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Involvement of Protein Kinase D1 in Signal Transduction from the Protein Kinase C Pathway to the Tyrosine Kinase Pathway in Response to Gonadotropin-Releasing Hormone*

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Running title: *Signal transduction of GnRH*

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Background: Gonadotropin-releasing hormone (GnRH) plays critical roles in the progression of sex hormone-dependent cancers.

Results: Activation of protein kinase D1 (PKD1) by protein kinase C was necessary for activation of the tyrosine kinase pathway.

Conclusion: PKD1 is involved in signal transduction in GnRH-induced activation of extracellular signal-regulated protein kinase.

Significance: Modification of PKD1 activity may be a new strategy for a therapy of sex hormone-dependent cancers.

and ErbB4 cleavage. Our experiments indicated that the novel PKC isoforms activated protein kinase D (PKD) after GnRH treatment. Knockdown and inhibitor experiments suggested that PKD1 stimulated the phosphorylation of Pyk2 by constitutively activated Src and Fyn for ERK activation. Taken together, it is highly possible that PKD1 plays a critical role in signal transduction from the PKC pathway to the tyrosine kinase pathway. Activation of the tyrosine kinase pathway may be involved in the progression of cancer.

ABSTRACT

The receptor for gonadotropin-releasing hormone (GnRH) belongs to the G-protein-coupled receptors (GPCRs), and its stimulation activates extracellular signal-regulated protein kinase (ERK). We found that the transactivation of ErbB4 was involved in GnRH-induced ERK activation in immortalized GnRH neurons (GT1-7 cells). We found also that GnRH induced the cleavage of ErbB4. In the present study, we examined signal transduction for the activation of ERK and the cleavage of ErbB4 after GnRH treatment. Both ERK activation and ErbB4 cleavage were completely inhibited by YM-254890, an inhibitor of G_{q/11} proteins. Down-regulation of protein kinase C (PKC) markedly decreased both ERK activation and ErbB4 cleavage. Experiments with two types of PKC inhibitors, Gö 6976 and bisindolylmaleimide I, indicated that novel PKC isoforms but not conventional PKC isoforms were involved in ERK activation

Gonadotropin-releasing hormone (GnRH) is secreted from hypothalamic neurons (GnRH neurons) and stimulates anterior pituitary gonadotrophs to synthesize and secrete the gonadotropins, luteinizing hormone, and follicle-stimulating hormone. Gonadotropins stimulate spermatogenesis, folliculogenesis, and ovulation; therefore, GnRH is the first key hormone of reproduction (for review, see 1). GnRH analogues are used extensively as treatments of male and female infertility, and sex hormone-dependent cancers, such as prostate cancer. Therefore, elucidation of the signal transduction mechanisms after stimulation of the GnRH receptor is extremely important for the development of various medical therapies, as well as an understanding of reproductive processes.

The GnRH receptor belongs to a class of G-protein-coupled receptors (GPCRs) that activate phospholipase C β (1). In addition to gonadotrophs, GnRH neurons also have a

GnRH receptor, and the autocrine action of GnRH is reportedly involved in the regulation of functions of GnRH neurons (for review, see 2). Because the mammalian brain contains only 800–1,000 GnRH neurons, it is necessary to use immortalized GnRH neurons in studies of signal transduction after stimulation of the GnRH receptor. Immortalized GnRH neurons (GT1-7 cells) retain many of the characteristics of native GnRH neurons, including the expression of GnRH receptors (3). GnRH treatment of GT1-7 cells activates mitogen-activated protein kinases (MAPKs), including extracellular signal-regulated kinase (ERK) (1). Signal transduction after ERK activation seems to be different between gonadotrophs and GnRH neurons. In the case of GnRH neurons, transactivation of the ErbB family of proteins after GnRH receptor stimulation is a major pathway for ERK activation (3).

The ErbB family of tyrosine kinases consists of four members, ErbB1/epidermal growth factor receptor (EGFR), ErbB2, ErbB3, and ErbB4 (4, 5). ErbB4 is expressed mainly in the nervous systems and heart (6, 7). Neuregulin 1 (NRG1) is a specific ligand for ErbB4, and it is interesting that *NRG1* and *ErbB4* have been identified as important susceptibility genes for schizophrenia (8-12). In order to elucidate the roles of NRG1 and ErbB4 in disease processes, it is extremely important to understand the molecular mechanisms involved in the regulation of ErbB4 in cell systems.

In the previous study, we found that GT1-7 cells expressed ErbB4 as well as EGFR, and that transactivation of both EGFR and ErbB4 was involved in the GnRH-induced activation of ERK in the cells (13). In addition, we found that GnRH treatment induced the cleavage of ErbB4 (13). Pretreatment of GT1-7 cells with GnRH completely inhibited ERK activation by NRG1 treatment, indicating that GnRH treatment induced the desensitization of ErbB4 via cleavage of the protein.

In the present study, we examined in detail the signal transduction mechanisms for the activation of ERK and the cleavage of ErbB4 after GnRH treatment in GT1-7 cells. The pharmacological and knockdown experiments suggested that protein kinase D (PKD) was activated by isoforms of a novel type of protein kinase C (novel PKC), and that PKD was involved in ERK activation but not ErbB4

cleavage. We found that Src and Fyn were constitutively activated in GT1-7 cells, whereas they activated Pyk2 only after GnRH treatment. Notably, it was interesting that PKD was necessary for the activation of Pyk2 by Src and Fyn. These results strongly suggested that PKD was involved in signal transduction between the PKC pathway and the tyrosine kinase pathway.

EXPERIMENTAL PROCEDURES

Materials – The following chemicals and reagents were obtained from the indicated sources: fetal calf serum from HyClone (Logan, UT, USA); des-Gly¹⁰, (D-Ala⁶)-LH-RH Ethylamide (GnRH), poly-L-lysine, mouse IgG, anti-ERK antibody (M5670), and phosphate-buffered saline from Sigma Chemical Co. (St Louis, MO, USA); DynaMarker Protein MultiColor from BioDynamics Lab. (Tokyo, Japan); Dulbecco's modified Eagle's medium from Nissui Pharmaceutical Co. (Tokyo, Japan); protease inhibitor (PI) cocktail and protein phosphatase inhibitor (PPI) cocktail (EDTA free) from Nacalai Tesque (Kyoto, Japan); anti-ErbB4 antibody (No. 4795), anti-Src antibody (No. 2108), anti-phospho-Src family (Tyr416) antibody (No. 2101), anti-Fyn antibody (No. 4023), anti-PKC isoform antibody sampler kit (No. 9960), anti-PKD1 antibody (No. 2052), anti-phospho-PKD (Ser744/748) antibody (No. 2054), anti-PKD2 antibody (No. 8188), anti-PKD3 antibody (No. 5655), and anti-Pyk2 antibody (No. 3292) from Cell Signaling Tec. (Danvers, MA, USA); anti-Fyn antibody (ab1881) from Abcam (Cambridge, UK); anti-PKD1 antibody (A20) (sc-638) and anti-phospho-Pyk2 (Tyr402) antibody (sc-101790) from Santa Cruz, (Santa Cruz, CA, USA); monoclonal anti-EGFR antibody (6F1) (ADI-CSA-330-E) from Assay Designs (Ann Arbor, MI, USA); anti-PKC ϵ antibody (GTX109028), anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (GTX100118), and anti-G α_q antibody (GTX104544), anti-G α_{11} antibody (GTX118876) from GeneTex Inc. (San Antonio, TX, USA); NF449 from Calbiochem (Darmstadt, Germany); Pertussis toxin (PT) from Seikagaku Biobusiness Corp. (Tokyo, Japan); bisindolylmaleimide I from Enzo Life Science (Farmingdale, NY, USA); dasatinib from BioBision (Milpitas, CA, USA); Gö 6976 and

CRT0066101 from Tocris Bio. (Minneapolis, MN, USA); anti-active ERK antibody (V8031) and phorbol 12-myristate 13-acetate (PMA) from Promega Corp. (Madison, WI, USA); and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) molecular weight standards from Bio-Rad (Richmond, CA, USA). YM-254890 was generously provided by Taiho Pharmaceutical Co., Ltd. (Tokyo, Japan) (14). Other chemicals were of analytical grade.

Cell culture and preparation of cell extracts – GT1-7 cells were kindly provided by Dr R. Weiner (University of California, USA) and Dr M. Kawahara (Musashino University, Japan) (15, 16). The cells were grown on 0.02% (wt/vol) poly-L-lysine-coated Petri dishes (Nunc, Roskilde, Denmark) as described previously (17). We chose the concentrations of signal transduction inhibitors (NF449, PT, YM-254890, bisindolylmaleimide I, Gö 6976, CRT0066101, dasatinib, PP2, and Src Inhibitor 1) as directed by the manufacturers. Cells were lysed in 1 × SDS-PAGE sample buffer containing 2% (wt/vol) SDS, 62.5 mM Tris-HCl, pH 6.8, 5% (vol/vol) 2-mercaptoethanol, 5% (vol/vol) glycerol, and 0.01% (wt/vol) bromophenol blue (18). The cell lysate was sonicated for 10 sec at room temperature, and heated to 98°C for 5 min to use as a cell extract. The cell extract was kept at -80°C until use.

SDS-PAGE and immunoblotting analysis – SDS-PAGE was performed in accordance with the method of Laemmli (18). Immunoblotting analysis was performed as described previously (17, 19). Immunoreactive proteins were detected using an enhanced chemiluminescence detection kit (GE Healthcare UK Ltd, Little Chalfont, UK) as directed by the manufacturer, and were quantified using an ImageQuant LAS4000 mini (GE Healthcare UK Ltd). We loaded MagicMark XP Western Protein Standard (Invitrogen, Carlsbad, CA, USA) onto all SDS-PAGE gels and estimated the apparent molecular weights of the standard proteins and proteins of interest by chemiluminescence. For reprobing, the membrane was incubated with stripping buffer containing 62.5 mM Tris-HCl, pH 6.7, 100 mM 2-mercaptoethanol, and 2% (wt/vol) SDS at 50°C for 30 min (17). For the quantification of ErbB4 cleavage, the ratio of the signal of the F80 fragment to that of native ErbB4 plus the F80 fragment was determined.

siRNA transfection – Small interfering RNAs (siRNAs) of mouse PKCα, PKCδ, PKCε, PKCη, PKCθ, PKD1, Pyk2, Gqα protein, G11α protein, and control siRNA were obtained from Qiagen (Valencia, CA, USA). The siRNAs used were as follows: PKCα-6, 5'-ATGAACTGTTTCAGTCTATAA-3'; PKCα-1, 5'-AAGCATTATCTTAGTGGATGA-3'; PKCδ-3, 5'-TTGAATGTAGTTATTGAAATA-3'; PKCε-3, 5'-TTGGCGGAACTCAAAGGCAAA-3'; PKCη, 5'-CACGATGAAGTTCAATGGCTA-3'; PKCθ, 5'-AAGCTTGATAATATCCTGTTA-3'; PKD1-1, 5'-CAGGAGGGTGATCTCATTGAA-3'; PKD1-2, 5'-TACAGCGAATGTAGTGTATTA-3'; Pyk2, 5'-CTGGATTATCATGGAAGTGA-3'; Gqα-7, 5'-CTGTGGGTTGTTGAAGATAAA-3'; Gqα-1, 5'-CTGGTGGATAGTATTATCCTA-3'; G11α-5, 5'-CCGGGAGGTCGATGTGGAGAA-3'; and G11α-1, 5'-CCGCATCGCCACAGTAGGCTA-3'. Each siRNA was introduced into GT1-7 cells using a Neon Transfection System Kit (Invitrogen) as directed by the manufacturer. Each siRNA was transfected into 0.5–1 × 10⁶ cells per 35-mm dish at a concentration of 40 nM. The cells were kept at 37°C in a CO₂ incubator for 48 h.

Subcellular fractionation – After treatment with GnRH or PMA, GT1-7 cells were collected in homogenization buffer containing 20 mM HEPES, pH 7.4, 1 mM Na₃VO₄, PI cocktail, and PPI cocktail. Cells were homogenized using three strokes of a homogenizer on ice, and NaCl was added to a final concentration of 100 mM. The nuclear fraction was pelleted by centrifugation at 800 g for 5 min, and the supernatant was centrifuged at 100,000 g for 1 h to obtain the cytosol fraction. The pellet was suspended in 1 × SDS-PAGE sample buffer and heated at 98°C for 5 min to use as the membrane fraction.

Immunoprecipitation – Immunoprecipitation (IP) was performed as

described previously (20). Cells were lysed on ice in IP buffer containing 25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 5% glycerol, PI cocktail, and PPI cocktail. The cell lysate was centrifuged at 13,000 g for 10 min at 4°C to obtain the supernatant (cell extract). The supernatant was incubated at 4°C overnight with an anti-Fyn antibody, anti-Src antibody, anti-PKD1 antibody, anti-phospho PKD antibody, anti-PKD3 antibody, or non-immune IgG. The antibody was immobilized on 50% (vol/vol) protein A Sepharose CL-4B (GE Healthcare UK Ltd) with shaking at 4°C for 90 min. The immunoprecipitate was eluted from protein A Sepharose CL-4B by adding SDS-PAGE sample buffer, boiling for 2 min, and centrifugation at 14,000 g for 2 min. The eluate was subjected to SDS-PAGE followed by immunoblotting analysis.

Other procedures – Protein concentrations were determined using the Qubit Protein Assay kit with the Qubit 2.0 Fluorometer (Invitrogen). We repeated the experiments at least three times with reproducible results. We combined all data from independent experiments and expressed values as the mean \pm standard error (SE). Statistical analysis was performed using a one-way analysis of variance plus Duncan's multiple range test. $P < 0.05$ was considered statistically significant.

RESULTS

Involvement of $G_{q/11}$ proteins in GnRH-induced ERK activation and ErbB4 cleavage – We reported previously that antide, a GnRH antagonist, completely inhibited the GnRH-induced activation of ERK and ErbB4 cleavage, indicating that both effects of GnRH were receptor mediated (13, 17). Because the GnRH receptor belongs to the GPCRs, we first examined the G proteins involved in GnRH effects using selective inhibitors of G proteins (Fig. 1). Pretreatment of GT1-7 cells for 30 or 60 min with 40 μ M NF449, an inhibitor of G_s , slightly inhibited ERK activation (Fig. 1A). Pretreatment of the cells for 24 h with 10 or 100 ng/ml PT, an inhibitor of $G_{i/o}$, also slightly inhibited ERK activation (Fig. 1B). These results may suggest that G_s and $G_{i/o}$ are involved in the GnRH-induced activation of ERK, at least in part, as reported previously (1). ErbB4 cleavage was not inhibited at all by either

compound. In contrast, pretreatment of the cells for 30 min with 10 nM YM-254890, an inhibitor of $G_{q/11}$, almost completely inhibited ERK activation and ErbB4 cleavage by GnRH treatment. PMA treatment induced ERK activation and ErbB4 cleavage, and YM-254890 did not inhibit the effects of PMA at all, even at 100 nM (Fig. 1C). These results indicated that G_q or G_{11} was mainly involved in GnRH-induced ERK activation and ErbB4 cleavage.

We next examined the effects of siRNAs for $G_q\alpha$ and $G_{11}\alpha$ on the effects of GnRH (Fig. 2). Transfection of $G_q\alpha$ or $G_{11}\alpha$ siRNA significantly decreased the protein levels of the corresponding α subunit, although small amounts of the subunits were still detected (Fig. 2). In these conditions, transfection of $G_q\alpha$ siRNA did not inhibit ERK activation or ErbB4 cleavage (Fig. 2A). We found that transfection of $G_{11}\alpha$ siRNA inhibited ERK activation by approximately 65%, whereas it inhibited ErbB4 cleavage by only approximately 10% (Fig. 2B). These results may suggest that $G_{11}\alpha$ was more involved in GnRH-induced ERK activation than $G_q\alpha$, and the remaining amount of $G_q\alpha$ or $G_{11}\alpha$ after transfection of the respective siRNA might be sufficient for ErbB4 cleavage. Transfection of a mixture of $G_q\alpha$ and $G_{11}\alpha$ siRNAs did not augment the inhibitory effects of $G_{11}\alpha$ siRNA alone (data not shown).

Involvement of PKC in GnRH-induced ERK activation and ErbB4 cleavage – Activation of PKC by GnRH receptor stimulation has been reported in various cell culture systems (21-23). Therefore, we next examined whether or not PKC was involved in ERK activation and ErbB4 cleavage after GnRH treatment. We pre-treated GT1-7 cells with 200 nM PMA for 20 h to down-regulate PKC and then with 50 nM GnRH or 200 nM PMA for 5 min (Fig. 3). It has been reported that the long-term treatment of cells with PMA down-regulated conventional PKC isoforms, such as PKC α , and novel PKC isoforms, such as PKC δ and PKC ϵ , but not atypical PKC isoforms (24, 25). We confirmed that PKC α , PKC δ , and PKC ϵ were completely down-regulated after pre-treatment with PMA (Fig. 3A). ERK activation by GnRH and PMA was almost completely inhibited by PKC down-regulation (Fig. 3B). The cleavage of ErbB4 by GnRH was markedly inhibited by

PKC down-regulation, and the cleavage by PMA was almost completely inhibited (middle panel in Fig. 3B). The results from three independent experiments are summarized in the bottom panel in Figure 3B. When the level of F80 fragment after GnRH treatment was taken as 100%, the level of the F80 fragment was $60.8 \pm 3.4\%$ after PMA treatment. When we down-regulated PKC, the levels decreased to $58.3 \pm 0.9\%$ and $6.5 \pm 1.1\%$ after GnRH and PMA treatment, respectively. These results strongly suggested that conventional PKC or novel PKC isoforms were necessary for PMA-induced ErbB4 cleavage, whereas the contribution of these isoforms to GnRH-induced ErbB4 cleavage was approximately 40%.

Gö 6976 is a selective inhibitor of conventional PKC isoforms, and it moderately inhibited ERK activation by PMA, whereas it had no inhibitory effect on GnRH-induced ERK activation (Fig. 4A). Bisindolylmaleimide I reportedly inhibits conventional PKC isoforms and novel PKC isoforms with IC_{50} of 20 nM and 0.2 μ M, respectively. Using this difference in IC_{50} , we tried to estimate which type of isoform was involved in the reactions (Fig. 4B). Bisindolylmaleimide I inhibited ERK activation by GnRH slightly and strongly at 0.2 and 10 μ M, respectively. In contrast, it strongly inhibited ERK activation by PMA at 0.2 μ M (Fig. 4B). These results may suggest that novel PKC isoforms were more involved in GnRH-induced activation of ERK than conventional PKC isoforms. Gö 6976 inhibited ErbB4 cleavage by GnRH and PMA slightly and strongly, respectively (middle panel in Fig. 4A). Bisindolylmaleimide I inhibited ErbB4 cleavage by GnRH partially, whereas it inhibited ErbB4 cleavage by PMA strongly (middle panel in Fig. 4B). The results with Gö 6976 and bisindolylmaleimide I from three independent experiments are summarized in the bottom panels in Figures 4A and B, respectively. In Figure 4B, when the level of the F80 fragment after GnRH treatment was taken as 100%, the level of the F80 fragment was $33.9 \pm 0.9\%$ after PMA treatment. GnRH-induced cleavage was decreased by bisindolylmaleimide I to $68.9 \pm 8.5\%$ and $54.3 \pm 1.2\%$ at 0.2 and 10 μ M, respectively. In contrast, PMA-induced cleavage was decreased to $4.4 \pm 1.0\%$ at 0.2 μ M. These results may suggest that conventional PKC

isoforms were mainly involved in PMA-induced ErbB4 cleavage. Bisindolylmaleimide I inhibited GnRH-induced ErbB4 cleavage to $45.7 \pm 1.2\%$ at 10 μ M. This inhibitory level was consistent with that of PKC down-regulation and these results indicated that the contribution of conventional and novel PKC isoforms to GnRH-induced ErbB4 cleavage was approximately 45%.

Using siRNA for each PKC isoform, we tried to identify the isoforms that were involved in ERK activation and ErbB4 cleavage (Fig. 5). Because PKC α , PKC δ , and PKC ϵ were clearly detected by immunoblotting analysis, we first examined the effects of siRNAs on these isoforms. Transfection of siRNAs for each isoform decreased the protein levels of PKC α , PKC δ , and PKC ϵ by approximately 75%, 88%, and 76%, respectively. Activation by GnRH of ERK1 but not ERK2 was inhibited by 40% after transfection of siRNA for PKC α . In contrast, GnRH-induced activation of ERK1 and ERK2 was inhibited by approximately 50% after transfection of siRNA for PKC δ (Fig. 5B). However, it was not inhibited by transfection of siRNA for PKC ϵ . In these conditions, the inhibition of GnRH-induced ErbB4 cleavage was not observed after transfection of siRNA for any isoforms (Fig. 5). Notably, ERK activation and ErbB4 cleavage by PMA were inhibited by approximately 50% and 60%, respectively, after transfection of siRNA for PKC α (Fig. 5A). However, transfection of siRNA for PKC δ or PKC ϵ did not inhibit ERK activation and ErbB4 cleavage by PMA. In addition, transfection of siRNA for PKC η and PKC θ , other novel PKC isoforms, had no inhibitory effects on GnRH-induced ERK activation and ErbB4 cleavage (data not shown).

We next examined the translocation of PKC α , PKC δ , and PKC ϵ after GnRH or PMA treatment (Fig. 6). Immunoblotting analysis of GAPDH and EGFR indicated that the cytosol fraction and the membrane fraction were clearly separated from each other. Treatment of the cells with PMA for 5 min induced the translocation of PKC α , PKC δ , and PKC ϵ . However, no clear translocation of any isoforms was observed after GnRH treatment. These results may suggest that the PKC δ isoform, which was localized to the plasma membrane before GnRH treatment, was involved in

GnRH-induced ERK activation.

Activation of PKD after GnRH treatment – PKD is reportedly activated by a variety of stimuli through the PKC-dependent pathway in many cell types (26-28). PKD translocated from the cytosol to the plasma membrane after the production of diacylglycerol, because an N-terminal cysteine-rich domain of PKD binds to diacylglycerol with high affinity (27). It was also reported that novel PKC isoforms induced the phosphorylation of PKD at Ser744 and Ser748, numbered according to mouse PKD1, in the activation loop to activate PKD (27, 29). Because our data indicated that PKC δ was involved mainly in GnRH-induced ERK activation, we decided to examine whether or not PKD was activated after GnRH treatment.

There are three isoforms in PKD (PKD1, PKD2, and PKD3) (for review, see 30). Therefore, we first examined which isoforms were expressed in GT1-7 cells (Fig. 7A). One antibody to PKD1 (No. 2052) detected a relatively broad band at the expected molecular weight. We noticed that GnRH treatment for 5 min reduced the protein level of PKD1. In addition, two types of siRNAs that targeted mutually different regions of the PKD1 transcript reduced the protein level by 75% and 75%, respectively, indicating that the antibody specifically detected PKD1. We used also another antibody to PKD1 (A20) (Fig. 7A). The antibody reacted with one protein strongly and another protein weakly. However, the apparent molecular weights of these proteins were higher than the previous report (e.g., see 31), and no protein levels were reduced after GnRH or siRNA treatment. From these results, we concluded that this antibody could not detect PKD1 in our experimental conditions. An antibody against PKD2 did not produce any immunoblotting reaction, indicating that GT1-7 cells did not express PKD2. An antibody against PKD3 reacted with one protein of the expected molecular weight (31). Of note, the apparent molecular weight of this protein increased slightly after GnRH treatment. No reduction in PKD3 protein level was observed after treatment with GnRH or siRNAs for PKD1 (Fig. 7A). These results indicated that GT1-7 cells expressed PKD1 and PKD3 but not PKD2. We then examined the phosphorylation of PKD and detected phosphorylation of both Ser744 and Ser748 after 5-min treatment with GnRH (Fig.

7B). This phosphorylation declined by 60 min and reached the basal level after approximately 8 h. The antibody cross-reacted with a protein of slightly lower apparent molecular weight. The level of this cross-reacting protein did not change after any of the treatments tested. We confirmed that GnRH and PMA treatments rapidly reduced the protein level of PKD1 (Fig. 7B). The reduction in PKD1 protein level was not recovered at least until 8 h. When the antibody against PKD3 or GAPDH was used for immunoblotting, no significant changes in immunoreactivity were observed for any treatment.

We next examined whether PKD1 or PKD3 was phosphorylated after GnRH treatment (Fig. 7C). There were no significant differences in the protein levels of PKD1 and PKD3 between the whole cell lysate and cell extract (input). These results suggested that almost all PKD1 and PKD3 were solubilized in the supernatant fraction. In addition, the protein level of PKD1 was reduced in the whole cell lysate after GnRH treatment. We immunoprecipitated phosphorylated PKD and performed immunoblotting analysis with antibodies against PKD1 or PKD3 (Fig. 7C). PKD3, but not PKD1, was observed after GnRH treatment. PKD3 was not detected after immunoprecipitation with non-immune rabbit IgG (Fig. 7C). These results might suggest that phosphorylated PKD after GnRH treatment in Fig. 7B was mainly PKD3. When we immunoprecipitated PKD1 and carried out immunoblotting analysis with anti-phospho PKD antibody, PKD1 was clearly detected after treatment with GnRH. Therefore, we confirmed that GnRH treatment induced the activation of PKD1 as well as PKD3. In Figures 7C and 7D, phosphorylation of PKD1 and PKD3 was not detected without GnRH treatment, further supporting our idea that the protein detected using anti-phospho PKD antibody without GnRH treatment at a lower apparent molecular weight in Figure 7B was not PKD. We then examined the translocation of PKD1 and PKD3 after GnRH treatment (Fig. 7E). The protein level of PKD1 decreased in both the nuclear and cytosol fractions, whereas it increased in the membrane fraction. The PKD3 protein level was not changed in the nuclear fraction, decreased slightly in the cytosol fraction, and increased in the membrane fraction. These results suggested that GnRH

treatment induced the translocation of PKD1 and PKD3 from the cytosol to the membrane, but not to the nucleus. We confirmed that PMA also induced the translocation of PKD1 to the membrane fraction and that PKD1 and/or PKD3 in the membrane fraction was phosphorylated after GnRH and PMA treatment (data not shown).

Involvement of $G_{q/11}$ proteins in the activation of PKD – For the next step, we examined the signal transduction pathway for the activation of PKD. First, we found that 10 nM YM-254890 almost completely inhibited PKD activation after GnRH treatment (Fig. 8A). In addition, a reduction in PKD1 protein level was not observed in the presence of YM-254890. These results indicated that G_q or G_{11} was necessary for the activation of PKD and reduction of PKD1 after GnRH treatment. As expected, YM-254890 did not inhibit the effects of PMA on the activation of PKD and the reduction of PKD1 protein level.

Involvement of PKC δ in the activation of PKD – Next, we examined whether or not PKC was involved in the activation of PKD. Down-regulation of PKC by 20-h pre-treatment with 200 nM PMA inhibited the activation of PKD by GnRH and PMA treatment almost completely (data not shown). Bisindolylmaleimide I at 0.2 μ M did not inhibit PKD activation clearly by either GnRH or PMA treatment (Fig. 8B). PKD activation by these treatments was partially inhibited by 2 μ M bisindolylmaleimide I, and PKD activation by GnRH was inhibited almost completely by 10 μ M bisindolylmaleimide I. These results strongly suggested that novel PKC isoforms were responsible for the activation of PKD. Notably, bisindolylmaleimide I did not inhibit the reduction in PKD1 protein level by either GnRH or PMA treatment. These results suggested that the activation of PKD1 was not prerequisite for its reduction.

In order to examine the involvement of PKC δ in PKD activation, we carried out knockdown of PKC δ and treated the cells with GnRH or PMA (Fig. 8C). The activation of PKD by GnRH and PMA was inhibited by approximately 59% and 58%, respectively, after the knockdown of PKC δ . In contrast, the knockdown of PKC ϵ had no effects on PKD activation. Next, we examined the effects of

knockdown of PKC δ on the translocation of PKD (Fig. 8D). The activation of PKD by GnRH was detected in the membrane fraction, and it was inhibited by approximately 56% after PKC δ knockdown. In contrast, the translocation of PKD1 was not inhibited even after PKC δ knockdown. These results indicated that PKC δ was necessary for the activation of PKD but not for its translocation to the membrane. The knockdown of PKC ϵ had no effects on the activation of PKD and its translocation.

Involvement of PKD in GnRH-induced ERK activation – As the next step, we examined whether or not PKD was involved in ERK activation and ErbB4 cleavage (Fig. 9). We first examined whether or not PKD1 was involved in ERK activation (Fig. 9A). It was interesting that the knockdown of PKD1 inhibited GnRH-induced activation of ERK by approximately 60%. In contrast, PMA-induced activation of ERK was inhibited by approximately 38% after the knockdown of PKD1. ErbB4 cleavage by GnRH and PMA was not inhibited at all by the knockdown of PKD1. These results indicated that the activation of PKD1 was involved in ERK activation but not ErbB4 cleavage after GnRH and PMA treatment. PKD1 was effectively knocked down by a given siRNA and the protein level of PKD1 was reduced after GnRH and PMA treatment. In addition, CRT0066101, a PKD inhibitor, inhibited GnRH-induced activation of ERK by 65% (Fig. 9B).

Activation of Pyk2 by GnRH and PMA treatment – It has been reported that the non-receptor tyrosine protein kinases, i.e. the Src family and Pyk2, were necessary for GPCR-mediated EGFR transactivation (22, 32, 33). It was also reported that GnRH treatment of GT1-7 cells activated Pyk2 through phosphorylation of Tyr402 (22). The previous study also reported that the activation was dependent on PKC (22). In our assay conditions, GnRH and PMA treatment increased the phosphorylation of Pyk2 at Tyr402, and PKC down-regulation inhibited phosphorylation (Fig. 10A). In addition, we found that YM-254890 strongly inhibited Pyk2 activation by GnRH, but not by PMA, suggesting that G_q or G_{11} was involved in GnRH effects (Fig. 10B).

We next examined whether or not PKD was involved in GnRH-induced Pyk2 activation (Fig.

10C). Transfection of siRNA for PKD1 effectively knocked down PKD1 by 75% (bottom panel in Fig. 10C). Knockdown of PKD1 inhibited GnRH-induced Pyk2 activation by approximately 60%, whereas it inhibited PMA-induced Pyk2 activation by approximately 30%. These results indicated that PKD1 was more involved in GnRH-induced activation of Pyk2 than PMA-induced activation of Pyk2. It was unexpected that Src was phosphorylated at Tyr416 without GnRH or PMA treatment, suggesting that the Src family was activated in the basal conditions (Fig. 10C). We confirmed that activation of the Src family was not affected by the knockdown of PKD1. PKD1 was effectively knocked down by a given siRNA and PKD1 protein level was reduced after GnRH and PMA treatment. Furthermore, we confirmed that CRT0066101 inhibited GnRH-induced activation of Pyk2 by 79%, but not activation of the Src family with/without GnRH treatment (Fig. 10D). We immunoprecipitated Src and Fyn and examined the phosphorylation of Tyr416 by immunoblotting analysis. We found that both Src and Fyn were phosphorylated in the basal conditions, and that phosphorylation was not increased after GnRH treatment (data not shown).

Involvement of PYK2 in ERK activation – It has been reported that transfection of a dominant-negative mutant of Pyk2 inhibited the activation of ERK by GnRH and PMA treatment of GT1-7 cells (22). In order to confirm the involvement of Pyk2 in the activation of ERK, a knockdown experiment of Pyk2 was carried out (Fig. 11A). We confirmed that the transfection of siRNA for Pyk2 decreased Pyk2 protein levels. Knockdown of Pyk2 inhibited ERK activation by GnRH and PMA by approximately 40% and 40%, respectively (Fig. 11A). In contrast, ErbB4 cleavage by GnRH and PMA was not inhibited by knockdown of Pyk2. These results suggested that Pyk2 was involved in ERK activation but not ErbB4 cleavage by GnRH and PMA.

Involvement of the Src family in ERK activation – It has been reported that the Src family activated Pyk2 after GnRH treatment of GT1-7 cells (22). We confirmed that GnRH-induced ERK activation was inhibited by approximately 94% in the presence of 250 nM dasatinib, an inhibitor of the Src family (Fig.

11B). PMA-induced ERK activation was inhibited by approximately 75% in the presence of 250 nM dasatinib (Fig. 11B). We confirmed that the activation of Src and Fyn was completely inhibited in the presence of 250 nM dasatinib; and the protein levels of Src and Fyn were not changed after any of these treatments (Fig. 11B). The activation of Pyk2 was also completely inhibited by 250 nM dasatinib, confirming that the Src family was necessary for the activation of Pyk2. In contrast, the activation of PKD and down-regulation of PKD1 after GnRH and PMA treatment were not inhibited at all in the presence of dasatinib, indicating that the Src family did not activate PKD (Fig. 11B). When the antibody against PKD3 was used for immunoblotting, no significant changes in immunoreactivity were observed for any treatment. In addition to dasatinib, 10 μ M PP2 and 2 μ M Src inhibitor I, other inhibitors of the Src family, inhibited GnRH-induced ERK activation by approximately 72% and 68%, respectively (data not shown). However, these inhibitors did not inhibit ErbB4 cleavage by GnRH and PMA at all (Fig. 11B for dasatinib).

Interaction of Src family with Pyk2 – It has been reported that GnRH treatment of GT1-7 cells induced the interaction of Src and Pyk2 (22). We immunoprecipitated Src or Fyn after GnRH treatment of GT1-7 cells to examine their interaction with Pyk2 (Fig. 11C). In our assay conditions, Pyk2 was not detected after immunoprecipitation of Src, whereas it was detected after immunoprecipitation of Fyn, only after GnRH treatment. These results may suggest that Fyn interacts with Pyk2 after GnRH treatment more strongly than Src.

DISCUSSION

It has been reported that PKC is involved in ERK activation after GnRH treatment of GT1-7 cells (22). Our experiments with two types of PKC inhibitors and siRNAs for PKC isoforms indicated that novel PKC isoforms were involved in ERK activation and ErbB4 cleavage after treatment of GT1-7 cells with GnRH. Furthermore, our experiments revealed that the signal transduction pathways are different between ERK activation and ErbB4 cleavage after the activation of novel PKC isoforms. Figure 12 shows the pathways that appear to play roles in signal transduction after GnRH treatment of GT1-7 cells.

PKD is reportedly activated by novel PKC isoforms after its translocation to the membrane (27). We confirmed that PKD in the membrane fraction was activated by GnRH treatment of GT1-7 cells. Knockdown experiments of novel PKC isoforms suggested that PKC δ was mainly involved in PKD activation. As reported previously (22), inhibitors of the Src family inhibited the activation of Pyk2 and ERK. It was interesting that the Src family was constitutively activated, whereas it activated Pyk2 only after GnRH treatment. In addition, a knockdown experiment suggested that PKD1 was involved in the activation of Pyk2 by the Src family. These results suggested that PKD1 relayed the signal from novel PKC isoforms to Pyk2 for ERK activation. In addition, CRT0066101 inhibited the activation of PYK2 and ERK. Recently, it was reported that PKD1 and PKD3 had different roles in cell growth (34). The roles of PKD3 in GnRH signaling should be examined carefully in a future study. To the best of our knowledge, this is the first report that PKD1 relays a signal from the PKC pathway to the tyrosine kinase pathway. It is worth examining how PKD1 induces the activation of Pyk2 by the Src family in a future study.

In previous reports, inhibition of the Src family by PP2 or overexpression of C-terminal Src kinase attenuated GnRH-induced ERK activation (22). We confirmed that dasatinib strongly inhibited the activation of ERK by GnRH. We also noticed that the effects of siRNA for Pyk2 on ERK activation were weaker than that of dasatinib. These results may suggest that the Src family activates ERK through Pyk2-independent pathways as well as a Pyk2-dependent pathway (Fig. 12).

We found that the Src family was constitutively activated in GT1-7 cells. It has been reported that Src and Fyn phosphorylated and activated PKC δ (Li et al., 1994; Denning et al., 1996). It has also been reported that Src and Fyn activated PKD (for review, see Rozengurt, 2011). These results suggested that the Src family was upstream of PKC δ and PKD in their cell systems. However, dasatinib did not inhibit the activation of PKD in GT1-7 cells, indicating that PKC δ and PKD were upstream of the tyrosine kinase pathway in the case of GnRH signaling (Figs. 11B and 12). It has been reported that GnRH treatment induced the

shedding of a precursor of heparin-binding EGF (HB-EGF), and HB-EGF stimulated EGFR and ErbB4 to activate ERK (3, 34). It will be worthwhile examining whether the Src family and Pyk2 are involved in the shedding of HB-EGF (Fig. 12).

We found that GT1-7 cells expressed PKD1 and PKD3. Our immunoprecipitation experiment clearly showed that PKD1 and PKD3 were activated by GnRH treatment. Because the PKD1 protein level was reduced rapidly, PKD isoforms that were detected using an anti-phospho-PKD antibody in Figure 7B might be mainly PKD3.

We considered that the reduction in PKD1 was due to down-regulation by proteolysis. It was unlikely that the anti-PKD1 antibody could not detect activated PKD1, because bisindolylmaleimide 1 completely inhibited the GnRH-induced activation of PKD1, but it failed to prevent the reduction in PKD1 protein level. In addition, it was also unlikely that PKD moved to other subcellular compartments from the cytosol, because we loaded proteins from all subcellular fractions onto SDS-PAGE gels.

It is well-known that the GnRH receptor in mammals is a type I receptor, and that it lacks the C-terminal cytoplasmic domain (for review, see 1). Because this C-terminal domain contains the arrestin-binding site, arrestin-dependent internalization may not occur, which incurs a risk of sustained activation of the receptor in the case of the GnRH receptor (for review, see 36). Instead of GnRH receptor down-regulation, PKD1 down-regulation may be a self-inhibitory mechanisms for ERK activation in the case of overly strong activation of the GnRH receptor.

It has been reported that calpain cleaved PKD after the treatment of mammary carcinoma cells with arachidonic acid (37). This previous study also reported that a 77-kDa fragment was concomitantly detected with PKD cleavage. In our study, we could not detect any fragments of PKD1, and the level of the decrease in PKD1 protein level seemed to be much more robust than that in reports on mammary carcinoma cells.

GnRH analogues stimulate the functions of GnRH neurons and anterior pituitary gonadotrophs through activation of the GnRH receptor (for review, see 1). GnRH analogues are used for the treatment of infertility to stimulate GnRH signaling in these cells. In

addition, GnRH analogues have been used for the treatment of sex hormone-dependent cancers for the purpose of down regulation of the GnRH signaling after overly strong activation of the GnRH receptor. Cleavage of ErbB4 and reduction of PKD1 may be the main molecular mechanisms for this down regulation of the GnRH signaling. In addition, a combination of GnRH analogues with activators and inhibitors of PKD may be effective for treatment of infertility and sex-hormone-dependent cancers, respectively.

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Conflict of Interests

The authors declare that they have no conflicts of interest with the contents of this article.

Author Contributions

S. H. and H. Y. designed the study and wrote the paper. S. H., N. M., S. T., and H. Y. performed the experiments.

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FOOTNOTES

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²The abbreviations used are: EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GnRH, gonadotropin-releasing hormone; GPCR, G-protein-coupled receptor; HB-EGF, heparin-binding epidermal growth factor; IP, immunoprecipitation; MAPK, mitogen-activated protein kinase; NRG1, neuregulin 1; PI-3 kinase, phosphatidylinositol-3 kinase; PI, protease inhibitor; PKC, protein kinase C; PKD, protein kinase D; PMA, phorbol 12-myristate 13-acetate; PPI, protein phosphatase inhibitor; PT, pertussis toxin; siRNA, small interfering RNA; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SE, standard error.

FIGURE LEGENDS

FIGURE 1. Effects of selective G protein inhibitors on gonadotropin-releasing hormone (GnRH)-induced extracellular signal-regulated kinase (ERK) activation and ErbB4 cleavage. A, GT1-7 cells were pretreated with or without 40 μ M NF449 for 30 or 60 min, and further treated with or without 50 nM GnRH for 5 min. Cell extracts (33 μ g) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 10% acrylamide, and immunoblotting analysis was performed using an anti-active ERK antibody (1:850). Cell extracts (33 μ g) were also subjected to SDS-PAGE in 7.5% acrylamide and immunoblotting analysis was performed with an anti-ErbB4 antibody (1:850). After the anti-active ERK antibody was stripped away, immunoblotting with an anti-ERK antibody was performed at a dilution of 1:1500. B, Cells were pretreated with or without 10 or 100 ng/mL pertussis toxin (PT) for 24 h, and further treated with or without 50 nM GnRH for 5 min. Cell extracts (29 μ g) were subjected to SDS-PAGE, and immunoblotting analysis of activated ERK, ERK, and ErbB4 was performed as described above. C, Cells were pretreated with or without 10 or 100 nM YM-254890 for 30 min, and further treated with or without 50 nM GnRH for 5 min or 200 nM phorbol 12-myristate 13-acetate (PMA) for 10 min. Cell extracts (19 μ g) were subjected to SDS-PAGE, and immunoblotting analysis of activated ERK, ERK, and ErbB4 was performed as described above. The positions of ERK1 (P-ERK1, ERK1), ERK2 (P-ERK2, ERK2), native ErbB4, and ErbB4-F80 are indicated.

FIGURE 2. Effects of G_{q/11} α small interfering RNAs (siRNAs) on GnRH-induced ERK activation and ErbB4 cleavage. A, GT1-7 cells were transfected with 20 nM control siRNA or G_q α siRNA.

After a 48-h incubation, the cells were treated with or without 50 nM GnRH for 5 min. Cell extracts (40 μ g) were subjected to SDS-PAGE in 10% acrylamide, and immunoblotting analysis was performed with anti-active ERK (1:850) and anti-G α (1:600) antibodies. Cell extracts (40 μ g) were also subjected to SDS-PAGE in 7.5% acrylamide and immunoblotting analysis was performed using an anti-ErbB4 antibody (1:850). After the anti-active ERK antibody was stripped away, immunoblotting with an anti-ERK antibody was performed at a dilution of 1:1500. B, Control siRNA or G α siRNA were transfected into GT1-7 cells at 20 nM, and treated with or without GnRH, as described above. Cell extracts (29 μ g) were subjected to SDS-PAGE, and immunoblotting analysis of activated ERK, ERK, G α , and ErbB4 was performed as described above. The positions of ERK1 (P-ERK1, ERK1), ERK2 (P-ERK2, ERK2), native ErbB4, ErbB4-F80, G α , and G α are indicated.

FIGURE 3. Dependence on the protein kinase C (PKC) pathway for GnRH-induced ERK activation and ErbB4 cleavage. A, GT1-7 cells were treated with or without 100 nM PMA for 20 hours. Cell extracts (28 μ g) were subjected to SDS-PAGE in 10% acrylamide, and immunoblotting analysis was performed using anti-PKC α (1:800), anti-PKC δ (1:750), or anti-PKC ϵ (1:1000) antibodies. B, After PMA pretreatment, cells were treated with or without 50 nM GnRH for 5 min or 200 nM PMA for 10 min. Cell extracts (28 μ g) were subjected to SDS-PAGE and immunoblotting analysis of active ERK, ERK, and ErbB4 as described in the legend for Fig. 1. The positions of each isoform of PKC (PKC), ERK1 (P-ERK1, ERK1), ERK2 (P-ERK2, ERK2), native ErbB4, and ErbB4-F80 are indicated. The amount of ErbB4-F80 was calculated by comparing that of ErbB4-F80 to total ErbB4 in the same sample. The mean \pm standard error (SE) was determined from three independent experiments. (* P <0.05 vs., GnRH or PMA stimulation without PMA pretreatment).

FIGURE 4. Effects of PKC inhibitors on the GnRH-induced ERK activation and ErbB4 cleavage. A, GT1-7 cells were pretreated with or without 0.5 μ M Gö 6976 for 30 min, and further treated with or without 50 nM GnRH for 5 min or 200 nM PMA for 10 min. B, Cells were pretreated with bisindolylmaleimide I (Bis) for 30 min and treated with or without 50 nM GnRH for 5 min or 200 nM PMA for 10 min. Cell extracts (36 μ g in A and 34 μ g in B) were subjected to SDS-PAGE, and immunoblotting analysis of active ERK, ERK, and ErbB4 was performed as described in the legend for Fig. 1. The positions of ERK1 (P-ERK1, ERK1), ERK2 (P-ERK2, ERK2), native ErbB4, and ErbB4-F80 are indicated. ErbB4 cleavage by GnRH was taken as 100%, and from this value, the other values were calculated. The mean \pm SE was determined from three independent experiments. A, (* P <0.05 vs., GnRH stimulation without Gö 6976, # P <0.05 vs., PMA stimulation without Gö 6976). B, (* P <0.05 vs., GnRH stimulation without Bis, # P <0.05 vs., PMA stimulation without Bis).

FIGURE 5. Requirement of PKC α and PKC δ for GnRH-induced ERK activation. GT1-7 cells were transfected with 20 nM of control siRNA, PKC α siRNA (A), PKC δ siRNA (B), or PKC ϵ siRNA (C). After a 48-h incubation, the cells were treated with or without 50 nM GnRH for 5 min or 200 nM PMA for 10 min. Cell extracts (40 μ g in A, 56 μ g in B, and 55 μ g in C) were subjected to SDS-PAGE, and immunoblotting analysis of active ERK, ERK, and ErbB4 was performed as described in the legend for Fig. 1. Cell extracts (40 μ g in A, 56 μ g in B, and 55 μ g in C) were also subjected to immunoblotting analysis of PKC α , PKC δ , and PKC ϵ as described in the legend for Fig. 3. The positions of ERK1 (P-ERK1, ERK1), ERK2 (P-ERK2, ERK2), native ErbB4, ErbB4-F80, and each isoform of PKC (PKC α , PKC δ , and PKC ϵ) are indicated.

FIGURE 6. Translocation of PKC isoforms from the cytosol to the plasma membrane by GnRH and PMA treatment. GT1-7 cells were treated with or without 50 nM GnRH for 5 min or 200 nM PMA for 10 min. The cytosol (Cyto) and membrane (Mem) fractions were obtained as described in the Experimental Procedures. The cytosol and membrane fractions were subjected to immunoblotting analysis using anti-PKC α , anti-PKC δ , and anti-PKC ϵ antibodies, as described in the legend for Fig. 3. The samples were also subjected to SDS-PAGE in 7.5% acrylamide, and immunoblotting analysis was

performed using an anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (1:1000). For immunoblotting of epidermal growth factor receptor (EGFR), samples were subjected to 7.5% acrylamide SDS-PAGE and an anti-EGFR antibody was used at a dilution of 1:800. The positions of each isoform of PKC (PKC α , PKC δ , and PKC ϵ), GAPDH, and EGFR are indicated.

FIGURE 7. Activation and degradation of PKD by GnRH and PMA treatment. A, Two types of siRNAs (PKD1 siRNAs 1 and 2) for PKD1 and control siRNA were transfected into GT1-7 cells at 20 nM. After a 48-h incubation, the cells were treated with 50 nM GnRH for 5 min. Cell extracts (23 μ g) were subjected to SDS-PAGE in 7.5% acrylamide, and immunoblotting analysis was performed using anti-PKD1, anti-PKD2, and anti-PKD3 antibodies at a dilution of 1:850. Immunoblotting of PKD1 (with the A20 antibody) was performed at a dilution of 1:600. The asterisks indicate non-specific cross-reacting proteins. B, Time course analysis of the effects of GnRH and PMA on PKD phosphorylation. GT1-7 cells were treated with or without 50 nM GnRH or 200 nM PMA for the indicated time periods. Cell extracts (47 μ g) were subjected to SDS-PAGE in 7.5% acrylamide, and immunoblotting analysis was performed using an anti-phospho-PKD (Ser744/748) antibody at a dilution of 1:850. After the anti-phospho-PKD (Ser744/748) antibody was stripped away, immunoblotting with an anti-PKD1 antibody was performed at a dilution of 1:850. Cell extracts were also subjected to SDS-PAGE in 7.5% acrylamide, and immunoblotting analysis of PKD3 was performed at a dilution of 1:850. Immunoblotting of GAPDH was performed as described in the legend for Fig. 6. An arrowhead indicates a protein cross-reacting with the antibody. C, Immunoprecipitation (IP) with an anti-phospho-PKD (Ser744/748) antibody was performed as described in the Experimental Procedures. Total cell lysates (Total) and cell extracts (Input) and IP samples were subjected to immunoblotting with anti-PKD1 and anti-PKD3 antibodies as indicated. D, IP with the anti-PKD1 antibody was performed as described in the Experimental Procedures. Immunoblotting with the anti-phospho-PKD (Ser744/748) antibody was performed as described above. E, GT1-7 cells were treated with or without GnRH, and the nuclear (Nucl), cytosol (Cyto), and membrane (Mem) fractions were obtained as described in the legend for Fig. 6. The fractions were subjected to immunoblotting analysis of PKD1 and PKD3 as described above. The positions of PKD (P-Ser744/748 PKD, PKD1, PKD2, and PKD3) and GAPDH are indicated.

FIGURE 8. Involvement of G $_{q/11}$ proteins and PKC in the activation of PKD. A, GT1-7 cells were pretreated with or without 10 and 100 nM YM-254890 for 30 min, and further treated with or without 50 nM GnRH for 5 min or 200 nM PMA for 10 min. Cell extracts (19 μ g) were subjected to immunoblotting analysis of activated PKD and PKD, as described in the legend for Fig. 7. B, Cells were pretreated with or without bisindolylmaleimide I (Bis) and treated with or without GnRH or PMA, as described in the legend for Fig. 4. Cell extracts (34 μ g) were subjected to SDS-PAGE, and immunoblotting analysis of active PKD and PKD was performed as described in the legend for Fig. 7. C, Cells were transfected with or without PKC α or PKC ϵ siRNA and treated with or without GnRH or PMA, as described in the legend for Fig. 5. Cell extracts (35 μ g) were subjected to SDS-PAGE, and immunoblotting analysis of activated PKD and PKD1 was performed as described above. D, Cells were transfected with or without PKC δ or PKC ϵ siRNA and treated with or without GnRH, as described above. The membrane fraction was obtained as described in the legend for Fig. 6, and samples (34 μ g) were used for immunoblotting analysis of activated PKD and PKD1, as described above. The positions of P-Ser744/748 PKD and PKD1 are indicated.

FIGURE 9. Effects of PKD knockdown on the GnRH- and PMA-induced ERK activation. A, GT1-7 cells were transfected with 20 nM of control siRNA or PKD1 siRNA. After a 48-h incubation, the cells were treated with or without 50 nM GnRH for 5 min or 200 nM PMA for 10 min. Cell extracts (30 μ g) were subjected to immunoblotting with anti-active ERK, anti-ERK, anti-ErbB4, and anti-PKD1 antibodies, as described in the legend for Fig. 1 and Fig. 7. B, Cells were pretreated with 10 μ M CRT0066101 for 6 h, and further treated with 50 nM GnRH for 5 min. Immunoblotting analysis of activated ERK and ERK was done as described in the legend for Fig.1. The positions of

ERK1 (P-ERK1, ERK1), ERK2 (P-ERK2, ERK2), native ErbB4, ErbB4-F80, and PKD1 are indicated.

FIGURE 10. Involvement of PKC, $G_{q/11}$ proteins, and PKD1 in the GnRH-induced activation of Pyk2. A, GT1-7 cells were pretreated with or without 100 nM PMA for 20 h, and the cells were treated with or without 50 nM GnRH for 5 min or 200 nM PMA for 10 min. Cell extracts (28 μ g) were subjected to SDS-PAGE in 7.5% acrylamide, and immunoblotting analysis was performed using an anti-phospho-Pyk2 antibody (1:600). After the anti-phospho-Pyk2 antibody was stripped away, immunoblotting with an anti-Pyk2 antibody was performed at a dilution of 1:1000. B, Cells were pretreated with or without 10 or 100 nM YM-254890 for 30 min, and further treated with or without 50 nM GnRH for 5 min or 200 nM PMA for 10 min. Cell extracts (19 μ g) were subjected to SDS-PAGE and immunoblotting analysis of activated Pyk2 and Pyk2 was performed as described above. C, Cells were transfected with or without PKD1 siRNA and treated with or without GnRH or PMA as described in the legend for Fig. 9. Cell extracts (50 μ g) were subjected to SDS-PAGE in 7.5% acrylamide, and immunoblotting analysis with an anti-phospho-Pyk2 antibody and an anti-Pyk2 antibody was performed as described above. Cell extracts (50 μ g) were also subjected to SDS-PAGE in 9% acrylamide, and immunoblotting analysis was performed using an anti-phospho-Src family antibody (1:850). After the anti-phospho-Src family antibody was stripped away, immunoblotting with an anti-Src antibody was performed at a dilution of 1:850. Immunoblotting analysis of PKD1 was performed, as described in the legend for Fig. 7. B, Cells were pretreated with 10 μ M CRT0066101 for 24 h, and further treated with 50 nM GnRH for 5 min. Immunoblotting analysis of phospho Pyk2, Pyk2, phospho-Src family, and Src was done as described above. The positions of Pyk2 (P-Tyr402 Pyk2, Pyk2), Src (P-Tyr416 Src, Src), and PKD1 are indicated.

FIGURE 11. Involvement of Pyk2 and Src family in gGnRH-induced ERK activation. A, GT1-7 cells were transfected with 20 nM of control or Pyk2 siRNA and treated with or without 50 nM GnRH for 5 min or 200 nM PMA for 10 min, as described in the legend for Fig. 5. Cell extracts (50 μ g) were subjected to SDS-PAGE, and immunoblotting analysis of active ERK, ERK, and ErbB4 was performed as described in the legend for Fig. 1. Cell extracts (50 μ g) were also subjected to immunoblotting analysis of Pyk2, as described in the legend for Fig. 10. B, GT1-7 cells were pretreated with or without dasatinib at 10 or 250 nM for 30 min and further treated with or without 50 nM GnRH for 5 min or 200 nM PMA for 10 min as described in the legend for Fig. 1. Cell extracts (35 μ g) were subjected to SDS-PAGE and immunoblotting analysis of active ERK, ERK, and ErbB4 was performed, as described in the legend for Fig. 1. Cell extracts (35 μ g) were also subjected to immunoblotting analysis of activated Pyk2 and Pyk2, as described in the legend for Fig. 10. Immunoblotting analysis of activated PKD, PKD1, and PKD3 was performed, as described in the legend for Fig. 7. An immunoblotting analysis of Fyn was performed using an anti-Fyn antibody at a dilution of 1:850. C, Immunoprecipitation (IP) with anti-Src or anti-Fyn antibodies was performed as described in the Experimental Procedures. Cell extracts and immunoprecipitated samples were subjected to immunoblotting with anti-Pyk2, anti-Src, and anti-Fyn antibodies as indicated. The positions of ERK1 (P-ERK1, ERK1), ERK2 (P-ERK2, ERK2), phosphorylated Src or Fyn (P-Tyr416 Src/Fyn), Src, Fyn, Pyk2 (Pyk2, P-Tyr402 Pyk2), PKD (P-Ser744/748 PKD, PKD1, and PKD3), native ErbB4, and ErbB4-F80 are indicated.

FIGURE 12. Schematic representation of the signaling pathways involved in GnRH-induced ERK activation (red arrows) and ErbB4 cleavage (blue arrows). Stimulation of the GnRH receptor in GT1-7 cells leads to the activation of ERK through transactivation of ErbB4 and EGFR (3, 13). When the GnRH receptor is stimulated by high concentrations of GnRH for a long time, ErbB4 is cleaved by TACE (13). The experiments with YM-254890 indicated that both ERK activation and ErbB4 cleavage are completely dependent on $G_{q/11}$ proteins. The contribution of each molecule to ERK activation (red letters) or ErbB4 cleavage (blue letters) is indicated as the approximate percentage suggested by the experiments with inhibitors or small interfering RNAs (siRNAs).

Fig. 1

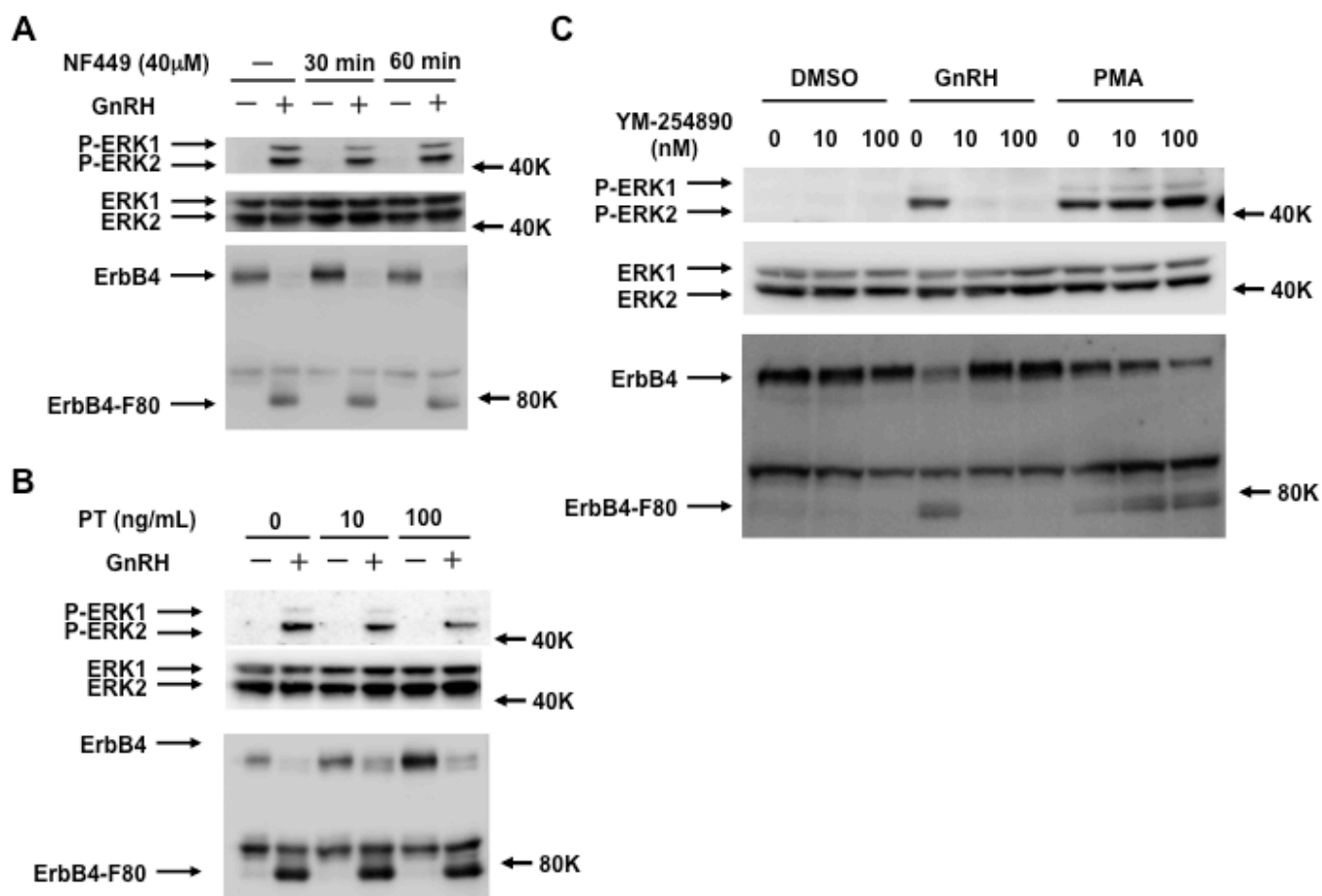


Fig. 2

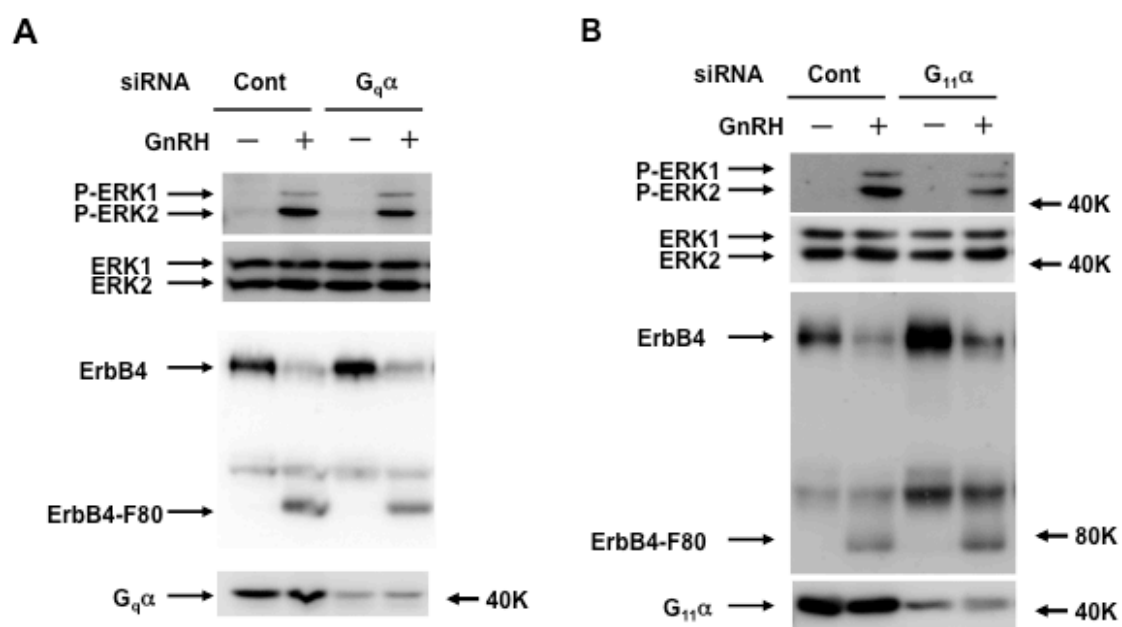


Fig. 3

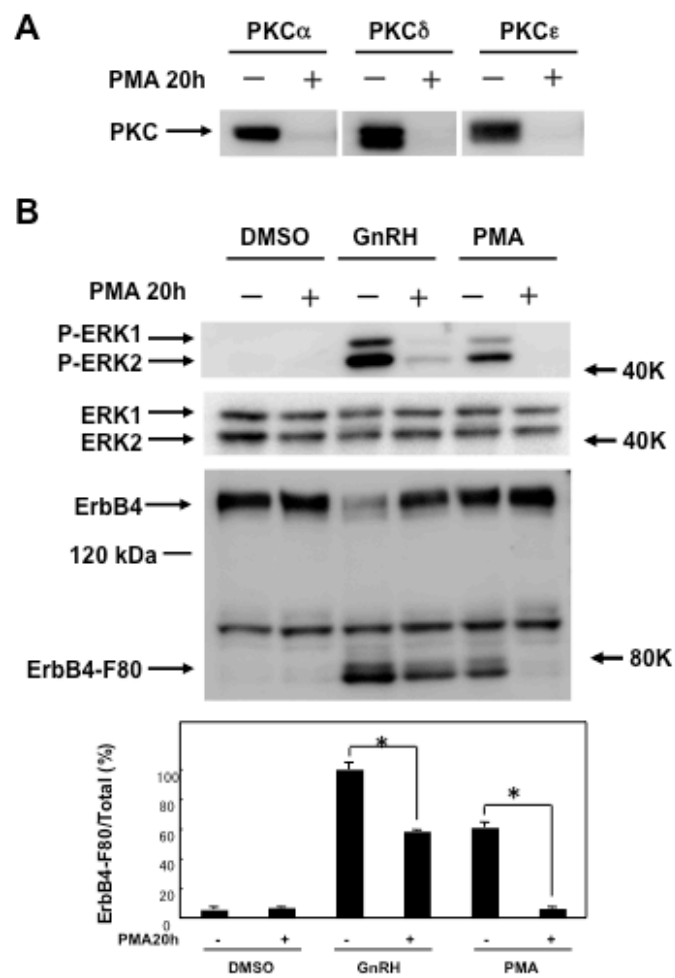
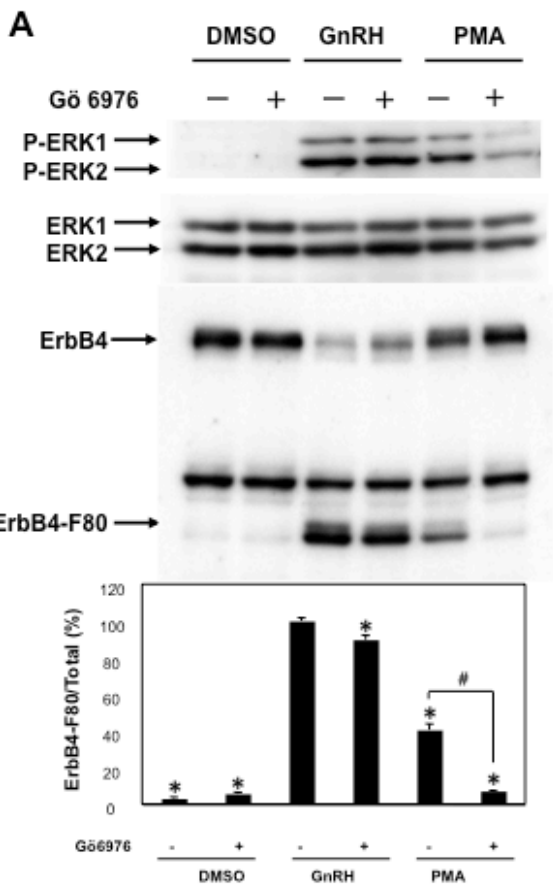


Fig. 4

A



B

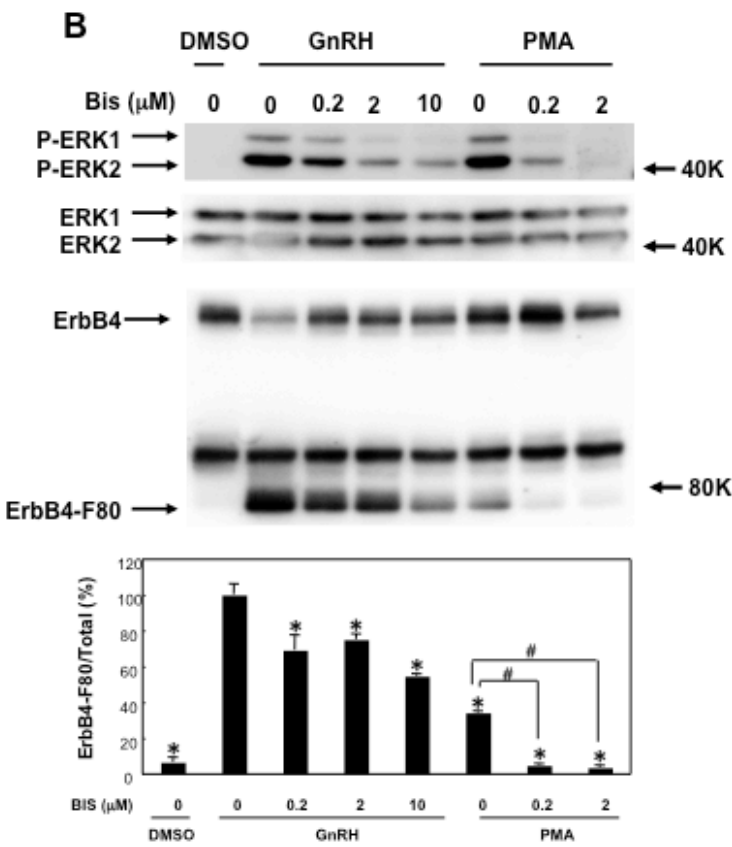


Fig. 5

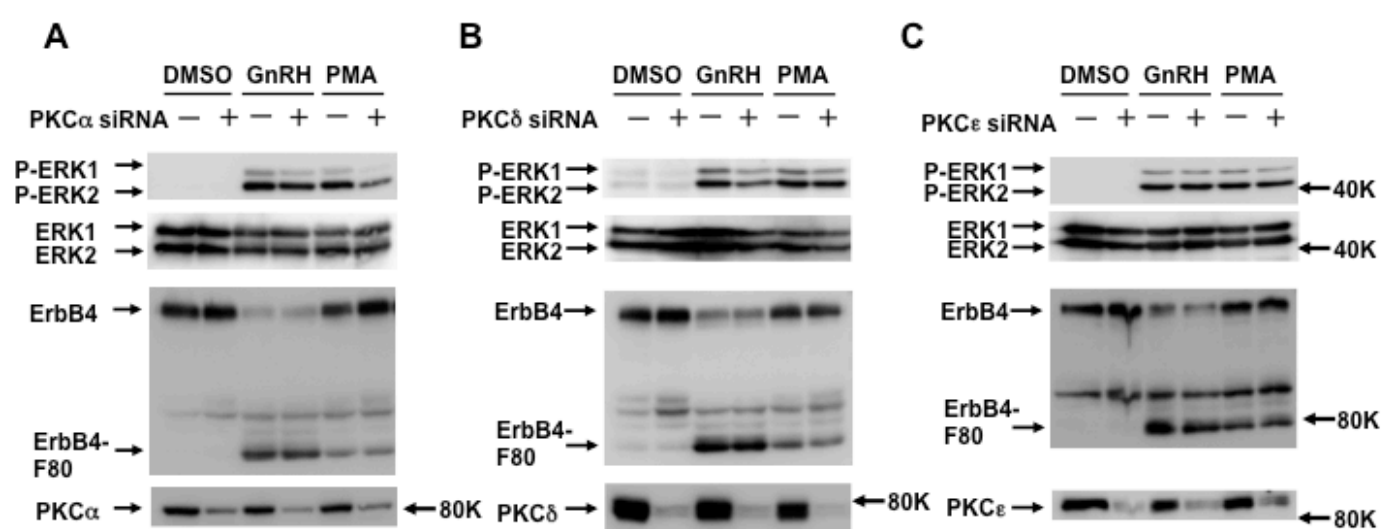


Fig. 6

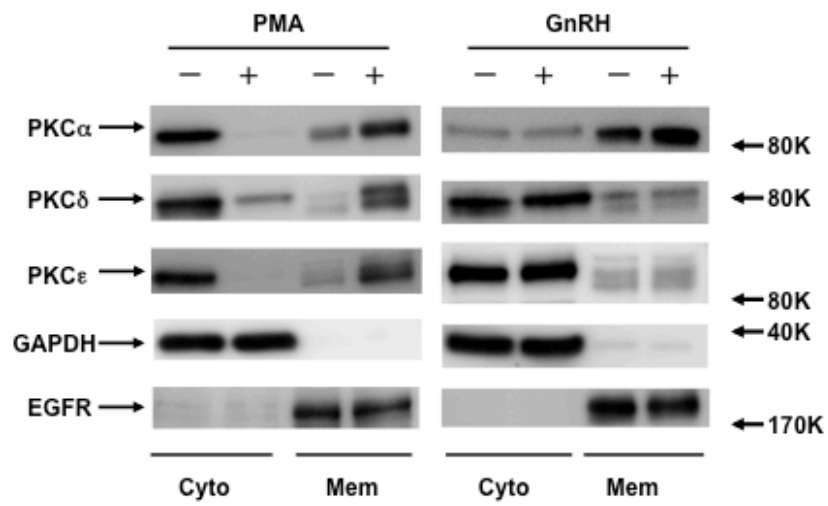


Fig. 7

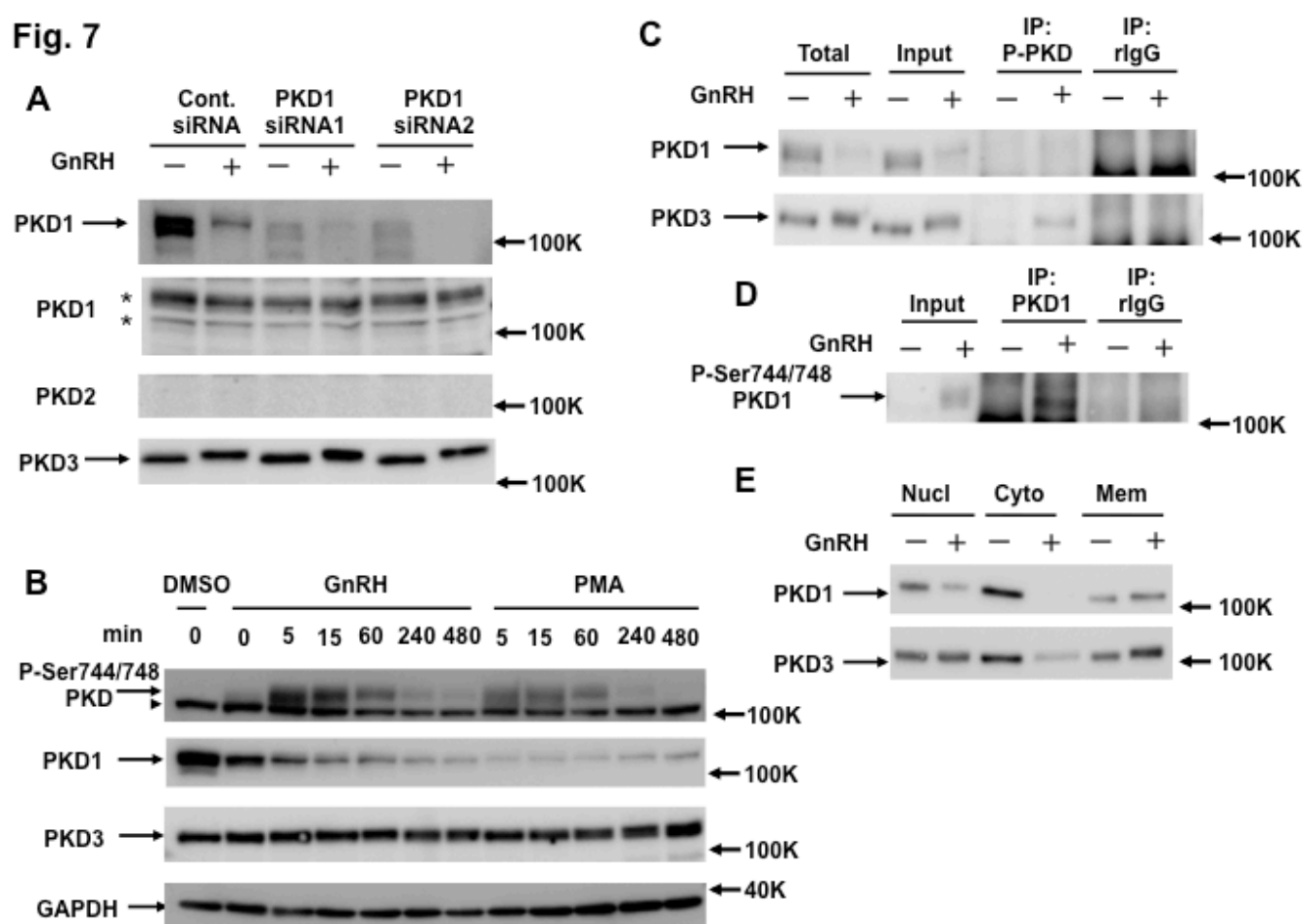


Fig.8

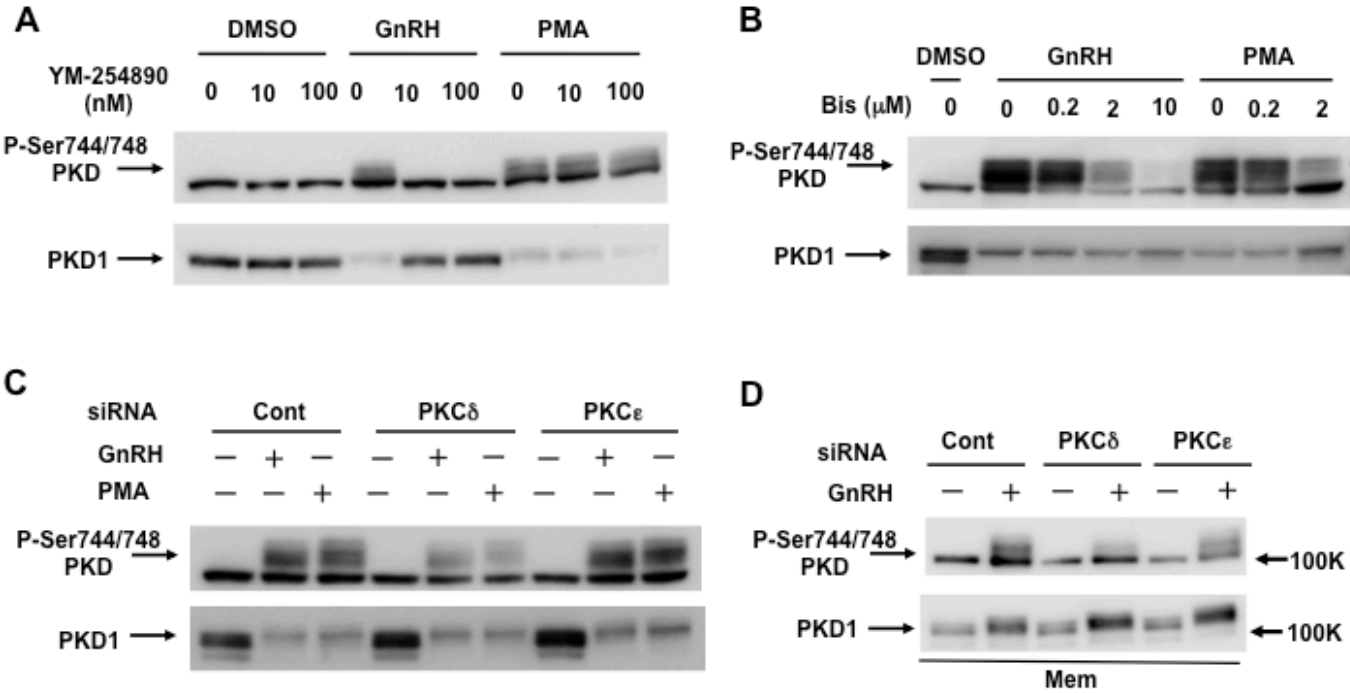


Fig. 9

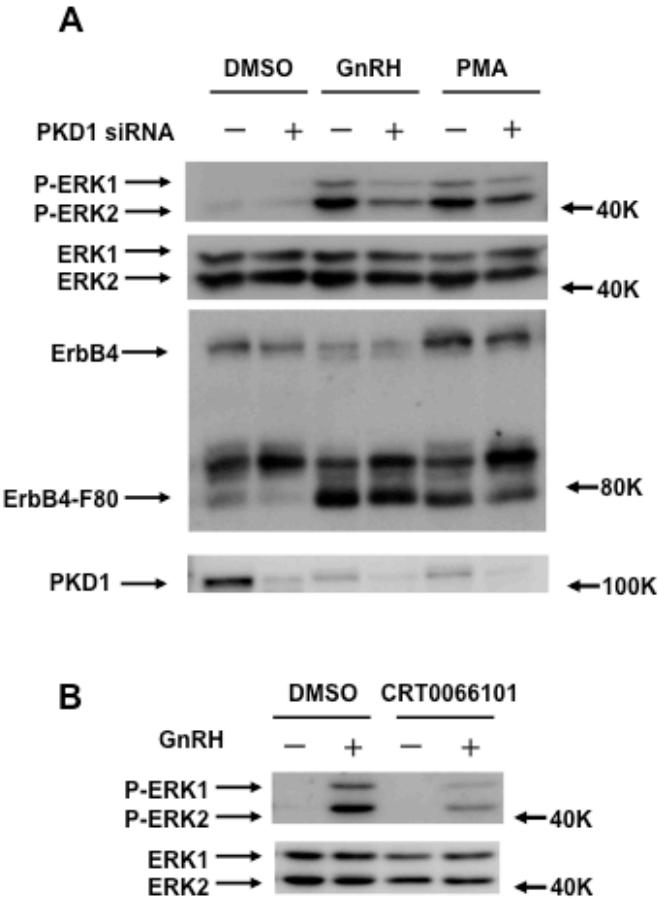


Fig. 10

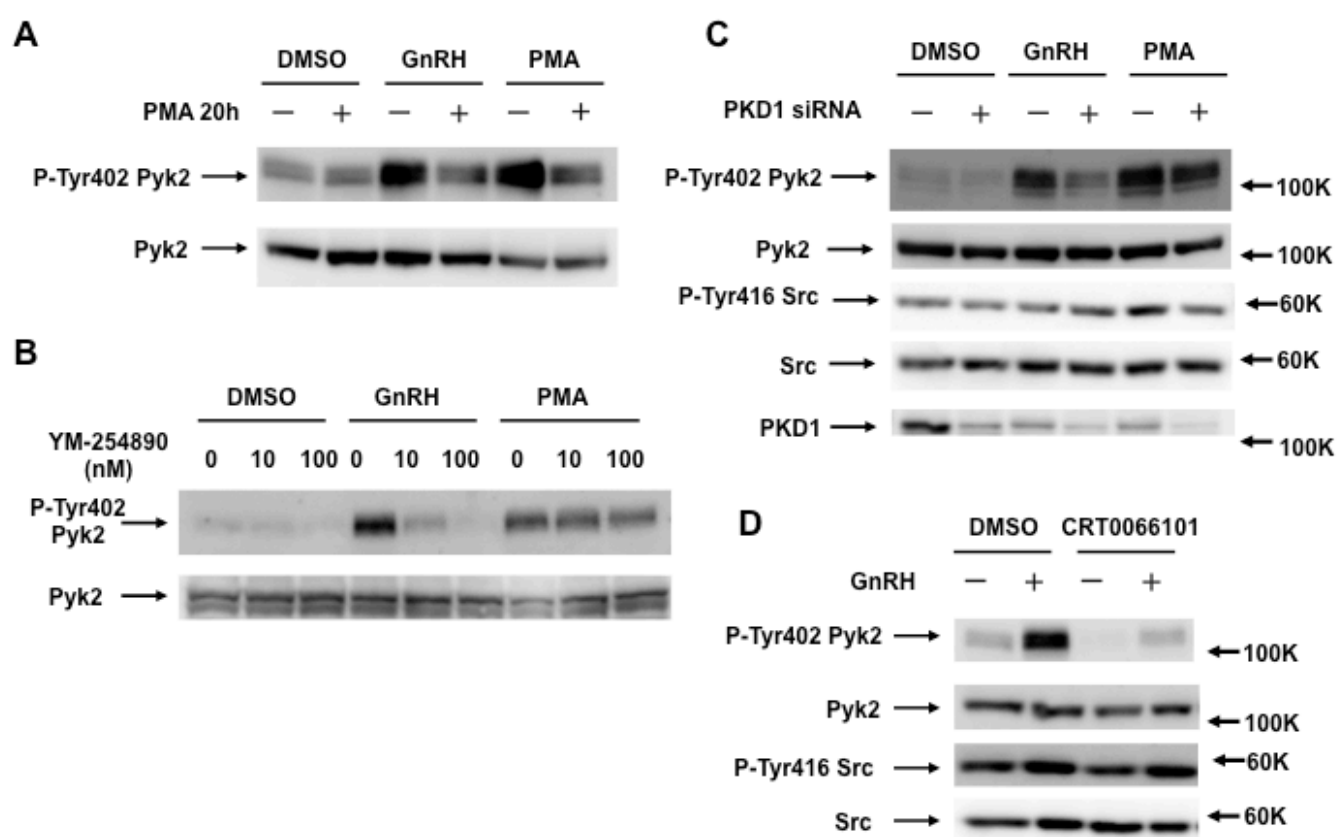


Fig. 11

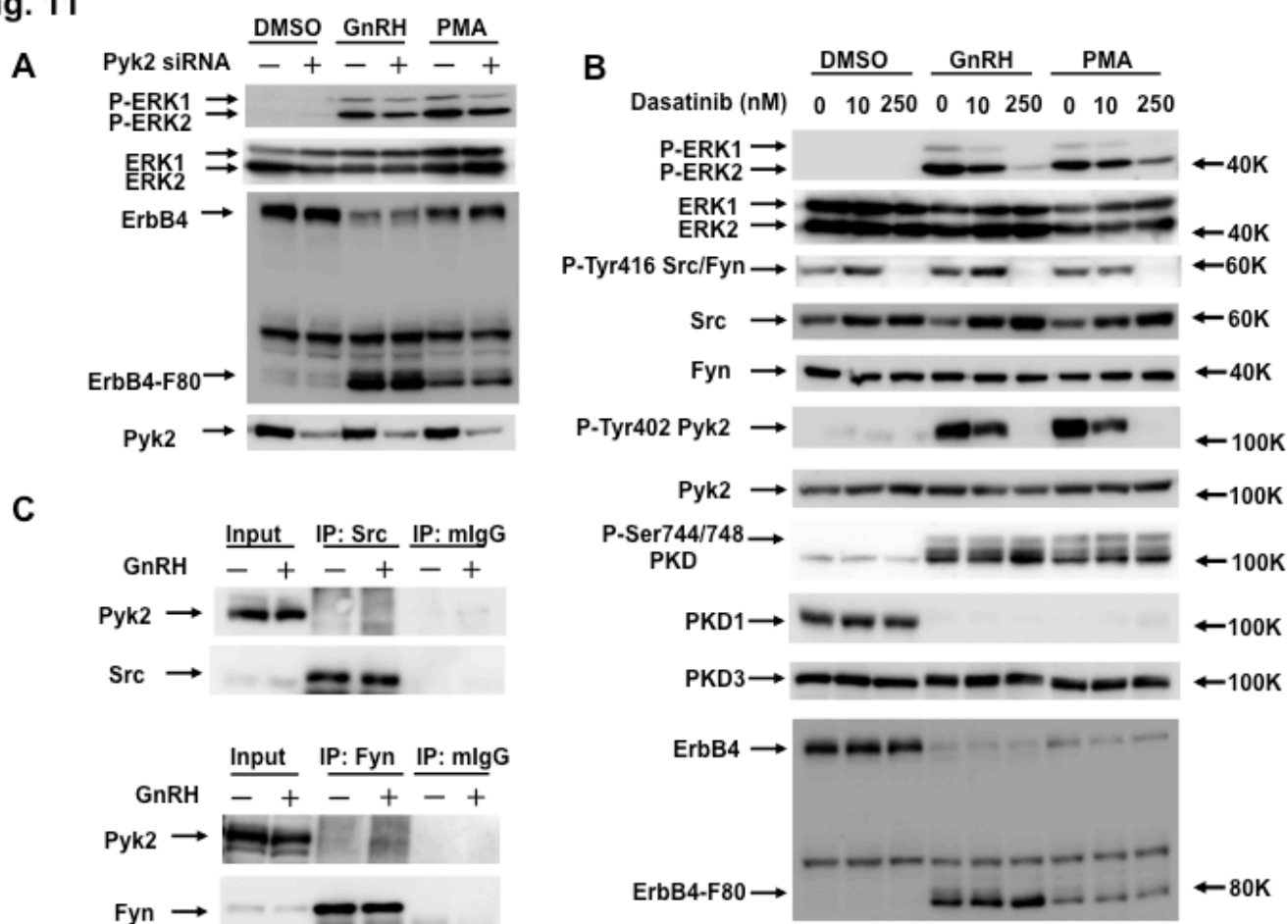


Fig. 12

