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Dependence of Stimulus-Transcription Coupling on Phospholipase D in Agonist-stimulated Pituitary Cells

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Stimulation of phospholipase D activity is frequently observed during agonist activation of Ca^{2+}-mobilizing receptors, but the cellular functions of this signaling pathway are not well defined. Pituitary gonadotrophs express Ca^{2+}-mobilizing receptors for gonadotropin-releasing hormone (GnRH) and endothelin (ET), activation of which stimulates luteinizing hormone secretion and transient expression of c-fos. In pituitary cells and αT3–1 gonadotrophs, GnRH action was associated with both initial and sustained diacylglycerol (DG) production, whereas ET-1 induced only a transient DG response. Also, phospholipase D activity, estimated by the production of phosphatidylethanol from phosphatidylcholine in the presence of ethanol, was stimulated by GnRH but not ET-1. Such formation of phosphatidylethanol at the expense of phosphatidic acid (PA) during GnRH-induced activation of phospholipase D significantly reduced the production of PA, DG, and cytidine diphosphate diacylglycerol. Inhibition of PA-phosphohydrolase activity by propranolol also decreased GnRH-induced DG production and, in contrast to ethanol, increased PA and cytidine diphosphate diacylglycerol levels. The fall in DG production caused by ethanol and propranolol was accompanied by inhibition of GnRH-induced c-fos expression, whereas agonist-induced luteinizing hormone release was not affected. In contrast to their inhibitory actions on GnRH-induced early gene expression, neither ethanol nor propranolol affected ET-1–induced c-fos expression, or GnRH- and ET-1–induced inositol trisphosphate/Ca^{2+} signaling. These findings demonstrate that phospholipase D participates in stimulus-transcription but not stimulus-secretion coupling, and indicate that DG is the primary signal for this action.

INTRODUCTION

Stimulation of both phospholipase C and phospholipase D is commonly observed in cells activated by plasma-membrane tyrosine kinase and G protein-coupled Ca^{2+} mobilizing receptors (Exton, 1990; Cook and Wakelam, 1992; Boarder, 1994). Several models for the coordinated activation of the two phospholipases have been proposed, but that involving protein kinase C in the integration of phospholipase D into the phospholipase C–dependent signaling pathway is the best defined (Billah *et al.*, 1989; Conricode *et al.*, 1992; Llahi and Fain, 1992; Balboa *et al.*, 1994; Zheng *et al.*, 1994). The phosphatidic acid (PA) formed during hydrolysis of phosphatidylcholine (PC) by phospholipase D, and the diacylglycerol (DG) produced during phospholipase C-mediated hydrolysis of PIP_2, are interconverted by the actions of DG-kinase and PA-phosphohydrolase. These products of receptor-stimulated phospholipase activity can be further metabolized through the cytidine diphosphate (CDP)-DG phosphoinositide cycle, or the lysophosphatidic acid and CDP-choline pathways. In the presence of ethanol, phospholipase D catalyzes a transphosphatidylation mechanism that leads to the formation of phosphatidylethanol (PEt), and this reaction is commonly used as a specific assay for phospholipase D activity in agonist-stimulated cells (Billah *et al.*, 1991; Liscovitch, 1991).

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In contrast to these well defined aspects of PC metabolism, the cellular functions of phospholipase D are still uncertain and the products of its activation that act as intracellular messengers remain speculative. Although PA is converted to DG, a potential activator of protein kinase C (Nishizuka, 1992), the fatty acid residues of the DG species produced from PC differ from those of DG derived from PIP_2 and may not be associated with activation of protein kinase C (Martin *et al.*, 1990). In addition, PA itself rather than DG has been suggested to act as a second messenger in the control of several cellular functions. These include the mediation of phospholipase D–dependent vesicular traffic, activation of phosphatidylinositol-4-phosphate kinase, and stimulation of Ca^{2+} entry and exocytosis (Putney *et al.*, 1980; Moritz *et al.*, 1992; Haslam and Coorssen, 1993; Stutchfield and Cockcroft, 1993; Cockcroft *et al.*, 1994; Jenkins *et al.*, 1994). In cells that do not secrete by exocytosis, both phospholipase D–derived DG and PA have been suggested to participate in the control of hormone release (Liscovitch and Amsterdam, 1989; Bollag *et al.*, 1990). Furthermore, DG, as well as PA and lysophosphatidic acid, have also been proposed to act as mitogens (Yu *et al.*, 1989; Fukami and Takenawa, 1992; Carnero *et al.*, 1994a) and PA induces early response gene expression in A431 human carcinoma cells (Moolenaar *et al.*, 1986). The fact that these cellular functions are also controlled by products of the phospholipase C pathway has complicated the analysis of the role of phospholipase D in their regulation.

This report examines the role of phospholipase D–derived signaling molecules in stimulus-transcription and -secretion coupling in anterior pituitary cells and immortalized αT3–1 gonadotrophs. Both cell types express gonadotropin-releasing hormone (GnRH) and endothelin (ET_A) receptors, activation of which is associated with robust increases in phospholipase C activity with markedly different kinetics. GnRH induces a biphasic increase in inositol phosphate/DG production in αT3–1 cells, with an early peak (within 1 min) followed by a second, sustained increase for about 60 min (Morgan *et al.*, 1987; Chang *et al.*, 1988; Horn *et al.*, 1991; Zheng *et al.*, 1994). In contrast, ET-1 induces a rapid and monophasic inositol phosphate response that lasts for only a few minutes (Stojilkovic *et al.*, 1990a, 1992a). Activation of GnRH receptors is associated with increased expression of early response genes in both αT3–1 gonadotrophs and cultured pituitary cells (Cesnjaj *et al.*, 1994). GnRH and ET-1 also stimulate the secretion of luteinizing hormone (LH) in cultured pituitary cells, whereas αT3–1 gonadotrophs constitutively produce glycoprotein hormone α-subunit and do not exhibit regulated secretion (Stojilkovic *et al.*, 1990a; Ben-Menahem *et al.*, 1992). The present findings indicate that agonist activation of GnRH but not ET_A receptors is associated with stimulation of phospholipase D activity and increased production of DG that leads to amplification of c-fos expression.

MATERIALS AND METHODS

Cell Culture

αT3–1 cells were grown in DMEM supplemented with 10% fetal bovine serum. After reaching confluence, the cells were subcultured by trypsin dissociation and plating in 60-mm petri dishes (10×10^6 viable cells in 10 ml medium). Before stimulation, on the 3rd or 4th day of culture and when the cells reached 70–80% confluence, the cultures were serum deprived by washing twice in DMEM, followed by a 12- to 16-h incubation in DMEM. Primary cultures of anterior pituitary cells were prepared from adenohypophyses obtained from normal random cycling adult female Sprague-Dawley rats (200–250 g; Charles River, Wilmington, MA). Isolated anterior pituitary cells were prepared by trypsin digestion and physical dispersion (Stojilkovic *et al.*, 1989) and cultured in M199 + 10% horse serum.

DG Assays

Cells were cultured in 4-well plates for 2–3 days, and their medium was replaced by DMEM containing 0.1% bovine serum albumin (BSA) 1–2 h before experiments and again immediately before stimulation. Agents were added in 0.5 ml DMEM/0.1% BSA and incubations were terminated by removing the medium and adding 0.5 ml of dry-ice cold methanol. The methanolic cell suspension was transferred to 15 ml polypropylene tubes. Lipids were extracted by a modification of the method of Bligh and Dyer (1959) and DG was assayed by a modification of the DG kinase assay described by Preiss *et al.* (1986). After addition of chloroform (2 ml), methanol (1.1 ml), and 1 M NaCl (1.75 ml), the tubes were vigorously vortexed and the water and lipid phases were separated by centrifugation ($800 \times g$ for 10 min). The lower phases were then transferred into a new set of tubes and evaporated under nitrogen. The dried lipid extracts were solubilized by sonication in 20 μl of a solution containing octyl-β-D-glucoside (7.5%) and cardiolipin (5 mM) in 1 mM DETAPAC solution (pH 6.6).

After incubation for 15 min at room temperature, 1 μCi [γ-^{32}P]ATP was added in 80 μl of an assay mixture containing final concentrations of 0.5 mM ATP, 0.08 U/ml DG kinase in *Escherichia coli* membrane extract, 2 mM dithiothreitol, 0.3 mM DETAPAC, 50 mM imidazole-HCl, 50 mM NaCl, 12.5 mM $MgCl_2$, and 1 mM EGTA (pH 6.6). The reaction was allowed to proceed for 30 min at 25°C with slow shaking and was stopped by adding 3.7 ml ice-cold chloroform-methanol-1% perchloric acid (PCA; 10:20:7, vol/vol/vol). After addition of 1 ml chloroform and 1 ml 1% PCA, the tubes were vigorously vortexed and centrifuged (5 min, $800 \times g$). The chloroform phases were quantitatively transferred into 12×75 mm glass tubes containing 50 μg phosphatidic acid (PA) and dried under nitrogen, then the lipid residues were dissolved in 250 μl aliquots of chloroform-methanol (19:1, vol/vol). Fifty-microliter aliquots of the samples were spotted on Silica Gel 60 thin layer chromatography (TLC) plates (Merck, Darmstadt, Germany), which were developed in hexane-ethyl ether-acetic acid (35:15:1, vol/vol/vol) for 40 min and then in chloroform-methanol-acetic acid-water (40:10:10:1, vol/vol/vol/vol) for 30 min in the same direction. After chromatography, the PA spots were identified by autoradiography and iodine vapor, scraped into vials with 250 μl water and 5 ml hydrofluor, and assayed for ^{32}P content in a Beckman LS 9000 Liquid Scintillation Counter (Irvine, CA). Standard amounts of 1-stearoyl-2-arachydonyl-*sn*-glycerol (0–1, 500 pmol/tube) were subjected to the same procedure as the αT_3-1 cell lipid extracts, including the two-step TLC sequence. The DG contents of the cell samples were determined from the log-logit transformation of the standard curve.

CDP-DG Assay

Culture wells were cultured in 4-well dishes for 2–3 days as described above and [^{3}H]CDP-DAG formation was detected by a modification of the method of Watson and Godfrey (1988). Cells were incubated in 0.45 ml of DMEM containing 0.1% BSA at 37°C for 60 min with [^{3}H]cytidine (5 μCi/ml). Fifty microliters of 100 mM LiCl was then added, followed 10 min later by 55 μl of medium with agonist. Incubations were continued for up to 60 min and stopped by addition of 0.5 ml dry ice-cold methanol. Cells were scraped from the plates and lipids were extracted by vigorous vortexing with 2.75 ml chloroform/methanol/water (1:2:0.75, vol/vol/vol). After mixing with chloroform (1 ml) and water (1 ml), the samples were centrifuged at 800 × *g* for 10 min. The lower phase was then transferred into a new set of 15-ml tubes and washed with 4 ml methanol/1 N HCl (1:1, vol/vol). Aliquots of the lipid phases containing [^{3}H]CDP-DG were dried under nitrogen and analyzed by liquid scintillation spectrometry after dissolving in Econofluor-2.

PA and PEt Assays

Two days after cell preparation, the medium in 35-mm culture dishes was changed to 1.1 ml DMEM containing 0.1% fatty acid-free BSA, L-glutamine, glucose (4.5 g/l), $NaHCO_3$ (1.4 g/l), and 5 μM [^{3}H]oleic acid. After 16–24 h of incubation, stimuli were added to the culture dishes in 120 μl of the above medium in the presence or absence of 0.5% ethanol for the indicated times. Treatments were terminated by placing the dishes on ice, followed by removal of the medium and rinsing the dishes with 1 ml ice-cold saline. Dry-ice cold methanol (0.75 ml) was then added and the cells were scraped from each dish, followed by washing with 0.75 ml cold methanol. Chloroform (1.5 ml) and a mixture of 0.1 N HCl/1 mM EGTA (1.5 ml) were added, and the tubes were vigorously mixed and centrifuged (400 × *g*). The chloroform phases were collected in 12 × 75 mm glass tubes containing standards of PA and PEt (10 μg each) and dried either under nitrogen or by centrifugation under vacuum.

Samples were redissolved in 50 μl of a mixture of chloroform/methanol (9:1, vol/vol) and spotted onto Silica Gel 60 TLC plates, which were developed in the organic phase of a mixture of ethyl acetate-2,2,4-trimethylpentane-acetic acid-water (13:2:3:10, vol/vol/vol/vol). PA and PEt standards (10 μg) were spotted on each TLC plate with the lipid samples extracted from the cells. PA and PEt spots were visualized either by autoradiography, for which the TLC plates were treated lightly three times with EN^{3}HANCE spray (DuPont-New England Nuclear, Boston, MA), or by iodine vapor staining. The regions corresponding to the appropriate standards were scraped into scintillation vials and extracted with 1 ml of methanol-HCl (150:1); Hydroflour (9 ml) was added after the iodine stains were extinguished. Samples were kept at room temperature overnight and their radioactivity was measured in a Beckman LS 9000 Liquid Scintillation Counter. The TLC background was determined in parallel to the experimental samples by running extracts of prelabeled but untreated and ethanol-free cultures.

Cytoplasmic Ca^{2+} Measurements

Pituitary cells were plated on cover slips coated with poly-L-lysine and placed in individual petri dishes (2 ml/dish), and incubated for 2 days at 37°C. Subsequently, cells were incubated with 2 μM fura 2-AM for 30 min and washed twice with modified medium 199. The chambers with coverslips were mounted at 24°C on the stage of an Axiovert 135 microscope (Carl Zeiss, Oberkochen, Germany) attached to an Attofluor Digital Fluorescence Microscopy System (Atto Instruments, Rockville, MD). Cells were examined under a 40× oil immersion fluorescence objective. The intensity of the incident light produced by a mercury arc lamp was reduced by a 1/16 neutral density filter and passed through an exciter filter (interchanging 340 and 380 nm). Light emission at 505 nm caused by the 340 and 380 nm beams was detected by a video camera. Mean numerical values of intensities in previously assigned rectangular regions within cells were recorded and ratioed. Changes in $[Ca^{2+}]_i$ are presented as a ratio of fluorescence obtained at 340 nm excitation divided by 380 nm excitation, F(340)/F(380). The calibration curve was constructed as described by Grynkiewicz *et al.* (1985) from two measurements of low and high Ca^{2+} standard solutions. Changes in cytosolic calcium were observed in about 30 cells simultaneously at a sampling rate of 1 point per second.

[^{3}H]Inositol Labeling and Stimulation of Pituitary Cells

On the second day of cell culture in 4-well plates, the medium was replaced by 0.5 ml inositol-free medium 199 with Hanks' salt solution containing 5 μCi *myo*[^{3}H]inositol, $NaHCO_3$ (1.4 g/l), and 0.1% BSA. After 24 h of incubation, the cells were washed three times with inositol-free medium 199 Hanks' salt solution containing 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.4) and 0.1% BSA, and stimulated with 100 nM GnRH. The reactions were performed at 37°C and terminated by the addition of 0.2 ml ice-cold perchloric acid (10% vol/vol). Samples were then kept on ice for 30 min and the cells were removed from each well by scraping, followed by washing with 0.1 ml water. The cell suspensions were transferred into 12 × 75 mm glass tubes and placed on dry ice for 30 min. After thawing, the suspensions were centrifuged at 4°C (800 × *g* for 15 min) and the supernatants were extracted in conical tubes containing 350 μl of a mixture of 1,1,2-trichlorotrifluoroethane and tri-*n*-octylamine (1:1, vol/vol) and 100 μl of 10 mM EDTA. The tubes were vortexed for 3 min and centrifuged for 5 min at 400 × *g*, and the upper phases were transferred into Eppendorf tubes containing 2 μl phenol-red. After neutralization, samples were applied to a high performance liquid chromatography column (Synchropak 100, Thomson, Vienna, VA) and inositol monophosphate (InsP), inositol bisphosphate ($InsP_2$), and inositol trisphosphate ($InsP_3$) were eluted with a linear gradient of ammonium phosphate (2 mM increase/min) as described previously (Zheng *et al.*, 1994). The radioactivity of the effluent was continuously monitored by an on-line radioactive flow detector (Flo-one Beta, IC, Radiomatic Instruments, Tampa, FL).

Secretory Responses

For static cultures, dispersed pituitary and αT3–1 cells were plated at 0.5 × 10^6 cells/well in 24-well plates (Falcon, Oxnard, CA). After 2 days the incubation medium was replaced by warmed Hanks' medium 199 with HEPES and 0.05% BSA, containing selected concentrations of GnRH, ET-1, and inhibitors of phospholipase C activity. After incubation for 3 h at 37°C in a water-saturated atmosphere of 5% CO_2 in air, 0.7 ml medium was carefully aspirated from each well and kept frozen. Column perifusions were performed on 3-day cultured cells under previously reported conditions (Stojilkovic *et al.*, 1990b). Briefly, 2 × 10^7 cells were incubated with preswollen cytodex-1 beads in 60-mm culture dishes, and perifused with Hanks' M199 containing 20 mM HEPES and 0.05% BSA for 60 min at a flow rate of 0.6 ml/min. After agonist stimulation, fractions were collected every minute and stored at −20°C. LH and αLH were determined by radioimmunoassay, using reagents and standards provided by the National Pituitary Agency (Baltimore, MD). LH and αLH responses in static culture were expressed as ng/10^6 cells, and in perifused cells as ng/ml.

Expression of c-fos

For studies on c-fos expression, cells were grown in DMEM (Biofluids, Rockville, MD) supplemented with 10% fetal bovine serum, as described (30). Before stimulation, 70% confluent cultures were serum deprived by washing twice with DMEM, followed by 12–16 h of incubation in serum-free medium. Total cellular RNA was isolated by the guanidine isothiocyanate method (Chomczynski and Sacchi, 1987), and 10- to 20-μg aliquots were electrophoresed on 1%

agarose gels and transferred to Nytran membranes (Schleicher & Schuell, Keene, NH) by capillary blotting. The filters were hybridized initially with a ^{32}P-labeled fos cDNA probe (Clontech, Paolo Alto, CA) and subsequently rehrybridized with a human glyceraldehyde phosphate dehydrogenase (GAPDH) cDNA probe (Clontech) to control for variations in gel loading and transfer efficiency. Hybridization was performed in a 50% formamide hybridization buffer at 42°C for 16 h, using cDNA probes labeled with [^{32}P]dCTP (6000 Ci/mmol, DuPont) by random hexamer extension (Amersham, Arlington Heights, IL); specific activities of 0.5–1 × 10^9 dpm/mg were routinely obtained. Blots were washed at high stringency and exposed to Kodak films at −70°C with intensifying screens for 12–24 h. The c-fos hybridization signals were quantitated with a Phosphorimager and Imagequant software (Molecular Dynamics, Sunnyvale, CA) and normalized to those of GAPDH.

RESULTS

GnRH induced a prolonged DG response in pituitary cells, with a maximum response that peaked at 15–30 min after stimulation (Figure 1, upper panel). A similar profile of DG production was observed in GnRH-stimulated αT3–1 gonadotrophs (Zheng *et al.*, 1994). In contrast, ET-1 induced transient DG formation, with a maximum response after 7 min (Figure 1, upper panel), a profile that was comparable to the GnRH-induced DG response in protein kinase C–depleted αT3–1 gonadotrophs (Zheng *et al.*, 1994). Because the phospholipase D pathway participates in sustained DG production in these cells, the transient effect of ET-1 on DG production indicates that this pathway is not activated by the ET_A receptor. In accord with this, GnRH but not ET-1 caused a significant increase in PEt production in both αT3–1 gonadotrophs and cultured pituitary cells in the presence of 50–300 mM ethanol. As shown in Figure 2, the GnRH-induced PEt response occurred in a time- and concentration-dependent manner. In contrast, addition of ET-1 did not cause a measurable increase in PEt production in the presence of 300 mM ethanol in either αT3–1 gonadotrophs or cultured pituitary cells. Thus, phospholipase D activity is stimulated by agonist activation of the GnRH but not the ET_A receptor in both αT3–1 gonadotrophs and normal pituitary cells.

The production of PEt in GnRH-stimulated cells was associated with a marked decrease in the formation of phospholipase D- but not phospholipase C-dependent products of phospholipid hydrolysis. As shown in Figure 1, bottom panel, GnRH-stimulated production of PA and DG was significantly reduced in the presence of 300 mM ethanol. GnRH-induced accumulation of CDP-DG in αT3–1 gonadotrophs was also reduced by ethanol in a concentration-dependent manner, with an IC_{50} of about 100 mM and maximum inhibition to about 50% of the agonist-stimulated level (Figure 3, A and B). In contrast, GnRH-induced $InsP_3$ (Figure 1, bottom panel) and $[Ca^{2+}]_i$ responses (Figure 4A) were not affected by ethanol. The lack of effect of ethanol on ET-1–induced inositol phosphate responses (Figure 4B) further indicated the selectivity of its action on the phospholipase D pathway. In summary, these data

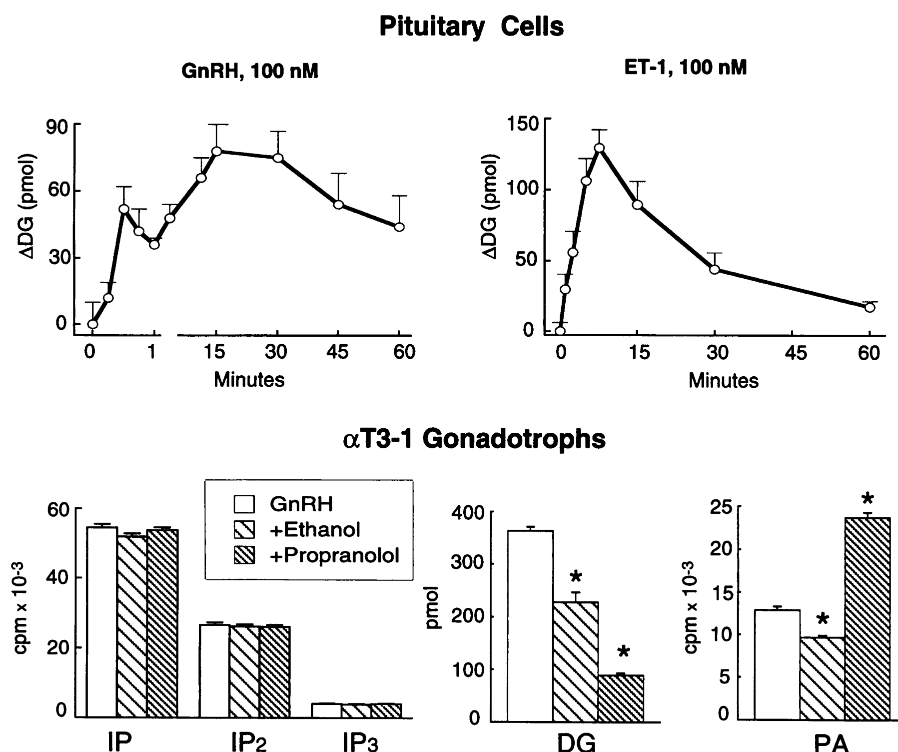

Figure 1. Agonist-induced inositol phosphate/diacylglycerol response in pituitary cells and αT3–1 gonadotrophs. (upper panel) Time course of GnRH- and ET-1–induced DG formation in pituitary cells. (bottom panel) Effects of ethanol (300 mM) and propranolol (50 μM) on $InsP_3$, DG, and PA formation in αT3–1 gonadotrophs.

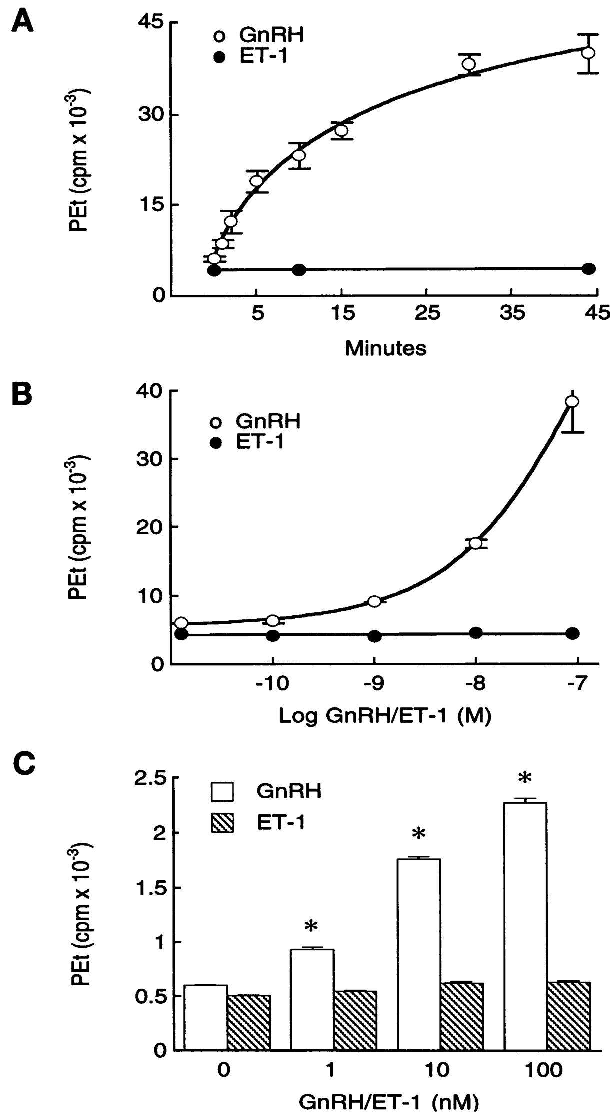

Figure 2. Gonadotropin-releasing hormone (GnRH)- and endothelin-1 (ET-1)–induced phosphatidylethanol (PEt) production in anterior pituitary cells and αT3–1 immortalized gonadotrophs. (A) Time-dependent effects of GnRH (100 nM) and ET-1 (100 nM) on PEt production in T3–1 gonadotrophs. (B and C) Concentration-dependent effects of GnRH and ET-1 on PEt production in αT3–1 gonadotrophs (B) and cultured pituitary cells (C). Cells were stimulated for 60 min. * $p < 0.01$ vs. control (untreated group), estimated by Student's *t*-test.

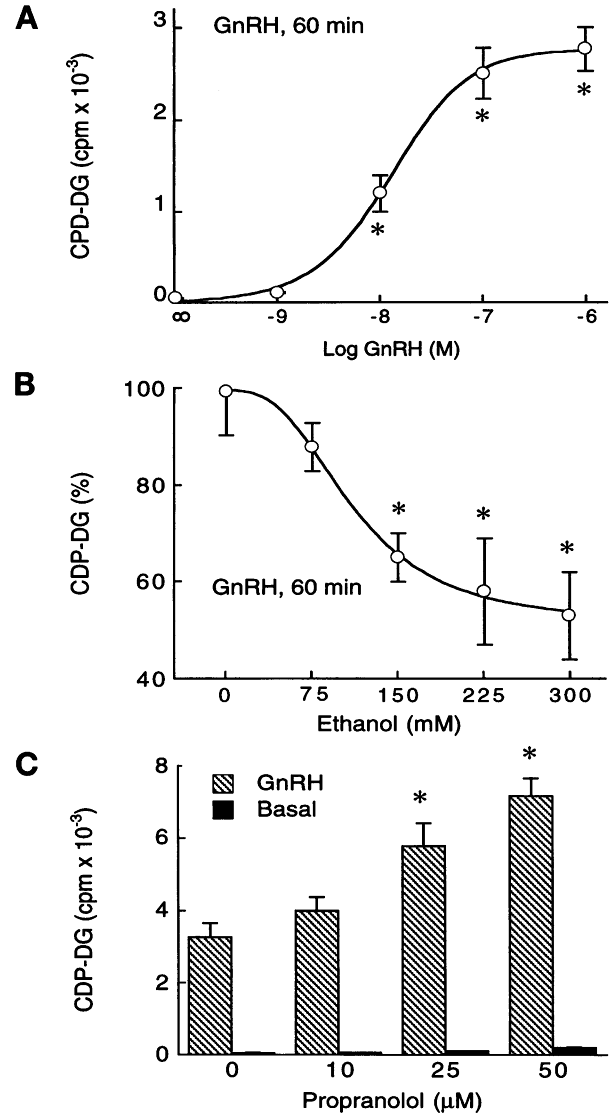

Figure 3. Characterization of cytidine diphosphate diacylglycerol (CDP-DG) responses in GnRH-stimulated αT3–1 gonadotrophs. (A) Concentration-dependent effects of GnRH on CDP-DG production. (B) Inhibitory effect of ethanol on GnRH (100 nM)-induced CDP-DG production. (C) Stimulatory effect of propranolol on GnRH (100 nM)-induced CDP-DG production. In all experiments, cells were stimulated for 60 min. * $p < 0.01$ vs. control (A, unstimulated cells; B and C, cells treated with GnRH only).

demonstrate that ethanol is an effective inhibitor of the phospholipase D–dependent PA response in GnRH-stimulated cells, and thus reduces both PA-phosphohydrolase–dependent production of DG and cytidylyltransferase-dependent formation of CDP-DG.

To further evaluate the role of PA-phosphohydrolase in phospholipase D–dependent signaling, agonist-stimulated αT3–1 gonadotrophs were treated with propranolol. In addition to acting as a β-adrenergic receptor antagonist, propranolol inhibits the activity of PA-phosphohydrolase at micromolar concentrations (Billah *et al.*, 1989; Lavie *et al.*, 1990; Carnero *et al.*, 1994b). In accord with this, GnRH-induced PA accumulation was markedly increased and DG production was reduced in propranolol-treated αT3–1 cells (Figure 1, bottom panel). Propranolol also increased the GnRH-induced CDP-DG response in a concentration-dependent manner (Figure 3C). In the presence of 50 μM propranolol, the GnRH-induced

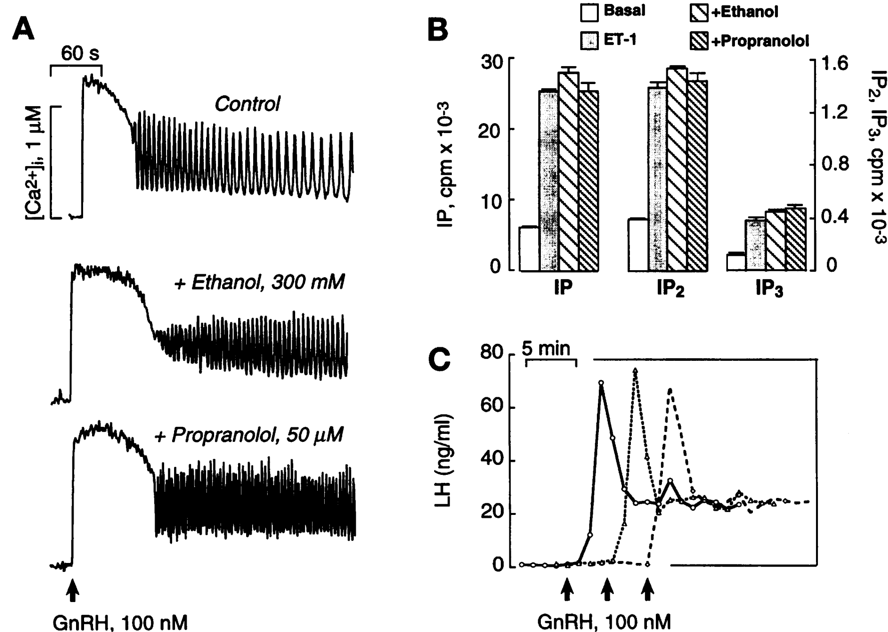

Figure 4. Lack of effects of ethanol and propranolol on inositol phosphate/diacylglycerol formation, Ca^{2+} signaling, and secretory responses in pituitary cells. (A) GnRH-induced Ca^{2+} responses in single gonadotrophs. The tracings shown are representative of 7–10 records per treatment. (B) $InsP_3$/DG formation in ET-1–stimulated pituitary cells in the presence or absence of ethanol (300 mM) and propranolol (50 μM). (C) GnRH-induced LH release in perifused pituitary cells, with or without ethanol and propranolol. Control (—); ethanol (300 mM, ·····) and propranolol (50 μM, - - -) were added 1 min before agonist application.

CDP-DG response doubled and DG formation was reduced by about 70%. On the other hand, the basal CDP-DG level was only slightly increased in propranolol-treated cells (Figure 3C), indicating that the background activities of phospholipases C and D are very low. Finally, propranolol did not significantly affect GnRH-induced $InsP_3$ responses in αT3–1 gonadotrophs (Figure 1, bottom panel) and $[Ca^{2+}]_i$ responses in pituitary cells (Figure 4A). The $InsP_3$ (Figure 4B) and DG responses in ET-1–stimulated pituitary cells were also unaffected by propranolol or ethanol, further indicating that the effects of these agents on phospholipase D products are not secondary to inhibition of the phospholipase C pathway.

These data demonstrate that ethanol and propranolol can be used not only to evaluate the status of phospholipase D activity, but also to manipulate PA, DG, and CDP-DG levels in GnRH-stimulated cells. Based on these observations, we examined the effects of such maneuvers on GnRH-induced gonadotropin secretion. For this purpose, basal and GnRH-induced LH release were measured in cultured pituitary cells in the presence of ethanol or propranolol. As shown in Figure 4C, ethanol and propranolol did not affect the GnRH-induced LH response in perifused pituitary cells during the first 15 min of stimulation. Also, ethanol had no effect on basal or GnRH-stimulated LH release in static cultures during a 3-h stimulation, and propranolol caused only a small increase in basal LH release at the highest (50 μM) concentration employed (Table 1). These results indicate that neither a simultaneous decrease in DG and PA production, nor a decrease in DG production in the presence of increased PA formation, had a detectable effect on stimulus-secretion coupling during the initial and sustained phases of agonist stimulation. Thus, the signaling molecules generated by phospholipase D are not essential for agonist-induced exocytosis in pituitary cells.

Table 1. Lack of effects of ethanol and propranolol on GnRH-stimulated LH release in cultured pituitary cells

Ethanol (mM)	Basal LH (ng/ml)	Stimulated LH (ng/ml)	Propranolol (μM)	Basal LH (ng/ml)	Stimulated LH (ng/ml)
0	14 ± 1	184 ± 6	0	15 ± 1	117 ± 9
75	16 ± 2	185 ± 8	6.25	13 ± 1	115 ± 5
150	15 ± 2	174 ± 12	12.5	18 ± 2	126 ± 2
225	18 ± 3	177 ± 15	25	17 ± 1	137 ± 4
300	13 ± 3	194 ± 10	50	32 ± 2*	121 ± 3

Data are the means ± SE of sextuplicate incubations in one of seven (ethanol) and five (propranolol) similar experiments. Cells were stimulated with 100 nM GnRH for 3 h, and basal LH release was subtracted from total LH release. *$p<0.01$ vs. unstimulated.

Table 2. Effects of GnRH and ET-1 on $InsP_3$, DG, and c-fos responses in pituitary cells and αT3-1 gonadotrophs

Cell type	Treatment	$InsP_3$ (cpm)	DG (pmol)	c-fos (fold-induction)
Pituitary cells	GnRH, 100 nM	684 ± 27	145 ± 12	7.1 ± 1
	ET-1, 100 nM	5450 ± 50*	249 ± 13*	7.2 ± 0.7
αT3-1 cells	GnRH, 100 nM	4126 ± 11	218 ± 15	7.7 ± 1.2
	ET-1, 100 nM	1027 ± 57*	137 ± 11*	2.9 ± 0.9*

Cells (10^6/well) were stimulated for 10 min in the presence of 10 mM LiCl. *$p<0.01$ vs. GnRH-treated.

In contrast to its lack of involvement in stimulus-secretion coupling, the phospholipase D pathway was found to participate in stimulus-transcription coupling in GnRH-stimulated gonadotrophs. During the initial period of stimulation, ET-1–induced DG and $InsP_3$ responses were several-fold higher than the corresponding GnRH-induced responses (Table 2). This difference reflects the expression of GnRH receptors only in gonadotrophs, which comprise about 20% of the pituitary cell population in ovariectomized rats, and of ET_A receptors in all secretory cells of the pituitary gland (Stojilkovic *et al.*, 1991a). On the other hand, the relative (peak-to-basal) increases in c-fos mRNA levels in ET-1 and GnRH-stimulated pituitary cells were similar (Figure 5A vs. Figure 5B; Table 2). Consistent with the lack of coupling of ET_A receptors to phospholipase D, ethanol had no effect on ET-1–induced c-fos expression in cultured pituitary cells (Figure 5A). Likewise, c-fos expression induced by PMA (activation of protein kinase C) was unaffected by ethanol and propranolol (Table 3). However, addition of ethanol caused a significant and concentration-dependent inhibition of GnRH-induced c-fos expression in cultured pituitary cells (Figure 5B). Furthermore, ethanol inhibited GnRH-induced c-fos expression in αT3–1 gonadotrophs with a calculated IC_{50} (105 mM) comparable to that observed for inhibition of the CDP-DG response (Figure 6A vs. Figure 3B).

Because all signaling molecules of the phospholipase D pathway are reduced in the presence of ethanol, these results do not identify the species that participate in the control of c-fos expression. However, in contrast to ethanol, propranolol differentially suppresses the formation of DG versus PA and CDP-

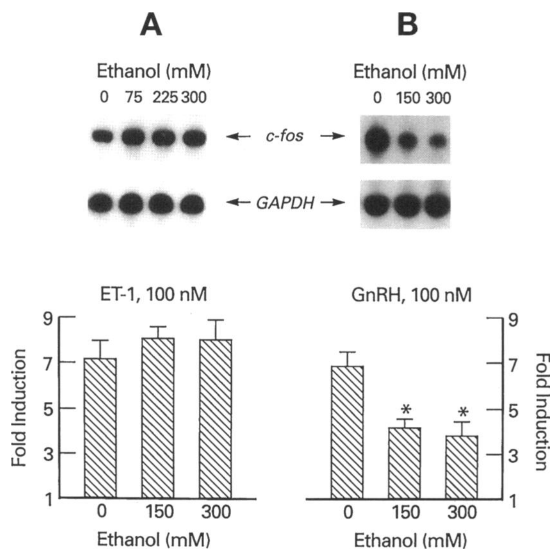

Figure 5. Dependence of agonist-induced c-fos expression on the phospholipase D pathway in cultured pituitary cells. (A) The lack of effect of ethanol on ET-1 (100 nM)-induced c-fos expression. (B) Concentration-dependent effect of ethanol on GnRH (100 nM)-induced c-fos expression. Cells were stimulated with either agonist for 30 min. * $p < 0.01$ vs. control (cells treated with GnRH only). GAPDH, glyceraldehyde 3-phosphate dehydrogenase. Fold induction was calculated as the stimulated over basal level of c-fos mRNA.

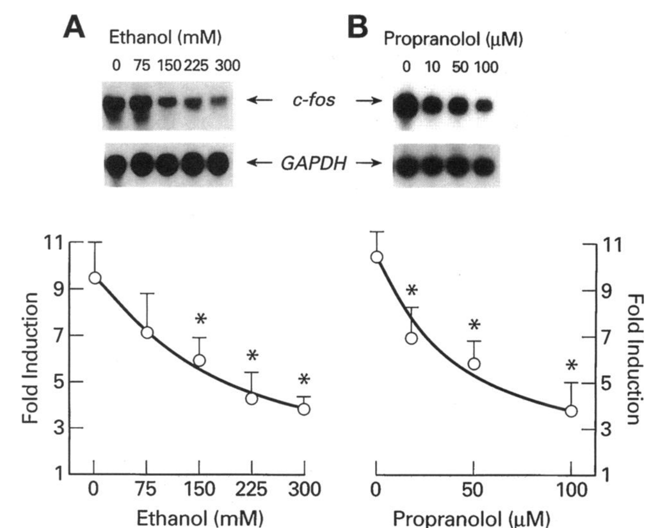

Figure 6. Dependence of GnRH-induced c-fos expression on the phospholipase D pathway in αT3–1 gonadotrophs. (A) Concentration-dependent effect of ethanol on GnRH (100 nM)-induced c-fos expression. Upper panel, representative experiment; lower panel, averaged data (means ± SE) of eight independent experiments. (B) Concentration-dependent effects of propranolol on c-fos expression. Upper panel, representative experiment; lower panel, averaged data, n = 4. * $p < 0.01$ vs. controls (cells treated with GnRH only).

Table 3. Effects of ethanol and propranolol on GnRH- and PMA-induced c-fos expression (fold induction) in αT3-1 gonadotrophs

Treatment	N	Control	+Ethanol (150 mM)	+Propranolol (50 μM)
GnRH, 100 nM	7	10.1 ± 1.5	5.1 ± 1*	5.3 ± 0.9*
PMA, 100 nM	4	13.5 ± 1.8	10.2 ± 2.7[a]	12 ± 2.4[a]

[a] nonsignificant.
* $p<0.05$.

DG. In parallel to the propranolol-induced inhibition of agonist-induced DG formation, GnRH- but not phorbol ester-induced c-fos expression was also significantly reduced. As shown in Figure 6B, propranolol inhibited c-fos expression over the same concentration range at which it reduced DG and CDP-DG formation (Figure 1 and Figure 6B vs. Figure 3C), but had no effect on PMA-induced c-fos expression (Table 3). These data indicate that DG rather than PA mediates phospholipase D–dependent c-fos expression in GnRH-stimulated cells.

DISCUSSION

Activation of phospholipase D is a common response in cells operated by Ca^{2+}-mobilizing receptors (Billah and Anthes, 1990; Thompson *et al.*, 1991). The integration of this pathway into GnRH-induced signaling responses has been demonstrated in ovarian granulosa cells (Liscovitch and Amsterdam, 1989), αT3–1 pituitary gonadotrophs (Netiv *et al.*, 1991; Zheng *et al.*, 1994), and cultured pituitary cells (this study). Thus, stimulation of phospholipase D activity during agonist binding to the GnRH receptor was independent of the cell type in which the receptor is expressed. In contrast, agonist stimulation of ET_A calcium-mobilizing receptors by ET-1 in pituitary cells and αT3–1 gonadotrophs is not associated with activation of phospholipase D. However, this is not typical of the ET receptor signaling pathway in general, because ET-1 stimulates phospholipase D in several other cell types expressing ET_A receptors (Van Der Bend *et al.*, 1992; Zhang and Abdel-Latif, 1992). The reason for the lack of coupling of ET_A receptors to phospholipase D activation in pituitary cells is not clear, but could be related to their extremely rapid desensitization in this cell type (Stojilkovic *et al.*, 1992b).

This difference in the coupling of the two Ca^{2+}-mobilizing receptors in pituitary cells was analyzed to determine the functional importance of the phospholipase D pathway, and to identify which products of this pathway act as intracellular messengers. The physiological importance of the phospholipase D signaling pathway was evaluated for two cellular responses, hormone secretion and c-fos expression. It is well established that activation of GnRH and ET_A receptors is associated with LH secretion (Stojilkovic *et al.*, 1990a, 1991a, 1994; Kanyicska *et al.*, 1991; Samson *et al.*, 1991). Recent studies have demonstrated that GnRH also induces robust c-fos responses in αT3–1 gonadotrophs, cultured pituitary cells, and GT1 neurons (Cesnjaj *et al.*, 1993, 1994). Activation of ET_A receptors in pituitary cells was also found to be associated with transient expression of c-fos. The time-course of c-fos induction was similar for both agonists and was comparable to that observed in other cell types (Vandenplas *et al.*, 1990; Collart *et al.*, 1991; Trejo and Brown, 1991; Werlen *et al.*, 1993), but the level of gene expression was lower in ET-1 than in GnRH-stimulated cells. These data suggest that activation of the phospholipase C pathway is sufficient to initiate c-fos expression, and that the phospholipase D pathway amplifies phospholipase C-induced gene expression.

To evaluate this hypothesis, cells were stimulated in the presence of ethanol or propranolol, agents that were shown to influence the phospholipase D–dependent formation of PA, DG, and CDP-DG in this and other studies (Martinson *et al.*, 1990; Stutchfield and Cockcroft, 1993). In the presence of ethanol, GnRH-induced formation of DG, CDP-DG, and PA was significantly reduced, and GnRH-induced c-fos expression in pituitary cells was inhibited in parallel with these changes. Inhibition of PA-phosphohydrolase activity by propranolol also decreased GnRH-induced DG production but, in contrast to ethanol, increased PA and CDP-DG levels. The concomitant inhibition of GnRH-induced c-fos expression in propranolol-treated cells suggests that DG is the messenger that mediates phospholipase D action on stimulus-transcription coupling.

These observations support the proposal that the coordinate actions of phospholipase C and D are required for sustained DG production, which is in turn responsible for prolonged activation of protein kinase C during agonist stimulation (Nishizuka, 1992). In GnRH-stimulated cells (Cesnjaj *et al.*, 1993, 1994), as in several other cell types (Gilman, 1988; Shaw *et al.*, 1989; Rivera *et al.*, 1990; Fujihara *et al.*, 1993), protein kinase C is an essential element in the control of c-fos transcription. In pituitary cells and GT1 neurons, the

protein kinase C activators, PMA and PDBu, can substitute for GnRH in stimulating c-fos expression. Also, GnRH- and phorbol ester-induced c-fos expression is attenuated by protein kinase C inhibitors, as well as in protein kinase C–depleted pituitary cells (Cesnjaj *et al.*, 1993, 1994). In general, c-fos induction is controlled by two regulatory elements that are located 60 and 300 bp upstream of the transcription initiation site. The first element integrates cAMP and Ca^{2+} signals through the common transcriptional factor CREB, and thus transduces signaling from both cAMP- and Ca^{2+}-mobilizing receptors. The second element mediates serum- and protein kinase C–dependent c-fos expression, and transduces signaling exclusively from Ca^{2+} mobilizing receptors (Doucet *et al.*, 1990; Sheng and Greenberg, 1990). Thus, it is likely that the phospholipase C–dependent actions of calcium signaling and protein kinase C on c-fos expression are reinforced and sustained by phospholipase D–mediated DG production.

Although DG/protein kinase C–dependent stimulation of c-fos expression could mediate the actions of the phospholipase D pathway in GnRH-induced cellular growth and differentiation, the lack of effects of ethanol and propranolol on LH release questions the importance of this pathway in regulated exocytosis. Because protein kinase C has been implicated in the control of sustained GnRH-induced LH release (Naor *et al.*, 1989; Stojilkovic *et al.*, 1991b), these observations imply that the DG produced by phospholipase D, in contrast to that derived from phospholipase C, does not activate the protein kinase C isozymes that are involved in the control of exocytosis. In accord with this, phosphatidylcholine contains mostly oleic and linoleic acids whereas phosphoinositides are relatively enriched in stearic and arachidonic acids (Billah and Anthes, 1990). Furthermore, there is a shift in DG composition from tetraenoic to more saturated fatty acids during the sustained phase of TRH stimulation in GH3 pituitary cells (Martin *et al.*, 1990). Thus, phospholipase D–dependent production of specific molecular forms of DG may activate a different set of protein kinase C enzymes than those responsive to DG released from phosphoinositides by phospholipase C.

Although ethanol and propranolol are not highly specific inhibitors, the concentrations used in the present work have been widely employed in studies on the phospholipase D pathway (Martinson *et al.*, 1990; Fukami and Takenawa, 1992; Kusner *et al.*, 1993; Stutchfield and Cockcroft, 1993; Cockcroft *et al.*, 1994; Gustavsson *et al.*, 1994; Lee *et al.*, 1994). No inhibitory effects of ethanol and propranolol on basal PA, DG, CDP-DG, and c-fos expression were observed in αT3–1 gonadotrophs or cultured pituitary cells. In regard to their possible actions on other elements of the intracellular signaling pathways, GnRH- and ET-1–induced $InsP_3/Ca^{2+}$ responses in these cells were not affected by ethanol or propranolol. Also, ET-1–induced c-fos expression is not inhibited by either compound, further suggesting that the operation of the phospholipase C pathway is not affected. Finally, phorbol ester–induced c-fos expression is not attenuated by ethanol and propranolol, arguing against possible inhibitory effects on protein kinase C. These observations indicate that appropriate concentrations of ethanol and propranolol exert relatively specific inhibitory actions on the phospholipase D pathway, and suggest that many of the effects of ethanol on cellular functions could be related to changes in this pathway.

In conclusion, these results have revealed a marked difference in the coupling of GnRH and ET_A receptors to phospholipase D in pituitary cells. The Ca^{2+} mobilizing action of ET is associated with an elevation in cytoplasmic DG concentration, but this response appears to be mediated exclusively by phospholipase C. In contrast, phospholipase D is integrated into the phospholipase C–dependent signaling pathways of the GnRH receptor, and both lipases participate in the DG response during sustained agonist stimulation. The difference in the coupling of the two receptors to their major effector enzymes, which provides an additional criterion of the selectivity of the actions of ethanol and propranolol, indicates that the phospholipase D signaling pathway participates in GnRH-induced c-fos expression but not in LH release. The parallelism between the changes in DG and c-fos expression in GnRH-stimulated cells suggests that DG rather than PA is the intracellular messenger of the phospholipase D–dependent pathway in stimulus-transcription coupling. Such phospholipase D–dependent activation of c-fos expression is probably also operative in other cell types in which the enzyme is integrated into the agonist-induced intracellular signaling cascade.

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