Epidermal Growth Factor Receptor Tyrosine Kinase Mediates Ras Activation by Gonadotropin-releasing Hormone*

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Gonadotropin releasing hormone (GnRH) contributes to the maintenance of gonadotrope function by increasing extracellular signal-regulated kinase (ERK) activity subsequent to binding to its cognate G-protein-coupled receptor. As the GnRH receptor exclusively interacts with Gα11 proteins and as receptor expression is regulated in a β-arrestin-independent fashion, it represents a good model to systematically dissect underlying signaling pathways. In αT3-1 gonadotropes endogenously expressing the GnRH receptor, GnRH challenge resulted in a rapid increase in ERK activity which was attenuated by the epidermal growth factor receptor (EGFR)-specific tyrosine kinase inhibitor AG1478. In COS-7 cells transiently expressing the human GnRH receptor, agonist-induced ERK activation was independent of free Gβγ subunits but could be mimicked by short-term phorbol ester treatment. Most notably, Gαq/11-induced ERK activation was sensitive to N17-Ras and to expression of the C-terminal Src kinase but also to other dominant negative mutants of signaling components localized upstream of Ras, like Shc and the EGFR. GnRH as well as phorbol esters led to Ras activation in COS-7 and αT3-1 cells, which was dependent on Src and EGFR tyrosine kinases, indicating that both tyrosine kinases act downstream of protein kinase C (PKC) and upstream of Ras. However, Src did not contribute to Shc tyrosine phosphorylation. GnRH or phorbol ester challenge resulted in PKC-dependent EGFR autophosphorylation. Furthermore, a 5-min phorbol ester treatment was sufficient to trigger tyrosine phosphorylation of the platelet-derived growth factor-β receptor in L cells. Thus, in several cell systems PKC is able to stimulate Ras via activation of receptor tyrosine kinases.

In many cells proliferation and differentiation is regulated by the extracellular signal-regulated kinase (ERK)3 subfamily of MAP kinases. ERKs represent distal members of a three-component kinase module through which extracellular stimuli are transmitted into the cell (1). The best studied signaling cascade is initiated by ligand-bound receptor tyrosine kinases (RTKs) which upon autophosphorylation and tyrosine phosphorylation of adaptor proteins like Shc and Grb2 recruit guanine nucleotide exchange factors for the monomeric GTPase Ras. Activated, i.e. GTP-loaded, Ras subsequently engages the ERK-MAPK cascade involving Raf, Ras, MEK, ERK kinase (MEK), and finally ERKs.

It has only recently been appreciated that besides classical growth factors like EGF and PDGF, agonists acting at G-protein-coupled receptors (GPCRs) also play a role in differentiation, proliferation, and even cellular transformation (2, 3). Both pertussis toxin (PTX)-sensitive and -insensitive heterotrimeric G-proteins mediate ERK activation, and distinct signaling pathways are ascribed to GTP-bound α and free Gβγ protein subunits. In many cells, Ras-dependent ERK activation via GPCRs appears to be mediated by Gβγ dimers released from PTX-sensitive G-proteins (4). However, Gβγ subunits derived from PTX-insensitive Gαq proteins have also been implicated in Ras-dependent ERK activation (5).

While Gαq-coupled receptors are unanimously viewed as coupling to ERKs in a Ras-dependent manner, previous studies have produced contradictory results as to Ras-dependent or -independent ERK activation via Gαq-coupled receptors in COS cells (5–7). In many cases, interpretation of results is complicated by the fact that many Gαq-coupled receptors have the propensity to concurrently activate Gαq and Gβγ proteins, particularly when expressed in heterologous cell systems (8).

In a variety of cellular systems GPCRs mediate the tyrosine phosphorylation of Shc adaptor proteins and their subsequent association with Grb2-SOS, thus initiating Ras-dependent stimulation of ERK activity (3, 4). Apart from non-receptor tyrosine kinases, RTKs like the PDGF and EGF receptors have also been invoked to participate in the signal transmission from GPCRs to ERKs (9, 10), and we have provided evidence that the receptor cross-talk is realized in a cell-specific manner (11). Most probably, the molecular mechanisms underlying ligand-independent activation (transactivation) of RTKs via GPCRs differ between Gαq- and Gαq-coupled receptors (11) and are far from being understood. ERK activation by Gαq-coupled receptors mediated by transactivation of RTKs naturally depends on Ras activation. However, it has recently been reported that in COS cells the agonist-bound m1 muscarinic receptor activates Ras via PKC by a novel mechanism clearly set apart from that initiated by activation of RTKs (7).

The termination of GPCR signaling involves Gβγ-directed receptor phosphorylation by receptor kinases and subsequent references to this.
binding of β-arrestins which inhibits further G-protein activation (12). Besides terminating receptor/G-protein coupling, β-arrestins have also been implicated in Src recruitment to the agonist-occupied receptor, thereby initiating Ras-dependent ERK activation in a second wave of signal transduction emanating from various GPCRs (13).

To gain further insight into the cellular mechanisms of ERK activation by Gαi-coupled receptors, we resorted to the gonadotropin-releasing hormone (GnRH) receptor because of several noteworthy receptor characteristics. In the pituitary gland GnRH interacts with a membranous receptor to regulate the synthesis and secretion of gonadotropin glycoprotein hormones and to secure the long-term maintenance of the gonadotropic phenotype (14). In pituitary gonadotropes expression of differentiation markers like glycoprotein hormone α- and β-subunits and the GnRH receptor require activation of the MAP kinase cascade (15–17) through a pathway critically involving protein kinase C (PKC) (18, 19). Cloning of cDNAs coding for mammalian GnRH receptors of various species demonstrated that GnRH interacts with a serpentine G-protein-coupled receptor (14) completely lacking a cytoplasmic C-terminal tail. In terms of receptor function this structural peculiarity results in the absence of GRK-mediated phosphorylation (20) and rapid desensitization and an exceptionally slow internalization rate hardly affecting the membranous receptor complement during short (5–10 min) incubations with agonist (21). A systematic comparison with the pituitary thyrotropin-releasing hormone receptor, furthermore, revealed that expression of the mammalian GnRH receptor at the plasma membrane is regulated in a β-arrestin-independent fashion (22). Finally, we demonstrated that the recombinant human receptor expressed in Chinese hamster ovary and COS-7 cells as well as the mouse receptor in gonadotropin αT3-1 cells initiate multiple signaling pathways by exclusively coupling to Gαi1 proteins.2 Thus, in the case of the GnRH receptor various paradigms explaining ERK activation by GPCRs have to be challenged, and an unequivocal interpretation of functional data is not impeded by Gαi-mediated effects as observed for various other primarily Gαi-coupled receptors (8).

In the present paper we dissect the signaling pathway underlying Gαi-mediated ERK activation in pituitary αT3-1 and transfected COS-7 cells. We provide direct evidence for PKC-mediated ERK activation subsequent to N17-Ras-sensitive Ras activation in response to GnRH receptor/Gαi1 coupling. GTP loading of Ras critically depends on PKC-induced activity of RTKs. In agonist-treated COS-7 and αT3-1 cells expressing the GnRH receptor, Src kinases contribute to Ras activation in concert with RTKs independently of Src tyrosine phosphorylation. PKC-dependent tyrosine phosphorylation of the EGF receptor (EGFR) in COS-7 and αT3-1 cells and of the PDGF-β receptor in L cells supports the concept that cross-talk between Gαi-coupling GPCRs and RTKs via PKC represents a general coupling mechanism.

### EXPERIMENTAL PROCEDURES

**Materials**

COS-7 cells were from ATCC, and αT3-1 cells were a gift of P. L. Mellon (23). Culture media and trypsin are from Biochrom. Fetal calf serum and phosphate-buffered saline were purchased from Life Technologies, Inc. GnRH, AG1478, AG1296, PP2, PD98059, staurosporin, GF109203X, and TPA were from Calbiochem. BAPTA/AM was purchased from Molecular Probes. [γ-32P]ATP was from NEN Life Science Products Inc. The enhanced chemiluminescence system (ECL) was from Amersham Pharmacia Biotech. Prestained protein marker (broad range) was obtained from New England Biolabs. All other reagents were obtained from Sigma.

**Methods**

**Tissue Culture and Transfections—**COS-7 and αT3-1 cells were grown in Dulbecco’s modified Eagle’s medium, L cells in α-modified minimal essential medium supplemented with 10% fetal bovine serum, 50 μg/ml streptomycin, and 50 units/ml penicillin. Cells were cultured at 37 °C gassed with 5% (COS-7 cells) or 5% (αT3-1 and L cells) CO₂. For transient transfections of COS-7 cells, a calcium phosphate co-precipitation method was applied. Twenty μg of total plasmid DNA were used for transfections of 100-mm dishes of subconfluent cells. The transfection mixture contained constant amounts of GnRH receptor cDNA (24) (5 μg/100-mm dish) and of other expression constructs supplemented with expression vector to keep the total amount of plasmid DNA constant. Cells were assayed 48 h after transfection. Under these controlled conditions co-transfection of COS-7 cells with different plasmids did not impair GnRH-induced phospholipase C activation (data not shown). Gonadotropic αT3-1 cells were transiently transfected by lipofection (FuGENE, Roche Molecular Biochemicals) according to the manufacturers suggestions. Prior to stimulation, cells were serum-starved for 18 h (COS-7 and L cells) or 8 h (αT3-1 cells). Cells were challenged by incubation with serum-free culture medium containing the concentrations of various substances as indicated. Basal values were determined in serum-free medium.

**Immunoprecipitations and Western Blotting Procedures—**Stimulation of cells was carried out for 5 min at 37 °C in serum-free medium in 100-mm dishes as described in the respective figure legends. Reactions were stopped by washing cells once with 10 ml of ice-cold phosphate-buffered saline (PBS). Cells were then lysed for 15 min in 800 μl of cold lysis buffer per dish (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM EDTA, 1 mM dithiothreitol, 40 μM Na₃P₂O₇, 500 μM Na₂VO₃, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, and 0.1 μM aprotinin). Lysates were preclarified by centrifugation, supernatants diluted with 600 μl of lysis buffer containing 0.1% Triton X-100 and subjected to immunoprecipitation at 4 °C overnight by using the appropriate antibodies. Subsequently, 100 μl of Protein A-Sepharose beads (12.5%, w/v) (Sigma) were added, incubated at 4 °C on a rotor for 3–4 h, and precipitated by centrifugation. Precipitates were washed three times with cold lysis buffer, resuspended in SDS sample buffer, boiled for 3 min, and resolved by SDS-polyacrylamide gel electrophoresis (PAGE).

**HA-ERK2** was immunoprecipitated using the monoclonal 12CA5 antibody (anti-HA antibody; Roche Molecular Biochemicals). Endogenously expressed ERK in αT3-1 cells was immunoprecipitated by a rabbit polyclonal anti-ERK2 antisemur C-14 (Santa Cruz Biotechnology). Shc immunoprecipitations were performed with a rabbit polyclonal anti-SHC antibody (Transduction Laboratories), EGFR immunoprecipitations with the monoclonal 108.1 antibody (25) generously provided by A. Ullrich. PDGF-β-R was immunoprecipitated with a rabbit polyclonal anti-PDGF-β-R antibody (Upstate Biotechnology) and precipitated by centrifugation. Precipitates were washed three times with cold lysis buffer, resuspended in SDS sample buffer, boiled for 3 min, and resolved by SDS-polyacrylamide gel electrophoresis (PAGE).

For Western blotting, the protein samples were transferred onto Biotrace polyvinylidene difluoride membranes (Pall), and tyrosine phosphorylation was determined with the monoclonal 4G10 anti-phosphotyrosine antibody (Upstate Biotechnology). Thereafter, blots were reprobed with the anti-Shc (Transduction Laboratories) and anti-EGFR antibody (Santa Cruz) to control for the amounts of immunoprecipitated Shc and EGFR proteins, respectively. The appropriate secondary horse-radish peroxidase-conjugated anti-mouse (Fc specific) or anti-rabbit antibodies (Sigma) were used at a 1:2,000 dilution and were visualized on x-ray film (Eastman Kodak) by enzyme-linked chemiluminescence (Amersham Pharmacia Biotech).

**ERK-MAP Kinase Activity—**HA-ERK2 and endogenously expressed ERK was immunoprecipitated from COS-7 and αT3-1 cells by incubating preclarified lysates with the appropriate antibody (see above). Immunocomplexes were washed three times with lysis buffer and two times with kinase buffer (40 mM Hepes, pH 7.5, 5 mM magnesium acetate, 2 mM dithiothreitol, 1 mM EGTA, and 200 mM Na₂VO₃). Immunocomplexes were resuspended in kinase buffer, and kinase reactions were performed in a 50-μl volume supplemented with 250 μM magnesium ATP (Sigma) and 2 μM protein kinase A inhibitor, fragment 6-22 amide (Sigma). The kinase assay was initiated by the addition of 5 μl of a 500 μM ATP solution containing 2 μCi of [γ-32P]ATP (NEN Life Science Products Inc.). Incubations were carried out at room temperature for 20 min and were terminated by the addition of 6 μl of 88% formic acid. Reaction mixtures were then spotted onto Whatman P81 chromatography paper squares and washed four times in 150 mM formic acid.

2 Grosse, R., Schmid, A., Schöneberg, T., Herrlich, A., Muhln, P., Schulte, G., and Gudermann, T. (2000) Proc. Natl. Acad. Sci. USA 97, 275, 9193–9200.
phosphoric acid. Incorporated radioactivity was determined by liquid scintillation spectrometry. Basal counts of independently performed experiments ranged from 3,000 to 7,000 cpm/reaction. The radioactivity of samples incubated without immune complex was between 150 and 250 cpm. All assays were performed in duplicates.

**GST Fusion Proteins Containing the Ras-binding Domain of Raf1—**

GST fusion proteins containing the minimal Ras-binding domain (RBD) of Raf1 (amino acids 51–131) were prepared with minor modifications as described (26). Briefly, cultures of E. coli (DH5α) transformed with the plasmid pGEX-2T/GST-RBD (26) kindly provided by A. Wittinghofer were grown to an A600 of 0.8, and fusion protein expression was induced by addition of 0.1 mM isopropyl-β-D-thiogalactoside (Sigma). After 2 h, the cells were collected by centrifugation, and the pellet was resuspended in ice-cold PBS containing 1.2 mM CaCl2, 0.5 mM MgCl2, 0.5 mM dithiothreitol, 0.1 μM aprotinin, and 1 μM leupeptin. After sonication of lysates, Triton X-100 was added to a final concentration of 1%, and lysates were incubated at 4 °C for 30 min. Lysates were then centrifuged (12,000 × g; 10 min at 4 °C), and glycerol was added to a final concentration of 10%. The fusion protein preparations were stored in aliquots at −80 °C for up to 4 weeks.

**Determination of Ras Activity—**

Activation of Ras was measured by a modification of the procedure described by de Rooij and Bos (27). After stimulation of COS-7 cells in 100-mm dishes, monolayers were washed with ice-cold PBS and lysed for 15 min by the addition of 600 μl of Ras/Ras2 buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.5 mM MgCl2, 0.5% sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS, 0.1 μM aprotinin, 1 μM leupeptin, and 1 mM phenylmethylsulfonyl fluoride). Lysates were cleared by centrifugation and incubated for 30 min at 4 °C with GST-RBD precoupled to glutathione-Sepharose 4B (Amersham Pharmacia Biotech). Sepharose beads were collected by centrifugation (5,000 rpm, 1 min at 4 °C), washed three times with RIPA buffer, and proteins were denatured in SDS sample buffer. Precipitates were resolved by SDS-PAGE on 15 or 12.5% acrylamide gels, and proteins were subsequently transferred to Biotrace polyvinylidene difluoride membranes (Pall). Ras proteins were probed with the monoclonal pan-Ras antibody (Oncogene) followed by a secondary horseradish peroxidase-conjugated anti-mouse IgG (Sigma) antiserum and visualized as described above.

**RESULTS**

**EGF Receptor Tyrosine Kinase Activity but Not Elevation of [Ca2+]i Is Required for Rapid GnRH-induced ERK Activation in the Gonadotrope αT3-1 Cell Line**

We recently demonstrated that in several different cell lines agonist-bound human and murine GnRH receptors exclusively interact with Gα11 proteins.2 Accordingly, GnRH-mediated ERK activation in the gonadotrope cell line αT3-1 is PTX-insensitive, however, inhibited after pharmacological blockade or down-regulation of PKC (18, 19). To further study signaling components involved in rapid ERK activation by GnRH challenge of αT3-1 cells, endogenous ERK isoforms were immunoprecipitated and subsequently analyzed in an in vitro kinase assay. When cells were stimulated with GnRH for 5 min, a 2-fold increase in ERK activity was observed (basal: 3292 ± 140 cpm; 200 nM GnRH: 6005 ± 390 cpm; mean ± S.D., n = 3) which could be suppressed to about 35% by a 20-min preincubation with the EGFR selective tyrosphostine AG1478 (27) (200 nM GnRH + 100 nM AG1478, 4268 ± 170 cpm). A partial inhibition of EGF-induced ERK activity by AG1478 was observed as well (10 ng/ml EGF, 5972 ± 150 cpm; 10 ng/ml EGF + 100 nM AG1478, 4390 ± 160 cpm). These findings lend support to the idea that EGFR tyrosine kinase activity may be an intermediate in rapid GnRH-induced ERK activation in gonadotropes.

To gain further insight into the contribution of the EGFR tyrosine kinase to GnRH-induced ERK activation in αT3-1 cells, time course experiments were performed (Fig. 1). GnRH-dependent ERK activity was detectable 1 min after agonist addition and reached a peak value after 5 min. ERK activity remained elevated for up to 30 min and declined to basal values 1 h after GnRH challenge (see Fig. 1). Inhibition of EGFR tyrosine kinase activity by 100 nM of the specific tyrosphostine AG1478 did not completely abolish GnRH-induced ERK activa-

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**FIG. 1. Effect of AG1478 on the kinetics of GnRH-induced ERK activity in αT3-1 cells.** Semiconfluent cells in 100-mm dishes were serum-starved for 8 h and stimulated with 200 nM GnRH for the times indicated. Preincubation with 100 nM AG1478 was started 20 min prior to stimulation. Reactions were stopped by the addition of ice-cold PBS, and endogenous ERK activity was determined by an in vitro kinase assay (see “Experimental Procedures”). Data shown are mean ± S.E. of at least three independent experiments each performed in duplicate.

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an in vitro kinase assay as described above. To assess a potential contribution of Gβγ released from activated Gq11 proteins to receptor-mediated ERK activation, GnRH receptor-expressing COS-7 cells were additionally transfected with a cDNA construct coding for a fusion protein between the extracellular and transmembrane domain of CD8 and the C-terminal domain of βARK (29) in order to sequester free βγ subunits. We had previously demonstrated the ability of the βARK-Ct construct to inhibit Gβγ-mediated PLCβ activation by LPA (30) and to ablate LPA-induced ERK activation (11). As shown in Fig. 3, expression of CD8-βARK-Ct had no effect on basal or GnRH stimulated ERK2 activity. Collectively, these data suggest that signaling from the GnRH receptor to ERK-MAP kinases is mediated by Gq11 subunits without a substantial contribution of Gβγ.

The role of PKC in ERK activation via Gq-coupled receptors is discussed controversially and may be defined by the type of cell under study (3). It has been suggested that PKC directly phosphorylates and activates Raf in a Ras-independent manner (31). In keeping with the latter hypothesis overexpression of a dominant negative Raf mutant, D Raf, lacking potential regulatory phosphorylation sites (32), resulted in substantial suppression of GnRH-evoked ERK2 activity in transiently transfected COS-7 cells (Fig. 4A). A comparable inhibitory effect was observed after expression of the Src inactivating enzyme C-terminal Src kinase (Csk). Csk phosphorylates an inhibitory tyrosine residue in the C-terminal tail of c-Src and is the major physiological inactivator of Src kinases (33). Expression of the non-functional mutant Csk222 did not as shown in Fig. 3, expression of CD8-βARK-Ct had no effect on basal or GnRH stimulated ERK2 activity. Collectively, these data suggest that signaling from the GnRH receptor to ERK-MAP kinases is mediated by Gq11 subunits without a substantial contribution of Gβγ.

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in ERK2 activity above basal values in COS-7 cells (Fig. 4D).

Characteristics of GnRH-stimulated Ras Activity in COS-7 and αT3-1 Cells—To directly monitor Ras activation via the GnRH receptor, we used a GST fusion protein containing the minimal Ras-binding domain of Raf-1 (GST-RBD) as an activation-specific probe for Ras in order to precipitate the GTP form of Ras from cell lysates (27). GnRH challenge of transiently transfected COS-7 cells caused an increase in GTP-bound Ras in the precipitate (Fig. 5A). Dominant negative mutants of the EGFR (HER1-CD533) and Shc (Shc-Y317F) effectively suppressed GnRH-dependent Ras activation (see Fig. 5A), thus illustrating that the EGFR and Shc are signaling intermediates upstream of Ras in the signal transduction pathway. The specificity of the constructs used was tested by a C-terminal truncated, kinase-deficient dominant negative PDGF-β-R (PDGF-β-R-CD504) (37) devoid of any inhibitory effect in COS-7 cells (see Fig. 5A). Inactivation of Src by Csk not only interfered with GnRH-elicited ERK activation (see Fig. 4A), but also ablated agonist-induced GTP loading of Ras, while the kinase-deficient Csk222 mutant was ineffective (see Fig. 5A).

As demonstrated at the ERK level before (see Fig. 4D), mitogenic signaling by the GnRH receptor can be fully mimicked by PKC activation via short-term phorbol ester treatment. In COS-7 cells, incubation with TPA at 50 ng/ml leads to rapid formation (within 5 min) of Ras-GTP which was suppressed by expression of Csk (Fig. 5B). Thus, our findings entertain the notion that PKC and Src kinases are downstream targets of the ligand-occupied GnRH receptor and, additionally, that Src operates distally from PKC.

In congruence with this contention, GnRH-mediated Ras activity was sensitive to down-regulation of PKC isoforms by long-term (18 h) pretreatment with phorbol ester (100 ng/ml TPA) (Fig. 5C). Inhibition of EGFR tyrosine kinase by AG1478 (100 nM) diminished GnRH-invoked Ras-GTP formation while the related PDGFR-specific tyrphostine AG1296 at 1 μM had no inhibitory effects (see Fig. 5C). Stimulation of COS-7 cells with 50 ng/ml EGF served as a positive control for maximal Ras activation.

Precipitation of endogenous GTP-loaded Ras proteins in gonadotropin αT3-1 cells revealed that in analogy to the situation in COS-7 cells, GnRH challenge leads to Ras activation which was blocked by preincubation of cells with specific inhibitors for PKC (GF109203X) and Src kinase (PP2) (Fig. 6A). Like in COS-7 cells, GnRH-mediated GTP loading of Ras could be fully mimicked by short-term (5 min) phorbol ester treatment (Fig. 6B). GnRH- and TPA-induced Ras activation was partially inhibitable by low concentrations (100 nM) of the EGFR kinase-specific tyrphostine AG1478 (see Fig. 6B). The latter observation was further corroborated by transiently expressing two different dominant negative EGFR mutants namely HER1Na8 (38) and HER1-CD533 in αT3-1 cells. The expression of both EGFR mutants inhibited phorbol ester-mediated Ras activation in gonadotrope-derived cells (Fig. 6C).

Our data strongly suggest that the small GTPase Ras plays a pivotal role in the signaling cascade connecting the GnRH receptor with ERKs. In addition, GnRH-mediated Ras activation appears to require PKC, Src, and EGFR tyrosine kinase activity. In gonadotropes PKC acts upstream of the EGFR whose tyrosine kinase activity contributes to phorbol ester-induced Ras activation.

Src-independent Shc Activation via the GnRH Receptor—There is mounting evidence for a role for members of the Src family of tyrosine kinases in signaling via GPCRs. For the
COS-7 cell model it has been postulated that Src is responsible for GPCR-initiated Shc phosphorylation (39) and that RTK- and GPCR-derived mitogenic signals converge at the level of the adaptor protein Shc setting in motion the tyrosine phosphorylation-dependent Shc-Grb2/SOS interaction (4). In line with this concept, Shc immunoprecipitations from agonist-treated GnRH receptor-expressing COS-7 cells revealed an increase in the tyrosine phosphorylation of all three Shc isoforms (Fig. 7). Inactivation of Src by Csk, however, did not impair GnRH-induced Shc tyrosine phosphorylation, and the pronounced Shc phosphorylation elicited by 10 ng/ml EGF remained unaffected as well (see Fig. 7). We did not try to further characterize a 110-kDa protein which was found to be co-immunoprecipitated with Shc and also phosphorylated on tyrosine residues in response to GnRH and EGF treatment (see Fig. 7). These data indicate that Src neither contributes to GnRH- nor to EGF-induced Shc activation while Src activity is required for GnRH-mediated ERK and Ras activation (see Figs. 4A and 5A). Thus, Src appears to act independently of Shc to contribute to Gq-mediated Ras activation.

Phorbol Esters Promote Tyrosine Phosphorylation of Shc and Association with the EGF Receptor—To further characterize the tyrosine phosphorylation of Shc in the course of Gq/PKC-mediated ERK activation, COS-7 cells were stimulated with various agonists, Shc proteins were immunoprecipitated, and the precipitates were subsequently probed with an anti-phosphotyrosine antibody. Treatment of cells with 25 μM LPA or 3 ng/ml EGF resulted in increased Shc tyrosine phosphorylation which upon prolonged exposure of the blot is best illustrated by the p66Shc isoform (Fig. 8). Whereas the forskolin-induced elevation of intracellular cAMP levels and subsequent activation of cAMP-dependent protein kinase was ineffective, short-term TPA treatment (100 ng/ml) led to an increase in the phosphotyrosine content of p66Shc (see Fig. 8). Interestingly, besides the appearance of a 110-kDa band (see also Fig. 7) another protein of approximately 170–180 kDa was also phosphorylated on tyrosine residues in response to TPA challenge (see Fig. 8). This high molecular weight band was
EGF Receptor Tyrosine Kinase Activation by GnRH

GnRH Transactivates the EGF Receptor via a PKC-dependent Mechanism in COS-7 and αT3-1 Cells—As agonist binding to the GnRH receptor resulted in Src-independent Shc tyrosine phosphorylation (see Fig. 5) and short-term phorbol ester challenge of COS-7 cells results in Shc/EGFR association (see Fig. 7), we set out to examine the possibility of a growth factor-independent EGFR activation (transactivation) mediated by the GnRH receptor. Subsequent to agonist stimulation, the EGFR was immunoprecipitated from transiently transfected COS-7 cells and probed with anti-phosphotyrosine antibodies. Fig. 9A shows that GnRH stimulation of cells leads to a rapid increase in the phosphorytrosine content of the EGFR after 5 min, albeit not as pronounced as after maximal EGF (10 ng/ml) stimulation (see Fig. 9A). Down-regulation of PKC isofoms by long-term (18 h) phorbol ester treatment as well as EGFR kinase inhibition by 100 nM AG1478 completely prevented GnRH-induced transactivation of the EGFR in COS-7 cells (see Fig. 9A). As expected, the low concentration of AG1478 did not completely abrogate EGFR tyrosine phosphorylation mediated by maximal growth factor stimulation. These findings show that activation of G_{q11} proteins via the GnRH receptor results in growth factor-independent activation of the EGFR. Most likely this phenomenon is the result of PKC activation and represents increased RTK autophosphorylation.

To examine whether transactivation of RTKs by the GnRH receptor can also be observed under more physiological conditions, αT3-1 cells were stimulated for 5 min with 1 μM GnRH or 3 ng/ml EGF. Fig. 9B shows that GnRH and EGF challenge leads to an increased tyrosine phosphorylation of the EGFR which was sensitive to nanomolar concentrations of the tyrphostine AG1478 in both cases. In addition, GnRH induced activation of the EGFR was inhibited by preincubation with 1 μM of the highly selective PKC inhibitor GF 109203X (bisindolylmaleimide I). Thus, in αT3-1 gonadotropes GnRH challenge leads to engagement of the ERK/MAPK cascade via PKC-mediated ligand-independent activation of the EGFR.

Protein Kinase C Mediates Transactivation of Receptor Tyrosine Kinases in L Cells—To test the hypothesis whether PKC-mediated autophosphorylation of RTKs is a general phenomenon, we decided to study PDGF-β-R activation in response to TPA in L cells. We previously showed that these cells do not express EGFRs and respond to LPA challenge with a ligand-independent activation of the PDGF-β-R (11). Stimulation of cells with 3 ng/ml PDGF-BB or 25 μM LPA resulted in tyrphostine AG1296-sensitive tyrosine phosphorylation of the immunoprecipitated PDGF-β-R (Fig. 10). Incubation of L cells with 100 ng/ml TPA also increased the phosphorytrosine content of the immunoprecipitated PDGF-β-R. This effect was observable as early as 5 min after TPA challenge and was completely prevented by 10 μM of the PDGFR-selective tyrosine kinase inhibitor AG1296 indicating a phorbol ester-induced increase in PDGFR autophosphorylation.

**DISCUSSION**

The results of these studies demonstrate that Ras and ERK activation by the G_{q11}-coupled GnRH receptor depends on EGFR and Src tyrosine kinase activity and illustrates that functionally both kinases act downstream of PKC in COS-7 as well as in gonadotrope αT3-1 cells. GnRH-mediated engagement of the ERK-MAPK cascade has been shown to play a crucial role in the regulation of gonadotrope gene expression (15–18). However, the mechanism underlying ERK activation via the G_{q}-coupling GnRH receptor is still unclear. Due to the lack of rapid receptor internalization (21) and the β-arrestin-independent regulation of receptor expression (22) the recently proposed coupling model of the β_{2}-adrenergic receptor to ERKs via β-arrestin-Src complex formation and receptor internaliza-

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**Fig. 7.** GnRH-induced Shc phosphorylation in COS-7 cells. COS-7 cells were co-transfected with 5 μg of GnRH receptor and 15 μg of CSK or CSK222 plasmid DNA. Serum-starved cells were stimulated for 5 min with 200 nM GnRH or 10 ng/ml EGF as a control. Immunoprecipitated Shc isoforms (IP αSHC) were subjected to 9% SDS-PAGE, followed by reprobing with anti-SHC antibodies (lower panel, blot αSHC) as described (see “Experimental Procedures”). The experiment was repeated three times with similar results.

**Fig. 8.** TPA-induced tyrosine phosphorylation of the EGFR and Shc in COS-7 cells. COS-7 cells were serum-starved and stimulated for 5 min with 25 μM LPA, 3 ng/ml EGF, 100 μM forskolin (FSK), or 100 ng/ml TPA. EGF receptor was co-immunoprecipitated with an anti-Shc antibody (IP αSHC), and tyrosine-phosphorylated proteins were detected by immunoblotting with an anti-phosphotyrosine antibody (blot α-P-Tyr(P)). Subsequently identified as the EGFR receptor (data not shown) co-precipitated with Shc proteins by anti-Shc antibodies. Our findings demonstrate that phorbol ester treatment of COS-7 cells is sufficient to cause tyrosine phosphorylation of Shc proteins and to induce the association of Shc with the tyrosine-phosphorylated EGFR.

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**Fig. 9.** TPA-induced tyrosine phosphorylation of the EGFR and Shc in COS-7 cells. COS-7 cells were serum-starved and stimulated for 5 min with 25 μM LPA, 3 ng/ml EGF, 100 μM forskolin (FSK), or 100 ng/ml TPA. EGF receptor was co-immunoprecipitated with an anti-Shc antibody (IP αSHC), and tyrosine-phosphorylated proteins were detected by immunoblotting with an anti-phosphotyrosine antibody (blot α-P-Tyr(P)).
Confluent L cells in 15-cm dishes were serum-starved for 16 h and stimulated with 3 ng/ml PDGF-BB, followed by SDS-PAGE and immunoblotting with an anti-phosphotyrosine antibody (α-Tyr(P)) followed by reprobing with polyclonal anti-EGFR antibodies (lower panel). Molecular weight markers are indicated on the right lower panel with polyclonal anti-EGFR antibodies (lower panel). Similar results were obtained in three independent experiments.

**FIG. 9.** GnRH-induced tyrosine phosphorylation of EGFR in COS-7 and αT3-1 cells. A, cells were transfected with human GnRH receptor cDNA. Serum starvation was carried out for 18 h in the absence or presence of 100 ng/ml TPA. Ten min prior to stimulation with 200 nM GnRH or 10 ng/ml EGF, cells were treated with 100 nM AG1478 as indicated. After lysis of cells, EGFR receptor proteins were immunoprecipitated using a monoclonal anti-EGFR antibody (IP αEGFR), and tyrosine phosphorylation was determined by immunoblotting with anti-phosphotyrosine antibody (α-Tyr(P)) followed by reprobing with polyclonal anti-EGFR antibodies (lower panel). Molecular weight markers are indicated on the right of each figure. Similar results were obtained in three independent experiments.

**FIG. 10.** Effects of phorbol ester stimulation on tyrosine phosphorylation of PDGFR in L cells. Confluent L cells in 15-cm dishes were serum-starved for 16 h and stimulated with 3 ng/ml PDGF-BB, 25 μM LPA, or 100 ng/ml TPA for the times indicated. Preincubations with 10 μM AG1296 were performed for 15 min prior to stimulation. Lysates were immunoprecipitated with an anti-PDGFR-β antibody (IP αPDGFR-β) followed by SDS-PAGE and immunoblotting with an anti-phosphotyrosine antibody (α-Tyr(P)).

A rise in [Ca\(^{2+}\)] is a characteristic signaling event that can be observed in GnRH-treated gonadotropes. An influx of Ca\(^{2+}\) through L-type voltage-gated Ca\(^{2+}\) channels is an essential prerequisite for GnRH-dependent ERK activity detectable at fairly late time points, e.g. 15 or 30 min after agonist challenge of gonadotropes, and the effect of Ca\(^{2+}\) most probably lies downstream of PKC (41). In neuroendocrine PC12 cells, Ca\(^{2+}\) transients alone are sufficient to trigger ERK activation (42, 43); the exact mechanism of action, however, is discussed controversially, but may involve the non-receptor tyrosine kinase Pyk2 (28).

While there is consensus with regard to the requirement of Ras for G\(_{q}\)-mediated ERK activation (3, 4), the participation of Ras in signaling to ERKs via G\(_{q}\)-coupled receptors is a much contended issue, and evidence for a Ras-independent or Ras-dependent ERK activation through G\(_{q}\)-coupled receptors has been presented (5, 6). The latter option is strengthened by the observation that signaling factors such as Shc, Grb2, and SOS, which act upstream of Ras, play important roles in G\(_{q}\)-mediated ERK activation in different cell systems (44–46). In contrast to a previous study (7) we show in the present paper that a dominant-negative Ras mutant, N17-Ras, interferes with G\(_{q}\)-induced ERK signaling, and we provide direct evidence that G\(_{q}\) activation increases the GTP-loaded fraction of Ras in signaling to ERKs via G\(_{q}\) receptor. The participation of Ras in GnRH signaling to ERKs in COS cells could rely on a significant overexpression of G\(_{q}\)-coupled receptors in a heterologous cell system which may allow for alternative Ras-independent routes to ERK. However, under the experimental conditions chosen by us we never exceeded a membranous receptor density of 400 fmol/mg protein (24) which closely corresponds to physiological B\(_{\text{max}}\) values determined in mouse and rat pituitaries (47). There are conflicting reports on a fully PKC-dependent (6), PKC-independent (48), or partially PKC-dependent (5) signaling to ERK-MAP kinases via G\(_{q}\)-coupled receptors. In the present study, inhibition of PKC activity by different inhibitors abrogated GnRH receptor-mediated ERK activation. The additional observation that short-term phorbol ester treatment of

**A**

![Image](http://www.jbc.org/)

**B**

![Image](http://www.jbc.org/)
COS-7 and αT3-1 cells could substitute for receptor agonist with regard to MAPK signaling further highlights the pivotal role of PKC in G<sub>i</sub>-induced ERK activity in COS-7 and αT3-1 cells. Direct activation of PKC by phorbol esters has been shown to trigger ERK activity in most cell types and thus to contribute to cellular responses such as cell differentiation and proliferation (49). The underlying mechanism, however, is still unclear. Apart from direct stimulation of Ras-GRP, a GEF predominantly expressed in the nervous system, by diacylglycerol and Ca<sup>2+</sup> (50), some PKC isoforms phosphorylate Raf. Functional consequences, for instance an increased Raf phosphorylation activity toward its natural substrate MEK, are discussed controversially (31, 51, 52). Our finding, that a dominant negative Raf mutant interferes with G<sub>i</sub>/PKC-mediated signaling to ERK, implicates an essential role of Raf in this signal transduction pathway, and it has recently been suggested that PKC might be involved in controlling the membrane association of Raf (52). The importance of Ras in transducing signals from PKC to ERKs is unclear, as despite PKC-induced Ras-GTP accumulation the dominant negative N17-Ras did not block Raf activation initiated by TPA or via the m1 muscarinic receptor (7). Thus, it was concluded that PKC activates Ras by a mechanism clearly set apart from that initiated by activation of RTKs (7).

Our findings do not support the latter conclusions, because in our hands N17-Ras attenuated ERK activation via the G<sub>i</sub>-coupled GnRH receptor in COS-7 cells. Both at the level of Ras and of ERK we show that intracellular signals emanating from the agonist-bound GnRH receptor can be interfered with by dominant negative mutants of signaling components like Shc (Shc-Y317F) or the EGFR (HER1-CD533), which are functionally localized upstream of Ras. Furthermore, GnRH challenge of transfected COS-7 cells resulted in increased tyrosine phosphorylation of Shc and in a PKC-dependent, growth factor-independent activation of the EGFR. Thus, in contrast to the model proposed by Marais et al. (7) our findings presented here favor a mechanism by which PKC leads to ERK activation via engagement of the canonical RTK-Ras-ERK cascade in analogy to classical growth factor signaling.

There is considerable evidence for a role for Src family tyrosine kinases in the signaling pathway from GPCRs to ERKs (4). Inhibition of Src kinase activity by expression of the Csk attenuated Shc tyrosine phosphorylation and ERK activation (39). While we observed a profound inhibitory effect of Csk on G<sub>i</sub>-dependent Ras and ERK activation, Src inactivation did not adversely affect Shc tyrosine phosphorylation elicited by the agonist-bound GnRH receptor, nor did it diminish EGF-dependent Shc tyrosine phosphorylation. Recently, Src has been suggested to be responsible for tyrosine phosphorylation of the EGFR which would then function as a scaffold for GPCR-mediated Ras activation (53).

Our current findings and previous data (11), however, stress the importance of RTK autophosphorylation for the transactivation process, which can be completely averted by preincubation with low concentrations of RTK-specific tyrosphostine inhibitors. In addition, pretreatment of cells with the Src tyrosine kinase inhibitor PP1, hardly affected EGFR transactivation upon LPA stimulation of COS-7 cells (54).

Both G<sub>i</sub> and G<sub>i</sub>-coupled receptors can mediate RTK transactivation (10, 54). While G-protein βγ subunits play a crucial role in G<sub>i</sub>-mediated signaling (11), our data show that PKC activation is necessary and sufficient to bring about EGFR transactivation in response to G<sub>i</sub> activation. We show that PKC-mediated transactivation of RTKs is a general phenomenon, because TPA is also able to initiate ligand-independent activation of the PDGF-β-R in L cells. So far, we do not have an explanation which would reconcile our data with the recently published observation that PKC suppresses EGF transactivation after angiotensin II stimulation of GN4 rat liver epithelial cells (55). Increased tyrosine phosphorylation of ErbB2 and ErbB3 in Fao rat hepatoma cells or the EGFR in PC12 and HEK 293 cells subsequent to short-term incubations with phorbol esters has also been observed (28, 56, 57); the underlying mechanism, however, remains elusive. We, furthermore, show that in gonadotropin αT3-1 cells phorbol ester-induced GTP loading of Ras is sensitive to the specific EGFR tyrosine kinase blocker AG1478 and to dominant negative EGFR mutants. Thus, EGFR tyrosine kinase activity is required for PKC-induced Ras activation, and PKC acts upstream of the EGFR.

Our data should not be interpreted to mean that RTK transactivation represents the only mechanism underlying PKC-mediated ERK activation. We show that the major role of EGFR transactivation in gonadotropes is to define the kinetics of GnRH-induced ERK activity. The delineation of distinct signaling components and their activation sequence as well as studies on the kinetics of GnRH-dependent ERK activation may provide further insight into regulation and gene expression of differentiated gonadotropes.

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Note Added in Proof—While this manuscript was being revised a general signaling pathway responsible for EGF receptor transactivation via G-protein-coupled receptors was described (Prenzel, N., Zwick, E., Daub, H., Leserer, M., Abraham, R., Wallasch, C., and Ulrich, A. (1998) Science 282, 402–409, in which cleavage of proHB-EGF by a metalloproteinase activated by PKC or intracellular calcium elevations is the underlying mechanism for EGF receptor activation.

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Epidermal Growth Factor Receptor Tyrosine Kinase Mediates Ras Activation by Gonadotropin-releasing Hormone

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