

Regulation of de novo phosphatidylinositol synthesis

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Abstract Mechanisms that function to regulate the rate of de novo phosphatidylinositol (PtdIns) synthesis in mammalian cells have not been elucidated. In this study, we characterize the effect of phorbol ester treatment on de novo PtdIns synthesis in C3A human hepatoma cells. Incubation of cells with 12-*O*-tetradecanoyl phorbol 13-acetate (TPA) initially (1–6 h) results in a decrease in precursor incorporation into PtdIns; however, at later times (18–24 h), a marked increase is observed. TPA-induced glucose uptake from the medium is not required for observation of the stimulation of PtdIns synthesis, because the effect is apparent in glucose-free medium. Inhibition of the activation of arachidonic acid substantially blocks the synthesis of PtdIns but has no effect on the synthesis of phosphatidylcholine (PtdCho). Increasing the concentration of cellular phosphatidic acid by blocking its conversion to diacylglycerol, on the other hand, enhances the synthesis of PtdIns and inhibits the synthesis of PtdCho. The TPA-induced stimulation of PtdIns synthesis is not the result of the concomitant TPA-induced G1 arrest, because G1 arrest induced by mevastatin has no effect on PtdIns synthesis. Inhibition of protein kinase C activity blocks the stimulatory action of TPA on de novo synthesis of PtdIns but has no effect on TPA-induced inhibition. Potential sites of enzymatic regulation are discussed.—Nuwayhid, S. J., M. Vega, P. D. Walden, and M. E. Monaco. Regulation of de novo phosphatidylinositol synthesis. *J. Lipid Res.* 2006. 47: 1449–1456.

Supplementary key words phorbol ester • cell cycle • protein kinase C • arachidonic acid • phosphatidic acid.

Phosphatidylinositol (PtdIns) is an essential acidic phospholipid found in the membranes of mammalian cells (1) as well as on the chromatin (2). A single molecular species composed of stearate (R') and arachidonate (R'') accounts for ~80% of the total PtdIns. There are two pathways for the synthesis of PtdIns: the de novo pathway and the salvage pathway, which converge at the level of phosphatidic acid (PtdOH). The de novo pathway uses dihydroxyacetone phosphate derived from glucose to make glycerol phosphate, which is subsequently acylated

at the *sn*-1 position with stearate and then at the *sn*-2 position with arachidonate to yield PtdOH. In the salvage pathway, diacylglycerol (DAG), derived from phospholipase C-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate (PtdInsP₂), is converted to PtdOH through the action of DAG kinase. In the first instance, there is a net increase in PtdIns, whereas in the second, there is not. The central element of both pathways is the production of PtdOH. For the salvage pathway, it appears that the conversion of DAG to PtdOH by DAG kinase is stimulated as a result of agonist-induced polyphosphoinositide turnover. Whether this results from an increase in DAG substrate and/or an increase in DAG kinase enzyme activity is unclear. This laboratory has previously demonstrated that the two pathways can be differentiated in situ on the basis of the inhibition by triacsin C, a specific inhibitor of arachidonate-specific long-chain fatty acyl-CoA synthetase (ACSL4) in situ (3, 4).

Although regulation of the salvage pathway via the activation of G-protein-coupled receptors has been analyzed extensively by this and other laboratories, little is known concerning the regulation of the de novo pathway in mammalian cells. The general consensus has been that the synthesis of PtdIns is constitutive and that the major regulatory mechanisms center on the subsequent generation of the polyphosphoinositides. Indeed, it has been demonstrated that a reduction in cellular PtdIns levels subsequent to inositol deprivation does not impinge on the levels of cellular polyphosphoinositides (5). However, recent studies in yeast and plants indicate that *pis1* gene expression is, in fact, regulated (6). Phosphatidylinositol synthase (PIS) is responsible for the final step in the PtdIns synthetic pathway. In this study, we demonstrate that treatment of C3A human hepatoma cells with phorbol ester causes profound biphasic changes in the de novo synthesis of PtdIns. We further characterize these changes with respect to cell cycle arrest and protein kinase C (PKC) activity and compare the phospholipid effects with the effects of 12-*O*-tetradecanoyl phorbol 13-acetate (TPA) on DNA synthesis and cell migration.

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Materials

Tissue culture media, sera, trypsin, and PBS (without Ca^{2+} and Mg^{2+}) were from Invitrogen. Glucose-free minimal essential medium was from Atlanta Biologicals. All radioactive compounds were purchased from American Radiolabeled Chemicals. Phorbol ester (12-tetradecanoyl phorbol 13-acetate), triacsin C, Ro 31-8220, and G6-6389 were obtained from Biomol. Baker-Flex silica gel IB2-F thin-layer chromatography sheets were purchased from Thomas Scientific, and Dowex AG 1 \times 8 resin was from Bio-Rad. Mevastatin and phospholipid standards were from Sigma-Aldrich. Ready-Safe liquid scintillation cocktail was from Beckman. The BCA protein determination reagent was from Pierce. The Amplex kit for measuring glucose levels was purchased from Amersham Biosciences.

Cell culture

HepG2/C3A cells were obtained from the American Type Culture Collection and grown in Ham's F-12 medium supplemented with 10% fetal bovine serum, 25 mM HEPES, and antibiotics (100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, and 0.25 $\mu\text{g}/\text{ml}$ Fungizone) in a humidified atmosphere of 5% CO_2 at 37°C. These cells are a subline on HepG2, originally isolated because of their high degree of differentiation for potential use in a liver-assist device (7). Compared with the parent line, C3A cells achieve a 60% lower final density and have a 10-fold higher albumin-to- α fetal protein ratio. They are capable of growing in glucose-free medium, whereas HepG2 cells are not. When glucose is removed from the medium, these cells can support gluconeogenesis. For experiments, cells were harvested with a solution of 0.05% trypsin and EDTA (0.02%) in 0.9% NaCl and replicately plated into 22 or 35 mm multiwell plastic dishes. Details of each experiment appear below and in the figure legends.

Quantitation of lipid radioactivity

Cells were incubated with [^3H]inositol (15 Ci/mmol), [^3H]choline (80 Ci/mmol), [^3H]glycerol (40 Ci/mmol), [^3H]arachidonic acid (200 Ci/mmol), [^3H]oleic acid (60 Ci/mmol), [^3H]cytidine (20 Ci/mmol), or [^{32}P]orthophosphate (carrier-free) as described in the figure legends. At the end of the experiment, the medium containing the radioactivity was removed and the cells were washed once with PBS, followed by the addition of 0.5 ml of methanol to each well. The methanol was then transferred to a glass 12 \times 75 mm tube containing 1.0 ml of chloroform and 0.4 ml of HOH. The mixture was vortexed for 10 s, and the layers were separated by centrifugation for 2 min. The upper, aqueous layer was removed, and an aliquot of the lower, chloroform layer was taken for further analysis. When the radioactive compound was choline, inositol, or cytidine, the chloroform aliquot was placed in a scintillation vial, dried, and quantitated using a Beckman LS6500 liquid scintillation counter. Determination of either glycerol or phosphate incorporation into specific phospholipids was accomplished by either thin-layer chromatography or Dowex chromatography, as described previously (8). Thin-layer solvent systems were as follows for the separation of individual phospholipids: *a*) chloroform-methanol-acetic acid-water (50:30:6:3) for the separation of PtdIns and phosphatidylcholine (PtdCho); *b*) chloroform-methanol-water (65:25:4) for the separation PtdIns, PtdCho, phosphatidylethanolamine (PtdEth), and cardiolipin (CL); and *c*) ether-benzene-ethanol-acetic acid (40:50:2:0.2) for the separation of neutral lipids from phospholipids. For the separation of PtdIns and PtdOH, Dowex chromatography was used (8).

Measurement of thymidine incorporation

Cells were incubated in 2 $\mu\text{Ci}/\text{ml}$ tritiated thymidine in serum-free minimal essential medium for 2 h. At the end of the incubation, cells were washed twice with 1 ml of ice-cold PBS and incubated in 1 ml of 5% TCA at 4°C for 30 min. After another wash with cold PBS, radioactive DNA was solubilized in 0.5 ml of NaOH (0.1 M) containing 0.2% SDS. Radioactivity was quantitated by scintillation counting.

Protein determination

Protein was measured using the BCA reagent from Pierce using the methodology suggested by the company.

Flow cytometry

C3A cells were grown in T25 flasks for 4 days to allow the formation of colonies. Cells were then treated as described in the figure legends. After trypsinization, cells were collected by centrifugation at 800 rpm for 10 min, medium was removed, and cells were suspended in 1 ml of HEPES-buffered saline solution (HBSS) buffer. For fixing, 1 ml of cells was added to 10 ml of ice-cold 70% ethanol while vortexing and fixed for at least 24 h at -20°C . After fixing, the ethanol was aspirated and cells were resuspended in 2 ml of HBSS/phosphate citrate buffer (1:3, pH 7.8) with 0.1% Triton X-100. After incubating for 30 min at room temperature, cells were centrifuged at 2,000 rpm for 5 min, buffer was removed, and the pellet was resuspended in 1 ml of propidium iodide solution, containing 20 μg of propidium iodide, 200 μg of EDTA, 5 μl of Igepal, and 200 μg of RNase PBS. Finally, cells were incubated in the dark at 37°C for 2 h before analysis. Flow cytometry was performed using a Becton Dickinson FACScan machine.

Glucose measurement

Glucose concentrations were measured using the Amplex Red glucose/glucose oxidase assay using the protocol suggested by Amersham Biosciences.

RESULTS

Effect of TPA on PtdIns synthesis in C3A cells

Figure 1 describes the incorporation of both inositol and choline into phospholipids as a function of treatment with TPA. At early times, incorporation of inositol into PtdIns is inhibited by 75%. This effect is reversed at 18 and 24 h, when a significant increase is observed. Incorporation of choline into PtdCho, on the other hand, is increased at both early and late times. Because a decrease in inositol incorporation could reflect an effect of TPA on the transport of radioactive inositol into the cell, whereas stimulatory effects on precursor incorporation could indicate increased turnover rather than increased de novo synthesis, additional experiments were carried out to assess both the de novo synthesis and turnover of PtdIns. Figure 2 shows the results of experiments in which cells were treated with TPA for 3 h, followed by incubation with either radioactive glycerol (A) or orthophosphate (B). Incorporation of both glycerol and phosphate into PtdIns is inhibited by treatment with TPA, indicating that the effect seen on inositol incorporation is not an artifact caused by the inhibition of uptake but reflects a cessation

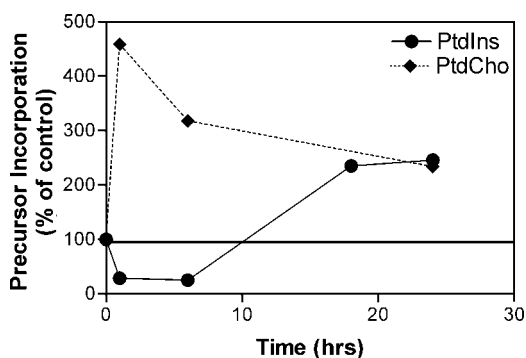


Fig. 1. Effect of 12-*O*-tetradecanoyl phorbol 13-acetate (TPA) on precursor incorporation into phospholipids in C3A cells. Cells were replicately plated in 12-well plastic dishes and allowed to grow for 48 h. Cells were then treated with DMSO (1%) or TPA (100 nM) for the times indicated in serum-free minimal essential medium containing 25 mM HEPES. After treatment, the cells were washed with serum-free minimal essential medium and incubated for another 2 h with either radiolabeled inositol or radiolabeled choline, and incorporation was quantitated as described in the text. Values shown represent results from three separate experiments. At each time point, the difference was statistically different from controls, with *P* values all <0.025.

of synthesis. With respect to PtdCho, glycerol incorporation is inhibited slightly, probably as a result of a decrease in glycerol specific activity attributable to a TPA-induced increase in glucose uptake (see below), whereas ortho-

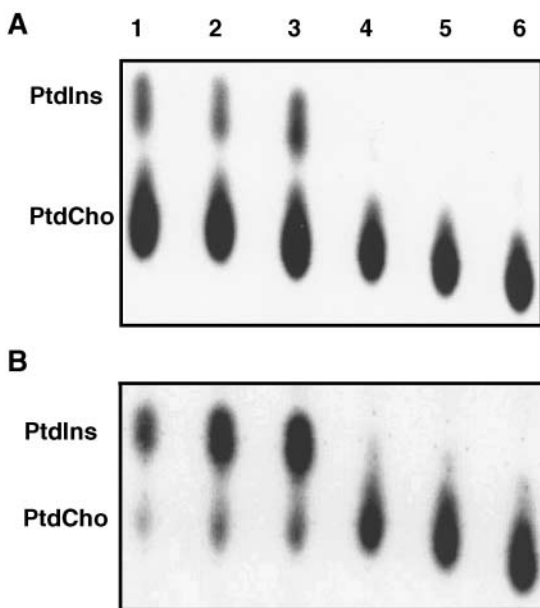


Fig. 2. Short-term effect of TPA on radiolabeled precursor incorporation into phospholipids in C3A cells. Cells were incubated with DMSO (1%; lanes 1–3) or TPA (100 nM; lanes 4–6) in serum-free minimal essential medium for 3 h, followed by washing with serum-free, glucose-free (A) or serum-free, phosphate-free (B) minimal essential medium and another 2 h incubation with either 20 μ Ci/ml radiolabeled glycerol in glucose-free medium (A) or 50 μ Ci/ml radiolabeled orthophosphate in phosphate-free medium (B). At the end of the incubation, lipids were extracted and separated by thin-layer chromatography as described in the text.

phosphate incorporation is increased, indicating that the early effect seen on choline incorporation is the result of increased turnover, probably attributable to enhanced phospholipase C and/or D activity, as has been reported previously (9–11). At 24 h, radioactive glycerol incorporation into PtdIns is increased (Fig. 3). The degree of stimulation by TPA is a function of cell density, with greater increases seen at lower densities. Incorporation of glycerol into PtdCho is also increased as a function of density (data not shown). Figure 4 confirms that the addition of TPA to C3A cells has no effect on PtdIns turnover at either early or late times. Total protein concentration is unaffected by the presence of TPA for 24 h (control, 0.87 mg/ml; TPA-treated, 0.87 mg/ml). In summary, treatment with TPA results in an early inhibition of PtdIns synthesis and increased turnover of PtdCho, followed at later times by an increase in the de novo synthesis of both PtdIns and PtdCho.

Effect of substrate availability on the synthesis of PtdIns

The glycerol backbone used for the synthesis of phospholipids and triglycerides is derived from glucose. Because changes in the rate of synthesis of phospholipids could result from an increased or decreased availability of glucose, experiments were carried out to determine the role of glucose availability in phorbol ester-mediated changes in the synthesis of PtdIns. Phorbol esters have been shown to enhance glucose uptake in adipose and muscle tissue (12). In C3A cells, TPA treatment enhances the disappearance of glucose from the extracellular medium in a manner similar to that seen with insulin (Fig. 5). Thus, we conclude that the inhibition of PtdIns synthesis at early times after treatment with TPA is not the result of a

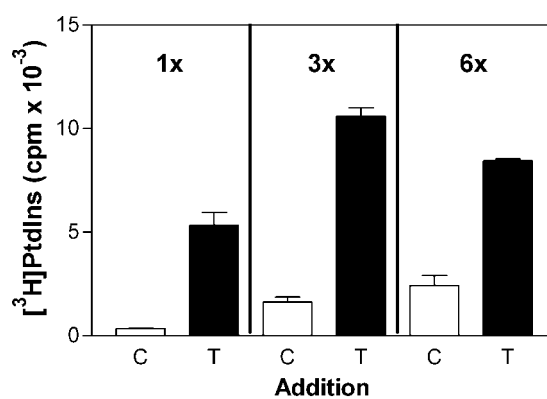


Fig. 3. Long-term effect of TPA on the incorporation of radiolabeled precursors into phospholipids in C3A cells. Cells were initially plated at three different densities (1 \times , 3 \times , and 6 \times) and subsequently incubated with DMSO (1%) or TPA (100 nM) in serum-free minimal essential medium for 24 h, followed by washing with serum-free, glucose-free minimal essential medium and another 2 h incubation with 20 μ Ci/ml radiolabeled glycerol in serum-free, glucose-free minimal essential medium. Lipids were extracted and deacylated. Dowex ion-exchange chromatography was used to isolate phosphatidylinositol (PtdIns). Details can be found in the text. Values shown represent means \pm SD of two separate determinations. All *P* values were <0.005.

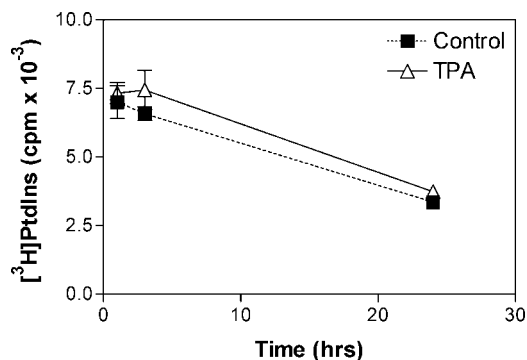


Fig. 4. Effect of TPA on the turnover of prelabeled PtdIns in C3A cells. Cells were grown for 48 h in minimal essential medium supplemented with fetal bovine serum (10%), antibiotics, and 5 $\mu\text{Ci/ml}$ [^3H]inositol. Cells were then washed with serum-free minimal essential medium and incubated for the times indicated in serum-free minimal essential medium containing 25 mM HEPES and either DMSO (1%) or TPA (100 nM) for the times indicated. Incorporation of radiolabel was determined as described in the text. Values shown represent means \pm SD of four separate determinations. There was no statistical difference between any of the values.

general limitation of glucose substrate. Because CTP is required for the synthesis of the intermediate, CDP-DAG, we wondered whether CTP availability is altered by TPA treatment at early times, perhaps as a result of the increased synthesis of CDP-choline for use in the TPA-induced resynthesis of PtdCho. To answer this question, we prelabeled cells with radioactive CTP (20 $\mu\text{Ci/ml}$) and then measured the accumulation of CDP-DAG in the absence and presence of TPA. There was no statistical difference between control (1,292 \pm 326 cpm/dish) and TPA-treated (1,712 \pm 328 cpm/dish) cells. In fact, the TPA-treated cells accumulated slightly more CDP-

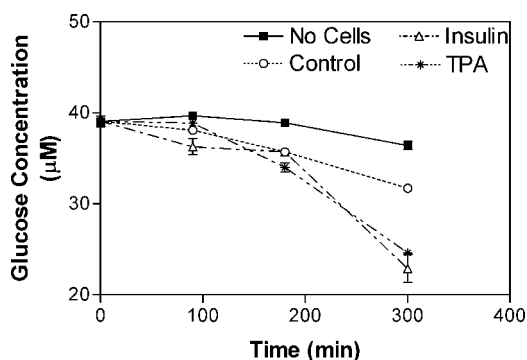


Fig. 5. Effect of TPA on glucose metabolism in C3A cells. Cells were replicately plated in 24-well plastic dishes and allowed to grow for 48 h. The medium was then changed to glucose-free minimal essential medium containing 40 μM glucose with the following additions: nothing (control), TPA (100 nM), or insulin (1 μM). At the times indicated, an aliquot of the medium was removed and the glucose concentration was measured as described in the text. Values shown represent means \pm SD of six separate determinations. The effect of TPA was significant at 180 min ($P < 0.005$) and 300 min ($P < 0.0005$).

DAG, suggesting that the inhibition was at the level of PIS. Further experimentation will be required to validate this conclusion.

To test whether the increase in PtdIns synthesis observed at later times could result from increased glucose availability, cells were incubated with or without TPA in the presence or absence of extracellular glucose for 24 h. **Figure 6** shows that TPA stimulates de novo PtdIns synthesis even in the absence of extracellular glucose, although not to the same degree. Thus, we conclude that enhanced glucose uptake cannot fully explain the ability of TPA to increase PtdIns synthesis.

Results from previous studies have suggested that neither CDP-DAG synthetase (CDS) nor PIS activity alone regulates the levels of cellular PtdIns (13). To determine whether the concentration of PtdOH was rate-limiting in the PtdIns synthetic pathway, cells were incubated with propranolol to inhibit PtdOH phosphatase activity and thus increase the concentrations of PtdOH available to the CDP-DAG pathway. The upper panel of **Fig. 7** shows that in the presence of propranolol, the incorporation of radiolabeled glycerol into PtdOH, PtdIns, and CL is increased by 230, 574, and 1,322%, respectively, whereas incorporation into PtdCho and PtdEth is decreased by 69% and 84%, respectively. We thus conclude that an increase in cellular concentrations of PtdOH is sufficient to drive the synthesis of lipids using the CDP-DAG pathway, including PtdIns and CL.

Because PtdIns is composed predominantly of a moiety containing arachidonic acid in the *sn*-2 position (14), we wondered whether the limitation of activated arachidonate might specifically interfere with the synthesis of PtdIns. Cells were treated with triacsin C to block the condensation of CoA with arachidonate, as described

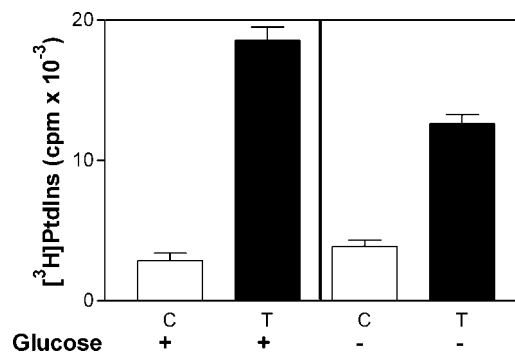


Fig. 6. Effect of glucose on the ability of TPA to increase precursor incorporation into PtdIns. Cells were replicately plated in 12-well plastic dishes and allowed to grow for 48 h. The medium was then changed to serum-free minimal essential medium with or without glucose and incubated for an additional 24 h in the presence (T) or absence (C) of TPA (100 nM). The cells were then washed in glucose-free, serum-free minimal essential medium and incubated in the same medium for an additional 2 h with 10 $\mu\text{Ci/ml}$ [^3H]glycerol. Incorporation of radioactivity into PtdIns was quantitated as described in the text. Values shown represent means \pm SD of three separate determinations. The effect of TPA was highly significant ($P < 0.0005$).

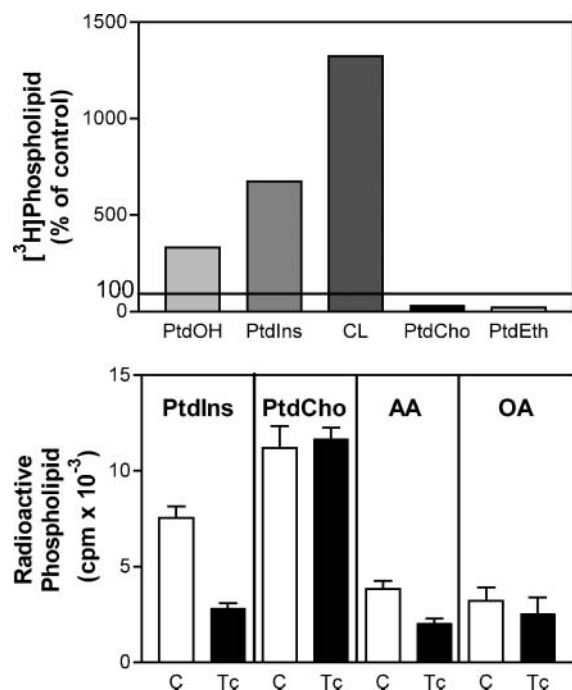


Fig. 7. Effect of propranolol (upper panel) and triacsin C (lower panel) on the incorporation of radiolabeled precursors into phospholipids in C3A cells. Upper panel: Cells were replicately plated in 12-well plastic dishes and allowed to grow for 48 h. The medium was then changed to serum-free, glucose-free minimal essential medium with 25 mM HEPES containing 10 μ Ci/ml radiolabeled glycerol with or without 250 μ M propranolol and further incubated for 2 h. Lipids were extracted and quantitated as described in the text. The effect of propranolol can be seen as the deviation from 100%. All deviations were statistically significant [phosphatidic acid (PtdOH), $P < 0.0125$; PtdIns, $P < 0.025$; cardiolipin (CL), $P < 0.01$; phosphatidylcholine (PtdCho), $P < 0.0025$; phosphatidylethanolamine (PtdEth), $P < 0.005$]. Lower panel: Cells were replicately plated in 12-well plastic dishes and allowed to grow for 48 h. The medium was then changed to either serum-free, phosphate-free F-12 medium containing 25 mM HEPES and 50 μ Ci/ml [³²P]orthophosphate for the labeling of PtdIns and PtdCho or serum-free minimal essential medium containing 25 mM HEPES and either 20 μ Ci/ml radiolabeled arachidonic acid (AA) or 20 μ Ci/ml radiolabeled oleic acid (OA) plus or minus 50 μ M triacsin C (Tc). After another 2 h incubation, lipids were extracted as described in the text and separated by thin-layer chromatography to yield either [³²P]PtdIns and [³²P]PtdCho or [³H]arachidonic acid- or [³H]oleic acid-labeled total phospholipid. Values shown represent means \pm SD of three separate determinations. Statistical significance for differences seen with triacsin C is as follows: PtdIns, $P < 0.0005$; PtdCho, no statistical difference; arachidonic acid, $P < 0.0025$; oleic acid, not determined.

previously (4), and the incorporation of radiolabeled orthophosphate into PtdIns and PtdCho was monitored. In addition, the incorporation of radiolabeled arachidonate and oleate into total phospholipid was also assessed. The lower panel of Fig. 7 shows that there is a 63% decrease in orthophosphate incorporation into PtdIns, whereas there is no effect on incorporation into PtdCho. Incorporation of radiolabeled arachidonate into phospholipids is inhibited by 48%, although there is no statistical difference in the

incorporation of radiolabeled oleate. These data suggest that concentrations of activated arachidonate might regulate the rate of cellular PtdIns synthesis.

Effect of G1 arrest on PtdIns synthesis

It has been reported that TPA causes growth inhibition, migration, and G1 arrest in HepG2 cells, the parent line of C3A (15–17). Similar findings apply to C3A cells. Results of flow cytometry experiments confirm that TPA treatment of C3A cells causes an increase in the percentage of cells in G1 phase (from 63.21% to 86.32%) and a decrease in the percentage in S phase (from 32.77% to 6.92%). We wondered whether the arrest in G1 was causally related to the increase in PtdIns synthesis. To test the possibility that G1 arrest induced PtdIns synthesis, cells were treated with mevastatin, an inhibitor of HMG-CoA reductase, that has been shown to cause cell cycle arrest in G1 (18), and both DNA and phospholipid synthesis were monitored. Incorporation of radiolabeled thymidine into DNA is inhibited by 59%, yet no effect is seen on the incorporation of glycerol into either PtdCho or PtdIns (Fig. 8). Thus, we conclude that the increase seen in phospholipid de novo synthesis as a result of long-term TPA treatment is not the result of G1 arrest.

Effect of PKC inhibition on the actions of TPA

To determine whether the effects of TPA on PtdIns synthesis are mediated by the activation of PKC, cells were preincubated with the PKC inhibitor G δ -6983, followed by treatment with or without TPA, and subsequently monitored for effects on PtdIns synthesis, migration, and DNA

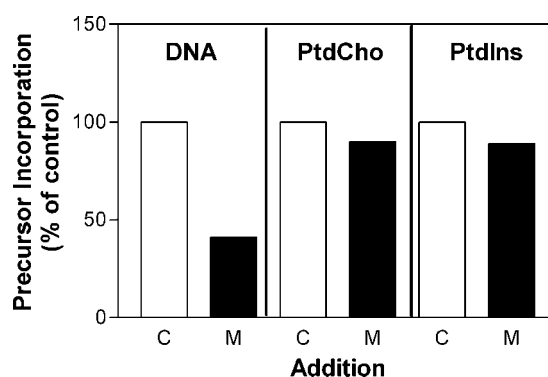


Fig. 8. Effect of G1 arrest with mevastatin (M; 25 μ M) on DNA and phospholipid synthesis in C3A cells. Cells were replicately plated in 12-well plastic dishes and allowed to grow for 48 h. The medium was then changed to serum-free minimal essential medium with 25 mM HEPES with or without mevastatin for 48 h. Cells were then washed with serum-free medium and incubated with either 2 μ Ci/ml radiolabeled thymidine in serum-free minimal essential medium with 25 mM HEPES or 20 μ Ci/ml radiolabeled glycerol in glucose-free minimal essential medium containing 25 mM HEPES. Cells were extracted and incorporation of radiolabel was quantitated as described in the text. Values shown represent means \pm SD of three (controls) or nine (mevastatin-treated) separate determinations. The effect of mevastatin on thymidine incorporation was significant ($P < 0.0001$), whereas there was no statistical difference in the phospholipid values.

synthesis. The presence of G δ -6983 in the medium inhibits the ability of TPA to induce migration (data not shown) and to stimulate PtdIns synthesis at 24 h (Fig. 9); however, the capacity of TPA to inhibit PtdIns synthesis at 2 h and to inhibit DNA synthesis at 24 h is unaffected by the PKC inhibitor (Fig. 9). A second, more general inhibitor of PKC (Ro 31-8220) yielded the same results (data not shown). These data suggest that different mechanisms are responsible for mediating the early and late effects of TPA on PtdIns synthesis, with the early inhibitory effect occurring independently of PKC and the later effect mediated through PKC activation.

DISCUSSION

To investigate the regulation of mammalian de novo PtdIns synthesis, we have developed a model composed of C3A human hepatoma cells in which de novo PtdIns synthesis is both increased and decreased as a function of time of treatment with phorbol ester. The effects are dramatic and are accompanied by a number of other physiological and biochemical changes, including the migration of cells from defined clusters and the inhibition of DNA synthesis. The inhibition of DNA synthesis results in an accumulation of cells in the G1 phase of the cell cycle. Similar results have been described for the parent cell line of C3A, HepG2 (15). In addition, we demonstrate here that TPA increases glucose uptake in C3A cells.

The biphasic effect of TPA on PtdIns synthesis involves an early inhibition of precursor incorporation followed by a later stimulation. The early inhibition is relatively specific, whereas the later stimulation appears to be a more general effect on phospholipid synthesis, affecting the synthesis of PtdCho as well. Preliminary studies (data not shown) suggest, in fact, that neutral lipid synthesis is also augmented. The early inhibitory effect does not

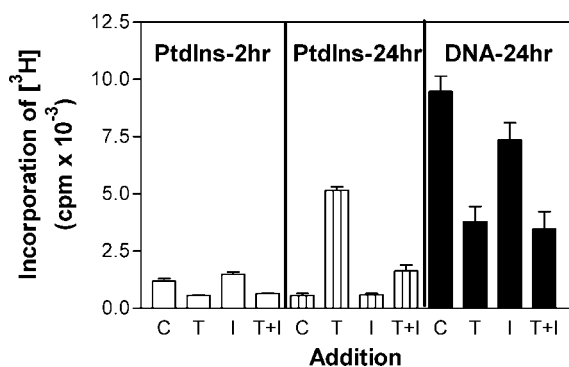


Fig. 9. Effect of the protein kinase C inhibitor (I) G δ -6983 (100 nM) on TPA (T; 100 nM)-induced changes in DNA and PtdIns metabolism. Methods were as described for Fig. 7. Cells were preincubated for 30 min with the inhibitor before the addition of TPA and radiolabeled precursors. Values shown represent means \pm SD of four separate determinations. The only significant effect of the inhibitor was to attenuate TPA-induced PtdIns synthesis ($P < 0.0005$).

require the activation of PKC activity, because neither Ro 31-8220 nor G δ -6389 is able to block the inhibition; however, these compounds do attenuate the stimulatory effect of phorbol ester seen at the later times. Glucose uptake from the medium is enhanced by TPA, ruling out a limitation of substrate availability as the cause of the early inhibition. Synthesis of CDP-DAG is not inhibited in the presence of TPA, excluding the possibility that turnover and resynthesis of PtdCho depletes the CTP pool required for the synthesis of PtdIns. Thus, it remains to be determined which aspect of the PtdIns synthetic pathway is responsible for mediating the inhibition seen in the presence of TPA.

In addition to the inhibition of PtdIns synthesis, DNA synthesis is also attenuated by phorbol ester treatment by a mechanism that is not sensitive to PKC inhibition (Fig. 9). It will be interesting to determine whether the early inhibition of PtdIns synthesis plays any role in the inhibition of DNA synthesis and cell cycle arrest in G1. Previous experiments using the inhibitor of PtdIns inostamycin suggest that PtdIns synthesis is required for the entry of NRK cells into S phase (19). The phorbol ester receptor responsible for the inhibition of both PtdIns and DNA synthesis remains to be determined. Possibilities include a variety of non-PKC moieties such as RasGRP3, the chimerins, MUNC13s, and DAG kinases β and γ (20).

The de novo synthesis of PtdIns is increased by TPA at later times (18–24 h) by a mechanism dependent on PKC activity. This increase is not accompanied by an increase in the turnover of existing PtdIns and is characterized by an increase in glycerol incorporation, indicating that de novo synthesis via the acylation of glycerol 3-phosphate, and not the phosphorylation of preexisting DAG, is responsible for the observed effect. The stimulation of phospholipid synthesis occurs in the absence of any increase in DNA or protein, so it cannot be attributed to a general effect on growth. The effect is apparent even in the absence of extracellular glucose, suggesting the likelihood of enhanced enzyme activity independent of substrate availability. Because the increase is not specific for PtdIns, enhancement of an enzyme activity before that which results in the production of PtdOH is suggested. Incubation of C3A cells with propranolol resulted in an increase in PtdOH concentrations as a result of the inhibition of the conversion of PtdOH to DAG, which is required for the synthesis of PtdCho and PtdEth. As expected, precursor incorporation into PtdCho and PtdEth was inhibited in the presence of propranolol, whereas that into PtdOH, CL, and PtdIns was enhanced (Fig. 7). These data confirm that increasing the levels of cellular PtdOH is sufficient to increase the synthesis of PtdIns. Thus, a TPA-induced increase in PtdIns synthesis could result from enhancement of the conversion of glycerol phosphate to PtdOH.

Alternatively, we wondered whether the availability of arachidonic acid might control the synthesis of PtdIns, because this lipid has been found to contain a high proportion of arachidonic acid in the *sn*-2 position. In mammalian liver, \sim 80% of the PtdIns contains arachidonic acid. Although tissue culture cells can lose some of

this specificity, they nevertheless also have arachidonic acid concentrated in PtdIns. Studies in HepG2 cells, the parent line of C3A, demonstrate that 36% of cellular PtdIns contains arachidonic acid when cells are grown in DMEM containing 10% fetal bovine serum. This value increases to 80% when the medium is supplemented with arachidonic acid (14). Although the C3A cells used in the studies described here were grown in the absence of added arachidonic acid, the growth medium used for these studies (F-12) contains linoleic acid, which can be converted to arachidonic acid by the cell. Triacsin C has been shown to specifically inhibit ACSL4 in intact cells (3, 4, 21). This appears to be the case for C3A cells as well. Treatment of cells with triacsin C specifically inhibited the synthesis of PtdIns but had no effect on the synthesis of PtdCho (Fig. 7). These data suggest that changes in ACSL4 activity might specifically alter the synthesis of PtdIns. Interestingly, ACSL4 has been shown to be overexpressed in some cancers, including liver and colon cancers (22–24). The relative levels of PtdIns in these tissues have not been measured.

Future investigations of the mechanisms responsible for the changes in PtdIns synthesis in C3A cells in response to TPA treatment will center on the activities of the five enzymes involved in the de novo synthesis of PtdIns. These are ACSL, glycerol-3-phosphate acyltransferase (GPAT), 1-acyl-*sn*-glycerol-3-phosphate acyltransferase (AGPAT), CDS, and PIS. Although only the final enzyme of the pathway, PIS, is absolutely specific for the synthesis of PtdIns, one cannot rule out the existence of a dedicated isoform (such as ACSL4, described above) or the compartmentalization of seemingly nonspecific enzymes as effective modes of rendering specificity. For example, it is well known that ACSL, GPAT, AGPAT, and CDS all have multiple isoforms that are found in different subcellular locations.

Several isoforms of ACSL have been described, distinguishable by their subcellular location (21) and substrate specificity (3). ACSL4, as described above, has a marked preference for arachidonic acid and is located primarily on the mitochondria-associated membrane as a peripheral, rather than an integral, membrane protein. The mitochondria-associated membrane is a subfraction of the endoplasmic reticulum that is closely associated with the mitochondria. ACSL1, on the other hand, has no preference for arachidonic acid and is localized to the endoplasmic reticulum, mitochondria-associated membrane, and cytosol. Because the activation of fatty acids by condensation with CoA is also a prerequisite for metabolism via other metabolic pathways, such as desaturation and oxidation, it has been suggested that the differential subcellular location of individual ACSL isoforms determines the fate of the product (25). Although the ACSL enzymes have been studied extensively with respect to their biochemical characteristics, location, and regulation by a variety of factors, including nutritional status and peroxisome proliferator-activated receptor ligands, their role in regulating phosphoinositide metabolism has not been investigated.


The conversion of glycerol-3-phosphate to lyso-PtdOH is considered the first committed step in the synthesis of

phospholipids (26). The enzyme activity responsible for this conversion exists in two unique isoforms: one is microsomal GPAT, which is associated with the endoplasmic reticulum, and the other is a mitochondrial form (mtGPAT1) associated with the outer mitochondrial membrane. Experiments in mtGPAT-deficient mice suggest the existence of a third form (mtGPAT2) present in the mitochondria but possessing the characteristics of the microsomal form (27). Overexpression of mtGPAT1 in rat hepatocytes results in a decrease in fatty acid oxidation and an increase in PtdCho, DAG, and triacylglycerol synthesis (28). No data are given for PtdIns. The second acylation step transfers arachidonic acid to the *sn*-2 position via the activity of AGPAT. This activity has been localized to the cytoplasmic surface of microsomal vesicles (29) as well as to the mitochondria and plasma membrane (30). Several isoforms have been cloned. AGPAT-2 shows a preference for arachidonoyl-CoA (31).

Acylation of glycerol phosphate yields 1-stearoyl,2-arachidonoyl-PtdOH, which is then converted to CDP-DAG by CDS. In rat liver, the majority of CDS activity is found in the microsomal fraction, with some in the mitochondria and nuclei (32). The product of CDS activity (CDP-DAG) can be used to synthesize phosphatidylglycerol as well as PtdIns, and in inositol auxotrophs deprived of inositol there is an increase in phosphatidylglycerol that accompanies the decrease in PtdIns synthesis (5). Because the intracellular levels of CDP-DAG are very low, it has been suggested that CDS is the rate-limiting enzyme (33); however, overexpression of this activity in COS7 cells resulted in only a slight (15%) increase in cellular PtdIns, although CDS in vitro activity was increased 15-fold (13). We have demonstrated previously that increasing substrate CDP-DAG is sufficient to increase PtdIns synthesis (34). In this study, we show similar results for increasing PtdOH substrate. Therefore, we conclude that an increase in the activity of ACSL, GPAT, AGPAT, or some combination thereof might be sufficient to cause an overall increase in the synthesis of PtdIns.

The final enzyme in the pathway, PIS, is the only one specific for the synthesis of PtdIns. In yeast, upregulation of the *pis1* gene, which codes for PIS, has been shown to be consistent with an overall increase in PtdIns levels (35). Plants have also been shown to regulate the expression of *pis1* (36). With respect to mammals, the *pis1* gene has been identified in rats (37) and humans (13), but data pertinent to the regulation of its expression are lacking. Although transient overexpression of *pis1* in COS-7 cells increases PIS activity in vitro by >20-fold, only a modest increase (8.2%) is seen in the rate of synthesis of PtdIns in cells in situ (13). This is increased to 59.6% in cells transfected with both *pis1* and *cds1*. In contrast, stable overexpression of *pis1* in NIH3T3 cells results in a 10-fold increase in the rate of PtdIns synthesis in situ and a 23-fold increase in activity in vitro (38). These stable *pis1* transfectants also exhibit increased levels of polyphosphoinositides (PtdInsP₂ and PtdInsP₃). They display a decrease in doubling time and accelerated G1 progression. Levels of cyclin D1 and E proteins are increased, as is Akt kinase activity. In addition, colony formation in soft agar is potentiated. The reason for the difference in the re-

sults generated using COS7 and NIH3T3 cells has not been resolved.

In summary, we have developed a tissue culture model in which PtdIns synthesis is regulated by treatment with phorbol ester. The large magnitude of the changes in PtdIns synthetic rates should facilitate further studies designed to determine the mechanism(s) that regulate the synthesis of this important lipid. In addition, it will be possible to assess the role of these regulatory changes in the control of cellular functions, such as growth, migration, carbohydrate metabolism, and the generation of inositol-containing second messengers. 

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REFERENCES

- Hawthorne, J. N. 1982. Inositol phospholipids. In *Phospholipids*. J.N. Hawthorne and G.B. Ansell, editors. Elsevier Biomedical Press, Amsterdam. 263–278.
- Albi, E., and M. P. Viola Magni. 2004. The role of intranuclear lipids. *Biol. Cell*. **96**: 657–667.
- Kang, M. J., T. Fujino, H. Sasano, H. Minekura, N. Yabuki, H. Nagura, H. Iijima, and T. T. Yamamoto. 1997. A novel arachidonate-preferring acyl-CoA synthetase is present in steroidogenic cells of the rat adrenal, ovary, and testis. *Proc. Natl. Acad. Sci. USA*. **94**: 2880–2884.
- Monaco, M. E., N. H. Moldover, and P. D. Walden. 2003. Analysis of hormone-stimulated phosphatidylinositol synthesis. *J. Cell. Physiol*. **194**: 151–161.
- Jackowski, S., D. R. Voelker, and C. O. Rock. 1988. Inositol metabolism and cell growth in a Chinese hamster ovary cell myoinositol auxotroph. *J. Biol. Chem*. **263**: 16830–16836.
- Gardocki, M. E., N. Jani, and J. M. Lopes. 2005. Phosphatidylinositol biosynthesis: biochemistry and regulation. *Biochim. Biophys. Acta*. **1735**: 89–100.
- Kelly, J. H. 1994. Permanent human hepatocyte cell line and its use in a liver assist device (LAD). U.S. Patent 5,290,684.
- Koreh, K., and M. E. Monaco. 1986. The relationship of hormone-sensitive and hormone-insensitive phosphatidylinositol to phosphatidylinositol 4,5-bisphosphate in the WRK-1 cell. *J. Biol. Chem*. **261**: 88–91.
- Antony, P., A. A. Farooqui, L. A. Horrocks, and L. Freysz. 2001. Effect of D609 on phosphatidylcholine metabolism in the nuclei of LA-N-1 neuroblastoma cells: a key role for diacylglycerol. *FEBS Lett*. **509**: 115–118.
- Antony, P., J. N. Kanfer, and L. Freysz. 2000. Phosphatidylcholine metabolism in nuclei of phorbol ester-activated LA-N-1 neuroblastoma cells. *Neurochem. Res*. **25**: 1073–1082.
- Antony, P., J. N. Kanfer, and L. Freysz. 2003. Retinoic acid specifically activates an oleate-dependent phospholipase D in the nuclei of LA-N-1 neuroblastoma cells. *FEBS Lett*. **541**: 93–96.
- Muhlbacher, C., E. Karnieli, P. Schaff, B. Obermaier, J. Mushack, E. Rattenhuber, and H. U. Haring. 1988. Phorbol esters imitate in rat fat-cells the full effect of insulin on glucose-carrier translocation, but not on 3-O-methylglucose-transport activity. *Biochem. J*. **249**: 865–870.
- Lykidis, A., P. D. Jackson, C. O. Rock, and S. Jackowski. 1997. The role of CDP-diacylglycerol synthetase and phosphatidylinositol synthase activity levels in the regulation of cellular phosphatidylinositol content. *J. Biol. Chem*. **272**: 33402–33409.
- Tanaka, T., J.-I. Morishige, T. Takimoto, Y. Takai, and K. Satouchi. 2001. Metabolic characterization of sciadonic acid (5c,11c,14c-eicosatrienoic acid) as an effective substitute for arachidonate of phosphatidylinositol. *Eur. J. Biochem*. **268**: 4928–4939.
- Gujdar, A., S. Sipeki, E. Bander, L. Buday, and A. Farago. 2003. Phorbol ester-induced migration of HepG2 cells is accompanied by intensive stress fibre formation, enhanced integrin expression and transient down-regulation of p21-activated kinase 1. *Cell. Signal*. **15**: 307–318.
- Sipeki, S., E. Bander, D. K. Ways, and A. Farago. 2002. Activation of Erk1/Erk2 and transiently increased p53 levels together may account for p21 expression associated with phorbol ester-induced transient growth inhibition in HepG2 cells. *Cell. Signal*. **14**: 115–121.
- Tsukada, Y., T. Tanaka, K. Miyazawa, and N. Kitamura. 2004. Involvement of down-regulation of Cdk2 in hepatocyte growth factor-induced cell cycle arrest at G1 in the human hepatocellular carcinoma cell line HepG2. *J. Biochem. (Tokyo)*. **136**: 701–709.
- Nagel, W. W., and B. L. Vallee. 1995. Cell cycle regulation of metallothionein in human colonic cancer cells. *Proc. Natl. Acad. Sci. USA*. **92**: 579–583.
- Imoto, M., T. Morii, A. Deguchi, and K. Umezawa. 1994. Involvement of phosphatidylinositol synthesis in the regulation of S phase induction. *Exp. Cell Res*. **215**: 228–233.
- Yang, C., and M. G. Kazanietz. 2003. Divergence and complexities in DAG signaling: looking beyond PKC. *Trends Pharmacol. Sci*. **24**: 602–608.
- Lewin, T. M., J. H. Kim, D. A. Granger, J. E. Vance, and R. A. Coleman. 2001. Acyl-CoA synthetase isoforms 1, 4, and 5 are present in different subcellular membranes in rat liver and can be inhibited independently. *J. Biol. Chem*. **276**: 24674–24679.
- Liang, Y. C., C. H. Wu, J. S. Chu, C. K. Wang, L. F. Hung, Y. J. Wang, Y. S. Ho, J. G. Chang, and S. Y. Lin. 2005. Involvement of fatty acid-CoA ligase 4 in hepatocellular carcinoma growth: roles of cyclic AMP and p38 mitogen-activated protein kinase. *World J. Gastroenterol*. **11**: 2557–2563.
- Sung, K. Y., S. Y. Hwang, M. K. Park, H. I. Bae, W. H. Kim, J. C. Kim, and M. Kim. 2003. Fatty acid-CoA ligase 4 is overexpressed in human hepatocellular carcinoma. *Cancer Sci*. **94**: 421–424.
- Cao, Y., K. B. Dave, T. P. Doan, and S. M. Prescott. 2001. Fatty acid CoA ligase 4 is up-regulated in colon adenocarcinoma. *Cancer Res*. **61**: 8429–8434.
- Lewin, T. M., C. G. Van Horn, S. K. Krisans, and R. A. Coleman. 2002. Rat liver acyl-CoA synthetase 4 is a peripheral-membrane protein located in two distinct subcellular organelles, peroxisomes, and mitochondrial-associated membrane. *Arch. Biochem. Biophys*. **404**: 263–270.
- Dircks, L. K., and H. S. Sul. 1997. Mammalian mitochondrial glycerol-3-phosphate acyltransferase. *Biochim. Biophys. Acta*. **1348**: 17–26.
- Lewin, T. M., N. M. J. Schwerbrock, D. P. Lee, and R. A. Coleman. 2004. Identification of a new glycerol-3-phosphate acyltransferase isoenzyme, mtGPAT2, in mitochondria. *J. Biol. Chem*. **279**: 13488–13495.
- Linden, D., L. William-Olsson, M. Rhedin, A. K. Asztely, J. C. Clapham, and S. Schreyer. 2004. Overexpression of mitochondrial GPAT in rat hepatocytes leads to decreased fatty acid oxidation and increased glycerolipid biosynthesis. *J. Lipid Res*. **45**: 1279–1288.
- Bell, R. M., and R. A. Coleman. 1980. Enzymes of glycerolipid synthesis in eukaryotes. *Annu. Rev. Biochem*. **49**: 459–487.
- Coleman, R. A., and D. P. Lee. 2004. Enzymes of triacylglycerol synthesis and their regulation. *Prog. Lipid Res*. **43**: 134–176.
- Eberhardt, C., P. W. Gray, and L. W. Tjoelker. 1997. Human lysophosphatidic acid acyltransferase. cDNA cloning, expression, and localization to chromosome 9q34.3. *J. Biol. Chem*. **272**: 20299–20305.
- Mok, A. Y. P., G. E. McDougall, and W. C. McMurray. 1992. CDP-diacylglycerol synthesis in rat liver mitochondria. *FEBS Lett*. **312**: 236–240.
- Nakagawa, Y., B. Rustow, D. Kunze, and K. Waku. 1989. The de novo synthesis of molecular species of phosphatidylinositol from endogenously labeled CDP diacylglycerol in alveolar macrophage microsomes. *Arch. Biochem. Biophys*. **268**: 559–566.
- Monaco, M. E., and J. R. Adelson. 1991. Evidence for coupling of resynthesis to hydrolysis in the phosphoinositide cycle. *Biochem. J*. **279**: 337–341.
- Gardocki, M. E., and J. M. Lopes. 2003. Expression of the yeast PIS1 gene requires multiple regulatory elements including a Rox1p binding site. *J. Biol. Chem*. **278**: 38646–38652.
- Lin, W. H., R. Ye, H. Ma, Z. H. Xu, and H. W. Xue. 2004. DNA chip-based expression profile analysis indicates involvement of the phosphatidylinositol signaling pathway in multiple plant responses to hormone and abiotic treatments. *Cell Res*. **14**: 34–45.
- Tanaka, S., J. Nikawa, H. Imai, S. Yamashita, and K. Hosaka. 1996. Molecular cloning of rat phosphatidylinositol synthase cDNA by functional complementation of the yeast *Saccharomyces cerevisiae* *pis* mutation. *FEBS Lett*. **393**: 89–92.
- Deguchi, A., K. Segawa, K. Hosaka, I. B. Weinstein, and K. Umezawa. 2002. Overexpression of phosphatidylinositol synthase enhances growth and G1 progression in NIH3T3 cells. *Jpn. J. Cancer Res*. **93**: 157–166.