

Gonadotropin-releasing Hormone-induced Activation of Diacylglycerol Kinase- ζ and Its Association with Active c-Src*

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Gonadotropin-releasing hormone (GnRH)-induced receptor activation has been demonstrated to entrain a wide variety of signaling modalities. Most signaling pathways are concerned with the control of serine, threonine, or tyrosine-protein kinases, however, in the current article we demonstrate that in both a model cell line and in gonadotropes, GnRH additionally mediates the activation of lipid-directed kinases. We have shown that there is a functional connection between protein-tyrosine kinase modulation and lipid kinase activation. In HEK293 cells stably expressing the Type I mammalian GnRH receptor, we employed a proteomic approach to identify novel protein binding partners for GnRH-activated c-Src. Using matrix-assisted laser desorption ionization time-of-flight mass spectrometry we identified a GnRH-induced association between c-Src and the lipid kinase, diacylglycerol kinase- ζ (DGK- ζ). Using reciprocal co-immunoprecipitation we show that there is a significant elevation of the association between catalytically active c-Src with DGK- ζ in both HEK293 cells and murine gonadotrope L β T2 cells. Employing lipid kinase assays we have shown that the catalytic activity of DGK- ζ is significantly heightened in both HEK293 and L β T2 cells by GnRH. In addition, we demonstrate that the activation of DGK- ζ exerts a functional role in the murine gonadotrope L β T2 cell line. Elevated expression of DGK- ζ resulted in a shortening of the time scale of ERK activation in these cells suggesting a potential role of endogenous DGK- ζ in controlling the induction of LH β transcription by ERK1/2.

pled receptors (GPCRs) specific for GnRH on pituitary gonadotrope cells and mediates the secretion of the gonadotropins, luteinizing (LH) and follicle-stimulating hormone. This process is controlled, in part, by the elevation of intracellular calcium and the activation of protein kinase C (for review see Ref. 1). GnRH initiates both of these processes via the activation of phospholipase C- β , which hydrolyzes phosphatidylinositol bisphosphate forming inositol trisphosphate and diacylglycerol (DAG). These two metabolites generate the intracellular signals required for gonadotropin secretion thus facilitating consistent reproductive function. Unlike most other GPCRs the Type I GnRH receptor does not possess an intracellular carboxyl terminus, which is typically involved in autoregulation of the receptors signaling activity (for review see Ref. 2). Therefore upon stimulation the Type I GnRH receptor chronically activates phospholipase C- β . It has, however, been demonstrated that there is a regulatory process upon GnRH-mediated signaling at the level of inositol trisphosphate receptor activation and subsequent intracellular calcium mobilization (3). The protracted GnRH receptor stimulation causes a physical down-regulation of the intracellular inositol trisphosphate receptors in the gonadotrope resulting in a curtailment upon any successive ligand-mediated intracellular Ca²⁺ pulses. However, the activity of the other primary GnRH-induced metabolite, DAG, does not seem to be modulated in the same manner despite its capacity to control many important intracellular processes such as modulation of Ca²⁺-channel and protein kinase C (PKC) activity. In the current article we demonstrate that upon GnRH receptor stimulation there is an activation of a protein intermediate that may control for excessive generation of DAG induced by the protracted Type I GnRH receptor signaling. Hence, GnRH induces activation of a diacylglycerol kinase (DGK) responsible for the conversion of DAG into non PKC-activating metabolites. DGKs have been identified in many organisms from bacteria (4), *Drosophila* (5), and mammals (for review see Ref. 6). DGKs in mammals have been identified in many different cell types and tissues (7). To date, nine mammalian isoforms of DGKs have been cloned, common to them all being their DAG catalytic domain and also cysteine-rich domains. These cysteine-rich domains are homologous to the C1A and C1B motifs of protein kinase C isoforms and are thought to be critical for binding of the DGKs to DAG or phorbol esters. DGK-mediated phosphorylation of DAG converts it to phosphatidic acid (PA). This metabolite itself has been shown to possess significant intracellular signaling effects, such as stimulation of DNA synthesis (8), modulation of PAK1 and phosphatidylinositol 5-kinase catalytic activity (9, 10), inhibition of RasGAP (11), and modulation of actin polymerization (12). Therefore, the primary actions of DGKs are to

Gonadotropin-releasing hormone (GnRH)¹ is the central regulator of the reproductive system and was first isolated as a decapeptide from mammalian hypothalamus. GnRH released from the hypothalamus activates heptahelical G protein-cou-

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¹ The abbreviations used are: GnRH, gonadotropin-releasing hormone; GPCR, G protein-coupled receptors; LH, luteinizing hormone; DAG, diacylglycerol; PKC, protein kinase C; DGK, diacylglycerol kinase; PA, phosphatidic acid; MAPK, mitogen-activated protein kinase; siRNA, small interfering RNA; HA, hemagglutinin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; ERK, extracellular signal-regulated kinase; wt, wild type; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; GFP, green fluorescent protein; SH, Src homology; TRITC, tetramethylrhodamine isothiocyanate; p90RSK, p90 ribosomal S6 kinase.

initially catalyze a reaction that would remove DAG, attenuating PKC signaling and in doing this yield a highly active metabolite, PA, that entrains other distinct ranges of intracellular signaling pathways.

In this manuscript we describe a novel signaling interaction between two proteins involved in several interconnected signaling cascades induced by GnRH receptor activation. By two-dimensional gel electrophoresis and mass spectrometry we have demonstrated that GnRH receptor activation induces a dynamic association between c-Src and the ζ isoform of DGK (DGK- ζ). This association appears critical for the GnRH-induced translocation of DGK- ζ from the cytoplasm to the plasma membrane in these cells. In addition, we have shown that the efficient translocation of the DGK- ζ isoform is dependent upon its ability to productively interact with the cells actin cytoskeleton. We have also demonstrated that the activity of DGK- ζ in the gonadotrope has an effect upon the longevity of signaling to mitogen-activate protein kinase isoforms (MAPKs) that control transcription of the β -subunits of the luteinizing and follicle-stimulating hormone gonadotropins.

EXPERIMENTAL PROCEDURES

Materials—The specific Src kinase inhibitor PP2, herbimycin-A, cytochalasin D, and latrunculin B were obtained from Calbiochem. GnRH I, rabbit muscle enolase, phosphatidylserine, and 1,2-*sn*-dioleoylglycerol were obtained from Sigma. Deepvent™ Taq polymerase was obtained from New England Biolabs. A murine c-Src-targeted SMART-Pool™ of validated siRNA duplexes was obtained from Dharmacon Plc. An N-terminal GFP-tagged DGK- ζ cDNA clone was generously donated by Isabel Mérida (National Centre for Biotechnology, CSIC, Madrid, Spain). A hemagglutinin (HA)-tagged p90 ribosomal S6 kinase (p90RSK) cDNA clone was generously donated by Michael Avruch (Harvard Medical School). A wild-type (wt) c-Src cDNA clone was generously donated by Sarah Parsons (University of Virginia). A myc-tagged extracellular signal-regulated kinase (ERK) 2 cDNA clone was obtained from Eisuke Nishida (Kyoto University).

cDNA Constructs—A kinase-deficient c-Src mutant (Lys²⁹⁵ → Met²⁹⁵) was generated by PCR mutation of wt c-Src in pcDNA3. Wild-type c-Src subcloned into pFLAG-CMV2 using primers 1 (5'-GCGGC-GAAGCTTATGGGGAGCAGCAAGAG-3') and 4 (5'-CGCGGCGATC-CCTATAGGTTCTCTCCAGGCT-3') were employed for the second round of PCR. Wild-type c-Src was mutated by first round PCR with primers 1 and 2 (5'-GCCGGCTTCAGAGTCATTATGGCCACTCT-3') together and then 3 (5'-AGAGTGGCCATAATGACTCTGAAGC-CCGGC-3') and 4 together. 100 ng of each of these PCR products were then employed in the second PCR round. The resultant constructs were ligated into pFLAG-CMV2 between HindIII and BamHI.

Cell Culture and Transfection—HEK293 cells stably expressing the rat Type I GnRH receptor (designated SCL60; Ref. 13) and murine gonadotrope L β T2 cells were maintained in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal calf serum, 2% glutamine, 1% penicillin (10,000 units/ml)/streptomycin (10,000 mg/ml) at 37 °C in a humidified 5% CO₂ atmosphere. Transient transfections were performed either using the Ca²⁺-phosphate precipitation (SCL60) method or using Superfect (Qiagen) for L β T2 cells (cDNA and siRNA duplexes) according to the manufacturers instructions. Cells were employed for experimentation 48 h post-transfection. Cells were serum-deprived prior to stimulation by incubation (16 h) in Dulbecco's modified Eagle's medium supplemented with only 2% glutamine and 1% penicillin/streptomycin. Agonist stimulations were performed at 37 °C following preincubation with chemical inhibitors as described in the figure legends.

Immunoprecipitation and Immunoblotting—Crude cytoplasmic protein extracts were prepared from SCL60 or L β T2 cells using a Nonidet P-40-based solubilization buffer as described previously (14). L β T2 nuclear extracts were separated by centrifugation from cytoplasmic extracts and solubilized in the Nonidet P-40-based buffer supplemented with 0.5% SDS. Endogenous c-Src was immunoprecipitated with 2 μ g of an anti-Src antibody (Src-2; Santa Cruz) incubated with the clarified whole cell lysate in addition to 20 μ l of a 30% slurry of protein G plus/protein A-agarose (Calbiochem). Src was also immunoprecipitated with 20 μ l of an agarose-preconjugated anti-Src slurry (Santa Cruz). Active c-Src was immunoprecipitated with 2 μ g of the anti-Tyr⁴¹⁸ phos-

phorylated Src antibody (BIOSOURCE) plus 20 μ l of a 30% slurry of protein G plus/protein A-agarose. Endogenous DGK- ζ was immunoprecipitated with 1 μ g of a goat anti-DGK- ζ polyclonal with 20 μ l of a 30% slurry of protein G plus/protein A-agarose. HA-tagged p90RSK was immunoprecipitated with 1.5 μ g of the anti-HA 12CA5 monoclonal serum (Roche Molecular Biochemicals) with 25 μ l of a 30% slurry of protein G plus/protein A-agarose added to the sonicated and clarified nuclear extracts. Immune complexes were processed as described previously (14). Immunoblotting of endogenous, active or inactive, ERK from clarified whole cell lysates was performed as described previously (14). Active p90 ribosomal S6 kinase (p90RSK) was detected using a 1:1000 dilution of a rabbit anti-human active p90RSK polyclonal (New England Biolabs) with a 1:15,000 dilution of alkaline phosphatase-conjugated polyclonal anti-rabbit IgG as a secondary antibody (Sigma). Protein phosphotyrosine status was determined using mouse anti-phosphotyrosine monoclonals PY99 or PY20 (Santa Cruz) at a 1:1000 dilution with a 1:15,000 dilution of alkaline phosphatase-conjugated polyclonal anti-mouse IgG as a secondary antibody (Sigma). Total c-Src protein was detected using a rabbit anti-human Src polyclonal IgG (Src-2, Santa Cruz) at a 1:1000 dilution with a 1:15,000 dilution of alkaline phosphatase-conjugated polyclonal anti-rabbit IgG as a secondary antibody (Sigma). Src Tyr⁴¹⁸ autophosphorylation was detected using anti-human polyclonal phosphospecific antisera (Bio-Source International) employed at a 1:1000 dilution with a 1:15,000 dilution of alkaline phosphatase-conjugated polyclonal anti-rabbit IgG as a secondary antibody (Sigma). Endogenous DGK- ζ was detected using a 1:1000 dilution of a goat anti-human DGK- ζ polyclonal with a 1:10,000 dilution of an alkaline phosphatase-conjugated anti-goat polyclonal as a secondary antibody. Each alkaline phosphatase-labeled protein was visualized using an enzyme-linked chemifluorescence reaction (Amersham Biosciences) and quantified using an Amersham Biosciences Storm 860 PhosphorImager.

c-Src *In Vitro* Kinase Assay—Cell monolayers, after specific stimulations, were placed on ice, washed once in ice-cold Dulbecco's phosphate-buffered saline and then lysed on 0.8 ml of the Nonidet P-40-based lysis buffer. *In vitro* c-Src kinase assays were performed as in Tsuganezawa *et al.* (15).

Cytoskeleton Preparation—Crude Triton X-100-insoluble cytoskeletal extracts were prepared according to Payrastré *et al.* (16). For lipid kinase assays upon cytoskeletal preparations sedimented material was washed twice with the extraction buffer without Triton-X-100.

Lipid Kinase Assays—Lipid kinase assays were performed upon immunoprecipitated DGK- ζ or crude cytoskeletal preparations as described in Payrastré *et al.* (Ref. 13; nu 15). Phosphorylated lipids were visualized upon a storage phosphorscreen and quantified using an Amersham Biosciences Storm 860 or Typhoon 9400 PhosphorImager.

Two-dimensional Gel Electrophoresis—Cell monolayers were stimulated with GnRH I (1 μ M, 10 min), placed on ice, washed once with ice-cold Dulbecco's phosphate-buffered saline, and cytoplasmic protein extracts were prepared using the previously described Nonidet P-40 lysis buffer. Clarified lysates were then pre-cleared by incubation with 20 μ l of protein A/protein G plus-agarose (4 °C, 1 h, continuous agitation), 25 μ l of an agarose-preconjugated anti-Src polyclonal antiserum was then added and incubated for 16 h at 4 °C with continuous agitation. Src immunoprecipitates were collected by centrifugation, washed once in ice-cold Dulbecco's phosphate-buffered saline, twice in a Tris sorbitol buffer (10 mM Tris-HCl, 250 mM D-sorbitol, pH 7.4), and solubilized in 50 μ l of CHAPS-urea buffer (8 M urea, 4% CHAPS, 40 mM Tris-HCl). Soluble proteins were loaded onto the first dimensional isoelectric focusing (pI 3 to 10) gel and run overnight. The second dimension was run along a polyacrylamide gradient (12–14%) and stained for 2 min with a standard silver solution (Amersham Biosciences). Isolation of silver-stained puncta was achieved by excision of the puncta from the gel and then by overnight tryptic digestion before drying down and subjection to MALDI-TOF mass spectrometry (Voyager, Micromass).

Confocal Laser Microscopy—Confocal laser microscopy was performed on a Zeiss LSM510 laser scanning microscope with a 40 \times 1.4 numerical aperture oil immersion lens. L β T2 cells, transiently transfected with the GFP-tagged DGK- ζ , were plated upon Matrigel-coated glass-bottomed 35-mm Petri plates (Mattek) and serum-deprived before stimulation. Plates were transferred to a heated chamber (37 °C) upon the microscope and were stimulated with GnRH I directly during capturing of confocal images, at the time points specified in the appropriate figure legends. Immunohistochemistry for c-Src was performed on fixed and permeabilized (described previously, see Ref. 17) L β T2 cells using a 1:100 dilution of an anti-Src polyclonal (Santa Cruz) with a 1:100 dilution of a TRITC-conjugated anti-rabbit polyclonal as a secondary

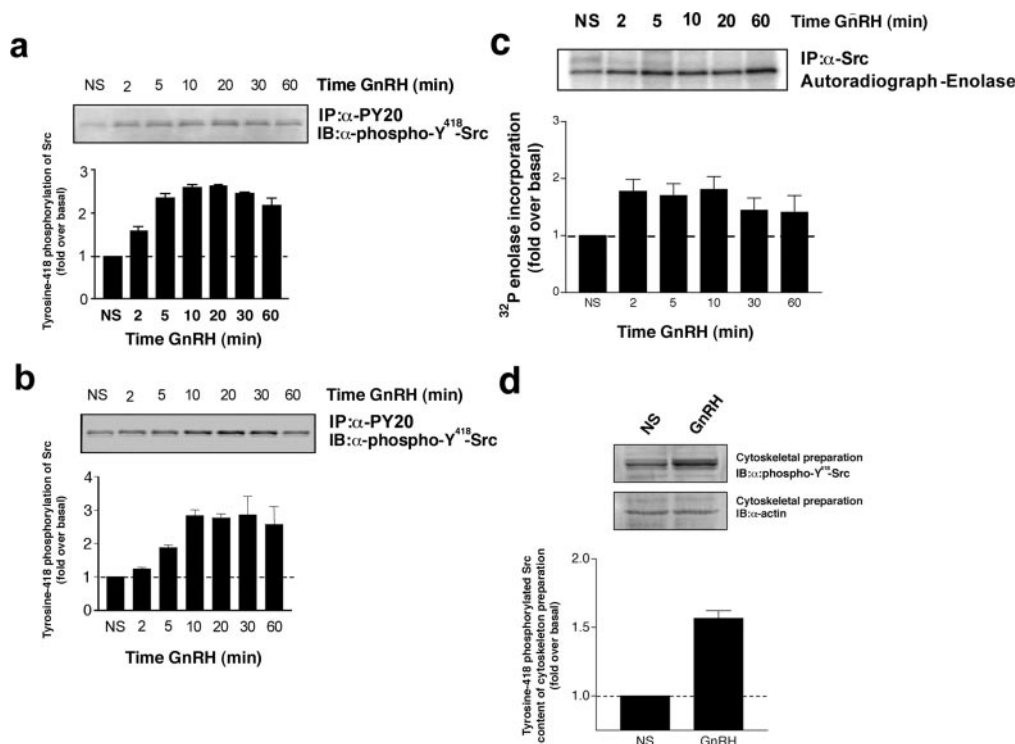


FIG. 1. GnRH-induced activation of c-Src. GnRH stimulation (100 nM) of serum-deprived SCL60 (*panel a*) and L β T2 cells (*panel b*) induces c-Src Tyr⁴¹⁸ autophosphorylation. *Panel c*, GnRH (100 nM) stimulation induces activation of c-Src catalytic activity, measured as tyrosine phosphorylation of acidified rabbit muscle enolase. *Panel d*, autophosphorylated active c-Src, co-sediments with actin-rich crude cellular cytoskeletal preparations upon GnRH I stimulation (100 nM, 10 min). Histograms associated with respective panels represent mean \pm S.E. from at least three individual experiments. NS, nonstimulated; IP, immunoprecipitated; IB, immunoblotted.

antibody (Sigma). FLAG-tagged c-Src was visualized with a 1:100 dilution of a Cy3-conjugated anti-FLAG monoclonal (Sigma).

RESULTS

GnRH Activation of c-Src—GnRH I stimulation of SCL60 and L β T2 cells resulted in a protracted activation of the tyrosine kinase activity of c-Src demonstrated by an increase in c-Src autophosphorylation (Fig. 1, SCL60, *panel a*; L β T2, *panel b*). We also directly measured the functional catalytic activity of c-Src by incubation of c-Src immunoprecipitates from GnRH-stimulated (100 nM) SCL60 cells with acid-treated rabbit muscle enolase as a substrate (Fig. 1, *panel c*). GnRH-activated c-Src was functionally linked to actin-rich cytoskeletal structures in the SCL60 cells (Fig. 1, *panel d*). We also noted a GnRH-induced elevation of the amount of total active/inactive c-Src associated with cytoskeletal preparations (data not shown). Hence, GnRH stimulation resulted in an elevation in the degree of active c-Src co-sedimenting with actin cytoskeletons recovered from Triton X-100 whole cell lysates. The modular SH3, SH2, and SH1 kinase domains of the Src family tyrosine kinases (18–20) allow these molecules to act as inducible linkers of a diverse array of signaling proteins (21–23). We employed a proteomic approach to investigate the ability of novel proteins to co-immunoprecipitate with c-Src upon its GnRH-induced activation.

Proteomic Analysis of GnRH-induced c-Src Binding Partners in SCL60 Cells—Anti c-Src immune complexes were prepared from GnRH-stimulated (1 μ M, 10 min) cell lysates and then subjected to two-dimensional gel electrophoresis. In Fig. 2 (*panel a*) GnRH activates the ERK1/2 forms of the mitogen-activated protein kinase (MAPK) family. A complete two-dimensional electrophoresis gel from the anti-c-Src immunoprecipitates is demonstrated in Fig. 2, *panel b*. Many additional proteins were observed in the section of the gel from the GnRH-

stimulated cells (GnRH) compared with the control section of the gel (non-stimulated, NS). In *panel c* (Fig. 2), a magnified inset of a gel section (from *black inset box* in *panel b*) is shown, displaying the specific protein that showed the greatest GnRH-induced increase in association with the immunoprecipitated c-Src. After excision from the gel and MALDI-TOFF mass spectroscopy, a peptide mass fingerprint was generated (*inset* in *panel d*). Using the estimated pI and molecular mass range of the protein, the mass ion profile of the excised spot was used to interrogate the Swiss-Prot data base (SwissProt 08:21:2003) using the MS-Fit Prospector protein analysis software.² The primary identified protein was human DGK- ζ , the peptide fingerprint coverage map of the complete sequence is shown in Fig. 2 (*panel d*).

GnRH Induces a Physical Association between c-Src and DGK- ζ in SCL60 and Gonadotrope L β T2 Cells—With GnRH I application (activating ERK1/2, Fig. 3, *panel a*, top immunoblot) an elevation in the total and active c-Src content of DGK- ζ immunoprecipitates (Fig. 3, *panel a*, quantified in *panels b* and *c*) was noted in SCL60 cells. In Fig. 4 it can be seen that GnRH stimulation of L β T2 cells results in an increase in the total (*panel a*) and active (*panel d*) c-Src content of DGK- ζ immunoprecipitates. Performing converse experiments (Fig. 4, *panel c*) it was also evident that with GnRH stimulation there was an increase in the DGK- ζ content of Src immunoprecipitates. In addition, GnRH stimulation increased the DGK- ζ content of Tyr⁴¹⁸-phosphorylated c-Src immunoprecipitates (Fig. 4, *panel d*). The time course of the GnRH-induced association was similar in both cell lines, *i.e.* association occurred within 5 min and persisted for at least 60 min (data not shown). Interestingly, we noted that DGK- ζ itself was not significantly tyrosine phospho-

² prospector.ucsf.edu.

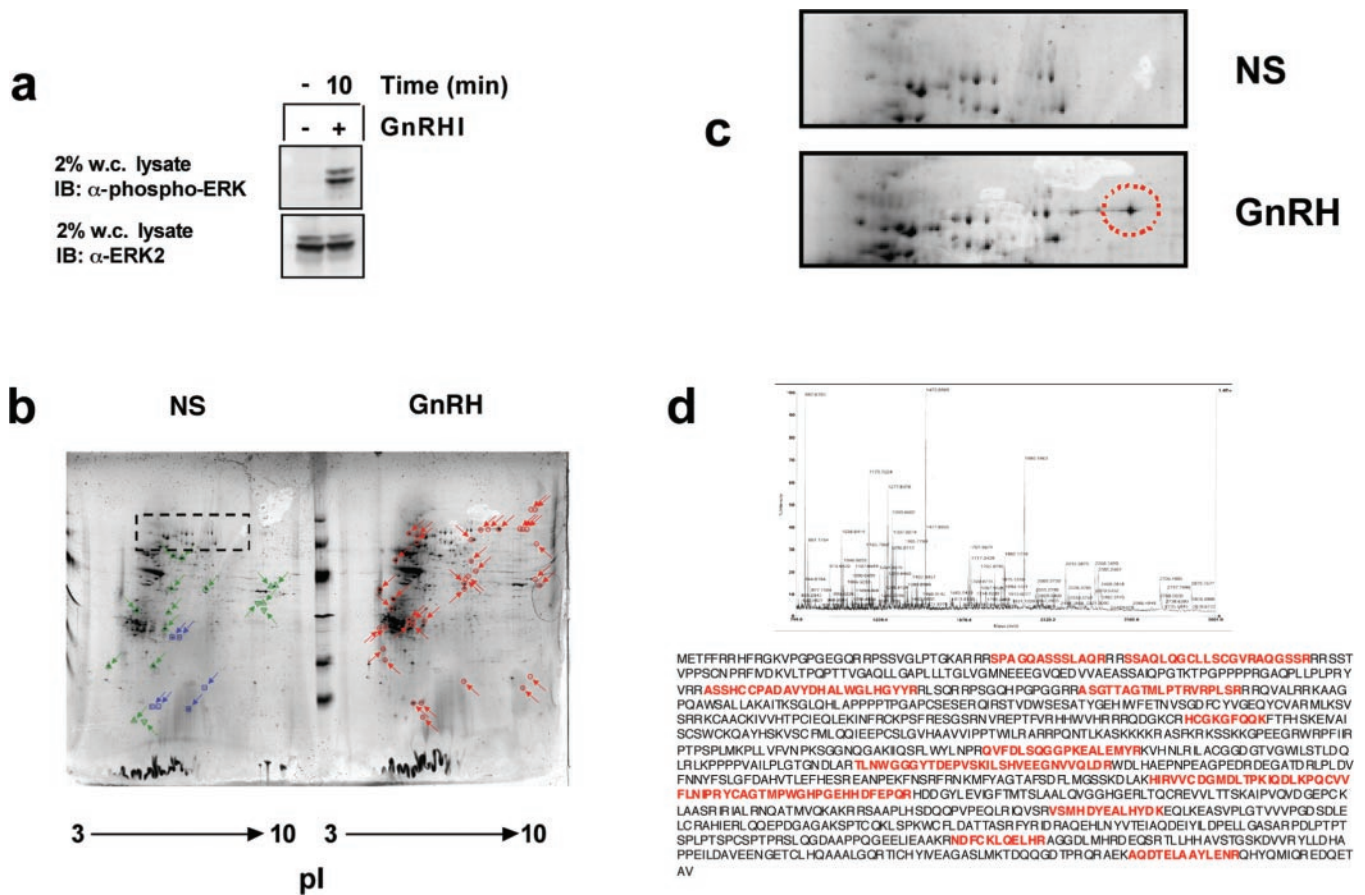


FIG. 2. Proteomic isolation of DGK- ζ as a GnRH-induced c-Src binding partner. *Panel a*, ERK1/2 activation in SCL60 cells stimulated with GnRH I (100 nM, 10 min). Anti-c-Src immunoprecipitates from stimulated GnRH were subjected to two-dimensional gel electrophoresis (*panel b*). Proteins associated with the c-Src immune complex with GnRH stimulation are labeled with silver. Proteins increased (red arrow), decreased (blue arrow), or not altered (green arrow) in their interaction with c-Src upon GnRH stimulation are highlighted. The dashed box depicts gel sections highlighted in *panel c*. *Panel c*, one particular protein (circled in red) demonstrating the most robust GnRH-induced alteration was isolated. The protein mass fingerprint for the isolated protein is shown in *panel d*, along with the peptide coverage map of the specific identified protein, human diacylglycerol kinase- ζ . NS, nonstimulated; IB, immunoblotted.

rylated in response to GnRH, suggesting it may not be a direct substrate for c-Src and their interaction may not be mediated via a direct phosphotyrosine-SH2 domain interaction.

GnRH Stimulates the Catalytic Activity of DGK- ζ —Immunoprecipitated DGK- ζ from GnRH stimulated (100 nM, 30 min) SCL60 (*Fig. 5, panel a*) or L β T2 (*Fig. 5, panel b*) cells induces the incorporation of 32 P into phosphatidic acid, demonstrating the GnRH-induced elevation of functional DGK- ζ kinase activity. We next investigated the effects of GnRH upon DGK- ζ subcellular localization, associated with its catalytic activity, directly in live cells by using a GFP-tagged form of the DGK- ζ .

GnRH-stimulated c-Src Controls DGK- ζ Activity—Pretreatment of SCL60 cells with inhibitors of Src catalytic activity, PP2 (5 μ M, 30 min) or herbimycin-A (1 μ M, 60 min), before GnRH I stimulation resulted in an inhibition of the GnRH-induced co-precipitation of c-Src and DGK- ζ (*Fig. 6, panel a*). Identical results were obtained from L β T2 cells (data not shown). The GnRH-induced catalytic activation of immunoprecipitated DGK- ζ (*Fig. 6, panel b*) was also attenuated by pretreatment of the cells with PP2 (5 μ M, 30 min) before the *in vitro* DGK- ζ kinase assay. Overexpression of wild-type c-Src (wt-Src) did not significantly affect the ability of GnRH to activate co-expressed myc-tagged ERK2, yet similar overexpression of a kinase-deficient c-Src mutant (Lys²⁹⁵ \rightarrow Met²⁹⁵; termed KD-Src) significantly attenuated the GnRH-induced ERK2 activation (*Fig. 6, panel c*). Co-expression of the KD-Src and not the wt-Src with a GFP-tagged DGK- ζ (DGK- ζ -GFP) significantly attenuated the ability of GnRH stimulation to

induce an association between DGK- ζ -GFP and endogenous c-Src (*Fig. 6, panel d*).

GnRH-mediated Control of DGK- ζ Translocation Involves Src—Expression of DGK- ζ -GFP in L β T2 cells did not affect cell survival or morphology. DGK- ζ -GFP was expressed highly in the cell nucleus and also widely across the cell cytoplasm (*Fig. 7, panel a, image 2*). GnRH I stimulation (100 nM) induced a rapid movement of the DGK- ζ -GFP to the plasma membrane (*Fig. 7, image 3*). This translocation continued (*Fig. 7, images 4–8*) until almost all of the DGK- ζ -GFP protein was cleared from the cytoplasm. Negligible movement of DGK- ζ from the nucleus to the cytoplasm occurred. At later time points (1–2 h), there was an eventual restoration of the presence of DGK- ζ in the cytoplasm (data not shown). We next investigated whether the catalytic activity of c-Src was involved in this GnRH-mediated control of the DGK- ζ rapid plasma membrane translocation. Control GnRH-induced DGK- ζ -GFP translocation (*Fig. 7, panel b, images 1–3*) was inhibited by PP2 pretreatment (5 μ M, 30 min) of the cells (*Fig. 7, panel b, images 4–6*). Wild-type Src supported (*Fig. 7, panel c, images 1–3*), while KD-Src significantly inhibited (*Fig. 7, panel c, images 4–6*) the GnRH-induced membrane translocation of the co-expressed DGK- ζ -GFP. In fixed L β T2 cells, when co-expressed, wt-Src associates and translocates with DGK- ζ -GFP upon GnRH stimulation (*Fig. 7, panel d, images 4–6*). When KD-Src was co-expressed with DGK- ζ -GFP, the GnRH-induced DGK- ζ translocation was lost and no membrane association of DGK- ζ -GFP and Src was observed (*Fig. 7, panel d, images 10–12*).

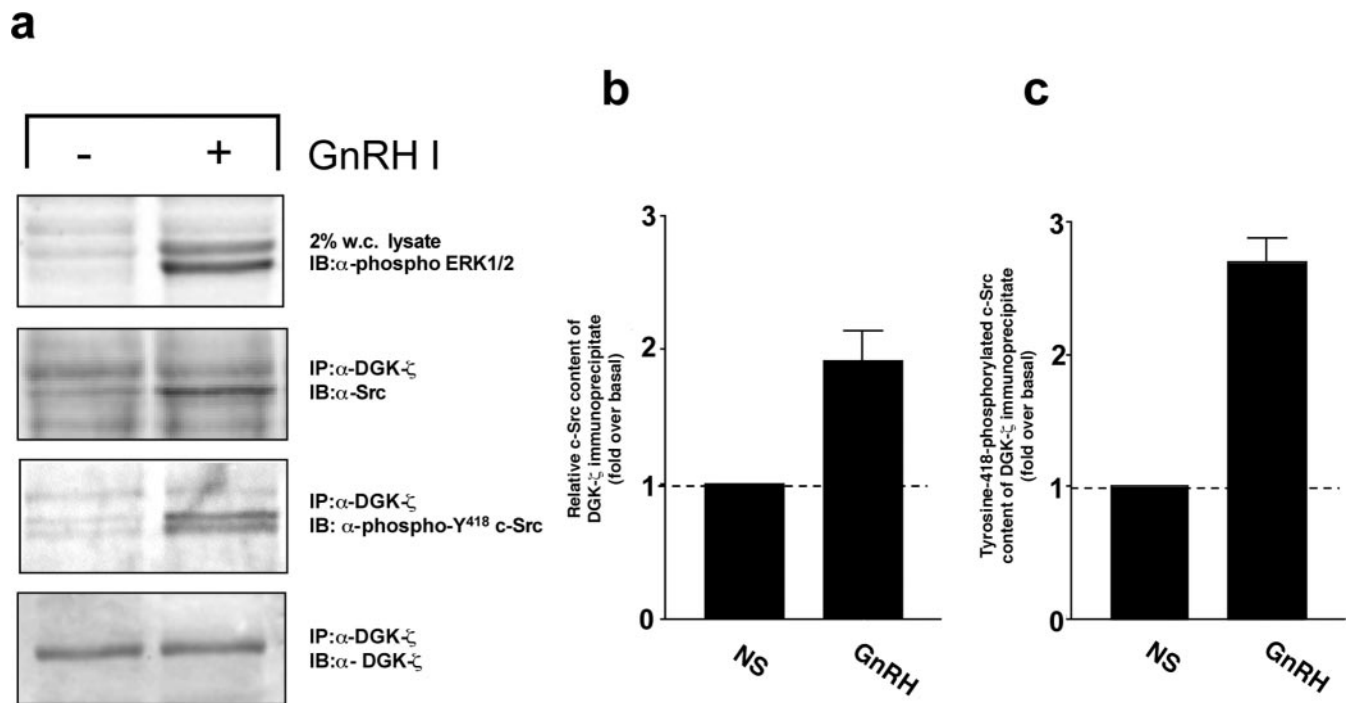


FIG. 3. GnRH induces an association between DGK- ζ and catalytically active c-Src in SCL60 cells. *Panel a*, GnRH I stimulation (100 nM, 10 min), activating ERK1/2, enhances the total (quantified in *panel b*) and active Src (quantified in *panel c*) content of DGK- ζ immunoprecipitates. Histograms in *panels b* and *c* represent relative total or active c-Src content of DGK- ζ immunoprecipitates data gathered from four separate experiments. The data are displayed as mean \pm S.E. NS, nonstimulated; IP, immunoprecipitated; IB, immunoblotted.

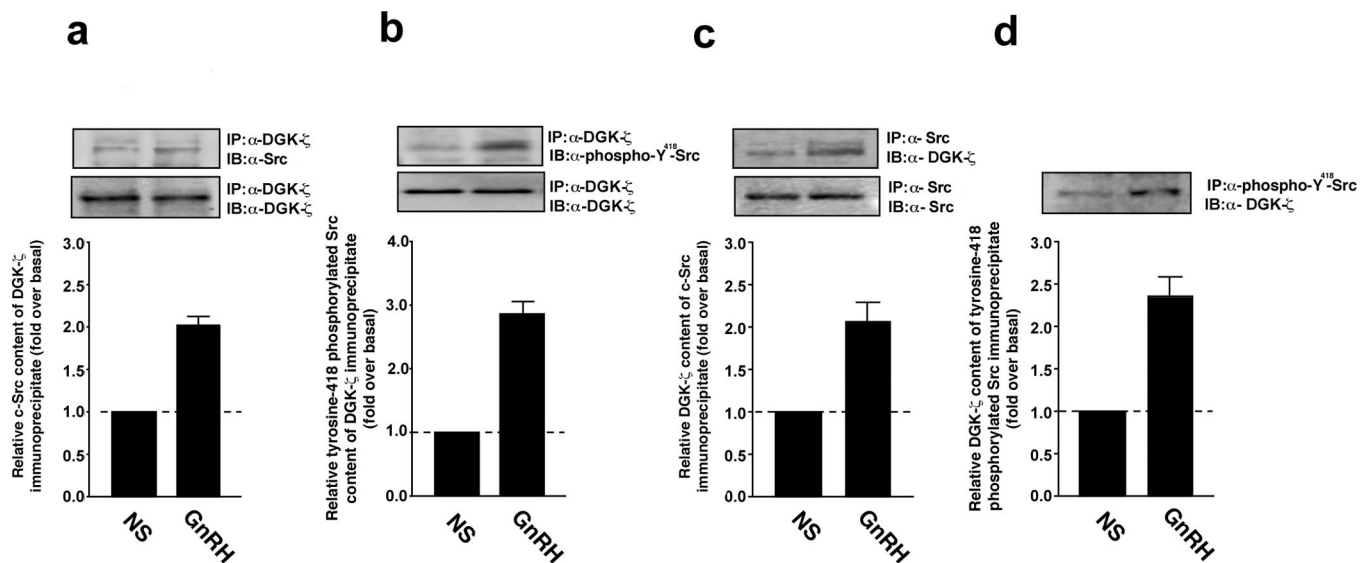


FIG. 4. GnRH induces a physical association between catalytically active c-Src and DGK- ζ in L β T2 murine gonadotropes. GnRH (100 nM, 10 min) increases the total (*panel a*) and active Src (*panel b*) content of DGK- ζ immunoprecipitates. GnRH also increases the DGK- ζ content of total (*panel c*) and active (*panel d*) c-Src immunoprecipitates. Histograms associated with respective panels represent mean \pm S.E. from at least three individual experiments. NS, nonstimulated; IP, immunoprecipitated; IB, immunoblotted.

Targeted disruption of murine c-Src with transfection of c-Src siRNA duplexes resulted in a reduction of c-Src expression in L β T2 cells (Fig. 8, *panel a*). Transfection of cells with nonsense siRNA duplexes ("control") did not affect c-Src expression levels. The reduction of cellular c-Src was dependent upon the concentration of transfected siRNA duplexes (Fig. 8, *panel b*). Diminution of c-Src protein levels induced by RNA interference inhibited GnRH-induced activation of myc-ERK2 to a similar degree as pretreatment of cells with PP2 (5 μ M, 30 min; Fig. 8, *panel c*). Transfection of L β T2 cells with control siRNA did not affect GnRH-induced translocation of DGK- ζ -GFP (Fig. 8, *panel d*, *images 1-7*), whereas transfection with c-Src siR-

NAs almost completely inhibited GnRH-induced DGK- ζ translocation (*images 8-14*).

Functional Cytoskeletal Dynamics Are Required for GnRH-induced DGK- ζ Plasma Membrane Translocation—Control GnRH-induced DGK- ζ translocation (Fig. 9, *panel a*, *images 1-6*) was inhibited by pretreatment with cytochalasin-D (*images 7-12*) or latrunculin-B (*images 13-18*). Disruption of the actin cytoskeleton with cytochalasin D or latrunculin B caused an inhibition of the GnRH-induced DGK- ζ -c-Src association suggesting that the dynamic generation of this protein complex is reliant upon a functional actin cytoskeleton (Fig. 9, *panel b*). In addition to this, we noted a significant, GnRH-induced,

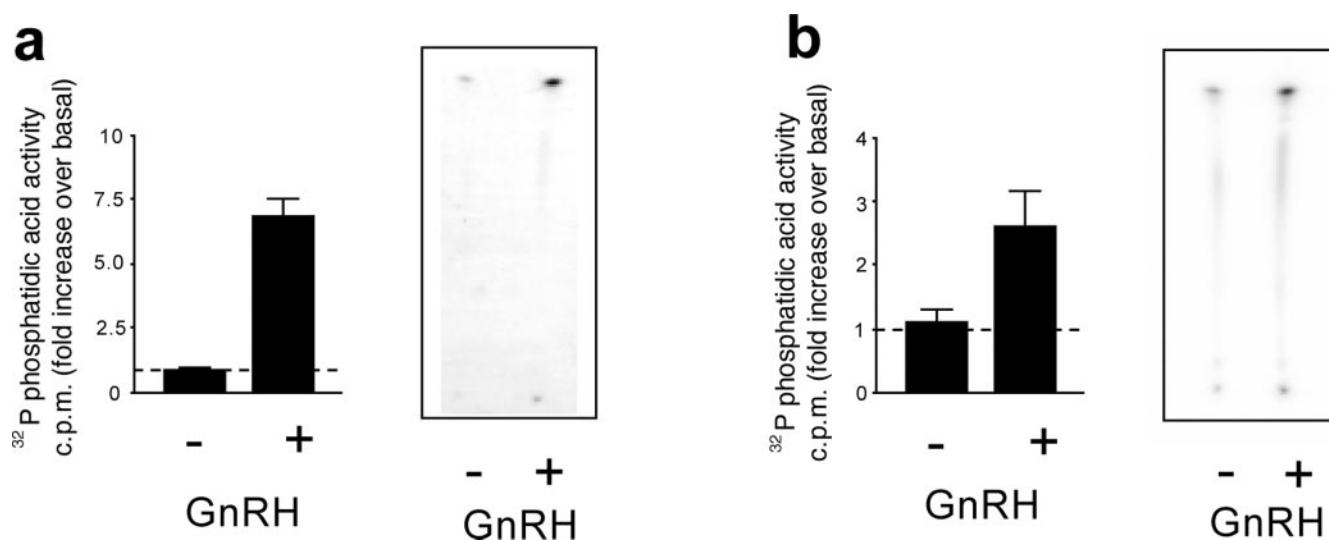


FIG. 5. GnRH induces the catalytic activation of the endogenous DGK- ζ in both SCL60 and L β T2 cells. In panel a (SCL60 cells) or panel b (L β T2 cells) GnRH I (100 nM, 10 min) induces a potent increase in the γ - 32 P incorporation into phosphatidic acid from cellular DGK- ζ immunoprecipitates. The data represented in the associated histograms are the mean \pm S.E. from three individual experiments.

elevation of the degree of DGK catalytic activity physically associated with the cells cytoskeletal structure (Fig. 9, panel c).

Overexpression of Wild-type DGK- ζ Results in an Alteration in the Functional Activation of MAPK in L β T2 Cells—DAG turnover by DGKs in cells may attenuate the temporal activity of PKC and also reduce the degree of L-type calcium channel activity. As both of these processes, *i.e.* PKC activity and Ca $^{2+}$ entry into the cell, are known to contribute to the downstream signaling of GnRH we investigated how modulation of DGK- ζ activity may affect the nature of signaling events in L β T2 cells. When DGK- ζ was significantly overexpressed, between 10- and 20-fold above endogenous levels (data not shown), we noted that the time course of GnRH-stimulated, co-transfected myc-tagged ERK2 activation was significantly attenuated (Fig. 10, panel a). In addition to this, we investigated the potential transcriptional effects of this attenuation of the MAPK activation profile employing an HA epitope-tagged form of the p90 ribosomal S6 kinase, a direct downstream substrate of active ERK. With GnRH stimulation (100 nM, 45 min) of cells expressing HA-p90RSK but not DGK- ζ -GFP there was a potent elevation in the amount of active p90RSK present in L β T2 nuclear extracts (Fig. 10, panel b). However, upon overexpression of GFP-tagged DGK- ζ , there was a considerable reduction in the level of active nuclear p90RSK, indicative of a reduction in the levels of active cellular ERK. This clearly demonstrates that the activity of DGK- ζ can therefore profoundly control the potential transcriptional activity of GnRH in the gonadotrope cells. GnRH activation of MAPKs in gonadotropes is vital for the control of gonadotropin hormone gene transcription and therefore the relative extent of DGK- ζ activity in these cells may contribute in part to the eventual generation and release of the gonadotropin hormones LH and follicle-stimulating hormone.

DISCUSSION

In the current article we have demonstrated that by using a proteomic approach it is possible to elucidate the coherent generation of multiprotein signaling complexes upon activation of GPCRs. As shown in several reports (24–27) the current view upon the basis of cell signaling is that multiprotein complex formation exerts the eventual effects of GPCR stimulation upon intermediary cell metabolism. The data presented here demonstrates for the first time that the mobilization and plasma membrane translocation of DGK- ζ induced by GnRH receptor activation is controlled by the concomitant activation

of the c-Src non-receptor tyrosine kinase. Activation of c-Src, along with its enhanced association with the cells cytoskeletal structure, provides a platform by which DGK- ζ can be shuttled from the cytoplasm to its substrate, DAG, produced at the sites of receptor activation. From overexpression studies we have also demonstrated that elevated degrees of DGK- ζ activation can limit GnRH-stimulated ERK1/2 activation. This effect has implications for the control of gonadotropin hormone transcription and thus control over the whole reproductive axis.

The control of DGK catalytic function and subcellular movement can occur through many mechanisms. Several DGK isoforms have been shown to translocate to the membrane in response to agents such as chemotactic factors (28) and adrenergic (29) or cholinergic agonists (30). One of the main mechanisms by which such extracellular ligands can induce this translocation is by elevating the plasma membrane levels of its substrate, DAG. It has been proposed that subpopulations of DGKs are shuttled to specific DAG pools at the plasma membrane (6). Van der Bend and co-workers (31) showed that the active forms of DGK were only localized to the DAG pools generated by transmembrane receptor activation. Such evidence suggests that cellular regulation of DGK activity occurs by altering the activity of DGKs by translocating them to specific membrane regions where DAG has accumulated. The activation of the non-receptor tyrosine kinase c-Src, by GPCRs, has been shown by many investigators to control the assembly of multiprotein signaling entities that profoundly affect important functions such as cell growth and migration (26, 32, 33). Through its activity state-dependent multidomain structure Src provides an ideal initiation point for constructing signaling molecule complexes. Thus possessing the capacity to bind to downstream effectors and substrates, c-Src allows GPCRs that control its activation to coherently assemble multiprotein signaling superstructures. As we have shown, GnRH stimulation of SCL60 and L β T2 cells induces a protracted activation of the non-receptor tyrosine kinase c-Src (Fig. 1). We assessed whether other members of the Src family of kinases were activated in these cell types. The tyrosine kinase Fyn was the only other Src family kinase significantly expressed in both cell types. GnRH stimulation failed to induce either activation of Fyn catalytic activity or association between Fyn and DGK- ζ (data not shown). c-Src undergoes significant ultrastructural changes during its activation process that involves tyrosine

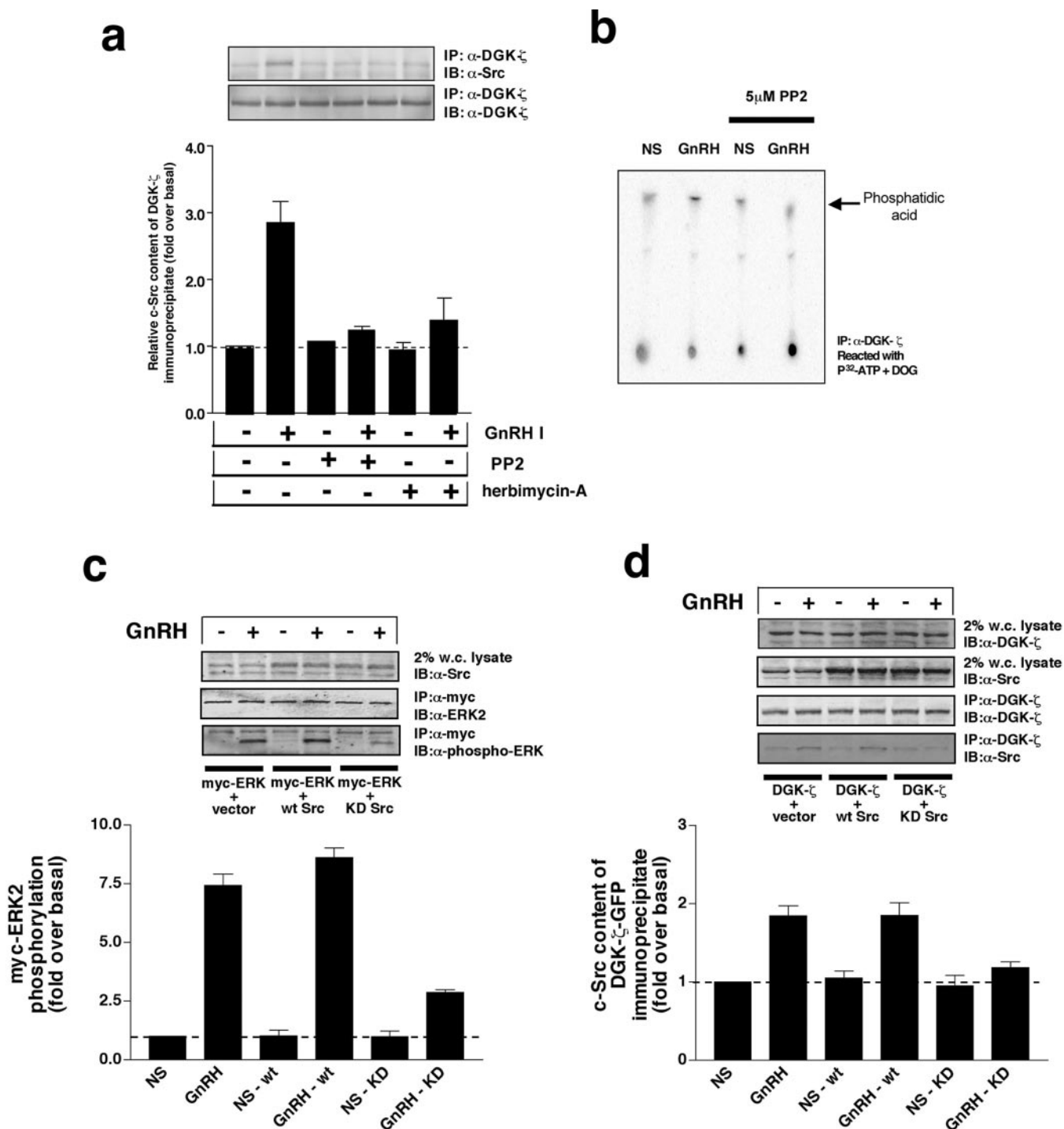


FIG. 6. Inhibition of tyrosine kinase activity disrupts GnRH-induced Src and DGK- ζ association and activity. *Panel a*, GnRH-induced (100 nM, 10 min) co-precipitation of c-Src with endogenous DGK- ζ is inhibited by pretreatment with the c-Src tyrosine kinase inhibitors, PP2 (5 μ M, 30 min) or herbimycin-A (100 nM, 60 min). *Panel b*, GnRH-induced (100 nM, 10 min) activation of endogenous, immunoprecipitated DGK- ζ lipid kinase catalytic activity is inhibited by cellular pretreatment with PP2 (5 μ M, 30 min). *Panel c*, GnRH-induced (100 nM, 10 min) activation of myc-ERK2 activation is supported by wt-Src co-expression and inhibited by KD-Src co-expression. *Panel d*, GnRH-induced (100 nM, 10 min) association of endogenous Src with DGK- ζ -GFP is not affected by overexpression with wt-Src but is significantly inhibited upon overexpression of KD-Src. Histograms associated with each panel depict mean \pm S.E. data from at least three individual replicates for the respective experiments. NS, nonstimulated; IP, immunoprecipitated; IB, immunoblotted.

autophosphorylation at tyrosine 418 and dephosphorylation at the carboxyl-terminal regulatory tyrosine residue. In addition, it is typical of c-Src activation that the activity of the kinase domain is maintained through the subsequent binding to either substrates or other proteins possessing appropriate interacting domains, such as polyproline regions (20). For example, the catalytic activity of Src family non-receptor tyrosine kinases can be greatly potentiated by its binding to the polyproline-rich

region of Nef-1 via its SH3 domain (34–36). Along with the GnRH-induced c-Src activation we noted an increase in the extent of active Src associated with the actin cytoskeleton (Fig. 1, *panel d*). Thus perhaps the GnRH-induced interaction with the cellular cytoskeleton may in some part dictate the time scale and extent of c-Src activity. When the cytoskeleton was functionally disturbed we found that GnRH-induced c-Src activation was largely abolished (17). The functional interaction

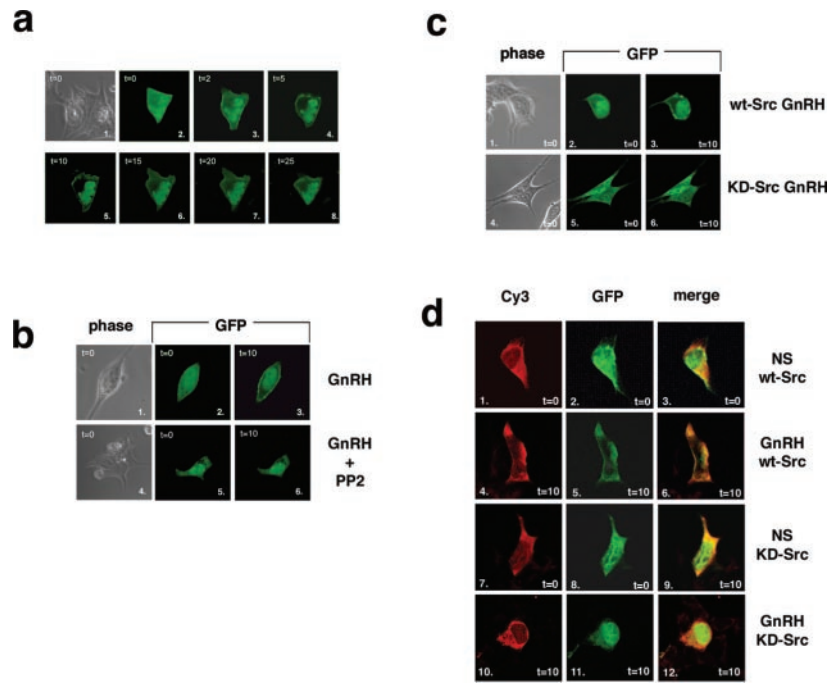


FIG. 7. GnRH-induced plasma membrane translocation of GFP-tagged DGK- ζ in L β T2 murine gonadotrope cells. Panel a, GnRH stimulation (100 nM) for the time period specified ($t = 0, 2, 5, 10, 15, 20$ and 25 min, respectively) mobilizes transfected DGK- ζ -GFP. Image 1 ($t = 0$ min) depicts the phase contrast image of the cell under observation, subsequent images (2–7) represent the input into the GFP-fluorescent channel. Translocation of DGK- ζ from the cytoplasm to the plasma membrane, but not the nucleus, is apparent from 2 min onwards (image 3) and begins to recede back into the cytoplasm by 20 min (image 7). Panel b, control GnRH-induced (100 nM, 10 min) DGK- ζ -GFP translocation (images 1–3) was significantly inhibited by pretreatment with PP2 (5 μ M, 30 min: images 4–6). Panel c, GnRH-induced (100 nM, 10 min) DGK- ζ -GFP translocation is supported by co-expression with wt-Src (images 1–3), whereas co-expression with KD-Src attenuates the GnRH-induced translocation (images 4–6). In fixed L β T2 cells (panel d), with no GnRH stimulation, wt-Src immunoreactivity (visualized with anti-FLAG-Cy3) and DGK- ζ -GFP are distributed throughout the cytoplasm (images 1–3). With GnRH stimulation (100 nM, 10 min) wt-Src and DGK- ζ -GFP are co-localized at the plasma membrane (images 4–6). Expression of KD-Src with DGK- ζ -GFP (images 7–12) inhibits the GnRH-induced (100 nM, 10 min) translocation of DGK- ζ -GFP.

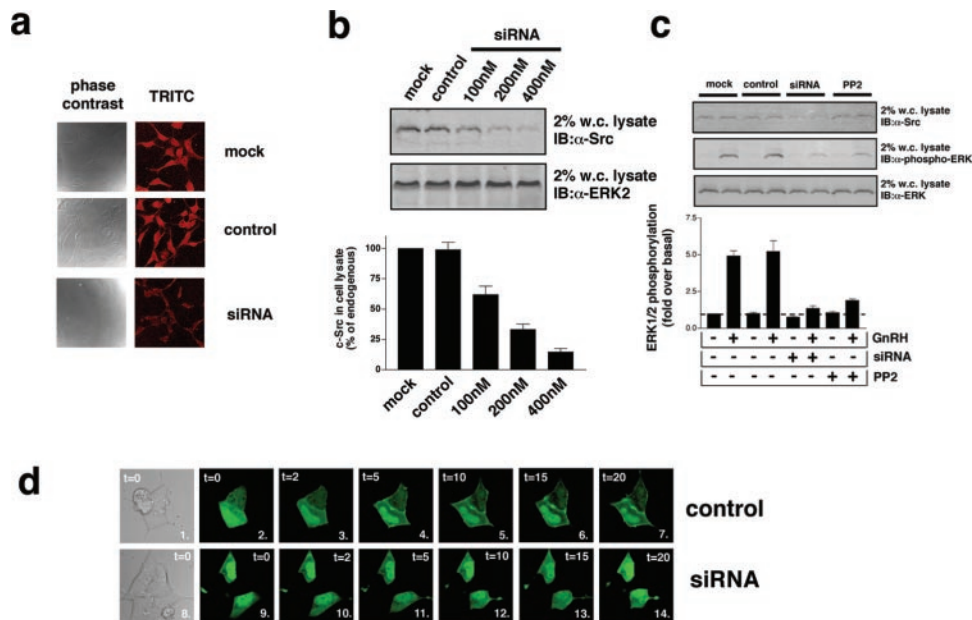


FIG. 8. c-Src siRNA duplexes attenuates GnRH-induced DGK- ζ -GFP translocation. Transfection of L β T2 cells with c-Src siRNA duplexes (siRNA: 200 nM) and not plasmid DNA (mock) or nonsense siRNA duplexes (control) reduces the expression of endogenous c-Src visualized with immunohistochemistry (panel a). Src siRNA transfection of L β T2 cells decreases cellular levels of Src in a concentration-dependent manner. Panel b, transfection with Src siRNA (200 nM) inhibited GnRH-induced (100 nM, 10 min) myc-ERK2 activation to a similar degree as PP2 pretreatment (5 μ M, 30 min). Panel d, GnRH-stimulated (100 nM) DGK- ζ -GFP translocation is not affected by control siRNA duplex (images 1–7) but is inhibited by transfection with Src siRNA duplexes (images 8–14). Histograms associated with various panels depict mean \pm S.E. data from three experimental replicates of the respective experiments. IB, immunoblotted.

between c-Src and other cytoskeleton-associated proteins may therefore be vital for control of downstream effects of GnRH receptor-induced tyrosine kinase signaling. We studied the

range of c-Src co-precipitating proteins after GnRH stimulation and noted an increase in the number of total proteins linked to c-Src. We subjected one protein, demonstrating a high GnRH-

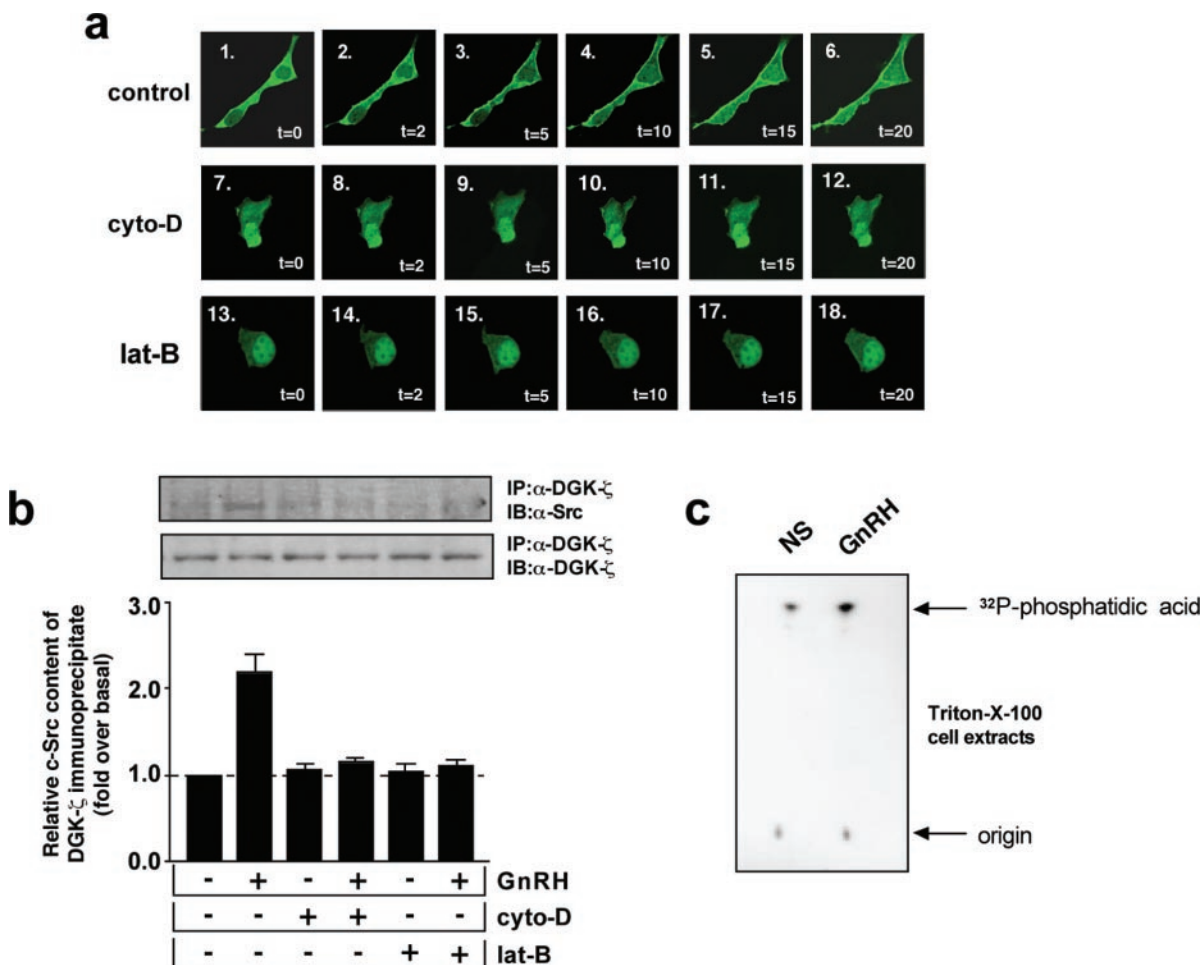


FIG. 9. Active cytoskeletal dynamics are required for GnRH-induced activation of DGK- ζ . Panel *a*, normal GnRH-induced (100 nM) DGK- ζ -GFP plasma membrane translocation (images 1–6, $t = 0$ –20 min) is inhibited by cellular pretreatment (60 min) with 1 μ M cytochalasin D (*cyto-D*; images 7–12; $t = 0$ –20 min) or 1 μ M latrunculin B (*lat-B*; $t = 0$ –20 min). Panel *b*, GnRH-induced (100 nM, 10 min) co-immunoprecipitation of c-Src with endogenous DGK- ζ is inhibited by pretreatment with cytochalasin D (1 μ M, 60 min) or latrunculin B (1 μ M, 60 min). The associated histogram represents mean \pm S.E. c-Src content of DGK- ζ immunoprecipitated data from three separate experiments. Panel *c*, GnRH stimulation promotes the association of catalytically active, inducing 32 P inclusion into phosphatidic acid, DGKs with crude Triton X-100 cytoskeletal preparations. NS, nonstimulated; IP, immunoprecipitated; IB, immunoblotted.

induced c-Src co-precipitation, to MALDI-TOFF mass spectrometry (Fig. 2). The protein was identified as human DGK- ζ . To verify this proteomic-based finding we demonstrated that with direct immunoprecipitation in SCL60 or L β T2 cells there was a GnRH-induced increase in association between active c-Src and DGK- ζ (Figs. 3 and 4). Only one other report has shown that DGK can interact with c-Src, yet this was the α -form of the DGKs, which shows considerable differences in its modular structure and primary sequence compared with DGK- ζ (37). It is of interest that the human DGK- ζ sequence possesses a canonical polyproline stretch between Pro⁷⁶¹–Pro⁷⁷⁹, such a large proline stretch, however, is not present in DGK- α . In addition, the sequence of DGK- α does not contain any consensus SH2 or protein tyrosine binding (PTB) domains (37). In accordance with Cutrupi *et al.* (37) we also found there was no significant agonist-induced tyrosine phosphorylation of the DGK. It seems likely that the large DGK- ζ polyproline-rich region may facilitate the c-Src-DGK- ζ interaction, however, this will be addressed in a subsequent publication. In addition to the GnRH-induced c-Src-DGK- ζ association, we also demonstrated that GnRH enhanced the catalytic activity of DGK- ζ in both SCL60 and L β T2 cells (Fig. 5). The stimulation of DGK- ζ activity by other GPCRs, such as the G α_q -coupled muscarinic acetylcholine receptor has been shown (30). Activation of DGK occurs, in part, via the production of DAG at the plasma mem-

brane, acting as a substrate for the activated kinase. As with hepatocyte growth factor-induced stimulation of DGK- α (37) we noted that catalytic activation of DGK- ζ was dependent upon receptor-induced c-Src activation (Fig. 9). Therefore c-Src and DGK activation may have some general synergy relying upon their physical association. This association may facilitate the subcellular translocation required for the eventual activation of DGK- ζ (31), which is mediated in part by its association with its substrate, DAG. Therefore any process that can expedite this association may then promote a significant increase in DGK- ζ activity. We demonstrated that the GnRH-induced physical association between c-Src and DGK- ζ and the activation of DGK- ζ itself was dependent on the catalytic activity of c-Src (Fig. 6). The GnRH-induced c-Src-DGK- ζ association may therefore facilitate the DGK- ζ translocation to the plasma membrane where it can interact with DAG thus generating the signaling metabolite PA. To this end we demonstrated that the GnRH-induced plasma membrane translocation of DGK- ζ was attenuated by pretreatment with the selective c-Src inhibitor PP2 (Fig. 7), expression of a kinase-deficient c-Src (Fig. 7), and by selective silencing of the murine c-Src gene (Fig. 8).

We have demonstrated that with GnRH stimulation there was a significant increase in the amount of active c-Src associated with the actin cytoskeleton (Fig. 1) and that this is accompanied by a GnRH-induced increase in the DGK catalytic

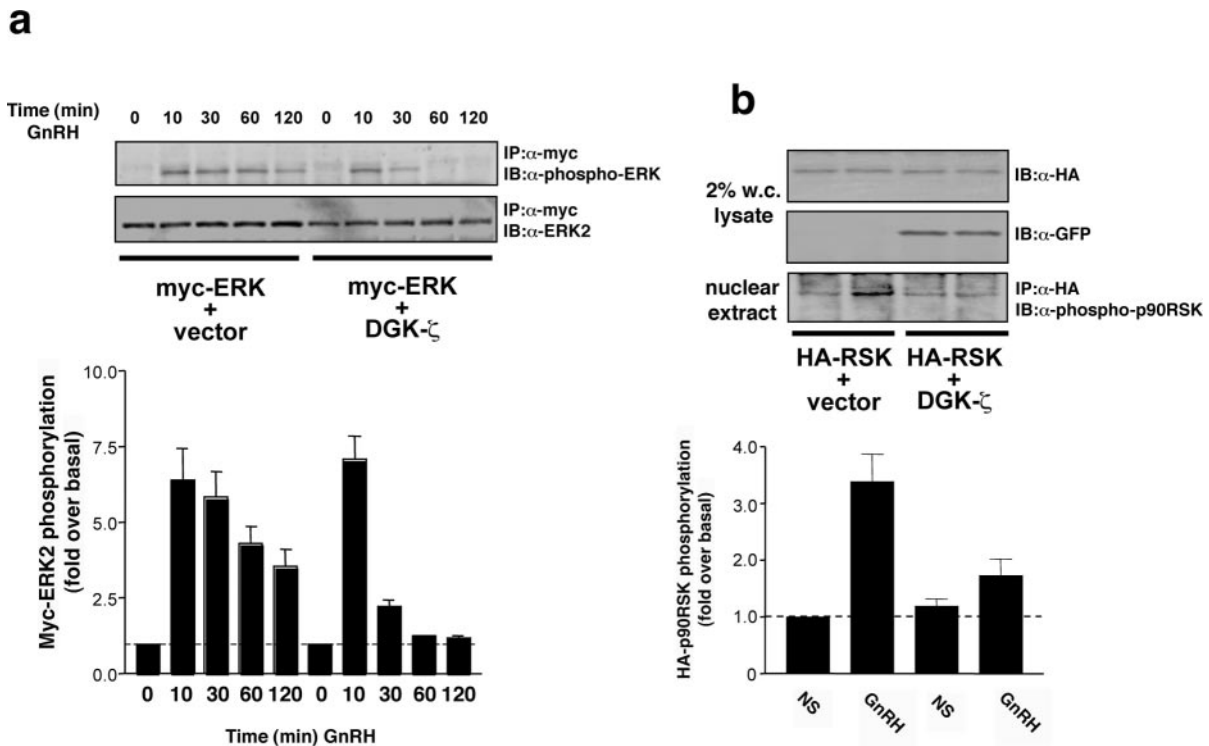


FIG. 10. Overexpression of wild-type DGK- ζ in murine gonadotropes causes alteration of the temporal nature of MAPK activation. *Panel a*, the temporal nature of GnRH-induced (100 nM, time periods specified) activation of transfected myc-ERK2 is attenuated upon co-transfection of DGK- ζ -GFP with myc-ERK2 instead of empty plasmid vector. The associated histogram represents myc-ERK2 time scale activation data, mean \pm S.E. *Panel b*, GnRH-induced (100 nM, 45 min) activation of nuclear p90RSK is inhibited when HA-p90RSK is co-transfected with DGK- ζ -GFP instead of empty plasmid vector. The upper two representative Western blots from 2% of the whole cell (*w.c.*) cytoplasmic L β T2 lysate are shown. The lowest Western blot represents an anti-HA immunoprecipitate from nuclear extracts immunoblotted for anti-active p90RSK. The associated histogram represents mean \pm S.E. p90RSK phosphorylation in nuclear extract data from three separate experiments. NS, nonstimulated; IP, immunoprecipitated; IB, immunoblotted.

activity in cytoskeletal preparations (Fig. 9). This suggests that upon GnRH stimulation, c-Src, in part via its association with both the cytoskeleton and DGK- ζ , may enable translocation of the DGK- ζ to the plasma membrane along the actin cytoskeleton, to allow it to subsequently interact with DAG. Supporting this hypothesis, disruption of the cytoskeleton with cytochalasin D or latrunculin B inhibited GnRH-induced translocation of DGK- ζ (Fig. 9). Therefore a rudimentary sequence of events may be surmised from our data, *i.e.* GnRH stimulation first activates c-Src, which subsequently facilitates the coupling of DGK- ζ to the actin cytoskeleton and then this complex can translocate to the cell membrane where it generates PA and down-regulates the levels of DAG. Alteration of the levels of plasma membrane-derived DAG may not just attenuate cell signaling but also entrain signaling pathways divergent from the original G α_q -PLC- β pathway. DAG can act as a membrane recruitment signal and activator for a series of signaling proteins, *e.g.* it has been shown that plasma membrane DAG is vital for the activation of some PKC isoforms (38), UNC-13 (39), RasGRP (40), and chimeras (41). The DAG-mediated control of these signaling proteins underlies its role in affecting cell proliferation, differentiation, and apoptosis. The regulation of these DAG-controlled signaling proteins is affected by the conversion of DAG to its metabolite PA. As mentioned previously, there are now many known subcellular effectors of PA (8–12), therefore DGKs may profoundly control subcellular localization of many kinases and their signaling capacity through a bidirectional control of both DAG and PA. When we studied the effects of DGK- ζ overexpression on downstream signaling in the L β T2 cells we noted a major alteration in the time course of GnRH-induced MAPK activation. With overexpressed DGK- ζ , accelerating DAG degradation and elevating PA levels, we

showed that the time course of ERK1/2 activation was significantly diminished (Fig. 10). When we assessed the protein kinase C dependence of the ERK1/2 stimulation at later time points we noted that there was a significant contribution of PKC to ERK1/2 activation, whereas at earlier time points there was a greater reliance upon tyrosine kinase activity (data not shown). This suggests that in these cells the temporal nature of ERK1/2 activation is, in part, controlled by the relative levels of DGK- ζ activity. We studied the resultant effect of this attenuated ERK signaling and demonstrated that there was a diminution of GnRH-induced active p90RSK in the nuclei of L β T2 cells overexpressing DGK- ζ -GFP compared with cells not overexpressing DGK- ζ -GFP (Fig. 10). Therefore the shortened time scale of GnRH-induced ERK activation attenuated the eventual nuclear p90RSK phosphorylation, which could translate into an alteration of the transcriptional activity of GnRH receptor activation. The primary role of ERK1/2 in these cells, and in anterior pituitary tissue, is to control the transcription of luteinizing hormone- β subunit, thus eventually controlling LH secretion and reproductive function. Therefore, it is possible that DGK expression and activity could have a role in controlling the signaling proteins involved in the cellular actions of the chronically signaling Type I GnRH receptor, which possesses no intrinsic control over the time course of its own G protein signaling.

In this article we have shown that upon GnRH receptor activation there is a rapid activation of c-Src, followed by its association with the actin cytoskeleton and also DGK- ζ . The GnRH-induced c-Src-DGK- ζ association affects the catalytic activation of this lipid kinase in both SCL60 and L β T2 cells. The formation of this multiprotein complex appears to underlie the mechanism by which GnRH induces the DGK- ζ plasma

membrane translocation observed in L β T2 gonadotropes. Thus the control of downstream, G α_q -mediated, signaling may be controlled by the relative expression and activity of DGK- ζ in these cells. Expression and activation of DGK- ζ may therefore affect the sensitivity of the pituitary to GnRH and affect the eventual control of reproductive function via the modulation of LH transcription and release into the systemic circulation.

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