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Activation and nuclear translocation of PKCδ, Pyk2 and ERK1/2 by gonadotropin releasing hormone in HEK293 cells☆

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Abstract

The mechanism of agonist-induced activation of Pyk2 and its relationship with ERK1/2 phosphorylation was analyzed in HEK293 cells stably expressing the gonadotropin releasing hormone (GnRH) receptor. GnRH stimulation caused rapid and sustained phosphorylation of ERK1/2 and Pyk2 that was accompanied by their nuclear translocation. Pyk2 was also localized on cell membranes and at focal adhesions. Dominant negative Pyk2 (PKM) had no effect on GnRH-induced ERK1/2 phosphorylation and c-*fos* expression. These actions of GnRH on ERK1/2 and Pyk2 were mimicked by activation of protein kinase C (PKC) and were abolished by its inhibition. GnRH caused translocation of PKCα and δ, but not of ε, ι and λ, to the cell membrane, as well as phosphorylation of Raf at Ser338, a major site in the activation of MEK/ERK1/2. Stimulation of HEK293 cells by EGF caused marked ERK1/2 phosphorylation that was attenuated by the selective EGFR receptor (EGF-R) kinase inhibitor, AG1478. However, GnRH-induced ERK1/2 activation was independent of EGF-R activation. These results indicate that activation of PKC is responsible for GnRH-induced phosphorylation of both ERK1/2 and Pyk2, and that Pyk2 activation does not contribute to GnRH signaling. Moreover, GnRH-induced phosphorylation of ERK1/2 and expression of c-*fos* in HEK293 cells is independent of Src and EGF-R transactivation, and is mediated through the PKC/Raf/MEK cascade.
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Keywords: c-*fos*; GnRH; ERK1/2 MAP kinases; HEK293 cells; Nuclear translocation; Pyk2; PKC

1. Introduction

The hypothalamic decapeptide, gonadotropin releasing hormone (GnRH), is a primary regulatory factor in the neuroendocrine control of reproduction, and is released in an episodic manner from hypothalamic GnRH neurons. The pulsatile delivery of GnRH to the anterior pituitary gland is essential to maintain the circulating gonadotropin profiles that are necessary for normal reproductive function. In addition to regulating pituitary gonadotropin release, GnRH has extrapituitary actions in neural and non-neural tissues and in several types of tumor cells [1].

The major signal transduction pathways in cells expressing GnRH receptors are initiated by activation of phospholipase C (PLC). The consequent calcium (Ca^{2+}) mobilization and activation of protein kinase C (PKC) by GnRH are key elements in the hypothalamic control of gonadotropin secretion from the anterior pituitary gland [1,2]. Activation of PKC and Ca^{2+} mobilization during GnRH receptor stimulation are also responsible for mediating downstream signals leading to activation of extracellularly regulated mitogen-activated protein kinases (ERK1/2 MAP kinases), which transmit signals from the cell surface to the nucleus to regulate gene transcription and other processes [2–7]. Although 11 PKC isoforms have been identified, there is no consensus about the specific PKC isoforms that are involved in GnRH-induced ERK1/2 activation [8–10].

GPCR-mediated phosphorylation of ERK1/2 can also occur through activation of tyrosine kinases of the Src family, focal adhesion kinases (FAKs), and receptor tyrosine kinases (RTKs). The RTKs involved in GPCR-mediated activation of ERK1/2 MAP kinases include the epidermal growth factor receptor (EGF-R), platelet-derived growth factor receptor (PDGF-R), and insulin-like growth factor (IGF-1) receptor [11–13]. The several GPCRs that mediate EGF-R transactivation during agonist stimulation include the AT_1 angiotensin receptor [14], the β-adrenoceptor [15], the P2Y2 purinoceptor [16], and receptors for endothelin-1, thrombin, lysophosphatidic acid (LPA) and bradykinin [17,18]. GPCR-mediated transactivation of the EGF-R initiates the

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ERK1/2 MAP kinase cascade through recruitment of adaptor proteins, such as the Shc/Grb2/Sos complex, which activate the small G protein, Ras [11,13–19]. Depending on the GPCR agonist and cell type, Ca^{2+}, PKC, G protein βγ subunits, and non-receptor tyrosine kinases including Src and Pyk2, have been implicated in GPCR-induced EGF-R transactivation [11,14,17,19]. Endogenous EGF-Rs are expressed in several of the cell types, including αT3-1 gonadotrophs, COS-7 cells, and HEK293 cells, that have been used in studies on GnRH signaling. However, the role of EGF-R transactivation in GnRH-induced ERK activation has been a subject of controversy and is not clearly defined [4,6,20].

GPCRs mediate both Ras-independent ERK1/2 activation via stimulation of PKC and Raf, and Ras-dependent ERK1/2 activation via receptor and non-receptor tyrosine kinases [7,11]. GnRH activates ERK1/2 MAP kinases in αT3-1 gonadotrophs and in COS-7 cells [2,4–6,21] and GH3 cells transfected with GnRH receptors [22]. GnRH also stimulates Jun N-terminal kinase (JNK) in αT3-1 gonadotrophs [23] and p38 MAP kinase in LβT2 gonadotrophs [24]. Activation of these MAP kinases by other GPCRs, such as angiotensin II [25,26], endothelin [27], adrenomedullin [28] and acetylcholine [29], is mediated by the proline-rich protein tyrosine kinase, Pyk2. In general, Pyk2 activation in conjunction with Src kinase appears to be a key element in GPCR-mediated transactivation of the EGF-R [30]. However, no information is available on the role of Pyk2, and the nature of its interaction with Src and EGF-R, during receptor stimulation by GnRH. In the present study, GnRH was found to stimulate phosphorylation and nuclear translocation of ERK1/2 and Pyk2 in HEK293 cells expressing the mouse GnRH receptor. These responses were mediated by activation of PKCα and δ but did not involve the activation of Src and the EGF-R.

2. Materials and methods

2.1. Materials

GnRH and its [D-Ala^{6}-*N*-ethylamide] agonist analog and [D-pGlu^{1}, D-Phe^{2}, D-Trp^{3,6}]GnRH antagonist were purchased from Peninsula Laboratories Inc. (Belmont, CA). The CDB peptide antagonist was provided by Dr. Hyun K. Kim (NICHD/NIH, Bethesda, MD). Lipofectamine and opti-MEM were obtained from Life Technologies Inc., *myo*-[^{3}H]inositol was purchased from Amersham Pharmacia Biotech, and PP2, AG1478, Go6983 and ionomycin were from Calbiochem. Poly-D-lysine coated cell culture plates were from Becton Dickinson Labware (Bedford, MA), and HEK293 cell culture medium was purchased from ATCC. C-terminal Src kinase (Csk) and dominant negative Pyk2 mutant (PKM) constructs were provided by Dr. Zvi Naor, University of Tel Aviv. PBS was purchased from Gibco-BRL, ECL reagent from Amersham and Pyk2 and ERK1/2 antibodies from Santa Cruz Biotechnology. Pyk2 antibodies recognizing phosphotyrosine at residues 402, 579, 580 were from Biosource International (Camarillo, CA), and PKC isoform antibodies were from BD Biosciences. Phospho-PKC and phospho-ERK antibodies were from Cell Signaling Technology. Texas Red-conjugated anti-mouse and anti-rabbit IgG were purchased from Vector Laboratories, and the PEEGFP-N1 vector from Clontech Laboratories Inc. (Palo Alto, CA).

2.2. GnRH receptor expression in HEK293 cells

The 1020 base-pair mouse GnRH receptor cDNA tagged with an N-terminal HA epitope was subcloned into the pEGFP-N1 vector between the *Bgl*11 and *Sma*1 sites. The HA-GnRH cDNA cloned into pEGFP-N1 MCS was expressed as a fusion protein bound to the N-terminus of the pEGFP. The pEGFP-N1 vector encodes a variant of wild-type GFP with enhanced fluorescence. For stable transfection, HEK293 cells were cultured in 24-well plates and transfected with 500 ng of subcloned HA-GnRH-GFP cDNA in opti-MEM containing 3 μl of lipofectamine 2000 for 2 h. After 24 h, cells were trypsinized and plated in 100 mm culture dishes at low density (100–500 cells per dish). On the next day, incubation was continued in culture medium containing 200 μM G418 for selection. Several colonies were obtained within 2 weeks of selection, and studies were performed on a stably transfected HEK293 cell line.

2.3. Intracellular Ca^{2+} measurements

For calcium measurement studies, HEK293 cells were plated on poly-L-lysine-coated cover slips. Prior to Ca^{2+} recording, cover slips were incubated for 20 min at 37 °C with 0.5 μM Fura-2 AM (Molecular Probes, Eugene, OR) in Phenol Red-free DMEM and then washed in Krebs-ringer buffer. They were then mounted on a cell chamber for microscopy and cells were examined under a 40× oil immersion objective during exposure to alternating 340 and 380 nm light beams. The intensity of light emission at 505 nm was measured and changes in [Ca^{2+}] were derived from the ratio of the two excitation wavelengths (F340/F380). Data were obtained at a sampling rate of two points per second.

2.4. Measurement of inositol phosphates (InsPs)

InsP assays were performed on HEK293 cells as previously described [31]. Briefly, equal numbers of HEK293 cells were plated in 24-well poly-D-lysine coated plates. Next day, cells were incubated overnight in inositol free DMEM containing 20 μCi/ml of *myo*-[^{3}H]inositol, then rinsed gently with inositol-free M199 medium and stimulated with 100 nM GnRH in the same medium containing 10 mM lithium chloride (LiCl) for up to 60 min. Reactions were stopped by adding ice-cold perchloric acid (10%) for 30 min on ice, and $InsP_2 + InsP_3$ in cell extracts were measured by anion exchange column chromatography as

previously described [32]. The radioactivity in each sample was measured by liquid β scintillation spectroscopy. Data indicate the mean CPM from four wells at each time point.

2.5. Immunocytochemistry

HEK293 cells were plated at low density on poly-L-lysine coated glass cover slips for 24 h, then rinsed and incubated in serum-free medium for 12–24 h. Cells were then stimulated with 100–200 nM GnRH or 250–500 nM PMA for the periods indicated. Following stimulation, cells were rinsed in warm PBS, fixed in 3.7% formaldehyde for 15 min, and permeabilized as previously described [33] or by immersing the cover slips in 100% methanol for 5 min. After incubation for 60 min at 37 °C in blocking medium (3% BSA and 3% normal goat or horse serum, depending on the species in which secondary antibody was made), the cells were incubated in primary antibody diluted 1:50 in blocking medium. The cover slips were then washed in PBS (3×, 5 min each) and incubated with Texas Red-labeled horse anti-mouse antibody diluted in blocking medium. For double labeling experiments, HEK293 cells were incubated with Cy5-labeled anti-rabbit or anti-mouse antibody. After final incubations the cells were washed gently with PBS, mounted in ProLong mounting medium (Molecular Probes, Eugene, OR), and examined with a Bio-Rad MRC 1024 confocal microscope.

2.6. Western blot analysis

HEK293 cells were grown in six-well culture plates to near confluence, then rinsed gently in warm serum-free medium and incubated in the same medium for 24 h. After treatment with selected agonists and inhibitors at 37 °C, cells were washed twice with ice-cold PBS and lysed in 100 μl of Laemmli sample buffer. After brief sonication, the suspension was heated for 5 min at 95 °C. The samples were then electrophoresed on a 8–16% gradient gel and transferred to PVDF membrane. After 60 min incubations in blocking buffer (5% dry milk in PBS), membranes were incubated overnight at 4 °C in the primary antibody. Phospho-ERK1/2 were detected using monoclonal mouse phospho-ERK1/2 antibody at 1:2000 dilution, and phosphorylated Pyk2 was detected using 1:1000 dilution of the indicated Pyk2 antibody. Next day, membranes were washed with 0.3% Tween-20 in PBS and incubated with peroxidase-conjugated secondary antibody for 1 h at room temperature, then washed in PBS as before and developed with ECL (Amersham Pharmacia).

3. Results

3.1. Characterization of GnRH-receptor expressing HEK293 cell line

Immunocytochemical analysis of the stably transformed non-permeabilized cells with HA epitope-specific antibody showed intense surface staining (Fig. 1A), consistent with expression of the GnRH-R in the plasma membrane. Coupling of the transfected GnRH-R-GFP protein to G_q was indicated by the ability of GnRH to stimulate InsP production within 5 min, with a progressive rise over 60 min (Fig. 1B, filled bars). In contrast, the untransfected control cells showed no InsP response to GnRH. Intracellular Ca^{2+} in vector-transfected control HEK293 cells showed no response to GnRH treatment for up to 150 s, but were markedly increased after treatment with 1 μM ionomycin (Fig. 1C). In stably transfected HEK293 cells, GnRH (100 nM) caused a rapid and prominent increase in cytosolic Ca^{2+} (Fig. 1D). These results indicate that the cell-surface GnRH-R of stably transfected HEK293 cells is effectively coupled to $G_{q/11}$ and the phosphoinositide/Ca^{2+} signaling pathway.

3.2. GnRH-induced ERK1/2 activation is PKC-dependent but independent of Src and EGF-R activation

GnRH has been shown to activate ERK1/2 MAP kinases through diverse signaling mechanisms in individual cell types. Western blot analysis showed rapid phosphorylation of ERK1/2 in response to GnRH stimulation in HEK293 cells, with an increase within 2 min to a maximum level after 5 min that was sustained for up to 60 min (Fig. 2A). Pretreatment of cells with the PKC inhibitor, Go6983 (which inhibits PKCα, β, γ, ζ and δ) completely abolished GnRH-mediated ERK1/2 phosphorylation (Fig. 2B). However, there was no effect of the Ca^{2+} chelator, BAPTA, indicating that GnRH-induced ERK1/2 in HEK293 cells is primarily dependent on PKC. The selective Src inhibitor, PP2, had no significant effect on GnRH-induced ERK1/2 phosphorylation (Fig. 2B).

Previous studies have shown that GPCR-induced ERK1/2 phosphorylation is often mediated through transactivation of the epidermal growth factor receptor (EGF-R) [30,34]. However, there are conflicting reports on the involvement of EGF-R in GnRH action [4,6]. The EGF-R is expressed in HEK293 cells, in which EGF caused marked phosphorylation of ERK1/2 that was abolished by the selective EGF-R kinase antagonist, AG1478 (Fig. 2C). In contrast, GnRH did not cause phosphorylation of the EGF-R (data not shown), and GnRH-induced ERK1/2 phosphorylation was not attenuated by selective inhibitors of the EGF-R (AG1478) and the PDGF-R (AG1295) (Fig. 2C), excluding a role of EGF-R and PDGF-R transactivation in this cascade. However, GnRH caused marked phosphorylation of Raf-1 at Ser338 (Fig. 2D), a site involved in the downstream activation of MEK/ERK1/2. These data suggest that GnRH-mediated activation of PKC in HEK293 cells is responsible for direct activation of the Raf/MEK/ERK cascade.

3.3. Role of PKC in GnRH-mediated ERK1/2 activation

HEK293 cells treated with the PKC activator, PMA, showed rapid phosphorylation of ERK1/2 (Fig. 3A).

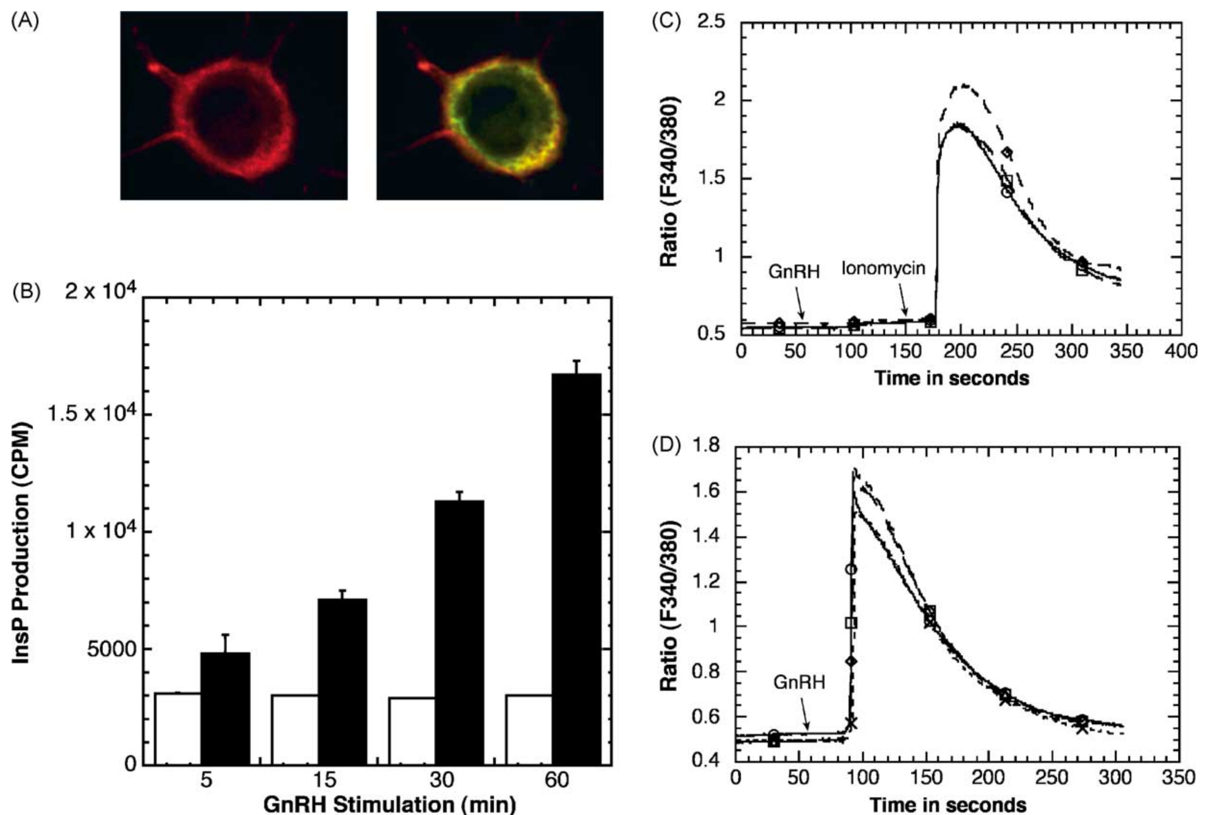

Fig. 1. Characterization of GnRH-R expressing HEK293 cell line. (A) Surface expression of GnRH-Rs. Cells were grown on glass cover slips at low density, fixed in 3.7% paraformaldehyde, and stained with anti-HA antibody against GnRH-R followed by Texas Red-conjugated anti-mouse antibody. The same cell also shows green labeling of GFP that was fused to GnRH-R. (B) Agonist-stimulated inositol phosphate production in transfected HEK293 cells. The ($InsP_2$ + $InsP_3$) fraction was isolated by anion exchange column chromatography and analyzed by liquid β scintillation spectrometry. The results from a representative experiment are shown as the mean ± S.E. of four replicates. Intracellular Ca^{2+} changes were determined in vector-transfected cells (C) and GnRH-R transfected HEK293 cells (D). Control cells did not respond to GnRH, but showed prominent Ca^{2+} increases after treatment with ionomycin ($n = 3$). Receptor-transfected cells showed prominent Ca^{2+} responses to GnRH.

Immunoblot analysis revealed that HEK293 cells contain PKCα, δ, ι, ε and λ isoforms (Fig. 3B). The specific PKC isoforms involved in GnRH action were determined by analyzing the translocation of these PKC isoforms to the cell membrane following GnRH treatment, which caused marked changes in the distribution of PKCα and δ, but not of the ι, ϵ and λ isoforms (Fig. 3C). In addition, PKCδ showed bright punctate nuclear staining (Fig. 3C). These effects of GnRH were abolished by pretreatment of HEK293 cells with a potent GnRH receptor antagonist (data not shown).

3.4. GnRH induces tyrosine phosphorylation of Pyk2

Several studies have suggested a role for the cytosolic proline-rich tyrosine kinase, Pyk2, in GPCR-mediated MAPK activation [35,36] and this has been established for G_q-linked GPCR agonists including angiotensin II (Ang II) [25,37,38], cholecystokinin [39], bradykinin [35], and LPA [35,38]. In HEK293 cells, GnRH stimulation caused marked phosphorylation of Pyk2 at Tyr402 as early as 2 min, and this was sustained for 60 min (Fig. 4A). The effect of GnRH on Pyk2 activation was mimicked by the PKC activator, PMA (100 nM) (Fig. 4B). Consistent with the predominant role of PKC in this response, the PKC inhibitor, Go6983, completely abolished GnRH-induced Pyk2 phosphorylation (Fig. 4C).

We next determined whether GnRH action on ERK1/2in HEK293 cells is dependent on Pyk2 activation. As shown in Fig. 4D, overexpression of dominant negative Pyk2 (PKM) had no effect on GnRH-induced ERK1/2 phosphorylation, excluding any involvement of Pyk2 in GnRH action in HEK293 cells. Since previous studies have shown that Pyk2 activation is mediated through Src activation [35], we also determined the effects of Csk on agonist-induced responses. Overexpression of Csk, which phosphorylates and inhibits Src family members, did not alter GnRH-induced ERK1/2 phosphorylation in HEK293 cells (Fig. 4D). Since both Src and Pyk2 have been implicated in GPCR-mediated transactivation of the EGF-R [30,37], their lack of involvement could account for the

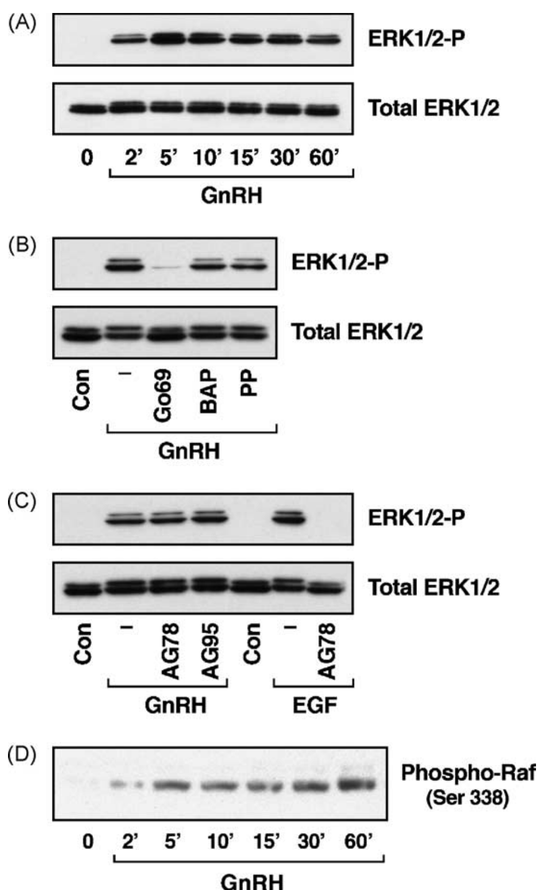

Fig. 2. (A) Time course of GnRH-induced ERK1/2 activation (ERK1/2-P) in stably transfected HEK293 cells expressing the mouse GnRH-R. Cells at 70–80% confluence were incubated in serum-free medium for 24 h and stimulated with 200 nM GnRH for 2 to 60 min. (B) Effects of treatment with the PKC inhibitor, Go6983 (Go69; 1 μM); the intracellular Ca^{2+} chelator, BAPTA (BAP; 10 μM); and the Src tyrosine kinase inhibitor, PP2 (PP; 5 μM). (C) Cells were pretreated with EGF-R and PDGF-R inhibitors, AG1478 (AG78; 500 nM) and AG1295 (AG95; 1 μM) before stimulation with GnRH, or with an EGF-R kinase inhibitor before exposure to EGF. (D) Time-dependence of Raf-1 phosphorylation in GnRH-treated cells. Cells were collected in Laemmli lysis buffer and analyzed by SDS-PAGE followed by Western blot analysis as described in Section 2.

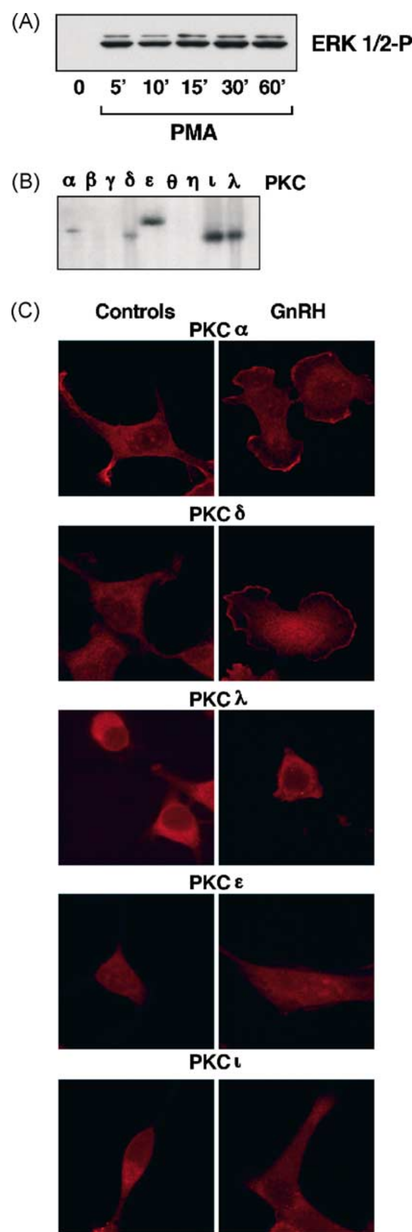

Fig. 3. (A) Time course of the stimulatory action of PMA (100 nM) on ERK1/2 activation. (B) Expression of PKC isoforms in HEK293 cells. Cell extracts were immunoblotted with anti-PKCα, β, γ, δ, ε, θ, η, ι and λ after SDS-PAGE. (C) Immunocytochemical analysis of GnRH-mediated translocation of PKC isoforms in HEK293 cells. After incubation in serum-free medium for 24 h, cells were stimulated with GnRH (200 nM) for 15 min and stained with anti-PKC antibodies as described in Section 2. After incubation with Texas Red-conjugated anti-mouse IgG, cells were washed, mounted, and analyzed by confocal microscopy. Pictures were taken using 40× oil objective at the same settings for control and GnRH stimulated cells. PKCα and δ exhibited translocation to cell membranes after GnRH stimulation.

absence of EGF-R transactivation by GnRH in HEK293 cells (Fig. 2C).

3.5. Immunocytochemical analysis of ERK1/2 and Pyk2 translocation

Immunocytochemical analysis to determine whether phosphorylation of ERK1/2 by GnRH alters the cellular redistribution of these kinases revealed sparse cytosolic staining of phospho-ERK1/2 in control cells. However, GnRH stimulation caused prominent translocation of phosphorylated ERK1/2 into the nucleus in a PKC-dependent manner (Fig. 5A). Pretreatment with the PKC inhibitor, Go6983, abolished GnRH-induced translocation of ERK1/2 into the nucleus. Treatment of HEK293 cells with PMA also caused rapid translocation of ERK1/2 into the nucleus. These results suggest that GnRH-induced translocation of ERK1/2 into the nucleus is a PKC-dependent process.

In an immunocytochemical analysis of the effects of GnRH-induced phosphorylation on the cellular redistribution

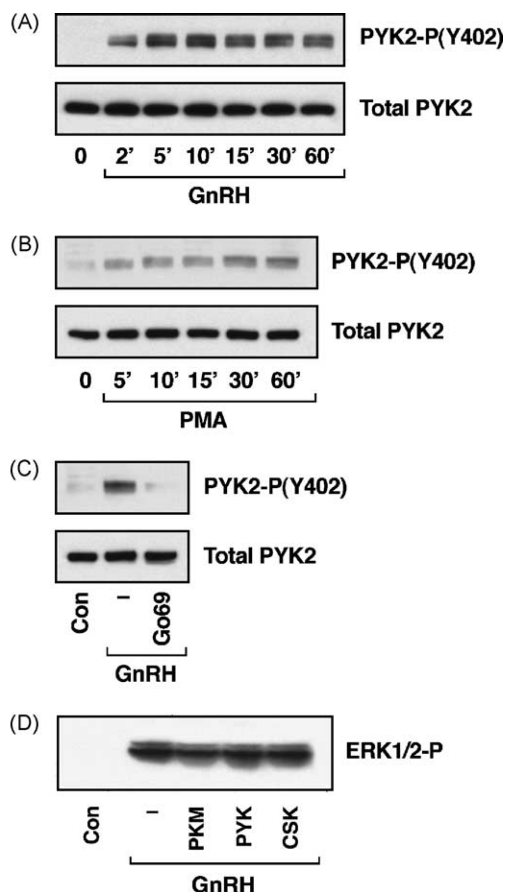

Fig. 4. PKC-dependence of GnRH-induced tyrosine phosphorylation of Pyk2 (Pyk2-P). HEK293 cells were stimulated with (A) GnRH (200 nM), or (B) PMA (100 nM) for the times indicated. (C) HEK293 cells were pretreated with the PKC inhibitor, Go6983 (Go69; 1 μM), for 15 min before stimulation with GnRH (200 nM for 5 min). (D) Lack of effects of dominant negative Pyk2 (PKM; 1 μg) and Src inhibitory kinase (Csk; 1 μg) on GnRH-induced ERK1/2 phosphorylation.

of Pyk2, application of phospho-Pyk2 antibodies revealed punctate staining throughout the cytoplasm and in the perinuclear region of unstimulated HEK293 cells (Fig. 5B). However, GnRH-stimulated cells exhibited prominent staining of the plasma membrane and bright punctate nuclear staining (Fig. 5B). Pretreatment of cells with the PKC inhibitor, Go6983, abolished the redistribution of Pyk2 in response to GnRH stimulation (data not shown). These results further confirm the role of PKC in GnRH-mediated Pyk2 activation. Immunocytochemical analysis of PMA-treated HEK293 cells showed phospho-Pyk2 labeling similar to that observed in GnRH-treated cells. After 10 min treatment with PMA (250 nM), bright punctate staining was evident in the nucleus and the perinuclear region, and also at the plasma membrane (Fig. 5B). These data demonstrate that GnRH-induced PKC activation enhances the translocation of Pyk2 to the cell membrane and also into the nucleus, as well as that of ERK1/2 into the nucleus. However, there is no overlapping functional interaction of these proteins in HEK293 cells.

3.6. *Colocalization of Pyk2 with paxillin at focal adhesions*

Earlier studies have reported localization of Pyk2 to focal adhesions [40,41] and near the sites of cell–cell contact [42]. In control HEK293 cells, Pyk2 was confined to the cytoplasm. However, GnRH-induced receptor activation caused rapid translocation of Pyk2 to the cell periphery and to focal adhesion-like spiny structures (Fig. 5C). The localization of Pyk2 at focal adhesions was confirmed by double labeling of transfected HEK293 cells with paxillin (a focal adhesion marker) and phospho-Pyk2 antibodies. The images shown in Fig. 5C demonstrate that Pyk2 is colocalized with paxillin at focal adhesions following GnRH stimulation. Both proteins were present in spine-like structures near the cell periphery. A similar staining pattern was observed in PMA-treated cells. In contrast, unstimulated HEK293 cells did not show paxillin staining in spine-like structures (Fig. 5C).

3.7. *GnRH-induced c-fos expression is mediated through PKC and the MEK/ERK pathway*

ERK1/2 phosphorylation by GPCRs and growth factors is known to transmit signals to the nucleus for transcriptional activation. In view of the nuclear localization of both ERK1/2 and Pyk2 in stimulated cells, we determined whether GnRH-induced phosphorylation of these proteins was associated with activation of nuclear events as exemplified by the expression of immediate early gene, c-*fos*. As shown in Fig. 6A, GnRH-induced expression of c-*fos* was abolished by inhibition of PKC and MEK, but was not altered by pharmacological inhibition of Src and the EGF-R, or by overexpression of dominant negative Pyk2 (Fig. 6B). These results demonstrate the parallel activation of ERK1/2 and Pyk2 by GnRH in HEK293 cells.

4. Discussion

Many of the genomic effects of GnRH in its neuroendocrine and other target cells are mediated by the activation of MAP kinases, which transmit GnRH-induced signaling from the cell surface to the nucleus for regulation of genes controlling the functions of GnRH neurons and pituitary gonadotropes [1,5]. However, the roles of intermediate signaling molecules such as Pyk2 and the EGF-R in this pathway have not been clearly defined. The use of cells transfected with GPCRs have provided useful insights into the mechanisms of receptor processing and signaling. In this study, the GnRH-mediated signal transduction pathway was analyzed in HEK293 cells expressing a HA-tagged GnRH-R fused to GFP. Our results show that GnRH causes rapid, marked, and sustained phosphorylation of ERK1/2 and Pyk2 in a PKC-dependent manner in HEK293 cells, and regulates the differential localization of these proteins in sub-cellular compartments. The use of pharmacological inhibitors and

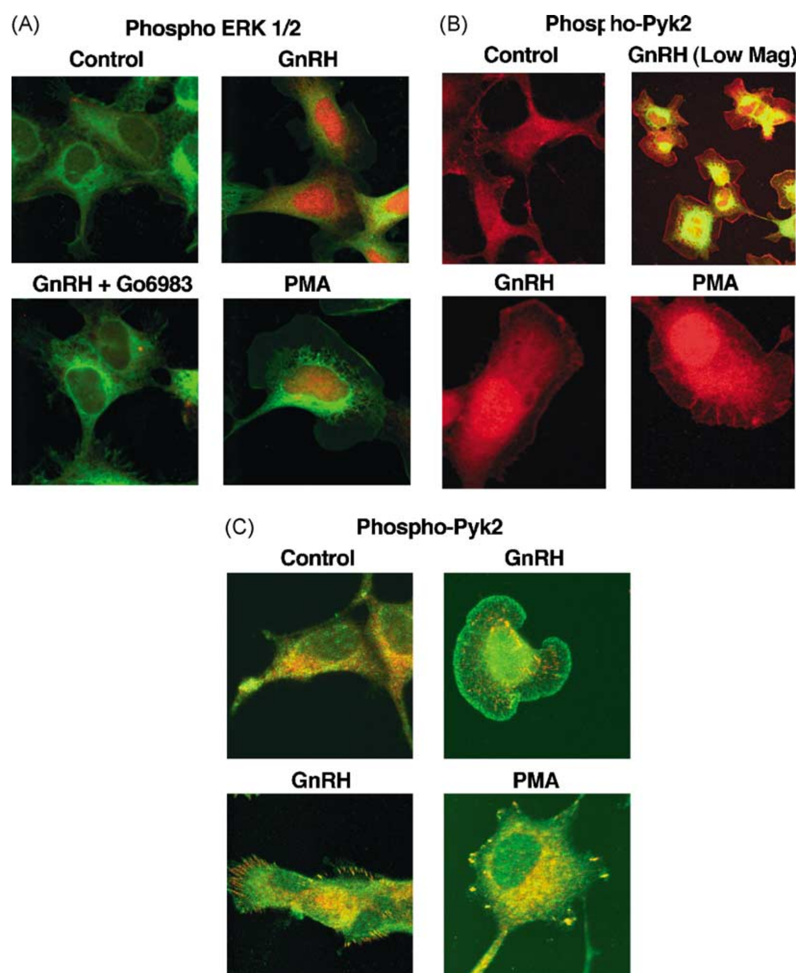

Fig. 5. Immunocytochemical localization of phospho-ERK1/2 and phospho-Pyk2 (Y402). HEK293 cells were stimulated with 200 nM GnRH for 15 min in the presence or absence of Go6983 (1 μM) or PMA (250 nM) for 5 min, fixed in paraformaldehyde, and treated with methanol for 5 min. The permeablized cells were then blocked and incubated with (A) phospho-ERK1/2 antibody or (B) Pyk2 (Y402 or Y579) antibody at 37 °C. After treatment with Texas Red-conjugated anti-IgG, cells were analyzed by confocal microscopy using a 40× oil objective. (C) Immunocytochemical colocalization of phospho-Pyk2 (Y402) and paxillin in HEK293 cells. After incubation in serum-free medium for 24 h, cells were stimulated with GnRH (200 nM) or PMA (250 nM) for 15 min, fixed in paraformaldehyde, permeablized with methanol for 5 min. Cells were then incubated in Texas Red-conjugated anti-mouse (paxillin) antibody for 1 h, followed by Cy5-conjugated anti-rabbit (Pyk2-402). For better visualization the blue color of Cy5 was changed to green color using Adobe Photoshop. Cells were analyzed by confocal microscopy using a 40× oil lens.

negative regulatory forms of specific signaling molecules excluded the involvement of Ca^{2+}, Src, Pyk2, and EGF-R transactivation in the control of this cascade.

Studies on the requirement for growth factor receptor transactivation in GnRH-induced ERK1/2 phosphorylation in various cell types have shown diverse patterns of signaling. While observations in immortalized pituitary gonadotrophs (αT3-1 cells) and COS-7 cells expressing GnRH receptors have suggested that GnRH-induced ERK1/2 activation involved transactivation of the EGF-R [4], other studies found no role of EGF-R transactivation in the phosphorylation of MAP kinase in αT3-1 cells [6] and HeLa cells expressing the GnRH-R [20]. We observed that EGF-Rs are abundantly expressed in HEK293 cells, and when stimulated undergo marked autophosphorylation leading to activation of ERK1/2. However, transactivation of the EGF-R kinase is not responsible for GnRH-induced ERK1/2 activation in HEK293 cells. This was inferred from the lack of an inhibitory effect of the selective EGF-R kinase antagonist, AG1478, on GnRH-mediated ERK1/2 activation. Moreover, GnRH failed to cause phosphorylation of the EGF-R in HEK293 cells (data not shown).

The several tyrosine kinases that have been implicated in cell signaling include Src family kinases, RTKs such as the EGF-R, the focal adhesion kinase (FAK) family, Pyk2, and JAK kinases. GnRH-mediated ERK1/2 activation was not affected by the selective Src inhibitor, PP2 (Fig. 2B) or by Csk (Fig. 4D), excluding a role of Src in GnRH action in HEK293 cells. However, GnRH caused marked phosphorylation of Pyk2, which belongs to the FAK family and is activated by tyrosine phosphorylation in response to several GPCRs [43], as well as by stress stimuli [44] and membrane

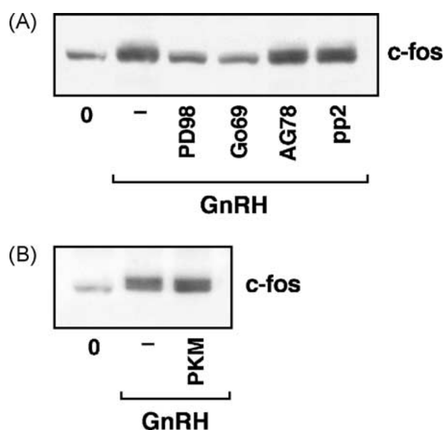

Fig. 6. Dependence of GnRH-induced c-*fos* expression on PKC and MEK. (A) HEK293 cells were pretreated with the MEK inhibitor, PD98059 (PD98; 10 μM), PKC inhibitor, Go 6983 (Go69; 1 μM), EGF-R kinase antagonist, AG1478 (AG78; 200 nM) and Src inhibitor (PP2; 5 μM), for 20 min, followed by stimulation with GnRH (200 nM) for 1 h. Cells were washed twice with ice-cold PBS and lysed in Laemmli lysis buffer. The cell lysates were processed and immunoblotted for c-*fos*. (B) Lack of effect of overexpression of dominant negative Pyk2 (PKM) on GnRH-induced c-*fos* expression during GnRH stimulation (200 nM) for 1 h.

depolarization [45]. Under various experimental conditions, Pyk2 has been shown to participate in the activation of all three major MAP kinases: ERK1/2 [35,36,46,47], P38 MAP kinase [27] and JNK [26,44]. Our data show that dominant negative Pyk2 (PKM) did not inhibit either GnRH-induced phosphorylation of ERK1/2 or expression of c-*fos* in HEK293 cells. However, GnRH-induced phosphorylation of Pyk2 resulted in its translocation from the cytosol to other subcellular compartments including cell membranes, nuclei and focal adhesions.

The GnRH receptor-mediated translocation of Pyk2 to focal adhesions was indicated by its co-localization with paxillin, a protein specifically associated with these sites (Fig. 6). In a recent report, nuclear accumulation of wild-type Pyk2 occurred in cells treated with leptomycin B, an inhibitor of the nuclear export [48]. At this point the functional significance of such nuclear staining is not known. Most of the protein-tyrosine kinases are found in the cytosol and the plasma membrane. However, a few tyrosine kinases such as Rak, WEE1, Abl, Fer and Fes are localized in the nucleus [49], and are believed to have a role in cell cycle progression, DNA repair and transcription [49]. Although the exact function of Pyk2 in the nucleus is unknown, it is possible that the agonist-activated enzyme has a role in certain transcriptional processes. Also, based on its known role in the regulation of cell adhesion [43,50], it is likely that activated Pyk2 influences cell migration, motility, invasion and attachment to the matrix.

GnRH-stimulated phosphorylation of Pyk2 and ERK1/2 in HEK293 cells is primarily dependent on PKC, since selective inhibition of PKC by Go6983 abolished the agonist-mediated activation of these proteins by GnRH (Figs. 2B, 4C and 5A). Moreover, the stimulatory effects of GnRH on phosphorylation of Pyk2 and ERK1/2 were mimicked by the PKC activator, PMA (Figs. 3A and 4B). Earlier studies on the role of PKC isoforms in GnRH action have demonstrated activation of PKCδ and ε [9], PKCβ2, δ, ε and ζ in αT3-1 cells [10], and PKCα and β in rat pituitary cells [8]. Our immunoblot and immunocytochemical analysis revealed that HEK293 cells contain both PMA-sensitive (α, δ and ε) and -insensitive (ι and λ) PKC isoforms (Fig. 3B). GnRH caused selective activation of PKCα and δ, but not that of ϵ and ι/λ, as demonstrated by the presence or absence of translocation of these proteins to the cell membrane (Fig. 3C). In addition, PKCδ underwent redistribution to the nucleus following stimulation with GnRH (Fig. 3C). A similar pattern of PKCδ translocation into the nucleus has been observed in hepatocytes treated with angiotensin II and PMA [51], and in cortical astrocytes stimulated by vasoactive intestinal peptide [52] and CHO cells by PMA [53]. We did not address the specific role of PKCδ in the nucleus of GnRH-activated HEK293 cells, but it is possibly involved in the regulation of gene expression [54].

The type 1 mammalian GnRH-R (type 1) lacks a C-terminal tail and does not exhibit agonist-induced phosphorylation. It is slowly internalized and poorly desensitized, and exhibits prolonged signaling behavior [55]. Consistent with this, GnRH caused sustained phosphorylation of ERK1/2 and Pyk2 that was accompanied by nuclear translocation of these proteins in HEK293 cells (Fig. 5). Translocation of agonist-activated ERK1/2 into the nucleus is important for cell growth and survival, since activated ERKs phosphorylate nuclear transcription factors [7]. GnRH stimulation was found to increase the expression of the immediate early gene, c-*fos*, in a PKC- and MEK-dependent manner (Fig. 6). Interestingly, dominant negative Pyk2 (PKM) had no effect on GnRH-induced activation of either ERK1/2 or c-*fos*, indicating that Pyk2 is not required for these GnRH-induced responses in HEK293 cells.

Studies utilizing cells transfected with GPCRs have provided useful insights into the mechanisms of receptor signaling. Obviously, the characteristics of cells expressing ectopic receptors and signaling proteins may differ from those of cells containing endogenous GPCRs [56]. For example, endogenously expressed GnRH-Rs in immortalized hypothalamic neurons (GT1-7 cells) preferentially interact with G_q, and also with G_s and G_i proteins [57,58]. However, GnRH-Rs expressed in HEK293 cells are solely coupled to $G_{q/11}$ as shown here and by other reports [59,60] and thus provide a useful model to study the signaling characteristics of GnRH-R associated with G_q activation. While GnRH-Rs expressed ectopically in HEK293 cells do not require Src and EGF-R transactivation, and cause sustained ERK1/2 phosphorylation (Fig. 2A–C), the stimulation of endogenous GnRH-Rs in GT1-7 cells activates Src/Pyk2 and EGF-R and thus causes transient ERK1/2 phosphorylation [58] without any detectable nuclear accumulation of activated ERKs [61]. Angiotensin II-mediated ERK phosphorylation in hepatocytes is also dependent on EGF-R transactivation

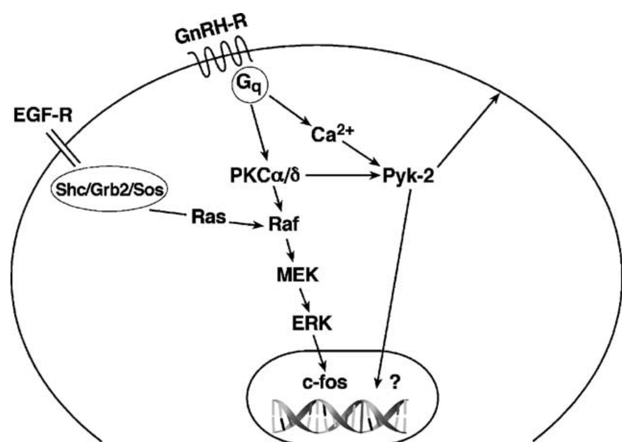

Fig. 7. Diagram of the signaling pathways activated by GnRH in HEK293 cells. GnRH stimulation leads to activation of G_q/PLC and of Ca^{2+}/PKC signaling. During GnRH action, PKCα and δ are translocated to cell membranes and cause phosphorylation and redistribution of activated Pyk2 and ERK1/2. However, GnRH-mediated ERK1/2 phosphorylation is independent of Pyk2, Src, and EGF-R transactivation in HEK293 cells.

and shows transient signaling similar to that induced by EGF [37]. It is evident that the signaling characteristics of GnRH in a particular cell type are largely determined by the nature of signaling proteins involved in the cascade. GnRH action in HEK293 cells is independent of Src and EGF-R activation but involves Raf-1 phosphorylation at Ser338, a major site responsible for activating MEK1/2. Based on these results, GnRH-mediated ERK1/2 phosphorylation in HEK293 cells appears to occur through direct activation of Raf/MEK/ERK1/2 by PKCα and δ. A summary of these signaling pathways is shown in Fig. 7.

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