 activation in the GT1-7 hypothalamic neuronal cell line is quite transient (18). The reasons for such short-lived signaling by GnRH and its impact on cellular activities including subcellular distribution of activated ERK1/2 are not known. An analysis of the differential effects of GnRH on the duration of ERK1/2 activation revealed that GnRH action in GT1-7 cells involves Src and EGFR transactivation that results in transient phosphorylation of ERK1/2, which does not undergo nuclear translocation. In contrast, ERK1/2 responses to activation of GnRHR expressed in HEK293 cells are independent of Src and EGFR but are dependent on PKC and lead to sustained activation and subsequent accumulation of ERK1/2 in the nucleus.

EXPERIMENTAL PROCEDURES

Materials—GnRH was obtained from Peninsula Laboratories, Inc. (Belmont, CA), EGF was from Invitrogen, and pertussis toxin was from List Biological Labs. 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 Kirkegaard and Perry Laboratories, Inc. Antibodies against Src, EGFR, phospho-EGFR (Tyr¹¹⁷³/Tyr¹⁰⁶⁸), and phosphotyrosine (PY20) were from Santa Cruz Biotechnology, Inc., and anti-phospho-EGFR (Tyr¹⁰⁶⁸) was from BIOSOURCE International. AG1478, Go6983, Ro 31-8220, PP2, BAPTA, and PMA were from Calbiochem, and LipofectAMINE was from Invitrogen. The Src negative regulatory kinase (Csk) was provided by Dr. Zvi Naor (University of Tel Aviv). Western blotting reagents and ECL were obtained from Amersham Biosciences or Pierce.

Cell Culture—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

0.584 g/liter L-glutamate and 4.5 g/liter glucose, mixed with 500 ml of F-12 medium containing 0.146 g/liter L-glutamate, 1.8 g/liter glucose, 100 μ g/ml gentamicin, 2.5 g/liter sodium carbonate, and 10% heat-inactivated fetal calf serum. DNA transfections were performed with LipofectAMINE according to the manufacturer's instructions.

Expression of GnRHR in HEK293 Cells—The 1020-base pair mouse GnRHR cDNA was tagged with an hemagglutinin epitope at its N terminus and subcloned into the pEGFP-N1 vector between *Bg*/III and *Sma*I sites. HEK293 cells were cultured in 24-well plate and transfected with 500 ng of subcloned hemagglutinin-GnRHR cDNA in Opti-MEM containing 3 μ l of LipofectAMINE 2000 for 2 h. After 24 h the cells were trypsinized and plated in a 100-mm culture dish at very low density (100–500 cells/dish). On the next day, incubation was continued in culture medium containing 200 μ M G418 for selection. Several colonies were obtained within 2 weeks, and the studies were performed on a stably transfected HEK293 cells.

Transfections—GT1-7 or HEK293 cells were transfected with Csk (2 μ g) using LipofectAMINE in Opti-MEM-1 (Invitrogen). After 5 h, the cells were switched to regular serum-containing medium for 24 h followed by replacement with serum-free medium overnight. The cells were treated with agonist and collected in Laemmli lysis buffer for immunoblot analysis.

Inositol Phosphate Measurements—The cells were labeled for 24 h in inositol-free Dulbecco's modified Eagle's medium containing 20 μ Ci/ml [³H]inositol as previously described (18) and then washed twice with 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.

Immunocytochemistry—The cells were plated at low density on poly-L-lysine-coated glass coverslips for 24 h, then rinsed, and incubated in serum-free medium for 12–24 h. The cells were then treated with GnRH or EGF for the time periods indicated. Following stimulation, cells were rinsed in warm PBS and fixed in 3.7% formaldehyde for 15 min. The cells were then permeabilized by immersing coverslips in 100% methanol for 5 min and then incubated at 37 °C in blocking medium (3% bovine serum albumin and 3% normal serum) for 60 min. Following incubation in primary antibodies diluted (1:50) in blocking medium, coverslips were washed with 3 \times PBS and incubated with Texas Red-labeled goat anti-mouse or Cy5-labeled anti-rabbit or anti-mouse antibodies. Finally, the cells were washed with PBS and mounted in ProLong mounting medium (Molecular Probes, Eugene, OR), and the images were taken with a Bio-Rad confocal microscope.

Immunoprecipitation—After treatment with inhibitors and drugs, the cells were placed on ice, washed twice with ice-cold PBS, lysed in RIPA lysis buffer containing 50 mM Tris, pH 8.0, 100 mM NaCl, 20 mM NaF, 10 mM sodium pyrophosphate, 5 mM EDTA, 1% Nonidet P-40, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 10 μ g/ml soybean trypsin inhibi-

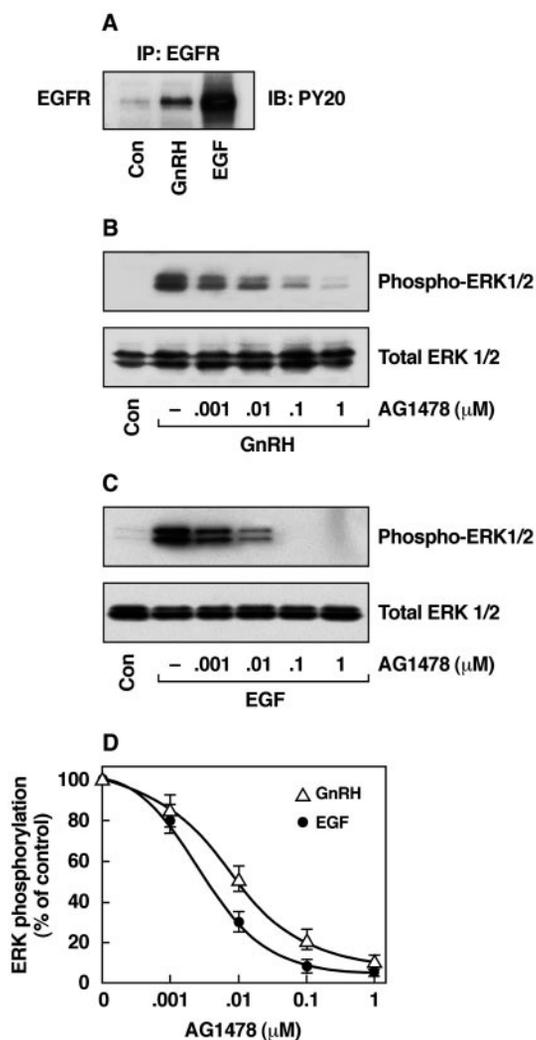


FIG. 2. EGFR transactivation mediates GnRH-induced ERK1/2 phosphorylation in GT1-7 cells. *A*, effects of GnRH and EGF on tyrosine phosphorylation of the EGFR in GT1-7 cells. The cells were stimulated with agonists for 2 min and collected in lysis buffer. The cell lysates were immunoprecipitated (IP) with anti-EGFR antibody as described under "Experimental Procedures" and immunoblotted (IB) with phosphotyrosine antibody (PY20). *Con* indicates untreated control cells. *B* and *C*, concentration-dependent inhibitory effects of the selective EGFR tyrosine kinase inhibitor, AG1478, on ERK1/2 phosphorylation by GnRH and EGF. GT1-7 Cells were treated with AG1478 for 20 min before stimulation with GnRH (*B*, 200 nM) or EGF (*C*, 50 ng/ml) for 5 min. The cells were washed with ice-cold PBS, lysed in Laemmli sample buffer, and analyzed by SDS-PAGE. *D*, quantitation of data (means \pm S.E.) exemplified by *B* and *C* ($n = 3$).

tor, 10 μ g/ml pepstain, and 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, and probe-sonicated (Sonifier Cell Disruptor). The solubilized lysates were clarified by centrifugation at 8000 \times *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 LB, and dissolved in Laemmli buffer. After heating at 95 $^{\circ}$ C for 5 min, the samples were centrifuged briefly, and the supernatants were analyzed by SDS-PAGE on 8–16% gradient gels.

Immunoblot Analysis—The cells were grown in 6-well plates and at 60–70% confluence were serum-starved for 24 h before treatment at 37 $^{\circ}$ C with selected agents. The media were then aspirated, and the cells were washed twice with ice-cold PBS and lysed in 100 μ l of Laemmli sample buffer. The samples were briefly sonicated, heated at 95 $^{\circ}$ 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. The blots were incubated overnight at 4 $^{\circ}$ C with primary antibodies and washed three times with Tris-buffered saline/0.1% Tween 20 before probing with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. The blots were then visualized with ECL (Amersham Biosciences

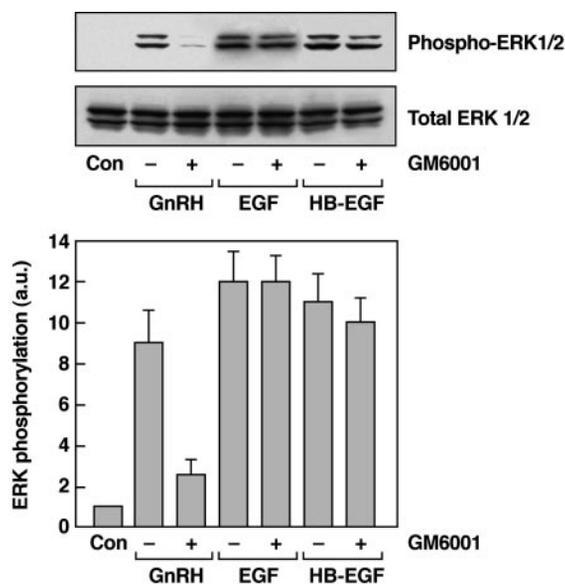


FIG. 3. Involvement of metalloproteases in GnRH-induced ERK1/2 activation. Serum-starved GT1-7 cells were pretreated with the metalloprotease inhibitor, GM6001 (200 nM), for 15 min and then stimulated with GnRH (200 nM), HB-EGF, or EGF (10 ng/ml) for 5 min. The cells were washed with ice-cold PBS, lysed in Laemmli sample buffer, and analyzed by SDS-PAGE for ERK1/2 phosphorylation ($n = 3$). *Con*, control.

or Pierce) and quantitated with a scanning laser densitometer. In some cases, the blots were stripped and reprobed with other antibodies.

RESULTS

Agonist stimulation of GT1-7 neuronal cells, which express endogenous receptors for GnRH and EGF, caused rapid but transient ERK1/2 activation, with a maximum response at 5 min and subsequent decline (Fig. 1*A*). In the same cells, EGF likewise caused marked but transient ERK1/2 activation (Fig. 1*B*). Both EGF and GnRH caused phosphorylation of the EGFR as measured by immunoprecipitation of the EGFR and immunoblotting with phosphotyrosine antibody (Fig. 2*A*). ERK1/2 activation by both GnRH and EGF was abolished by the selective EGFR kinase inhibitor, AG1478, consistent with the need for EGFR transactivation during GnRH-induced ERK1/2 signaling in GT1-7 cells (Fig. 2, *B–D*).

Recent studies have shown that the soluble EGF-like ligand, HB-EGF, is generated by proteolytic processing of the proHB-EGF precursor by metalloproteases in response to a variety of external stimuli (2, 13, 15). To evaluate the involvement of metalloproteases in transactivation of the EGFR by GnRH, GT1-7 cells were pretreated with the selective metalloprotease inhibitor, GM6001, and stimulated with GnRH. As shown in Fig. 3, GM6001 abolished GnRH-induced activation of ERK1/2. However, GM6001 had no inhibitory effect on ERK1/2 activation induced by EGF or HB-EGF stimulation. These findings indicate the specificity of GM6001 and also demonstrate the dependence of GnRH signaling on metalloprotease action upstream of the EGFR.

We next examined the effects of GnRH on ERK1/2 signaling in HEK293 cells transfected with the GnRHR. These cells express endogenous EGFRs and have been used in recent studies to analyze the signaling properties of the GnRHR (23, 24). As in GT1-7 cells, ERK1/2 activation by EGF was transient, reaching a peak between 2 and 5 min and declining thereafter toward basal levels (Fig. 4*A*). EGF stimulation also caused marked phosphorylation of the EGFR at Tyr¹¹⁷³ that was attenuated by AG1478 in a concentration-dependent manner (Fig. 4, *B* and *C*).

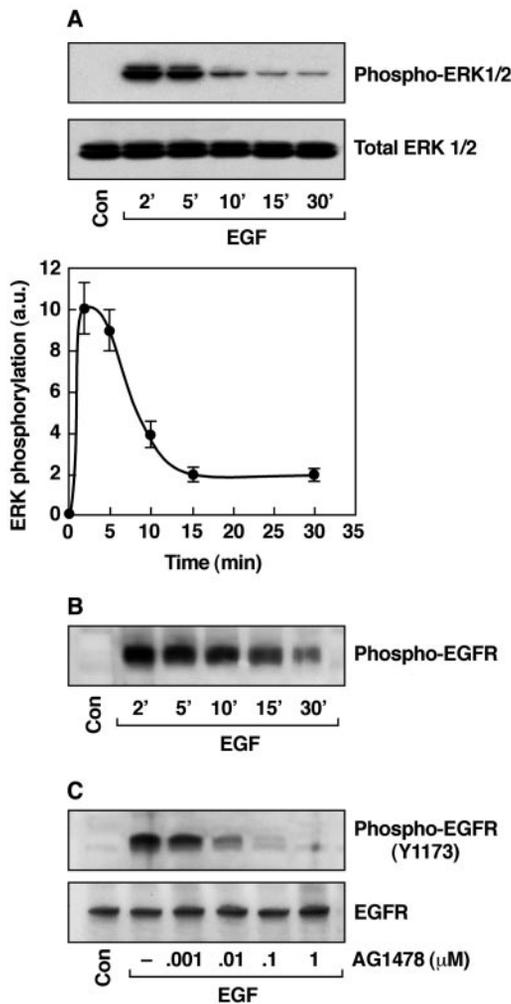


FIG. 4. EGF causes transient phosphorylation of EGFR and ERK1/2 in HEK293 cells. *A* and *B*, time course of the effects of EGF (50 ng/ml) on phosphorylation of ERK1/2 and EGFR at Tyr¹¹⁷³ in HEK293 cells stably expressing GnRHR. *C*, concentration-dependent inhibitory effects of AG1478 on EGF-stimulated phosphorylation of the EGFR at Tyr¹¹⁷³ in HEK293 cells. After washing with ice-cold PBS, the cells were lysed in Laemmli sample buffer and analyzed for phosphorylation of EGFR at Tyr¹¹⁷³ with anti-phospho-EGFR antibody. *Con*, control.

In contrast to GT1-7 cells, GnRH stimulation of HEK293 cells caused rapid and sustained ERK1/2 activation (Fig. 5A). EGF, but not GnRH or PMA, caused marked phosphorylation of the EGFR (Fig. 5B). Moreover, GnRH-mediated ERK1/2 activation was not affected by the selective EGFR kinase antagonist, AG1478 (Fig. 6A), which abolished the effect of EGF on ERK1/2 phosphorylation (Fig. 6B). These findings demonstrate that GnRH-induced ERK1/2 activation in HEK293 cells is independent of EGFR transactivation.

To determine the effects of transient and sustained activation of ERK1/2 on its translocation to the nucleus, the cellular distribution of GnRH-activated ERK1/2 in GT1-7 and HEK293 cells was examined by confocal microscopy. As shown in Fig. 7 (A and B), nuclear translocation of ERK1/2 occurred only during the sustained ERK1/2 activation induced by GnRH in HEK293 cells and was not observed in GT1-7 cells. In comparison, EGF failed to cause nuclear translocation of ERK1/2 in GT1-7 cells (Fig. 7C) following EGF stimulation. These data show that the transient GnRH-induced ERK1/2 phosphorylation in GT1-7 cells that occurs through transactivation of the EGFR does not cause translocation of activated ERK1/2 into the nucleus. However, in HEK293 cells, GnRH causes sus-

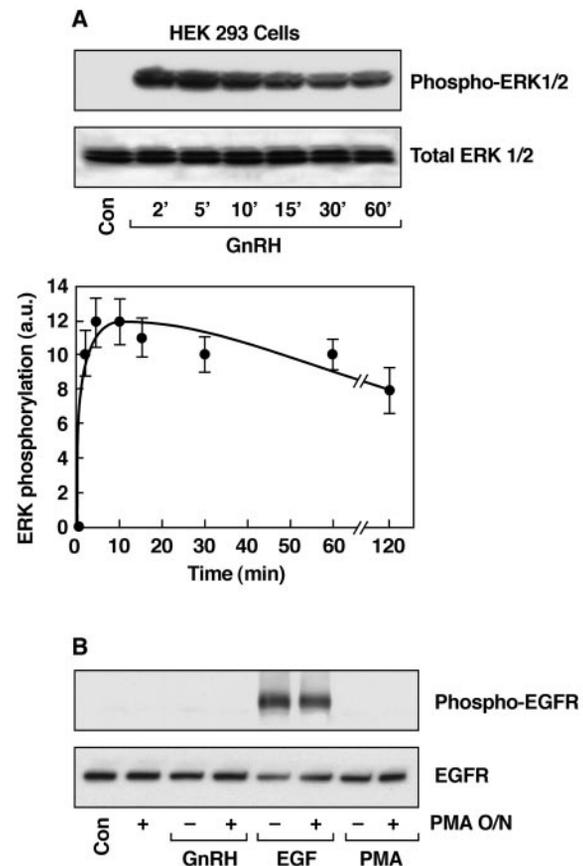


FIG. 5. GnRH causes sustained ERK1/2 phosphorylation in HEK293 cells. *A*, time course of the effects of GnRH on ERK1/2 phosphorylation in HEK293 cells transfected with GnRHR. The cells were stimulated with GnRH (200 nM) for the periods indicated, washed twice with ice-cold PBS, and lysed in Laemmli sample buffer. Immunoblot analysis was performed as described under "Experimental Procedures." The data are the means of four independent experiments. *B*, effects of GnRH, EGF, and PMA on phosphorylation of the EGFR in control and PKC-depleted (*PMA*, 1 μ M) overnight (*O/N*) HEK293 cells. The cells were stimulated with GnRH (200 nM), EGF (10 ng/ml), and PMA (100 nM) for 5 min. Phosphorylation of the EGFR was detected using phospho-specific antibody against EGFR Tyr¹⁰⁶⁸. The blots were reprobbed with EGFR antibody to show the equivalent protein loading (*lower panel*). The data are representative of two similar experiments. *Con*, control.

tained ERK phosphorylation responses that are independent of EGFR transactivation and are accompanied by prominent nuclear translocation of the activated ERK1/2.

To explore the cause(s) of the differential signaling of GnRH in these two cell types, we examined the mechanism of GnRH-induced ERK1/2 activation. Depletion of PKC by prolonged treatment with PMA (1 μ M for 16 h) blocked ERK1/2 phosphorylation induced by GnRH in both GT1-7 and HEK293 cells (Fig. 8A), consistent with the major role of PKC in GnRH signaling. However, PKC depletion had no effect on the EGF-induced phosphorylation of EGFR and ERK1/2 in these cell types. These data suggest that PKC is a common upstream mediator of GnRH-induced ERK1/2 activation in both cell types. To confirm that PKC activation in GT1-7 cells causes phosphorylation of ERK1/2 through EGFR transactivation, we pretreated the cells with AG1478 and measured ERK1/2 phosphorylation induced by PMA. This revealed that ERK1/2 activation by PMA (100 nM for 10 min) was significantly attenuated by AG1478 in GT1-7 cells but not in HEK293 cells (Fig. 8B). These data suggest that GnRH-mediated PKC activation signals through the EGFR only in GT1-7 cells.

Because Src kinase has been implicated in GPCR-mediated

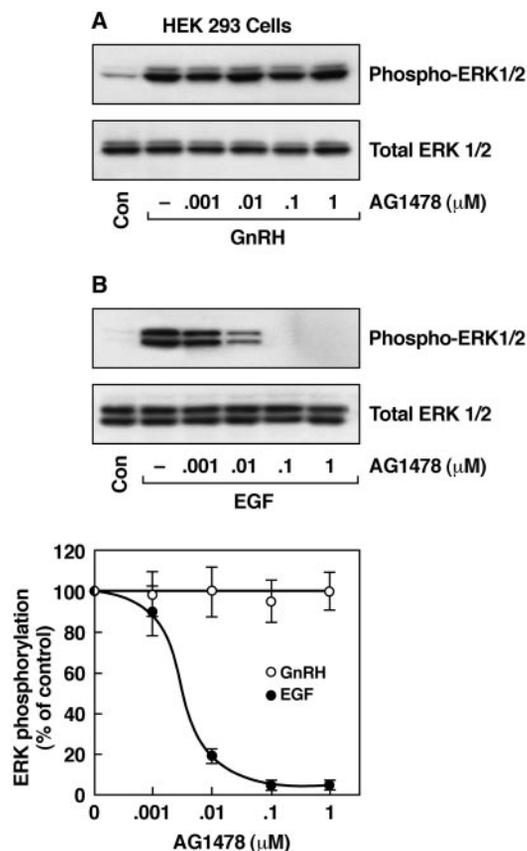


FIG. 6. Absence of EGFR transactivation by GnRH in HEK293 cells. *A* and *B*, effects of EGFR tyrosine kinase inhibition by AG1478 on ERK1/2 phosphorylation by GnRH and EGF in HEK293 cells. The cells were treated with increasing concentrations of AG1478 for 20 min before stimulation with GnRH (*A*, 200 μM) and EGF (*B*, 50 ng/ml) for 5 min. The lower panels show total ERK1/2. Quantitation of the relative effects of AG1478 on ERK1/2 activation by GnRH and EGF is shown in the bottom panel ($n = 3$). Agonist-stimulated phosphorylation in cells without AG1478 treatments was taken as 100% (control), and the data are presented as a percentage of control. *Con*, control.

EGFR transactivation, we examined the effects of Src inhibition on GnRH-mediated ERK1/2 activation. Inhibition of Src by the selective Src kinase inhibitor, PP2, or overexpression of Csk attenuated agonist-induced ERK1/2 activation in GT1-7 cells (Fig. 9A). However, EGF-induced ERK1/2 activation was not affected by PP2 or Csk overexpression (data not shown). In contrast to its action in GT1-7 cells, Src inhibition had no effect on GnRH-mediated ERK1/2 activation in HEK293 cells (Fig. 9B). These results show that Src kinase is required for GnRH-mediated ERK1/2 activation in GT1-7 cells and acts upstream of the EGFR. In contrast, GnRH does not utilize Src and EGFR transactivation in HEK293 cells and therefore does not mimic the short term signaling characteristics of EGF-induced ERK1/2 activation.

Phosphorylated ERK1/2 is often translocated to the nucleus and elicits a variety of transcriptional responses (3). However, neither GnRH nor EGF caused nuclear translocation of ERK1/2 in GT1-7 cells, as shown in Fig. 7. Interestingly, GnRH is known to increase *c-fos* expression in these cells, indicative of transcriptional activation (26). To determine whether other intermediate signaling molecules are involved in the transmission of mitogenic signals from activated cytosolic ERK1/2 to the nucleus, we examined the interaction of ERK1/2 with p90 ribosomal S6 kinase(s) (p90^{RSK} or RSK1), which undergoes translocation to the nucleus after phosphorylation by ERK1/2 (27). Both EGF and GnRH markedly stimulated phosphorylation of RSK-1 at Ser³⁶⁰/Thr³⁶⁴ in GT1-7 cells in a MEK-depend-

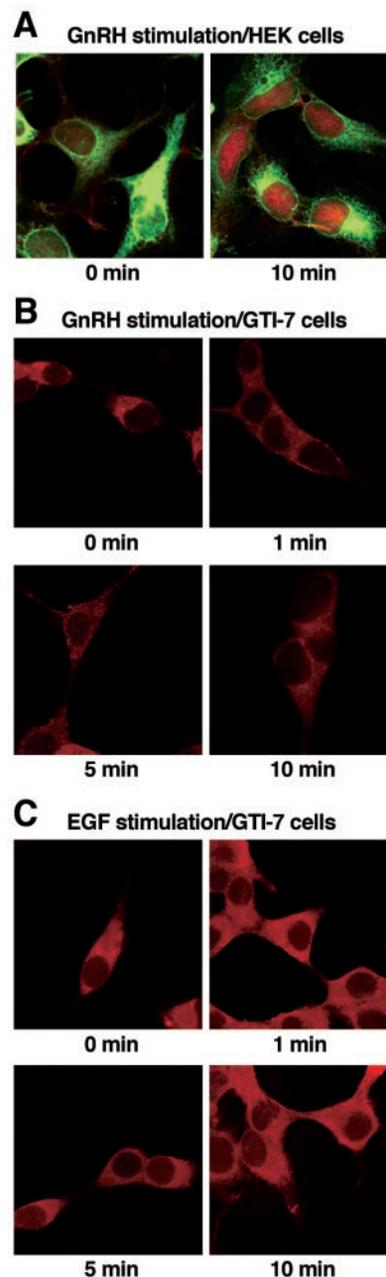


FIG. 7. Nuclear translocation of ERK1/2 in agonist-stimulated HEK293 but not GT1-7 cells. *A* and *B*, serum-starved cells were stimulated with GnRH (200 nM) and EGF (50 ng/ml) for the periods indicated, and the cellular distribution of ERK1/2 was examined by confocal microscopy as described under "Experimental Procedures." The HEK293 cells shown in *A* are stably transfected with GFP-tagged GnRH receptors. GnRH causes marked translocation of ERK1/2 into the nucleus in HEK293 cells (*A*) but not in GT1-7 cells (*B*). *C*, lack of ability of EGF to cause ERK1/2 translocation into nuclei of GT1-7 cells.

ent manner. GnRH-stimulated RSK-1 phosphorylation was attenuated by inhibition of PKC and Src (Fig. 10A). Similarly, EGF-induced RSK-1 phosphorylation was inhibited by AG1478 and the MEK inhibitor, PD98059 but not by the Src inhibitor, PP2. As expected, RSK-1 phosphorylation by GnRH was also abolished by the EGFR kinase antagonist, AG1478 (Fig. 10B). These data indicate that GnRH-induced RSK-1 phosphorylation in GT1-7 cells is mediated by PKC, Src, and EGFR transactivation and that Src acts upstream of the EGFR.

To determine whether RSK-1 is translocated to the nucleus following GnRH stimulation, we visualized its distribution in GT1-7 cells. Under basal conditions, RSK-1 was predominantly

FIG. 8. Role of PKC in agonist-induced ERK1/2 phosphorylation. A, PKC depletion by overnight (O/N) treatment with 1 μ M PMA abolishes the phosphorylation of ERK1/2 induced by GnRH (200 nM for 5 min) but not that induced by EGF (50 ng/ml for 5 min) in both GT1-7 cells and HEK293 cells. B, EGFR kinase inhibition by AG1478 abolishes ERK1/2 phosphorylation by PMA in GT1-7 cells but not in HEK293 cells. The cells were pretreated with AG1478 (100 nM) for 20 min followed by stimulation with PMA (100 nM) for 10 min. The cells were washed, lysed in Laemmli sample buffer, and subjected to immunoblot analysis. Con, control.

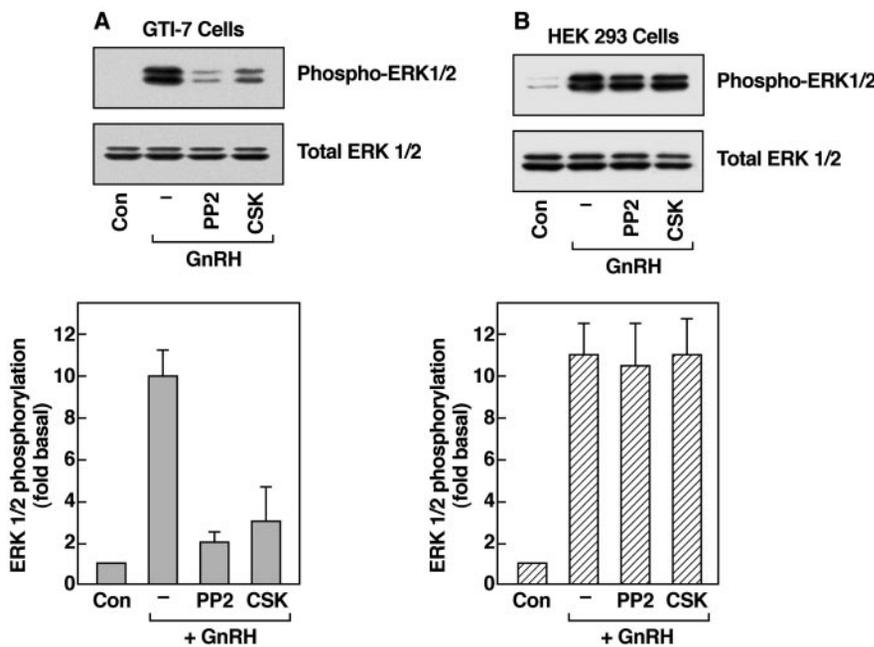
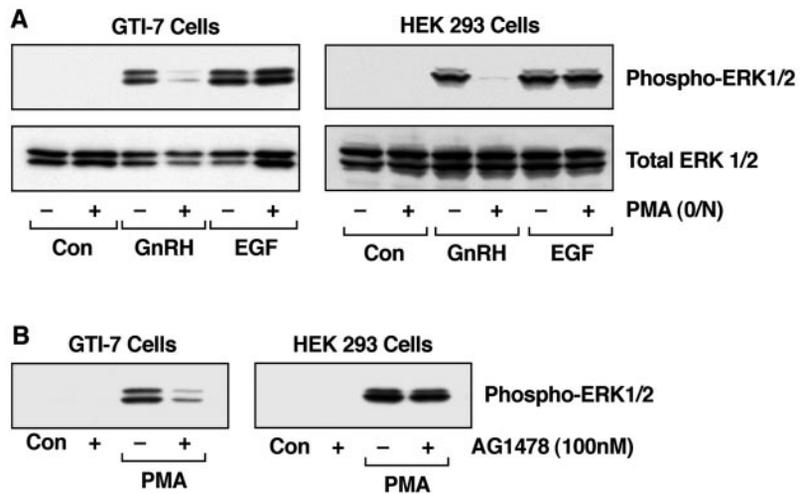


FIG. 9. Effects of Src kinase inhibition on GnRH-induced ERK1/2 activation. A and B, cells transfected with Csk (1 μ g) or pretreated with the selective Src kinase inhibitor PP2 (5 μ M) for 20 min were stimulated with GnRH (200 nM) for 5 min. The cells were washed and lysed in Laemmli sample buffer, and lysates were analyzed by SDS-PAGE and immunoblotted using phospho-ERK-specific antibodies. The blots were stripped and reprobed with ERK1/2 antibody. The quantitated data are shown as the means \pm S.D. ($n = 3$). A, GT1-7 cells. B, HEK293 cells. Con, control.

present in the cytosol. However, GnRH stimulation caused marked accumulation of RSK-1 in the nucleus (Fig. 11). These results suggest that the agonist-induced changes in *c-fos* gene expression observed in GT1-7 cells (26) could be mediated by ERK1/2-dependent phosphorylation of RSK-1.

DISCUSSION

Our results show that, depending on cell type, GnRH-induced ERK1/2 activation can occur through both the G_q /PKC/Raf pathway and G_q /PKC/Src/EGFR activation, with correspondingly distinct effects on the duration and cellular distribution of the activated ERK1/2. Although endogenous GnRHRs in GT1-7 cells mediate Src- and metalloprotease-dependent EGFR transactivation that results in transient ERK1/2 activation, those expressed in HEK293 cells are not coupled to Src and EGFR activation and elicit sustained ERK1/2 responses. The finding that EGF stimulation of endogenous EGFRs in both GT1-7 and HEK293 cells causes transient ERK1/2 activation without nuclear translocation suggests that GnRH-induced signaling characteristics, such as the kinetics of ERK1/2 activation, are largely determined by the extent to which this response is dependent on EGFR transactivation.

Following RTK phosphorylation, the steps involved in

GPCR- and RTK-mediated ERK1/2 activation are generally similar (1, 2, 14, 16). Thus, the signaling pathways initiated by GPCR-mediated transactivation of the EGFR, or directly by EGF binding, are indistinguishable (14, 16, 18). EGFR activation leads to the recruitment and activation of other signaling proteins such as phospholipase $C\gamma$ and/or adaptor proteins such as Shc, Grb2, and Sos. The latter catalyzes the exchange of GTP for GDP on Ras, an effect that eventually culminates in the activation of ERK1/2. EGF is known to induce phosphorylation of several tyrosine residues in its receptor, some of which mediate specific functions of the EGFR (19). In GT1-7 cells, ERK1/2 activation by both GnRH and EGF was abolished by the selective EGFR kinase antagonist, AG1478. Moreover, GnRH caused phosphorylation of the EGFR, albeit of lesser magnitude than EGF, at Tyr¹¹⁷³, a major target site for Src action (28, 29) and at Tyr¹⁰⁶⁸, a site involved in Grb2 recruitment (17). Grb2 is known to link EGFR tyrosine kinase to activation of the Ras/Raf/ERK1/2 cascade (30). The involvement of scaffolding and adaptor proteins in response to GnRH stimulation in GT1-7 cells might be a key determinant of the fidelity of ERK1/2 signaling and the precise control of biological responses.

The type 1 mammalian GnRHR lacks a C-terminal tail and

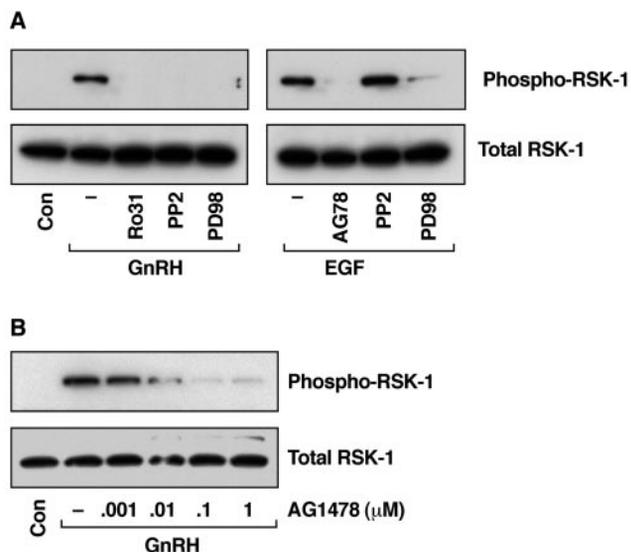


FIG. 10. Mechanism of RSK-1 phosphorylation in GT1-7 cells. A, effects of inhibition of PKC, Src, and MEK on GnRH-induced phosphorylation of RSK-1. GT1-7 cells were treated with the PKC inhibitor, Ro318220 (*Ro31*; 1 μ M), EGFR antagonist, AG1478 (*AG78*; 100 nM), the Src inhibitor, PP2 (5 μ M), and the MEK inhibitor, PD98059 (*PD98*; 20 μ M) before stimulation with GnRH (200 nM) or EGF (50 ng/ml) for 5 min. B, concentration-dependent inhibition by AG1478 of RSK-1 phosphorylation by GnRH (200 nM for 5 min). The cells were washed, lysed in Laemmli lysis buffer, and analyzed for agonist-induced RSK-1 phosphorylation at Ser³⁶⁰/Thr³⁶⁴. The blots were stripped and reprobed with RSK-1 antibody (*lower panel*). The data are representative of three independent experiments. *Con*, control.

does not exhibit agonist-induced phosphorylation. It is slowly internalized and desensitized and hence exhibits prolonged signaling behavior (25). This feature is particularly evident in pituitary gonadotrophs (10) and transfected HEK293 cells (23, 24). In HEK293 cells, GnRH causes sustained ERK1/2 activation that is independent of Src and EGFR activation (Figs. 5 and 9). In contrast, GnRH-mediated ERK1/2 activation in GT1-7 cells is Src-dependent, is mediated through generation of HB-EGF by metalloprotease activation, and reflects the transient signaling characteristics of the EGFR (Figs. 1 and 9). Transactivation of the EGFR by GnRH is not a general feature of GnRH target cells and does not occur in HEK293 cells (22) and α T3-1 pituitary gonadotrophs (31). Thus, the duration of GnRH-induced neuronal signaling to ERK1/2 correlates with the transient phosphorylation of the EGFR during GnRH action, despite sustained GnRH receptor activation. As noted above, EGF causes transient ERK1/2 activation in both GT1-7 and HEK293 cells (Fig. 1 and 6). ERK1/2 activation, in particular that mediated by RTKs, is closely regulated through multiple forms of negative feedback control mechanisms. The short duration of EGF-induced ERK signaling could be attributable to rapid internalization and degradation of the EGFRs (4, 19) and to negative feedback phosphorylation of Sos by a kinase downstream of MEK that results in dissociation of the Shc-Grb2-Sos complex (4, 21).

To determine the impact of the differential pattern of ERK1/2 activation by GnRH in the two cell types, we investigated downstream signaling characteristics such as the compartmentalization of agonist-activated ERK1/2. Consistent with the relationship between sustained ERK1/2 signaling and nuclear translocation, nuclear localization of the GnRH-activated ERK1/2 was observed only in HEK293 cells (Fig. 7), a finding similar to that observed in pituitary L β T2 gonadotrophs (10). In contrast, GnRH stimulation of GT1-7 cells caused only transient ERK1/2 phosphorylation without nuclear translocation of activated ERK1/2 (Figs. 1 and 7). Our obser-

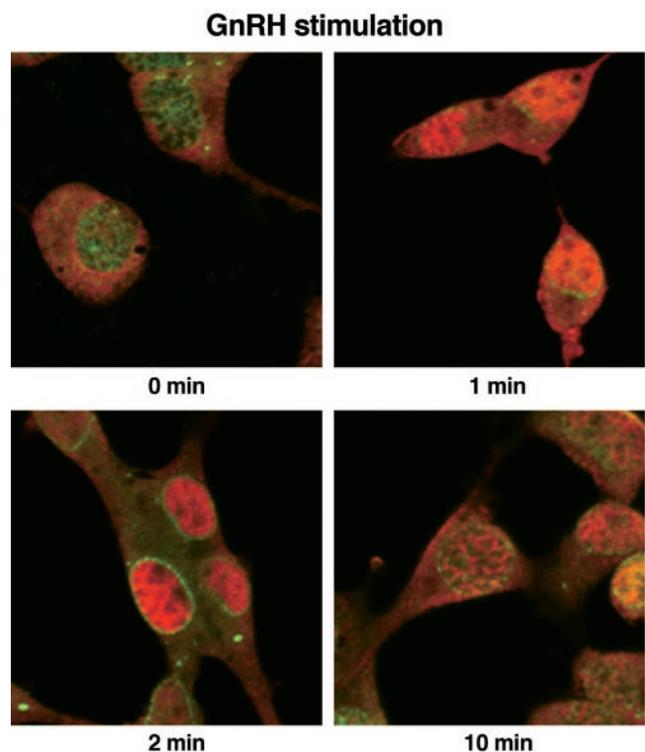


FIG. 11. GnRH causes nuclear translocation of RSK1 in GT1-7 cells. The cells were stimulated with GnRH (200 nM) for the periods indicated, and translocation of activated RSK1 into the nuclei was determined by confocal microscopy. RSK-1 in control cells is mostly present in the cytosol (*red*) with negligible amounts in the nuclei (*green*). GnRH stimulation causes translocation of RSK-1 into the nuclei (*red*).

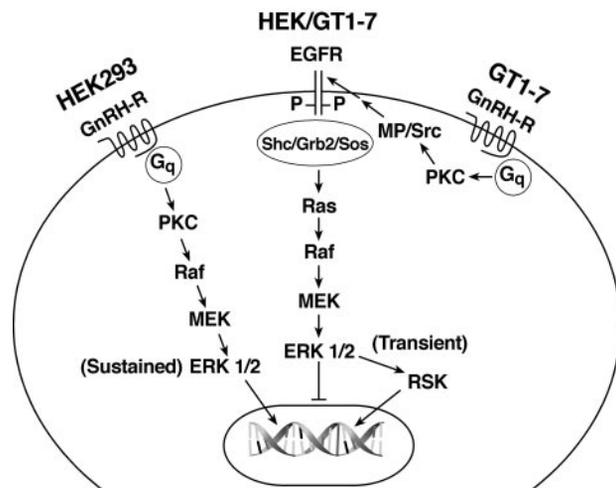


FIG. 12. Comparison of signaling pathways utilized by GnRH in GT1-7 and HEK293 cells. GnRH causes transient ERK1/2 activation through Src, metalloprotease (MP), and EGFR transactivation in a PKC-dependent manner. The transient ERK1/2 activation by EGF and GnRH through transactivation of the EGFR does not cause nuclear translocation of ERK1/2 in GT1-7 cells. Thus, the GnRH-mediated transcriptional activation in these cells is mediated by ERK1/2-dependent phosphorylation of RSK1. In contrast, GnRH causes ERK1/2 activation independent of Src and EGFR transactivation in HEK293 cells transfected with GnRH-R. The PKC-dependent ERK1/2 phosphorylation by GnRH in these cells is sustained and accompanied by marked translocation of ERK1/2 into the nucleus.

vations on the differential localization of ERK1/2 are consistent with recent studies (32) in which ERK1/2 activated by angiotensin II through the G_q/PKC pathway (*e.g.* in HEK293 cells) was translocated to the nucleus, whereas ERK1/2 activated

through the Src/Ras pathway was not, as seen in GT1-7 cells. It appears that the involvement of Src and Ras proteins is an important factor in modifying the characteristics of GPCR signaling. In fact, Boglari *et al.* (33) found that overexpression of dominant negative Ras in PC12 cells caused sustained signaling accompanied by nuclear translocation of ERK1/2, whereas low expression of dominant negative Ras resulted in transient signaling without accumulation of ERK1/2 into the nucleus.

The mechanism of translocation of agonist-activated ERK1/2 in various subcellular compartments is not fully understood. Recently, Tohog *et al.* (11) showed that although angiotensin II-induced activation of G_q /PKC pathway in COS-7 cells caused translocation of activated ERK1/2 into the nucleus, overexpression of β -arrestins caused retention of agonist-activated ERK1/2 in the cytosol. However, this does not account for the differential subcellular localization of activated ERK1/2 in GT1-7 cells, because GnRH signaling characteristics including GnRHR endocytosis are not affected by under- or overexpression of β -arrestins (25, 34). Although studies utilizing cells transfected with GPCRs or signaling molecules have provided useful insights into the mechanisms of receptor signaling, the characteristics of cells containing overexpressed ectopic receptors and signaling proteins may differ from those of cells expressing endogenous GPCRs (35, 36). For example, in GT1-7 cells the GnRHR preferentially interacts with G_q and also with G_s and G_i proteins (37), whereas GnRHRs expressed in HEK293 cells are solely coupled to $G_{q/11}$ (23, 24). Taken together, it is likely that the differential coupling of G proteins in the two cell types accounts for the engagement of distinct signaling proteins with variable consequences for the duration of ERK1/2 phosphorylation and the subsequent cellular distribution during GnRH stimulation.

In general, ERK1/2 activation elicited by GPCRs and RTKs is important for cell survival and growth responses that can be dependent on or independent of transcriptional activation processes (3, 38, 39). Most GPCRs, and growth factors such as nerve growth factor and fibroblast growth factor, are known to cause translocation of activated ERK1/2 into the nucleus and subsequent changes in target gene transcription and DNA synthesis. However, EGF stimulation elicits variable responses in terms of nuclear translocation of ERKs, cell proliferation, and cell survival (40–43). EGF is well known to exert neurotrophic and neuromodulatory effects (44, 45). Our preliminary data show that both GnRH and EGF cause activation of cell survival signaling proteins such as protein kinase B/Akt in GT1-7 cells.² It is possible that the involvement of EGF in GnRH action in GT1-7 neuronal cells is related to the generation of cell survival signals.

Recently, Liu *et al.* (10) reported that GnRH causes nuclear accumulation of ERK1/2 and activation of the *c-fos* and *LH* genes in $L\beta T2$ gonadotropes. Although GnRH failed to induce nuclear translocation of ERK1/2 in GT1-7 cells, it caused rapid induction of nuclear transcription factors such as *c-fos* (26). The mechanism of such transcriptional activation in the absence of measurable nuclear accumulation of activated ERK1/2 is not known. To determine whether other intermediate signaling molecules are involved in the transmission of mitogenic signals from phosphorylated ERK1/2 in the cytosol to the nucleus, we examined the interaction of ERK1/2 with RSK1, which undergoes translocation to the nucleus after phosphorylation by ERK1/2. The RSKs are cytoplasmic serine/threonine kinases that are phosphorylated by activated ERK1/2 and translocate to the nucleus, where they phosphorylate transcription factors including cAMP-responsive element-binding protein, c-Fos, c-

Jun, and serum response factor (27). Our data show that both EGF and GnRH markedly stimulated phosphorylation of RSK-1 at Ser³⁶⁰/Thr³⁶⁴ in GT1-7 cells through EGFR transactivation in a MEK/ERK1/2-dependent manner (Fig. 10). Moreover, GnRH promoted translocation of RSK-1 into the nucleus. Thus, it is probable that the nuclear effects of both GnRH and EGF in GT1-7 cells are mediated through the intermediary protein, RSK-1 (Fig. 11).

A substantial body of data has demonstrated the absolute dependence of GPCR-mediated ERK1/2 activation on EGFR transactivation in various cell types by specific agonists, including angiotensin II in vascular smooth cells (46) and hepatocytes (14), bradykinin in PC-12 cells (17), and bombesin, lysophosphatidic acid, and carbachol in COS-7 cells (16). One of the key regulators during GPCR-mediated EGFR transactivation is Src kinase (1, 12, 14, 18). Consistent with this, our results show that ERK1/2 activation is dependent on Src kinase as well as EGFR activation in GT1-7 cells but not in HEK293 cells (Figs. 2 and 9). Such cross-talk also does not have a major role in HEK293 cells transfected with other GPCRs such as angiotensin AT₁ (47), lysophosphatidic acid, and thrombin receptors (48). The reason why GnRH-mediated ERK1/2 activation is independent of EGFR transactivation in these cells is not clear. Our preliminary data indicate that overexpression of constitutively active c-Src causes phosphorylation of the EGFR in HEK293 cells.² Therefore, it is possible that the lack of EGFR transactivation by GnRH in HEK293 cells is due to inadequate activation of Src or Src-related kinases. This assumption is supported by earlier studies showing that ERK1/2 activation by PMA is independent of Src activation in HEK293 cells (49). In conclusion, our results demonstrate that GnRH-induced ERK1/2 activation in GT1-7 cells occurs through Src and EGFR transactivation, which mediates transient responses similar to those elicited by EGF. However, GnRHRs expressed ectopically in HEK293 cells do not require Src and EGFR transactivation and cause sustained ERK1/2 phosphorylation. Thus, the exclusive routing of GPCR-mediated signals through EGFR transactivation restricts their characteristics to those of EGF stimulation in the individual cell types examined in this study. These pathways are summarized in the diagram shown in Fig. 12. It is possible that such EGF-induced signaling accounts for the time course of ERK phosphorylation elicited by agonist activation of GPCRs expressed in diverse cell types.

REFERENCES

- Gschwind, A., Zwick, E., Prenzel, N., Leserer, M., and Ullrich, A. (2001) *Oncogene* **20**, 1594–1600
- Pierce, K. L., Luttrell, L. M., and Leftkowitz, R. J. (2001) *Oncogene* **20**, 1532–1539
- Marinissen, M. J., and Gutkind, J. S. (2001) *Trends Endocrinol. Metab.* **22**, 368–376
- Kao, S.-C., Jaiswal, R. K., Kolch, W., and Landreth, G. E. (2001) *J. Biol. Chem.* **276**, 18169–18177
- Qui, M. S., and Green, S. H. (1992) *Neuron* **9**, 705–717
- Marshall, C. J. (1995) *Cell* **80**, 179–185
- Liang, C.-C., and Chen, H.-C. (2001) *J. Biol. Chem.* **276**, 21146–21152
- Pouyssegur, J., Volmat, V., and Lenormand, P. (2002) *Biochem. Pharmacol.* **64**, 755–763
- Stanciu, M., and DeFranco, D. B. (2002) *J. Biol. Chem.* **277**, 4010–4017
- Liu, F., Austin, D. A., Mellon, P. L., Olefsky, J. M., and Webster, N. J. G. (2002) *Mol. Endocrinol.* **16**, 419–434
- Tohog, A., Pierce, K. L., Choy, E. W., Leftkowitz, R. J., and Luttrell, L. M. (2002) *J. Biol. Chem.* **277**, 9429–9436
- Dikic, I., Tokiwa, G., Lev, S., Courtneidge, S. A., and Schlessinger, J. (1996) *Nature* **383**, 547–550
- Prenzel, N., Zwick, E., Leserer, M., Abraham, R., Wallasch, C., and Ullrich, A. (1999) *Nature* **402**, 884–888
- Shah, B. H., and Catt, K. J. (2002) *Mol. Pharmacol.* **61**, 343–351
- Eguchi, S., Dempsey, P. J., Frank, G. D., Motley, E. D., and Inagami, T. (2001) *J. Biol. Chem.* **276**, 7957–7962
- Daub, H., Wallasch, C., Lankenau, A., Herrlich, A., and Ullrich, A. (1997) *EMBO J.* **16**, 7032–7044
- Zwick, E., Hackel, P. O., Prenzel, N., and Ullrich, A. (1999) *Trends Pharmacol. Sci.* **20**, 408–412

² B. H. Shah and K. J. Catt, unpublished results.

18. Shah, B. H., Soh, J. W., and Catt, K. J. (2003) *J. Biol. Chem.* **278**, 2866–2875
19. Schlessinger, J. (2000) *Cell* **103**, 211–225
20. Zwick, E., Bange, J., and Ullrich, A. (2002) *Trends Mol. Med.* **8**, 17–23
21. Brightman, F. A., and Fell, D. A. (2000) *FEBS Lett.* **482**, 169–174
22. Hislop, J. N., Everest, H. M., Flynn, A., Harding, T., Uney, J. B., Troskie, B. E., Millar, R. P., and McArdle, C. A. (2001) *J. Biol. Chem.* **276**, 39685–39694
23. Willars, G. B., Heding, A., Vrecl, M., Sellar, R., Blumenrohr, M., Nahorski, S. R., and Eidne, K. A. (1999) *J. Biol. Chem.* **274**, 30146–30153
24. Heding, A., Vrecl, M., Bogerd, J., McGregor, A., Sellar, R., Taylor, P. L., and Eidne, K. A. (1998) *J. Biol. Chem.* **273**, 11472–11477
25. McArdle, C. A., Franklin, J., Green, L., and Hislop, J. N. (2002) *J. Endocrinol.* **173**, 1–11
26. Cesnjaj, M., Krsmanovic, L. Z., Catt, K. J., and Stojilkovic, S. S. (1993) *Endocrinology* **133**, 3042–3045
27. Frodin, M., and Gammeltoft, S. (1999) *Mol. Cell. Endocrinol.* **151**, 65–77
28. Wright, J. D., Reuter, C. W., and Weber, M. J. (1996) *Biochim. Biophys. Acta* **1312**, 85–93
29. Grosse, R., Roelle, S., Herrlich, A., Hohn, J., and Gudermann, T. (2000) *J. Biol. Chem.* **275**, 12251–12260
30. Tari, A. M., and Lopez-Berestein, G. (2001) *Semin. Oncol.* **28**, 142–147
31. Benard, O., Naor, Z., and Seger, R. (2001) *J. Biol. Chem.* **276**, 4554–4563
32. Seta, K., Nanamori, M., Modrall, J. G., Neubig, R. R., and Sadoshima, J. (2002) *J. Biol. Chem.* **277**, 9268–9277
33. Boglari, G., Erhardt, P., Cooper, G. M., and Szeberenyi, J. (1998) *Eur. J. Cell Biol.* **75**, 54–58
34. Vrecl, M., Anderson, L., Hanyaloglu, A., McGregor, A. M., Groarke, A. D., Milligan, G., Taylor, P. L., and Eidne, K. A. (1998) *Mol. Endocrinol.* **12**, 1818–1829
35. DeGraff, J. L., Gagnon, A. W., Benovic, J. L., and Orsini, M. J. (1999) *J. Biol. Chem.* **274**, 11253–11259
36. Zhang, J., Ferguson, S. S. G., Barak, L. S., Menard, L., and Caron, M. G. (1996) *J. Biol. Chem.* **271**, 18302–18305
37. Krsmanovic, L. Z., Mores, N., Navarro, C. E., Tomic, M., and Catt, K. J. (2001) *Mol. Endocrinol.* **15**, 429–440
38. Cantley, L. C. (2002) *Science* **296**, 1655–1657
39. Bonni, A., Brunet, A., West, A. E., Datta, S. R., Takasu, M. A., and Greenberg, M. E. (1999) *Science* **286**, 1358–1363
40. Ochoa, A., Domenzain, C., Clapp, C., and Martinez de la Escalera, G. (1997) *J. Neurosci. Res.* **49**, 739–749
41. Tanimura, S., Nomura, K., Ozaki, K., Tsujimoto, M., Kondo, T., and Kohno, M. (2002) *J. Biol. Chem.* **277**, 28256–28264
42. Adachi, T., Kar, S., Wang, M., and Carr, B. I. (2002) *J. Cell. Physiol.* **192**, 151–159
43. Yao, Z., Flash, I., Raviv, Z., Yung, Y., Asscher, Y., Pleban, S., and Seger, R. (2001) *Oncogene* **20**, 7588–7596
44. Abe, K., and Saito, H. (2000) *Neurosci. Lett.* **282**, 89–92
45. Yamada, M., Ikeuchi, T., and Hatanaka, H. (1997) *Prog. Neurobiol.* **51**, 19–37
46. Eguchi, S., Numaguchi, K., Iwasaki, H., Matsumoto, T., Yamakawa, T., Utsunomiya, H., Motley, E. D., Kawakatsu, H., Owada, K. M., Hirata, Y., Marumo, F., and Inagami, T. (1998) *J. Biol. Chem.* **273**, 8890–8896
47. Turner, N. A., Ball, S. G., and Balmforth, A. J. (2001) *Cell Signal.* **13**, 269–277
48. Della Rocca, G. J., Maudsley, S., Daaka, Y., Lefkowitz, R. J., and Luttrell, L. M. (1999) *J. Biol. Chem.* **274**, 13978–13984
49. Della Rocca, G. J., van Biesen, T., Daaka, Y., Luttrell, D. K., Luttrell, L. M., and Lefkowitz, R. J. (1997) *J. Biol. Chem.* **272**, 19125–19132