Neuropeptide-induced transactivation of a Neuronal Epidermal Growth Factor Receptor Is Mediated by Metalloprotease-dependent Formation of Heparin-binding Epidermal Growth Factor*  

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Numerous external stimuli, including G protein-coupled receptor agonists, cytokines, growth factors, and steroids activate mitogen-activated protein kinases (MAPKs) through phosphorylation of the epidermal growth factor receptor (EGF-R). In immortalized hypothalamic neurons (GT1-7 cells), agonist binding to the gonadotropin-releasing hormone receptor (GnRH-R) causes phosphorylation of MAPKs that is mediated by protein kinase C (PKC)-dependent transactivation of the EGF-R. An analysis of the mechanisms involved in this process showed that GnRH stimulation of GT1-7 cells causes release/shedding of the soluble ligand, heparin binding epidermal growth factor precursor (HB-EGF), as a consequence of metalloprotease activation. GnRH-induced phosphorylation of the EGF-R and, subsequently, of Shc, ERK1/2, and its dependent protein, p90^RSK-1 (p90 ribosomal S6 kinase 1 or RSK-1), was abolished by metalloprotease inhibition. Similarly, blockade of the effect of HB-EGF with the selective inhibitor CRM197 or a neutralizing antibody attenuated signals generated by GnRH and phorbol 12-myristate 13-acetate, but not those stimulated by EGF. In contrast, phosphorylation of the EGF-R, Shc, and ERK1/2 by EGF and HB-EGF was independent of PKC and metalloprotease activity. The signaling characteristics of HB-EGF closely resembled those of GnRH and EGF in terms of the phosphorylation of EGF-R, Shc, ERK1/2, and RSK-1 as well as the nuclear translocation of RSK-1. However, neither the selective Src kinase inhibitor PP2 nor the overexpression of negative regulatory Src kinase and dominant negative Pyk2 had any effect on HB-EGF-induced responses. In contrast to GT1-7 cells, human embryonic kidney 293 cells expressing the GnRH-R did not exhibit metalloprotease induction and EGF-R transactivation during GnRH stimulation. These data indicate that the GnRH-induced transactivation of the EGF-R and the subsequent ERK1/2 phosphorylation result from ectodomain shedding of HB-EGF through PKC-dependent activation of metalloprotease(s) in neuronal GT1-7 cells.

Cells utilize a wide variety of signaling pathways in transducing signals from plasma membrane receptors to mitogen-activated protein kinases (MAPKs), especially the extracellular signal-regulated kinases 1 and 2 (ERK1/2). The MAPKs are involved in cell survival, growth, secretion, chemotaxis, and motility (1, 2). Recent studies have suggested that MAPK activation by external stimuli, such as G protein-coupled receptor (GPCR) agonists, cytokines, growth hormones, steroids and environmental stresses, occurs through transactivation of receptor tyrosine kinases (RTKs), in particular the epidermal growth factor receptor (EGF-R). RTK tyrosine phosphorylation leads to recruitment of specific signaling proteins and adaptor molecules, culminating in sequential activation of the Ras/Raf/MEK/ERK cascade (3, 4). Although the mechanism of ERK activation by RTKs is well defined, the steps leading from GPCR stimulation to RTK tyrosine phosphorylation and activation are only partially understood (5).

A major potential mechanism of the cross-communication between GPCRs and RTKs is the activation of metalloproteases that cause proteolytic cleavage of the transmembrane, proheparin-binding EGF precursor to yield the soluble ligand HB-EGF, which binds to and activates the EGF-R (6). The signaling characteristics downstream of EGF-R phosphorylation in response to stimulation by either EGF or HB-EGF are indistinguishable. Thus, GPCRs that cause MAP kinase activation through transactivation of the EGF-R mimic the signaling pathways downstream of the EGF-R (7–11).

Metalloproteases are a large family of proteolytic enzymes that include the matrix metalloproteases, the ADAMs (a disintegrin and metalloprotease), and the ADAMTS (ADAM with thrombospondin type I motif/s). Metalloproteases contribute to both normal and pathological tissue remodeling by regulating the processing of matrix proteins, cytokines, growth factors, and adhesion molecules to generate fragments with enhanced or reduced biological effects (6, 12, 13). In this context, increasing evidence points to members of the ADAM family of metalloproteases as the key enzymes responsible for the processing of growth factor precursors. For example, the action of ADAM12 on HB-EGF processing in cells stimulated by GPCR agonists, including angiotensin II, endothelin, and phenylephrine, has been shown to cause myoid cell growth and cardiac hypertrophy (14). Similarly, ADAM10 mediates EGF-R transactivation by stimulation of bombesin receptors transiently expressed in COS cells (15). Although there is substantial evidence for metalloprotease-dependent EGF-R transactivation, the mechanism by which metalloproteases mediate this effect is not fully understood. The precise role of metalloproteases in the signaling of GPCR agonists has been the subject of much debate.

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1 The abbreviations used are: MAPK, mitogen-activated protein kinase; ERK1/2, extracellular signal-regulated kinase 1 and 2; MEK, MAPK/ERK kinase; ADAM, a disintegrin and metalloprotease; CRM, diphtheria toxin mutant; EGF, epidermal growth factor; EGF-R, EGF-receptor; GnRH, gonadotropin-releasing hormone; GPCR, G protein-coupled receptor; HA, hemagglutinin A; HEK293, human embryonic kidney 293 (cells); HB-EGF, heparin-binding EGF; PBS, phosphate-buffered saline; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; Pyk2, proline-rich tyrosine kinase 2; ROS, reactive oxygen species; RSK-1, p90 ribosomal S6 kinase; RTK, receptor tyrosine kinase; TGF, transforming growth factor.
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evidence that ectodomain shedding through metalloproteases by various GPCRs occurs upstream of RTKs (6, 8, 14), recent studies suggest that the GPCR agonist lysophosphatidic acid (LPA) requires prior activation of the EGFR and the Ras/Raf/MEK/ERK cascade to induce metalloprotease activation (16, 17).

We have recently defined an essential role of EGFR-R transactivation in GnRH-induced MAPK signaling in GT1-7 neuronal cells (11), and our preliminary results suggested metalloprotease involvement in this cascade (18). It is well established that metalloprotease activation can cause the release of several ligands and growth factors that can interact with RTKs. These include HB-EGF, transforming growth factor α (TGF-α), fibroblast growth factor (FGF), and tumor necrosis factor α (TNF-α) (19). However, the exact mechanism(s) of metalloprotease-dependent transactivation of the EGFR-R and the subsequent MAPK activation by GnRH have not been identified.

Gonadotropin-releasing hormone (GnRH), a primary regulatory factor in the neuroendocrine control of reproduction, is released in an episodic manner to stimulate pituitary gonadotropic secretion and also exerts autocrine regulatory actions on hypothalamic GnRH neurons (20). GnRH released from the hypothalamus acts on anterior pituitary gonadotropes to stimulate the synthesis and release of the pituitary gonadotropins, follicle-stimulating hormone (FSH), and luteinizing hormone (LH) (21). The GnRH receptor belongs to the class of GPCRs that are coupled to heterotrimeric Gs proteins. GnRH-induced activation of ERK1/2 in immortalized hypothalamic neurons (GT1-7 cells), which closely resemble native hypothalamic neurons in terms of their signaling characteristics and pulsatile neurosecretion (20, 22), occurs through transactivation of the EGFR-R (11). However, the formation of metalloprotease-dependent release of HB-EGF and its role in agonist-induced MAPK signaling have not been investigated in neuronal cells. Here we show that GnRH-mediated transactivation of the EGFR-R and the subsequent phosphorylation of Shc, ERK1/2, and RSK-1 are attenuated by the inhibition of metalloprotease activity and blockade of the effects of HB-EGF. Moreover, HB-EGF mimics the effects of EGFR as shown by tyrosine phosphorylation of the EGFR-R and the activation of downstream signaling molecules in GT1-7 cells.

EXPERIMENTAL PROCEDURES

Materials—GnRH was obtained from Peninsula Laboratories, Inc. (Belmont, CA), EGF from Invitrogen, and pertussis toxin from List Biological Labs. Protein assay kits were from Pierce Biotechnology. ERK1/2 and anti-phospho-ERK1/2 (Thr-202/Tyr-204) antibodies were from Cell Signaling Technology (Beverly, MA), and secondary antibodies conjugated to horseradish peroxidase were from Kirkegaard and Perry Laboratories (Gaithersburg, MD). Antibodies against Src, EGFR-R, She, phospho-EGF-R (Tyr-1173), and phosphotyrosine (Tyr(P)-20) were from Santa Cruz Biotechnology. Recombinant human HB-EGF and anti-HB-EGF antibodies were from R&D Systems. Anti-phospho-Pyk2 (Tyr-402) antibody was from either Calbiochem or BIOSOURCE, and anti-Pyk2 (Tyr-1068) was from Bio-Rad. Mouse monoclonal HA tag antibody was from Covance, Berkeley, CA. AG1478, Go6983, GM6001, PD2, PMA, diphtheria toxin CRM mutant, and anti-Shc/p66 phospho-specific antibody were from Calbiochem. Antibodies against Pyk2 were from BD Transduction Laboratories, and LipofectAMINE was from Invitrogen. Western blotting and ECL reagents were from Amersham Biosciences or Pierce Biotechnology.

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 20 μg/ml essential F-12 medium and transfected with 500 ng of subcloned HA-GnRH-GFP cDNA as described previously (5) and then washed twice with F-12 medium containing 200 μg/ml G418 for selection. Several colonies were obtained within 2 weeks of selection, and studies were performed on a stably transfected HEK293 cell line.

Inositol Phosphate Measurements—Cells were labeled for 24 h in inositol-free Dulbecco's modified Eagle's medium containing 20 μCi/ml 32P. After incubation, the radioactive medium was aspirated, and the cells were washed twice with 100-mm culture medium containing 200 μg/ml G418 for selection. Several colonies were obtained within 2 weeks of selection, and studies were performed on a stably transfected HEK293 cell line.

Immunoprecipitation—After treatment with inhibitors and drugs, cells were placed on ice, washed twice with ice-cold PBS, lysed in lysis buffer (LB) containing 50 mM Tris, pH 8, 150 mM NaCl, 1 mM NaF, 0.25% sodium-deoxycholate, 1 mM EDTA, 1% Nonidet P-40, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml pepstatin, and 1 mM 4-(2-aminophenyl)benzenesulfonyl fluoride, and probe-sonicated (Sonifier cell disruptor). Solubilized lysates were clarified by centrifugation at 8000 × g for 10 min, precleared with antibodies, 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 °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 were grown in 6-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 then centrifuged, and the supernatants were subjected to polyvinylidene difluoride membranes. Blots were incubated overnight at 4 °C with primary antibodies and washed three times with Tris-buffered saline containing Tween 20 (TBST) before probing with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Blots were then visualized with ECL reagent (Amersham Pharmacia or Pierce) and quantitated with a scanning laser densitometer. In some cases, blots were stripped and reprobed with other antibodies.

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

RESULTS

In GT1-7 neuronal cells, which express endogenous receptors for both GnRH and EGF, activation of ERK1/2 by either of these ligands was abrogated by the selective EGF-R kinase inhibitor AG1478, consistent with the dependence of GnRH-induced ERK1/2 activation upon transactivation of the EGF-R (Fig. 1A). This was confirmed by the ability of GnRH to cause tyrosine phosphorylation of the EGF-R as revealed by immunoprecipitation of the EGF-R and immunoblotting with phosphotyrosine antibody (PY20) (Fig. 1B).

Recent studies have shown that metalloprotease induction by GPCRs or RTKs causes the release of cytokines and growth factors such as TGF-α or β and HB-EGF. Whether this mechanism is operative in neurons during GnRH action is not
known. To evaluate the involvement of metalloproteases in GnRH-induced transactivation of the EGF-R, GT1-7 cells were pretreated with the metalloprotease inhibitor GM6001 and stimulated with GnRH or EGF. As shown in Fig. 2A, GM6001 abolished GnRH-induced phosphorylation of the EGF-R and the subsequent activation of ERK1/2 and its dependent protein, RSK-1. However, GM6001 had no inhibitory effect on EGF-R phosphorylation and ERK1/2 activation induced by EGF stimulation (Fig. 2B). These findings demonstrate the specificity of GM6001 and also indicate the dependence of GnRH signaling on metalloprotease action upstream of the EGF-R.

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 external stimuli. HB-EGF, in turn, binds to the EGF-R and causes its activation and phosphorylation (6, 7, 23). To find out if this mechanism is operative in GT1-7 cells, we determined whether GnRH-induced ERK1/2 activation results from the release of TGF-α or HB-EGF through proteolytic processing by metalloproteases. As shown in Fig. 3A, a diphtheria toxin mutant (CRM) that selectively inactivates HB-EGF attenuated the effects of GnRH and HB-EGF. Furthermore, the addition of the anti-HB-EGF antibody (20 μg/ml) diminished the ERK1/2 activation induced by GnRH and HB-EGF (Fig. 3B). In contrast, EGF-induced ERK1/2 activation was not affected by CRM or the antibody against HB-EGF (Fig. 3C). In GT1-7 cells, TGF-α had no effect on ERK1/2 activation, thus excluding its involvement in this cascade (data not shown). Taken together, these data demonstrate that GnRH-induced activation of the ERK1/2 pathway through activation of the EGF-R requires the formation of HB-EGF through GM6001-sensitive metalloprotease(s).

EGF-induced activation of EGF-R tyrosine kinase leads to the recruitment of adaptor molecules such as Shc, Grb2, and Sos, which initiate ERK1/2 signaling through the Ras/Raf/MEK pathway (4, 24). To determine whether HB-EGF likewise causes ERK activation through phosphorylation of the EGF-R, we examined the signaling pathways activated by HB-EGF in GT1-7 cells. As shown in Fig. 4, A–C, HB-EGF treatment caused significant but transient phosphorylation of the EGF-R and its adaptor protein, Shc, as well as of ERK1/2 and RSK-1. The stimulatory effects of HB-EGF on phosphorylation of the EGF-R and ERK1/2 were abolished by AG1478, a selective antagonist of EGF-R kinase (Fig. 5), but not by metalloprotease inhibition (Fig. 6). These data clearly indicate that HB-EGF release is responsible for GnRH-induced transactivation of the EGF-R in GT1-7 cells.

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Fig. 4. HB-EGF mimics the signaling characteristics of EGF-R activation. A–C, time courses of the effects of HB-EGF on phosphorylation (P−) of EGF-R, Shc, ERK1/2, and RSK-1. Serum-starved GT1-7 cells were treated with HB-EGF (10 ng/ml) for the time periods indicated (prime symbol (′) represents minutes) and immunoblotted with antibodies against EGF-R (Tyr-1068), Shc (Ser-36), ERK1/2 (Thr-202/Tyr-204), and RSK-1 (Thr-360/Ser-364). Bottom section in each panel shows the non-phosphorylated proteins.

Fig. 5. HB-EGF causes ERK1/2 activation through phosphorylation of the EGF-R. Inhibitory effects of the selective EGF-R kinase antagonist, AG1478, on HB-EGF-induced phosphorylation (P−) of EGF-R (A) and ERK1/2 (B). GT1-7 cells were pretreated with increasing concentrations of AG1478 and stimulated with HB-EGF (10 ng/ml) for 5 min. After washing with ice-cold PBS, cells were collected in Laemmli lysis buffer and analyzed for phosphorylation of the EGF-R and ERK1/2. Con, control.

GPCR agonists such as angiotensin II and GnRH, which cause ERK1/2 activation through EGF-R transactivation, resemble those of EGF stimulation. For example, both GnRH and EGF failure to cause nuclear translocation of activated ERK1/2 in GT1-7 cells but act through RSK-1 to induce transcriptional responses in the nucleus (18). To determine whether HB-EGF mimics the effects of EGF stimulation, we examined its actions on the phosphorylation and cellular localization of RSK-1 in GT1-7 cells. Immunocytochemical analysis showed that HB-EGF treatment stimulated phosphorylation and nuclear translocation of RSK-1 in GT1-7 cells in a manner similar to that of GnRH and EGF. As expected, phosphorylation and nuclear translocation of RSK-1 was abolished by the selective EGF-R antagonist AG1478, confirming the ability of HB-EGF to transduce signals through activation of the EGF-R in GT1-7 cells (Fig. 7).

Although GPCR-mediated transactivation of RTKs is well documented in several cell types, there is no consensus about the nature of the upstream mediators involved in this cascade. In GT1-7 cells, PKC is the predominant second messenger during GnRH-induced ERK1/2 activation (11). Consistent with this, ERK1/2 activation by the PKC activator, PMA, was attenuated by the metalloprotease inhibitor GM6001 (Fig. 8A), indicating the potential role of PKC in agonist-induced release of HB-EGF in GT1-7 cells. Furthermore, PMA-induced ERK1/2 activation was attenuated by the selective EGF-R kinase antagonist AG1478 (Fig. 8B). Moreover, PKC depletion by prolonged PMA treatment (1 μM for 16 h) abolished ERK1/2 activation by GnRH but not by HB-EGF or EGF (Fig. 8C). These data further provide evidence in support of the intermediary role of PKC in metalloprotease induction in GT1-7 cells.

The mechanism of GPCR-mediated metalloprotease activation is not fully understood, and several signaling molecules have been implicated in this process in various cell types (6, 8, 25, 26). We have recently shown that both Src and Pyk2 have important roles in GnRH-induced ERK1/2 activation in GT1-7 cells (11). In addition, reactive oxygen species (ROS) have been shown to cause activation of Src/Pyk2 and EGF-R by angiotensin II in non-neuronal cells such as vascular smooth muscle cells (26, 27) and cardiac fibroblasts (28). However, no information is available on the role of ROS in GnRH action. We observed that the antioxidant N-acetylcysteine (NAC), which acts by increasing intracellular glutathione levels, decreased ERK1/2 phosphorylation by GnRH in a concentration-dependent manner but had no effect on EGF-induced responses (Fig. 9). Moreover, overexpression of dominant negative Pyk2 had no effect on HB-EGF-induced ERK1/2 responses (data not shown) in contrast to its inhibition of ERK1/2 by GnRH (11). These data indicate that intermediary signaling molecules such as ROS, Src, and Pyk2 exert important regulatory actions upstream of agonist-induced HB-EGF generation in GT1-7 cells.

As in GT1-7 cells, EGF-Rs are endogenously expressed in renal HEK293 cells, and their stimulation causes marked activation of the ERK1/2 cascade (18, 29, 30). However, although GnRH-induced ERK1/2 phosphorylation in GT1-7 cells is primarily mediated through transactivation of the EGF-R, this mechanism is not operative in HEK293 cells expressing the GnRH receptor (18). Consistent with this, metalloprotease inhibition had no effect on ERK1/2 activation induced by GnRH or EGF in these cells (Fig. 10, A and B). Similarly, GnRH-induced ERK1/2 signaling was independent of EGF-R transactivation (Fig. 10C). However, EGF-induced ERK1/2 activation...
was abolished by AG1478 (Fig. 10D). These data indicate that the lack of EGF-R transactivation in HEK293 cells is attributable to the inability of GnRH to cause release of HB-EGF via metalloprotease activation, in contrast to its action in GT1-7 cells.

**DISCUSSION**

GnRH binds to specific receptors on hypothalamic GT1-7 cells to initiate signaling cascades leading to the expression of specific genes, increased synthesis and release of gonadotropic hormones, and regulation of reproductive function. Many of the cellular actions of GnRH are dependent on the phosphorylation and activation of MAPKs, including ERK1/2, c-Jun N-terminal kinase, and p38 MAPK, through diverse signaling pathways (31, 32). Earlier studies in αT3-1 pituitary gonadotropes gave conflicting results on the involvement of the EGF-R in mediating the activation of ERK1/2 by GnRH (33, 34). However, the present data have clearly demonstrated the dependence of GnRH signaling on EGF-R transactivation in hypothalamic
GT1-7 neurons. In fact, a number of diverse extracellular stimuli, unrelated to EGF-like ligands, can also activate the EGF-R. These include several GPCR agonists, cytokines, adhesion receptor ligands (integrin), membrane depolarizing agents (KCl), steroid hormones (estrogen), and environmental stresses (radiation, heat shock, and trauma) (7–10, 35, 36). Not all of these stimuli cause activation of metalloprotease(s) and production of HB-EGF to activate the EGF-R (29, 37, 38). However, the present observations have defined the intermediary action of metalloprotease-dependent shedding of HB-EGF in transactivation of the EGF-R by GnRH in GT1-7 neuronal cells.

The roles of upstream mediators such as PKC, Ca2+, Src, and Pyk2 have been demonstrated during GPCR-induced MAPK activation in several cell types (7–11, 39–42). Our results in GT1-7 cells show that GnRH-induced transactivation of the EGF-R and the subsequent ERK1/2 phosphorylation are caused by cell-surface cleavage and ectodomain shedding of neuronal HB-EGF through activation of GM6001-sensitive metalloproteases, which is a PKC-dependent process. The HB-EGF generated by GnRH, in turn, causes activation and phosphorylation of the EGF-R and the consequent phosphorylation of Src, ERK1/2, and RSK-1 (Fig. 4). The role of metalloprotease-dependent shedding of HB-EGF in GnRH-induced ERK activation in neuronal cells has not been previously identified. Our findings show that inhibition of metalloprotease activity attenuates the activation of EGF-R and ERK1/2 by GnRH, but not by HB-EGF and EGF (Fig. 2), indicating that the release of soluble HB-EGF by induction of GM6001-sensitive metalloproteases after GnRH stimulation is responsible for EGF-R transactivation.

There is considerable heterogeneity in the mechanism of HB-EGF formation through metalloprotease induction. Ectodomain shedding is induced by various GPCRs (6, 42), GTP-Y-S (43), PMA (6, 44), Ca2+ (9, 27), Src (35, 40), and derivatives of arachidonic acid metabolism such as prostaglandins and leukotrienes (42) and ROS (43–45). Although substantial evidence supports the role of metalloprotease-dependent shedding of HB-EGF in transducing signals from GPCRs to the EGF-R (6, 14), some studies have excluded their involvement in this process (29, 37, 38). Likewise, we did not observe GnRH-mediated induction of metalloproteases and activation of EGF-R in HEK293 cells expressing the GnRH receptor (Fig. 10). The exact reason(s) for this discrepancy are not yet clear but may be related to the differential expression of specific metalloproteases in particular cell types or to a lack of Src activation by GnRH in these cells.

Metalloproteases are well known to contribute to both normal and pathological tissue remodeling by regulating the processing of matrix proteins, cytokines, growth factors, and adhesion molecules (6, 13). In this regard, the involvement of metalloproteases in reproductive processes, such as rupture of the mature ovarian follicle and structural luteolysis by GnRH, has been well documented (46, 47). Metalloproteases also have an important role in the normal development of the nervous system and contribute to a number of pathological conditions such as neuroinflammation, stroke, cerebral hemorrhage and edema, demyelination, and multiple sclerosis (14, 19, 43, 48, 49). However, HB-EGF produced in the brain has been reported to be a potential neurotrophic and neuroprotective agent. It is also involved in neuronal survival and proliferation through the activation of MAPKs (50, 51) and in central nervous system repair after hypoxic/ischemic injury (50, 52). Thus, the processing/release of HB-EGF by GnRH stimulation of metalloproteases might have potential implications for the survival and repair of GT1-7 hypothalamic neurons.

GnRH-induced ERK1/2 activation in pituitary gonadotropes are dependent on both PKC and calcium (31, 53). However, in GT1-7 cells this process is primarily dependent on the activation of a multiprotein signaling complex consisting of PKCα/ε and the non-receptor tyrosine kinases Src and Pyk2 (11). To determine whether these signaling proteins contribute to metalloprotease activation in GT1-7 cells, we examined the effects of metalloprotease inhibition on phosphorylation of the EGF-R and ERK1/2 by GnRH and the PKC activator, PMA. These studies showed that the metalloprotease inhibitor GM6001 attenuated agonist-induced

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