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Thyrotropin-Releasing Hormone Increases Phospholipase D Activity Through Stimulation of Protein Kinase C in GH_3 Cells

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Activation of phospholipase D was investigated after treatment of GH_3 cells with thyrotropin-releasing hormone. Thyrotropin-releasing hormone treatment resulted in both time- and dose-dependent increases of phospholipase D activity, translocation of protein kinase C-α and -βI isozymes from cytosol to membrane within 30 min, and approx 43-fold increase of phosphatidylinositol-specific phospholipase C activity. Intracellular calcium concentration was rapidly increased and diacyglycerol level remained high up to 3 h after the treatment. Pretreatment of the cells with U73122, a potent inhibitor of phosphatidylinositol-specific phospholipase C, inhibited thyrotropin-releasing hormone–induced phospholipase D activation. Protein kinase C activity was down-regulated by pretreatment of the GH_3 cells with either protein kinase C inhibitors (RO320432, GF109203X) or preincubation of the cells with phorbol myristrate acetate (500 n*M*) for 24 h. This treatment largely abolished the thyrotropin-releasing hormone–induced activation of phospholipase D, thus further confirming the involvement of protein kinase C in the activation. These results suggest that thyrotropin-releasing hormone–induced phospholipase D activation may be due to phosphatidylinositol-specific phospholipase C, and activation of protein kinase C isozymes is responsible for this stimulation.

Key Words: Thyrotropin-releasing hormone (TRH); phospholipase D (PLD); protein kinase C (PKC); GH_3 cell.

Introduction

The hypophysiotropic neuropeptides, thyrotropin-releasing hormone (TRH), act on their respective adenohypophysial target cells to increase the secretion of thyrotropin and prolactin. TRH regulates pituitary hormone secretion through postrecepter mechanisms, involving inositol phosphate generation with Ca^{2+} mobilization and diacylglycerol (DAG) formation with protein kinase C (PKC) activation *(1,2)*. The initial response of pituitary target cells to these neuropeptides is the activation of a phosphoinositol-specific phospholipase C (PI-PLC) via guanine nucleotide-binding regulatory proteins (G-proteins), specifically, $G_{\alpha q}$ and $G_{\alpha 11}$ *(3)*. PLC in turn mediates hydrolysis of phosphatidylinositol-4,5-diphosphate [PtdIns(4,5)P_2] to generate inositol-1,4,5-triphosphate [Ins(1,4,5)P_3] and diacylglycerol *(4)*. Ins(1,4,5)P_3 leads to oscillatory or biphasic elevations in the cytoplasmic calcium concentration *(4)*. In the next step of intracellular signaling pathway, Ca^{2+} and DAG transiently activate PKC, which plays a very important role in maintenance of cellular responses such as growth, proliferation, and hormonal secretion *(5–9)*.

PKC is a major regulator of phospholipase D (PLD) activity in many cell types. PLD is an enzyme to hydrolyze phosphatidylcholine (PC) to phosphatidic acid (PA) and choline. PA and its metabolite, lysophosphatidic acid (LPA), function as second messengers acting on various protein kinases in the control of cell proliferation *(10)*. In addition, PA is subsequently converted to DAG by the action of phosphatidate phosphohydrolase *(11)*. It has been shown that DAG from PLD activation is either saturated or monounsaturated, while the lipids generated by PLC activation are predominantly polysaturated, and PLD-derived DAG does not activate PKC in porcine aortic endothelial cells *(12)*.

PLD is a ubiquitous enzyme in mammalians and activated by agonists such as neurotansmitters, hormones, growth factors, and cytokines. There have been some reports on PLD activation in hormone-secreting cells, and a recent study has shown that PLD activity is increased in thyroid cell lines *(13)*. However, there has been no report to date about TRH effect on PLD activity in GH_3 cells. In the present study, we attempted to investigate the mechanism involved

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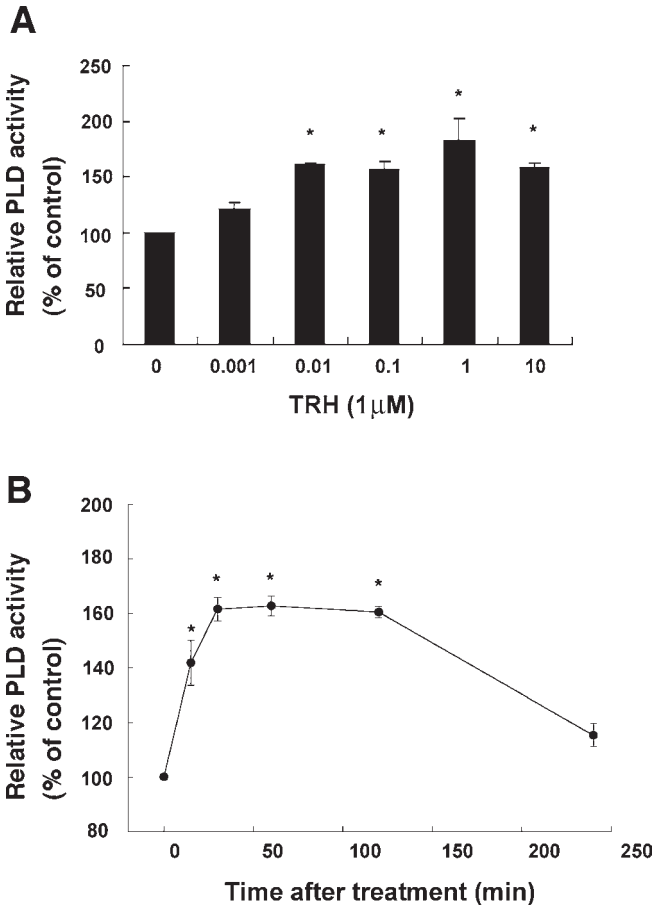

Fig. 1. Concentration- and time-dependent activation of PLD by TRH. (**A**) GH_3 cells (1.5×10^6 cells/well) were labeled with 1 μCi/mL [^{3}H]palmitic acid and then stimulated with different concentrations of TRH for 30 min. PLD activities were then determined by estimating the amount of [^{3}H]PBt in the presence of 1-butanol. (**B**) TRH-stimulated [^{3}H]PBt formation was measured at different times after the addition of 1 μ*M* TRH. Results are mean ± SD from four independent experiments. Significantly different from the control values at $*p < 0.01$.

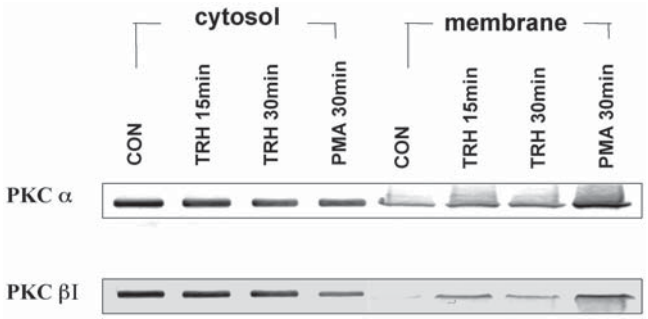

Fig. 2. Effects of TRH on PKC translocation in GH_3 cells. Serum-starved cells on 100-mm dishes were stimulated with 1 μ*M* TRH for 15 min or 30 min. As a positive control for PKC translocation to plasma membrane, cells treated with 500 n*M* PMA for 30 min were also prepared. The cells were then lysed, and subjected to subcellular fractionation into cytosolic and membrane fractions by centrifugation at 100,000*g*, and each fraction containing 30 μg of protein was analyzed by 10% SDS-PAGE and subsequent Western blotting using anti-PKCα and -βI polyclonal antibody.

in TRH-induced PLD activation in GH_3 cells and possible crosstalk between PKC activation and PLD activity.

Results

To determine the effect of TRH on PLD activity, GH_3 cells labeled with [^{3}H]palmitic acid were treated with TRH, and [^{3}H]phosphatidylbutanol (PBt) produced by PLD-catalyzed transphosphatidylation was quantitated by TLC analysis. As shown in Fig.1, treatment of the GH_3 cells with TRH increased PLD activity in both a dose- and time-dependent manner, up to 1 μ*M* (Fig. 1A) and 120 min (Fig. 1B), respectively. PLD activity maximumally increased 1.8-fold at 1 μ*M* TRH and 60 min.

Among various PKC isotypes, PKC α and PKC β are known to activate PLD *(14)*. Therefore, in an attempt to elucidate the possible involvement of PKC in PLD activation by TRH, we investigated the effects of TRH on PKC translocation in the GH_3 cells. As shown in Fig. 2, TRH treatment increased the amounts of PKC α and PKCβI in membrane fraction after 30 min, and induced translocation of these PKC isozymes from the cytosol to the membrane fraction. Phorbol 12-myristate 13-actate (PMA) was also used as a positive control for PKC translocation. The subcellular distributions of other PKC isoforms were not changed by TRH treatment (data not shown).

DAG is known to be an activator of PKC. Therefore, to investigate TRH-induced DAG release, the GH_3 cells were labeled with [^{3}H]palmitic acid for 18 h and then stimulated with TRH for the indicated time periods. TLC analysis revealed that the significant amount of DAG was released in 15 min after TRH treatment, and the increased DAG level remained high for 3 h after the treatment (Fig. 3A). These results suggested that TRH activated PLD activity through increase of the DAG level, subsequently activating PKC α and PKC βI.

To address the possibility of TRH for the induction of phosphoinositide-specific phospholipase C (PI-PLC) in TRH-induced DAG generation, we examined the hydrolysis of PI by PI-PLC after TRH treatment. Treatment of the cells with TRH for 30 min increased the inositol phosphates level by about 43-fold of control values, whereas fetal calf serum (10%) and LPA (1 μg/mL) induced 5.6- and 2.4-fold increases, respectively (Fig. 3B).

Because PLD activity can be modulated by cytosolic Ca^{2+} concentration *(15)*, cytosolic free Ca^{2+} concentration was monitored in TRH-treated GH_3 cells. As shown in Fig. 3C, TRH induced a rapid but transient increase of Ca^{2+} con-

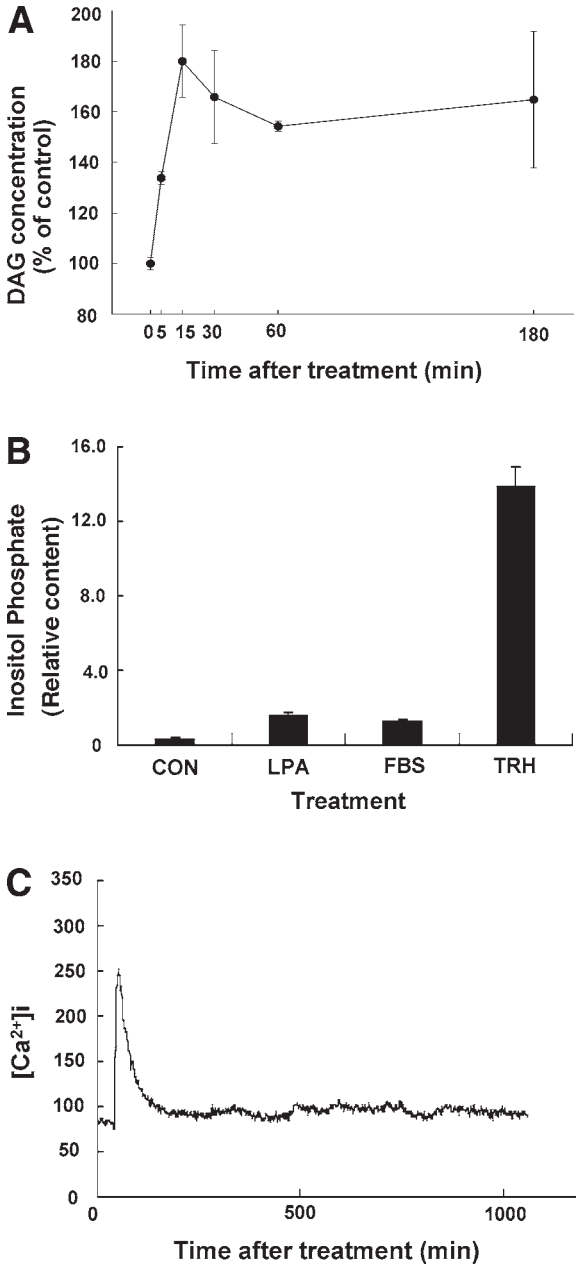

Fig. 3. Effects of TRH on DAG, PI-PLC activity and intracellular Ca^{2+} levels in GH_3 cells. (**A**) GH_3 cells (1.5×10^6 cells/well) were labeled with 1 μCi/mL [^{3}H]palmitic and stimulated with 1 μ*M* TRH in the presence of 0.3% (v/v) 1-butanol for indicated time periods. DAG level was determined, as described in *Materials and Methods*. Results are mean ± SD from four independent experiments. (**B**) GH_3 cells on six-well plates were serum starved and labeled with 2 μCi/mL of *myo*-[^{3}H]inositol for 18 h in inositol-free medium. After treating the cells with 20 m*M* LiCl for 10 min, the cells were stimulated with 1 μ*M* TRH for 30 min. As a positive control for PI-PLC activation, the cells were treated with 10% (v/v) FBS and LPA for 15 min. The amount of inositol phosphates was determined, as described under Materials and Methods. Results are mean ± SD from two independent experiments. (**C**) Serum-starved cells were labeled with 5 μCi/mL fluo-3AM for 1 h and serial incubation with 1 μ*M* TRH was performed. $[Ca^{2+}]_i$ was monitored using a laser scanning confocal microscope, as explained under *Materials and Methods*. Results are expressed as relative arbitary fluorescence intensity (I_F).

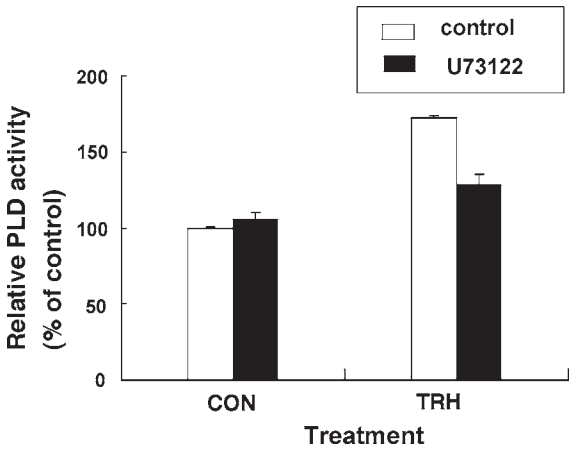

Fig. 4. Effects of U73122 on TRH-mediated PLD activity. GH_3 cells (1.5×10^6 cells/well) were labeled with 1 μCi/mL [^{3}H]palmitic acid and then pretreated with U73122 (10 μ*M*) for 30 min before measurement of relative PLD activity. PLD activities were determined by estimating the formation of [^{3}H]PBt in the presence of 1-butanol. Results are mean ± SD from three independent experiments.

centration. The data are in good agreement with the result obtained with PI-PLC activity, and TRH treatment was responsible for the Ca^{2+} release from intracellular Ca^{2+} storage organelles *(16)*. Taken together, the data in Fig. 3 strongly indicated that the PI-mediated signaling pathway was involved in this TRH signaling. Assay of PLD activity, using PBt as a PLD activity marker, revealed that the PBt contents decreased by 36% in the U73122 (50 μg/mL, a PI-PLC inhibitor) treated cells compared to those of untreated cells (Fig. 4), suggesting that DAG released by PI-PLC after TRH treatment plays a major role in PKC/PLD activation pathway.

To determine whether PLD was activated via PKC activation, two separate experiments were performed. In the first approach, TRH-mediated PLD activation was studied in the GH_3 cells, following desensitization of PKC by long-term stimulation with PMA. As shown in Fig. 5A, PKC down-regulation decreased the extent of PMA-mediated PLD activation by approx 76%, and also decreased the TRH-mediated PLD activation by about 82%. The second approach was to inhibit PKC activity with PKC inhibitors such as RO320432 and GF109203X. As shown in Fig. 5B, when the cells were pretreated with RO320432 (1 μ*M*) or GF109203X (1 μ*M*) for 15 min, the maximum level of TRH-induced PLD activity was decreased by these inhibitors by about 83% and 67%, whereas PMA-induced PLD activity was inhibited by 85% and 73%, respectively.

Discussion

There are many reports describing the role of PLD in neuronal, endocrine, or secretory cells, and it is well known that PLD plays a critical role in growth, mitosis, and secretion of endocrine cells. PLD in cell membranes hydrolyzes

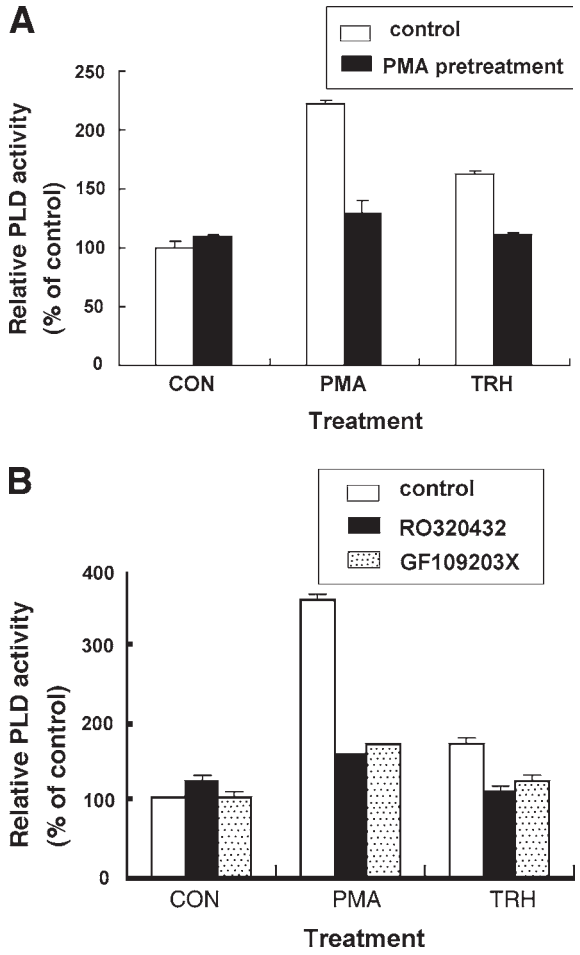

Fig. 5. Effects of PKC downregulation on TRH mediated PLD activation (**A**) GH_3 cells (1.5×10^6 cells/well) were treated in the absence or presence of 500 n*M* PMA for 24 h. The cells were labeled with 1 μCi/mL [^{3}H]palmitic acid and then pretreated with 0.3% (v/v) 1-butanol for 15 min before stimulation with 1 μ*M* TRH or 500 n*M* PMA. PLD activities were determined by estimating the formation of [^{3}H]PBt in the presence of 1-butanol. Results are mean ± SD from four independent experiments. (**B**) GH_3 cells (1.5×10^6 cells/well) were labeled with 1 μCi/mL [^{3}H]palmitic acid and then pretreated with RO320432 or GF109203X (1 μ*M*, each) for 15 min before measurement of relative PLD activity. PLD activities were determined by estimating the formation of [^{3}H]PBt in the presence of 1-butanol. Results are mean ± SD from three independent experiments.

PC to PA and choline, and PA is then converted to DAG and LPA. These two end-products play important roles in cellular growth, differentiation, and secretion *(8)*. In the present study, we demonstrated that TRH stimulated PLD activity in GH_3 cells, which contain TRH receptors in the cell membranes. Furthermore, TRH-mediated PLD activation occurred as a consequence of PKC activation : TRH induced the translocation of PKCα and PKCβI from cytosol to membrane (Fig. 2), since this translocation of PKC has been regarded as a process in activation of the PKC. These conclusions were derived from two separate experiments. First, TRH-induced PLD activity was almost completely abrogated by prior long-term exposure to PMA, which has been known to down-regulate PKC (Fig. 5A) *(17)*. Second, pretreatment of GH_3 cells with PKC inhibitors (RO320432 and GF109203X) also inhibited TRH-induced PLD activation (Fig. 5B). Theses results strongly support the notion that PKC activation participates in the stimulation of PLD in GH_3 cells.

It is well known that DAG, a physiological activator of PKC, can be generated by PI-PLC *(12)*. In GH_3 cells, TRH bind to their receptors with an apparent K_d of 10 n*M* *(18)*, and stimulate $G_{\alpha q}$ and $G_{\alpha 11}$ proteins *(19)*. Subsequently, TRH rapidly induce hydrolysis of PtdIns(4,5)P_2 by PI-PLC to generate Ins(1,4,5)P_3 and DAG *(3)*. When PI-PLC pathway is turned on, intracellular Ca^{2+} and DAG concentrations rapidly increase *(20)*. In the present study, TRH was found to effectively increase the inositol phosphates level in GH_3 cells about 43-fold over the control value, and also intracellular DAG level and cytosolic free Ca^{2+} concentration. Furthermore, preincubation of the cells with U73122, a potent inhibitor of PI-PLC, markedly inhibited TRH-induced PLD activity. These observations suggested that DAG, produced by TRH-induced PI-PLC activation, was responsible for stimulating PKC/PLD activation.

PKC is a family of isoforms that play key roles in the process of proliferation/apoptosis, differentiation, or hormone release, and the function of the enzyme is regulated at multiple levels: transcription, phosphorylation, and subcellular targeting. Although the effects of PKC activation on GH_3 cells are not known, recent studies suggested that DAG/PKC branch of PLC-mediated signaling pathway is involved in the recovery of TRH-induced inhibition of rat ERG channel *(21)*. In GH_4 rat pituitary tumor cell lines, several members of the PKC family mediate TRH-stimulated rPRL promoter activation *(22)*. In addition, the present finding provided new insights that PKC is involved in TRH-induced phosphorylation of the EGF receptor. Therefore, it is highly likely that the role of PKC in GH_3 cells is to mediate various hormonal effects of TRH.

Much evidence indicates that PKC is the major mediator of agonist effects upon PLD. It is known that treatment of many types of cells with agonists, which induce PtdIns(4,5)P_2 hydrolysis, or with phorbol esters stimulates PLD activity, implying the regulation of the enzyme by PKC *(23)*. However, the mechanism by which PKC regulates PLD in intact cells remains unclear. The stimulatory effects of the PKC isozymes do not require ATP *(24)* and are not associated with protein phosphorylation either *(25)*. Thus, the activation of PLD seems to occur through a non-phosphorylating protein–protein interaction. The failure of other PKC isozymes (PKCδ, PKCε) to activate PLD indicates that regulatory domain sequences, unique to the α- and βI-isozymes, are involved in the interaction *(26)*.

Besides the PKC activation, PLD is known to play a critical role in hormonal secretion *(27)*. In the hormone-secret-

ing cells, vesicle budding and binding are needed, and PLD can change phospholipid composition in hormone-secreting-cell membranes, consequently playing an important role in vesicle transport in the Golgi *(28,29)*. Moreover, ADP-ribosylation factor, which plays an important role in vesicle trafficking, has been known to activate PLD *(30)*. These findings raise the possibility of an important role played by PLD in the process of hormone transport and secretion. Furthermore, PLD1 have recently been shown to be an important component of the exocytotic machinery in neuroendocrine cells *(31)*. Therefore, the predicted effect of the local increase of the PA level at exocytotic sites due to PLD activation would be to promote membrane bending, particularly in the presence of Ca^{2+}, to facilitate hemifusion and subsequent formation of the exocytotic fusion pore. However, the exact physiological role of PLD in the process of hormone secretion in GH_3 cells is not fully understood.

In conclusion, we demonstrated for the first time that TRH stimulated PLD activity in GH_3 tumor cells via stimulation of PKC. In addition, DAG, produced by PI-PLC, played an essential role in TRH-induced PKC/PLD activation. These results, therefore, provide more clear insight into the mechanism of TRH action in pituitary tumor cells.

Materials and Methods

Materials

Materials for tissue culture such as fetal bovine serum (FBS), penicillin, streptomycin, and Dulbeco's minimal essential media (DMEM, low glucose) were obtained from Gibco-BRL (Gaithersburg, MD), and TRH and phorbol 12-myristate 13-acetate (PMA) from Sigma (St. Louis, MO). The PKC inhibitors RO320432 and GF109203X were obtained from the Calbiochem (Nottingham, UK), AG1-X8 resin from Bio-Rad (Hercules, CA), and [^{3}H]palmitic acid and *myo*-[^{3}H]inositol were from Du Pont–New England Nuclear (Boston, MA). Polyclonal anti-PKC-α and -βI were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), L-α-PBt was from Avanti Polar Lipid Inc. (Alabaster, AL), and the silica gel 60A plates for TLC were purchased from Whatman (Clifton, NJ). All other chemical agents were of analytical grade.

Cell Culture

GH_3 cells, rat somatomammotropic pituitary tumor cell, were obtained from American Type Culture Collection (ATCC CCL-82.1). The cells were cultured at 37°C in DMEM medium supplemented with 10% FBS, 100 units/mL penicillin and 100 µg/mL streptomycin at 37°C in a humidified atmosphere of 95% air–5% CO_2.

Determination of PLD Activity and Measurement of DAG

PLD activity was determined as previously described by measuring [^{3}H]PBt produced via PLD catalyzed transphosphatidylation in [^{3}H]palmitic acid labeled cells *(32)*. Briefly, GH_3 cells (1.5×10^6 cells/well) cultured on six-well plates were metabolically labeled with 1 µCi/mL [^{3}H]palmitic acid in serum free media for 18 h. The cells were then pretreated with 0.3% (v/v) 1-butanol for 15 min before stimulation with TRH or other test agents. After the stimulation, the cells were quickly washed with ice-cold phosphate buffered saline and suspended in 0.5 mL of ice-cold methanol. Lipids were extracted according to the method of Bligh and Dyer *(33)*. PBt was separated from other phospholipids by thin layer chromatography on Silica G-60 plates, using a solvent system of ethyl acetate/iso-octane/acetic acid/water (110:50:20:100, v/v), and DAG was separated with solvent system of toluene/ether/ethanol/concentrated NH_4OH (50:30:20:0.2, v/v) *(34)*. The regions corresponding to the authentic PBt bands and DAG bands were identified with 0.002% (w/v) primulin in 80% (v/v) acetone, scraped and counted using a scintillation counter.

Determination of PI-PLC Activity

GH_3 cells on six-well plates were serum starved and labeled with 2 µCi/mL of *myo*-[^{3}H]inositol for 18 h in inositol-free medium. The cells were then washed with fresh serum-free medium and preincubated for 1 h at 37°C. After treating with 20 m*M* LiCl for 10 min, the cells were stimulated with TRH or other test agents. It should be noted that in the presence of LiCl, the hydrolysis of PI by PI-PLC is known to increase inositol phosphates *(35)*. Incubations were terminated by removal of the medium and washing with ice-cold PBS. The cells were then resuspended in ice-cold methanol. The water-soluble phase was separated as described by Bligh and Dyer, dried in a Speed Vac concentrator, and redissolved in 1 mL of water. After applying the water-soluble phase to a 1-mL bed volume of AG 1-X8 resin (200–400 mesh, fomate form), the column was washed with 10 mL of 5 m*M* Na_2B_4O and 60 m*M* $HCOONH_4$. Total inositol phosphates were eluted by the addition of 2 ml of 0.1 m*M* HCOOH and 1.0 *M* $HCOONH_4$. The radioactivity of the eluate was determined by a scintillation counter. The PLC activity was determined as a ratio of the amount of the eluted inositol phosphates in total phospholipids.

Translocation of PKC

Serum-starved cells were incubated with TRH (1 µ*M*) for 15 or 30 min, scraped into ice-cold phosphate buffered saline, and harvested by microcentrifugation. The cells were then resuspended in a buffer solution (50 m*M* NaCl, 1 m*M* $MgCl_2$, 2 m*M* EDTA, 1 m*M* DTT, 1 m*M* PMSF, 10 µg/mL aprotonin, 10 µg/mL leupeptin, 50 m*M* Hepes, pH 7.5), and disrupted by sonication. The cytosolic fraction was separated from the particulate fraction by centrifugation at 100,000*g* for 1 h. Proteins were resolved on 10% SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked for 1 h with TTBS (Tris-buffered saline containing 0.01% Tween 20) containing 5% (w/v) non-fat dry skim milk and incubated for

additional 1 h with anti-PKC antisera (1 μg/mL). Unbound primary antibodies were removed by three washes (10 min each) with TTBS. The membranes were incubated with alkaline phosphatase conjugated-anti-rabbit IgG (KPL, Gaithersburg, MD) for 1 h, followed by washes in TTBS, and developed using phosphatase substrate system (KPL).

Determination of Cytosolic-Free Ca^{2+} Concentration

GH_3 cells were cultured on microscope cover slides and serum-starved for 18 h. The cells were incubated for 1 h with 5 μ*M* fluo-3AM (Molecular probe) in serum-free DMEM and washed with serum-free DMEM. Each cover slide was then mounted on a confusion chamber (self-designed), and stained cells were then observed under confocal laser scanning microscope (Bio-Rad MRC-1024). The cells were then scanned every second with a 488-nm excitation argon laser and a 515–nm emission filter. TRH in serum-free DMEM was added to cells using a 1-mL syringe. The time-series scanning images were collected and analyzed for the changes of Ca^{2+} concentration in a single cell level.

Statistical Analysis

All test incubations were performed in duplicate, and data are presented as mean ± SD. The significance of differences was assessed by unpaired t-test.

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