Inositol Metabolism and Cell Growth in a Chinese Hamster Ovary Cell myo-Inositol Auxotroph*

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The intracellular concentrations of polyphosphoinositides and inositol phosphates were determined, and their role in growth factor-initiated cell division was investigated in a Chinese hamster ovary cell inositol auxotroph (CHO-K1-Ins). Metabolic labeling experiments during inositol starvation of CHO-K1-Ins cells showed that 1) the lipid-linked inositol component was maintained at the expense of the soluble inositol pool, 2) the decreasing cellular content of phosphatidylinositol was replaced by phosphatidylglycerol, and 3) the concentrations of inositol polyphosphates and polyphosphoinositides were conserved at the expense of inositol and phosphatidylinositol. These data show that homeostatic mechanisms exist for the maintenance of the polyphosphoinositide and inositol phosphate pools at the expense of inositol and phosphatidylinositol. The addition of α-thrombin to growth-arrested (serum-starved) CHO-K1-Ins cells stimulated the incorporation of "Hthyidine into DNA to the same extent as that observed following serum readdition. γ-Thrombin was also an effective mitogen, but active site-inhibited α-thrombin was not. Both α- and γ-thrombin, but not catalytic site-inhibited α-thrombin, initiated phosphatidylinositol turnover in vivo and increased phosphatidylinositol 4,5-bisphosphate phospholipase C activity in vitro. Serum and insulin were potent CHO-K1-Ins cell mitogens, but neither triggered phosphatidylinositol turnover in vivo nor activated phospholipase C in vitro. The activation of phospholipase C plays a determinant role in thrombin-initiated cell cycle progression in Chinese hamster ovary cells, although other growth factor-signaling pathways exist that are independent of polyphosphoinositide catabolism. The metabolism of PtdIns1 is receiving increased attention due to the involvement of this phospholipid in stimulus-response coupling. PtdIns-P2 is a major substrate for hormone-activated phospholipase C and is the precursor for diacylglycerol and Ins-P3 second messengers (for reviews see Refs. 1-4). Diacylglycerol stimulates protein phosphorylation via the activation of protein kinase C, and the liberated Ins-P3 triggers the mobilization of Ca2+ from intracellular stores, thereby initiating a host of Ca2+-regulated functions. In addition, PtdIns contains a high percentage of arachidonic acid in the sn-2-position, and the hydrolysis of this phospholipid by phospholipase A2 releases the substrate for the lipoxygenase and cyclooxygenase pathways (for review see Ref. 5).

Conditional lethal mutants of lower eukaryotes (6-9) defective in Ins synthesis strongly support the concept that the lipid-linked forms of Ins are necessary for normal cell growth and function. Many types of mammalian cells require an exogenous Ins supplement for growth in culture (9, 10); however, CHO cells are able to synthesize sufficient Ins de novo and do not require Ins-supplemented media. Ins-requiring CHO cells have been isolated by Kao and Puck (11) and Esko and Raetz (12), and the phospholipid composition of the latter auxotrophs was examined during Ins starvation. Ins deprivation results in the disappearance of PtdIns and the accumulation of phosphatidylglycerol (13). During the first 2-3 days of starvation the cells continue to grow, and cell physiology is not obviously affected by these alterations in phospholipid composition; however, when the PtdIns content falls to 0.5% of the total phospholipid there is a precipitous loss of cell viability. This cell system allows the Ins pools to be radiolabeled to known specific activity and thus offers a distinct advantage in the study of the metabolism of the important phosphorylated forms of PtdIns and Ins. Our aim was to investigate the metabolism of these minor components during Ins deprivation and investigate the role of PtdIns-P2 catabolism in the initiation of cell cycle progression in this cell line.

EXPERIMENTAL PROCEDURES

Materials—Sources for supplies were: Du Pont-New England Nuclear, Ptd[2-3H]Ins-P2 (specific activity 3.6 Ci/mmol), [methyl-3H]thymidine (specific activity 86.6 Ci/mmol), [32P]orthophosphate (carrier-free), and tritium-labeled Ins-P, Ins-P2, and Ins-P3 standards; Amersham Corp., [2-3H]Ins (specific activity 15.6 and 16.2 Ci/mmol) and ACS scintillation fluid; Gibco, fetal bovine serum and Ins-free ham's F-12 medium; Analtech, thin-layer chromatography plates; Fisher, HPLC-grade potassium phosphate; Whatman, Partisil-10 SAX HPLC column; Sigma, phospholipid standards, insulin, neomycin, sodium cholate, and buffers. Thrombin derivatives (14, 15) were the generous gift of Dr. John W. Forsion II, Division of Laboratories and Research, New York State Department of Health, Albany, NY, and were stored in 0.75 M NaCl. All other solvents and chemicals were reagent grade or better.

Strains and Growth Conditions—Strains CHO-K1 and CHO-K1-
Ins were obtained from F.-T. Kao and T. T. Puck (11) and were routinely grown in Ins-free Ham's F-12 medium supplemented with 100 μM Ins, 10% dialyzed fetal calf serum, 100 units/ml penicillin, 50 μg/ml streptomycin, and 2 mM glutamine. Cell number was determined by harvesting the dishes by trypsinization and counting the cells using either a hemacytometer or a Coulter counter.

Phosphoinositide Analysis—Phosphoinositides and inositol phosphates were separated using a two-phase system (16). For example, a 100-μl reaction mixture was mixed with 360 μl of chloroform:methanol:2% HCl (1:2:0.2, v/v) followed by 120 μl of chloroform and 120 μl of 2 M KCl. The sample was vortex-mixed and centrifuged for 5 min in a Beckman Microfuge-12 to separate the phases. Approximately 97% of Ins-phosphoinositides were found in the lower organic layer, and >90% of the inositol phosphates were found in the upper aqueous layer. Solvents were added in the same ratio to extract larger or smaller sample volumes. When inositol phosphates were determined, KCl was left out of the extract and the aqueous phase was evaporated in a Speed-Vac concentrator and resuspended in 100 μl of water.

PtdIns species were separated by thin-layer chromatography on Silica Gel H layers that were sprayed with 5 mM EDTA and activated at 100 °C for 30 min prior to development. The solvent system was chloroform:methanol:ammonium hydroxide:water:2% HCl (45:35:1:5:8:30±16, v/v). The distribution of tritium on the thin-layer plates was quantitated using the Bioscan 2000 positionally sensitive detector. HPLC separation of inositol phosphates was performed using a Perkin-Elmer series 400 liquid chromatograph equipped with a 4.5 x 250-mm Partisil-10 SAX strong anion exchange column. The column was eluted with a linear gradient of 5% HCl to 0.05% HCl over 30 min at a flow rate of 1 ml/min. Fractions were collected every 0.5 min, and a 200-μl aliquot was removed for scintillation counting. Retention times for [3H]iniositol phosphate standards were: Ins, 6 min; Ins-P, 15 min; Ins-P2, 20 min; and Ins-P3, 30 min.

The absolute amount of each Ins-containing compound was determined by multiplying the total nanomoles of [3H]Ins in the extract (either organic or soluble) times the percent of the total radioactivity recovered in each metabolite following chromatographic fractionation. Protein was determined using the Bio-Rad protein assay kit and γ-globulin as the standard.

Membrane Isolation and Phospholipase C Assay—Confluent cultures of CHO-K1-Ins in 150-mm dishes were washed 3 times with 4 ml of ice-cold phosphate-buffered saline containing 0.1% glucose. Next, 4 ml of cold hypotonic buffer (0.01 M Tris-HCl, pH 7.4, 0.01 M NaCl) were added, and the dishes were scraped with a rubber policeman, homogenized for 50 strokes with a Dounce (B pestle) homogenizer, and 0.25 ml/ml sucrose, 0.01 M Tris-HCl, pH 7.4, 0.01 M NaCl was added, and the dish was kept on ice for 20 min. The cells were scraped from the dish with a rubber policeman, homogenized for 50 strokes with a Dounce (B pestle) homogenizer, and 0.25 ml/ml extract of 1.25 mM sucrose, 0.01 M Tris-HCl, pH 7.4, 0.01 M NaCl was added. The extract was centrifuged at 3,000 rpm in a JA-20 rotor, and the supernatant was layered on top of a sucrose cushion (15% sucrose, 0.01 M Tris-HCl, pH 7.4, 0.01 M NaCl) in a SW 28 rotor for 30 min at 25,000 rpm in an SW 28 rotor. The total membrane pellet was resuspended in 50 mM bis-Tris, pH 6.0.

The phospholipase C assay method was essentially as described previously (16, 17). Ptd[3H]Ins-P2 was dried in a test tube under a stream of nitrogen and dissolved in 5% sodium cholate to yield a solution containing 13,900 cpm of Ptd[3H]Ins-P2/μl. The detergent and substrate were added to the incubation mixture simultaneously (3 μl/assay). The final concentrations of components added to the phospholipase C assay were: Ins-Tris-HCl (0.1 M, pH 7.4), sodium cholate (0.88 M), Ptd[3H]Ins-P2 (40,000 cpm, 0.48 μM), and membrane protein (10 μg/assay) in a final volume of 40 μl. The reaction mixtures were incubated for 10 min at 37 °C, the reaction was stopped by the addition of chloroform:methanol:HCl (1:2:0.2, v/v), and the assay mixture was extracted as described above. Two aliquots of the aqueous phase were determined to contain the amount of [3H]Ins-P2 produced. The formation of [3H]Ins-P2 was linear with time up to 10 min and linear with protein up to 10 μg/assay. The aqueous phase from control incubations (no protein or no activator) typically contained 3% of the total radioactivity in the assay. A rate of 10 pmol x min^-1 x mg^-1 protein corresponds to the production of 2,300 cpm of [3H]Ins-P2/μg protein.

[3H]Thymidine Incorporation—CHO-K1-Ins cells from a stock culture were plated at a density of 4 x 10^6 cells/well into a 6-well cluster plate along with 3 ml of Ham's F-12 containing 10% dialyzed fetal calf serum and 100 μM Ins. Cells were grown for 72 h (subconfluent) and media were changed to Ham's F-12 containing 100 μM Ins. Following serum starvation for 24 h, the media were changed to 3 ml of Ham's F-12 containing 100 μM Ins, 6 μCi of [3H] thymidine, and the indicated concentration of mitogen. After 24 h in the presence of [3H]thymidine, the cells were trypsinized, transferred to a centrifuge tube, and the cells were pelleted and resuspended in 100 μl of phosphate-buffered saline containing 50 μg/ml bovine serum albumin. The suspension was made 10% in trichloroacetic acid, precipitated with the addition of cold acetone, and centrifuged for 10 min. The precipitate collected on a glass fiber filter which was washed with 1 ml of 5% cold trichloroacetic acid followed by 3 ml portions of 1% trichloroacetic acid, and counted in 3 ml of ACS. Incorporation of [3H]thymidine into precipitable material was 88% of the total cell-associated label, and the amount of label taken up by the cells was proportional to the amount of label determined by the filter disc assay. The incorporation of [3H]thymidine was indicative of an increase in the cell number. Under conditions of maximum mitogenic stimulation, the cell population doubled within the 24-h assay period.

Metabolic Labeling—To determine the phospholipid composition of CHO-K1-Ins cells during Ins starvation, cultures were grown to confluence in Ham's F-12 medium containing 10% dialyzed fetal calf serum, 100 μM Ins, and 3 μCi/ml [3P]orthophosphate. The cells were then harvested and seeded into 60-mm dishes containing Ham's F-12 medium, 10% dialyzed fetal calf serum, and 5 μCi/ml [3P]orthophosphate either with or without 100 μM Ins. At 24-h intervals, cells from duplicate dishes with or without Ins were harvested, and the phospholipids were extracted. Total cellular phospholipids were separated by two-dimensional thin-layer chromatography using chloroform:methanol:acetic acid (85:25:10, v/v) in the first dimension and chloroform:methanol:formic acid (65:25:10, v/v) in the second dimension. The amount of [3P] incorporation was determined by removing the appropriate area of silica gel and scintillation counting.

In the compositional experiments where CHO-K1-Ins cells were grown on different concentrations of Ins, cells were seeded at 2 x 10^6 cells/90-mm dish in Ham's F-12 medium containing dialyzed fetal calf serum and the indicated concentration of [3H]Ins (specific activity 80 Ci/mol). Duplicate dishes were harvested for determination of cell number and for extraction and analysis of Ins-derived metabolites. The Ins starvation experiments were performed by first obtaining a confluent culture of CHO-K1-Ins cells that were grown in Ham's F-12 medium with 10% dialyzed fetal calf serum and 100 μM [3H]Ins (specific activity 80 Ci/mol). The cells were harvested and seeded at a density of 2.5 x 10^5 cells/100-mm dish in medium without Ins. At 24-h intervals duplicate dishes were harvested, extracted, and Ins-derived metabolites were analyzed by HPLC and thin-layer chromatography as described above.

For the mitogen-induced PtdIns turnover experiments, cells were seeded into 100-mm dishes and grown to a density of 5 x 10^5 cells/dish (subconfluent). The medium was then replaced with Ham's F-12 supplemented with 5 μM [3H]Ins (specific activity 1.6 Ci/mmol) and serum-starved for 24 h. The [3H]Ins medium was then removed, the cells were washed twice with phosphate-buffered saline and collected as described above. The cells were then overlaid with Ham's F-12 medium containing 10 mM HEPES, pH 7.4. LiCl was added to a final concentration of 10 mM, and 10 min later the appropriate mitogen was added. Incubations in the presence of mitogens were for 10 min, and the cells were rapidly cooled on ice. The cells were removed by scraping with a rubber policeman in phosphate-buffered saline, extracted, and the soluble Ins phosphate pool analyzed by HPLC as described above.

Data Presentation—Each data point represents the average of duplicate determinations, and each experiment was performed twice.

RESULTS

Growth and Phospholipid Composition of CHO Cell Ins Auxotrophs—CHO-K1-Ins cells exhibited an absolute Ins requirement for growth in Ins-free Ham's F-12 medium supplemented with dialyzed fetal calf serum. The maximum yield of cells was obtained in medium containing 100 μM Ins. CHO-K1-Ins cells were grown in medium supplemented with 100 μM Ins and were plated at a density of 2.5 x 10^5 cells/60-mm dish and incubated either in the presence or absence of 100 μM Ins. Cell number in duplicate dishes was determined at 24-h intervals. We observed that the growth rates of CHO-K1-Ins cells under these two conditions were the same for the first 48 h in culture. However, by 72 h the Ins-starved cells stopped growing, lost viability, and detached from the dish.
resulting in a lower cell number at 96 h. Esko and Raetz (13) reported that during Ins starvation PtdIns was replaced by phosphorylcholine in their CHO Ins auxotroph, and we observed the same phenomenon in the CHO-K1-Ins cell line. Cells were prelabeled with 3 μCi/ml [3H]orthophosphate either with or without 100 μM Ins. At 24-h intervals, cell samples were harvested, and the lipids were extracted and analyzed by two-dimensional thin-layer chromatography as described under "Experimental Procedures." As in the CHO Ins auxotroph isolated by Esko and Raetz (13), PtdIns was 8% of the phospholipid pool in CHO-K1-Ins cells and decreased to 0.8% of the total at 72 h, at which point the cells began to lose viability. During this 72-h period phosphorylcholine increased from 1% of the total pool to 9.9%. These data demonstrated that the CHO-K1-Ins auxotroph used in this study had properties that were indistinguishable from the independently isolated Ins-dependent CHO cell line isolated by Esko and Raetz (13).

**Lipid-linked Ins Content Was Maintained at the Expense of the Soluble Pool**—CHO-K1-Ins cells were grown for 72 h in the presence of a series of increasing concentrations of [3H]Ins ranging from 10 to 200 μM, and the cells were harvested at a density of 3 × 10^6 cells/dish (Fig. 1). Although the lowest concentration of Ins used in this experiment would not support the growth of the Ins auxotroph to a confluent monolayer, the cells grew to the same rate and to the same density under all experimental conditions shown in Fig. 1. At 10 μM Ins the cells were at the point where a precipitous decrease in viability would be observed if growth in this concentration of Ins was allowed to continue. The content of total cellular Ins increased as the Ins supplement in the medium increased. The amount of lipid-linked Ins climbed sharply between the 10 and 50 μM Ins supplement and then remained constant up to 200 μM. Between 50 and 200 μM Ins the increase in the total cellular Ins occurred exclusively in the soluble Ins pool. HPLC analysis demonstrated that this increase was in the Ins component and not the inositol phosphates (not shown).

**Conservation of Polyphosphoinositides and Inositol Phosphates during Ins Starvation**—The metabolism of polyphosphoinositides and inositol phosphates was assessed during Ins deprivation. CHO-K1-Ins cells were prelabeled by growth for 72 h in the presence of 100 μM [3H]Ins (specific activity 80 Ci/mol), then subcultured in Ins-free medium and incubated for the indicated times up to 96 h. Cell growth and viability during Ins starvation were the same as described in the first paragraph under "Results." There was a consistent decrease in the cellular Ins content during starvation from 21.5 to 2.1 nmol/mg protein (Fig. 2). Less than 5% of the total label was found in the medium indicating that the cells efficiently conserved Ins. PtdIns-P_2 (0.3%) and PtdIns-P_0 (0.5%) were minor components of the lipid-linked Ins pool in cells grown in 100 μM Ins, and a component comigrating with acyl-GPI comprised 1.2% of the total Ins-labeled phospholipids (Fig. 3A). The absolute amounts of the lipid-linked Ins species were: PtdIns, 12.65 nmol/mg protein; 2-acyl-GPI, 0.15 nmol/mg protein; PtdIns-P, 0.06 nmol/mg protein; and PtdIns-P_0, 0.04 nmol/mg protein. At 72 h, the total content of lipid-linked Ins had decreased 10-fold; however, the percentage of PtdIns-P_2 (4.0%) and PtdIns-P_0 (2.2%) had increased substantially (Fig. 3B). The absolute amounts of lipid-linked Ins species after 72 h of Ins starvation were: PtdIns, 1.5 nmol/mg protein; 2-acyl-GPI, 0.01 nmol/mg protein; PtdIns-P, 0.06 nmol/mg protein; and PtdIns-P_0, 0.08 nmol/mg protein. These data show that the polyphosphoinositide pools were maintained in the face of large decreases in the cellular PtdIns content.

The composition of the soluble Ins pool was also analyzed during Ins starvation (Fig. 4). In cells grown in 100 μM Ins, the total soluble Ins pool was 8.6 nmol/mg protein, and Ins was the major component and the inositol phosphates were minor species (Fig. 4A). The absolute amounts of soluble Ins species were: Ins, 8.5 nmol/mg protein; Ins-P, 0.03 nmol/mg protein; Ins-P_0, 0.03 nmol/mg protein; and Ins-P_2, 0.04 nmol/mg protein. After the starvation for 72 h, Ins decreased to 23% of the total and Ins-P_2 (41%) became the dominant component (Fig. 4B). The percentage of tritium label in the Ins-P and Ins-P_2 areas of the chromatogram also increased significantly. The absolute amounts of soluble Ins metabolites after 72 h of Ins starvation were: Ins, 0.103 nmol/mg protein; Ins-P, 0.063 nmol/mg protein; Ins-P_0, 0.099 nmol/mg protein; and Ins-P_2, 0.185 nmol/mg protein. These data indicate that large changes in the intracellular Ins concentration do not perturb the homeostatic mechanisms that regulate the cellular content of lipid-linked Ins.

**Fig. 1. Conservation of lipid-linked Ins at the expense of soluble Ins.** CHO-K1-Ins cells were seeded at a density of 2 × 10^6 cells/60-mm dish and incubated for 72 h in Ham's F-12 medium containing 10% dialyzed fetal calf serum and the indicated concentration of [3H]Ins (specific activity 80 Ci/mol). In all cases the growth of the cells was the same resulting in the same number of cells/dish at harvest. The amount of lipid-linked and soluble Ins label was determined using the extraction procedure described under "Experimental Procedures."

**Fig. 2. Decrease in cellular Ins content during Ins starvation.** CHO-K1-Ins cells were prelabeled with 100 μM [3H]Ins (specific activity 80 Ci/mol) and then plated in Ham's F-12 medium containing 10% fetal calf serum without Ins. Samples were taken at the indicated times for determination of total cellular Ins and protein as described under "Experimental Procedures."
PtdIns-P₃ Catabolism and Cell Growth

2.0

A. 100 μM Ins

Counts X 10⁻⁴

PtdIns-P₃ (98.1%)
Acyl-GPI (12.2%)
PtdIns-P₂ (0.3%)
PtdIns-P (0.2%)

B. Minus Ins (72 h)

Counts X 10⁻³

PtdIns-P₃ (93.2%)
PtdIns-P₂ (4.0%)
PtdIns (2.2%)
Acyl-GPI (0.7%)

Relative Mobility

FIG. 3. PtdIns-P₃ and PtdIns-P were conserved during Ins starvation. CHO-K1-Ins cells were grown to confluence in medium containing 100 μM [³H]Ins (specific activity 80 Ci/mol). Cells were then subcultured in the absence of Ins and cell samples extracted to determine the content and composition of lipid-linked Ins species. Panel A, separation of tritiated PtdIns species extracted from cells grown in 100 μM Ins (zero time). Panel B, separation of tritiated PtdIns species extracted from cells grown in Ins-free medium for 72 h. Thin-layer chromatography and radioactivity imaging were performed as described under "Experimental Procedures."

the concentration of inositol phosphates increased during Ins starvation.

Thrombin-dependent Mitogenesis and PtdIns Turnover in CHO-K1-Ins Cells—Thrombin is a potent fibroblast mitogen and activates PtdIns turnover (18-21). CHO cells possess high levels of cell surface thrombin binding sites (22). Therefore, we used the ability to uniformly label the Ins pools of CHO-K1-Ins cells to examine thrombin-induced alterations in PtdIns catabolism and determine the relationship between phospholipase C activation and stimulation of DNA synthesis in this cell line. CHO-K1-Ins cells were grown in medium containing 100 μM Ins in 100-mm dishes to a density of 5 × 10⁵ cells/dish (subconfluent). The medium was then removed and replaced with Ham's F-12 supplemented with 100 μM Ins and lacking fetal calf serum. After a 24-h incubation in the absence of serum, cells were assayed for their ability to incorporate [³H]thymidine into DNA in response to different mitogens. [³H]Thymidine incorporation in the absence of added mitogen averaged 2142 cpm/10⁵ cells (subconfluent). Thrombin stimulated [³H]thymidine incorporation into CHO-K1-Ins cells 7-9-fold (Fig. 5). Both α- and γ-thrombin were effective although more [³H]thymidine was incorporated in response to γ-thrombin than α-thrombin (Fig. 5). Cell numbers were also determined in these experiments, and the number of cells approximately doubled following thrombin, insulin, or serum addition. To determine the effect of mitogens on PtdIns catabolism in vivo, CHO-K1-Ins cells were labeled with 5 μM [³H]Ins (specific activity 1.6 Ci/mmol) during the serum starvation period. The addition of either α- or γ-thrombin triggered the accumulation of phosphorylated Ins species (Fig. 6) indicating the activation of phospholipase C. Ins-P was the most abundant inositol phosphate detected due to the prevention of its hydrolysis by LiCl (Fig. 7). Ins-P₃ was also increased...
Our results illustrate that CHO-K1-Ins cells possess homeostatic mechanisms that regulate and maintain the intracellular concentrations of polyphosphoinositides and inositol phosphates. The least regulated metabolite was Ins. When Ins was scarce it was efficiently incorporated into phospholipid in order to maintain the levels of lipid-linked Ins, and when Ins was abundant, the amount of lipid-linked Ins remained constant while the size of the Ins pool increased significantly (Fig. 2). Physiological mechanisms exist that preserve the level of PtdIns-P2 and PtdIns-P since the cellular content of these phosphorylated derivatives was maintained even though the content of PtdIns decreased 10-fold (Fig. 3 and see "Results"). Similarly, inositol phosphates were only minor components of the soluble pool in cells grown in an abundance of Ins but were the major components in cells that were deprived of Ins (Fig. 4 and see "Results"). Cells with lower than normal concentrations of polyphosphoinositides and inositol phosphates were not observed, suggesting that when the levels of these components fell below the set point, the cells were no longer viable. PtdIns can also function as a

**FIG. 6.** α- and γ-Thrombin stimulation of PtdIns catabolism. CHO-K1-Ins cells were prelabeled with [3H]Ins, serum-starved, exposed to 10 mM LiCl for 10 min followed by the indicated concentration of growth factor for 10 min, and the hormone-induced accumulation of [3H]Ins-P (thrombin-treated minus control) was determined by extraction and HPLC as described under "Experimental Procedures."

**FIG. 7.** Stimulation of inositol phosphate production by α-thrombin. CHO-K1-Ins cells were prelabeled with [3H]Ins, serum-starved, and exposed to 10 mM LiCl for 10 min followed by 200 nM α-thrombin for 10 min. Cells were rapidly cooled on ice, harvested, extracted, and the composition of the soluble Ins pool analyzed by ion-exchange HPLC as described under "Experimental Procedures."

**DISCUSSION**

showing that PtdIns-P2 hydrolysis was stimulated. However, due to the presence of active phosphatases we were unable to determine whether Ins-P or was formed by the degradation of Ins-P3 or the cleavage of PtdIns in vivo. Both α- and γ-thrombin also enhanced PtdIns-P2 phospholipase C activity associated with isolated CHO-K1-Ins cell membranes (Fig. 8). Basal PtdIns-P2 phospholipase C activity (4.6 pmol/min/mg) was increased to a maximum of 17.4 pmol/min/mg by the addition of 400 nM α- or γ-thrombin to the assay. The biochemical characteristics of thrombin-activated PtdIns-P2 phospholipase C were the same as we described previously (17). The dose responses for mitogenesis, PtdIns turnover in vivo, and PtdIns-P2 phospholipase C activity in vitro were comparable, indicating that these three events were related (Figs. 5–8). Active site-inhibited α-thrombin (DFP-α-thrombin) was not a CHO cell mitogen, did not stimulate inositol phosphate accumulation in vivo, and did not activate membrane-associated PtdIns-P2 phospholipase C (not shown). We also observed that insulin and serum did not activate PtdIns turnover in vivo nor PtdIns-P2 phospholipase C activity in vitro (not shown).

To determine if PtdIns catabolism plays a determinant function in thrombin-mediated mitogenesis, neomycin was employed as an inhibitor of PtdIns-P2 hydrolysis (19, 23, 24). Neomycin was a potent inhibitor of α-thrombin-stimulated PtdIns-P2 phospholipase C activity in vitro, and neomycin also blocked α-thrombin-dependent mitogenesis. At 6 mM neomycin, α-thrombin-dependent [3H]thymidine incorporation was inhibited >95% whereas insulin-initiated mitogenesis was inhibited 20%, and the cells' response to serum was unaffected by this concentration of neomycin. The reason that more neomycin was required to inhibit mitogenesis than phospholipase C activity measured in vitro was attributed to the inability of neomycin to readily penetrate cell membranes (25). These results indicate that PtdIns catabolism is required for α- or γ-thrombin to induce DNA synthesis in CHO-K1 cells but that other mitogenic pathways also exist that are independent of PtdIns breakdown.
source of arachidonic acid (5), raising the possibility that cell death may occur when the PtdIns content falls below the level required to maintain the production of eicosanoids from this specific precursor. Ins is also incorporated into two other lipid-linked forms that may be important for normal cell growth and function. A PtdIns glycan structure is thought to be hydrolyzed in response to insulin, thus generating a phospholipid scaffold second messenger (28-30). A similar PtdIns-glycan structure (31) attaches proteoglycan sulfate (32) and ornithine decarboxylase (33) to the membrane, and the hydrolysis of this phospholipid anchor mediates the release of these proteins in response to growth factors. At present it is not possible to establish a direct causal relationship between any one of these important metabolites and the loss of cell viability.

The hydrolysis of PtdIns-P2 by a phospholipase C was required for thrombin-dependent initiation of cell cycle progression in CHO-K1-Ins cells. Our data show that 100-400 nM α- or γ-thrombin stimulated the incorporation of [3H] thymidine into DNA, the catabolism of PtdIns in vivo (Fig. 7), and the activation of PtdIns-P2 phospholipase C in vitro (Fig. 8). The correlation between the dose-response for [3H] thymidine incorporation and PtdIns-P2 phospholipase C activity (in vivo and in vitro) (Figs. 5 and 8) and the inhibition of these events by neomycin suggests a causal relationship between the mitogenesis and PtdIns-P2 hydrolysis. Phospholipase C involvement in α-thrombin-induced mitogenesis has been reported by a number of previous investigators (18-21), and there is evidence for two distinct pathways triggered by α-thrombin (34-40). The high-affinity pathway is activated by either α-thrombin or DFP-α-thrombin (but not γ-thrombin) and is coupled through the inhibitory guanine nucleotide binding protein to the inhibition of adenylate cyclase and the production of eicosanoids (40). There is also evidence that this high-affinity pathway activates PtdIns-P2 phospholipase C via a G-protein (39, 41-44) that is modified by pertussis toxin in some cell systems (42-44) but not in others (39, 41). A separate low-affinity system that stimulates PtdIns-P2 phospholipase C activity is triggered by either α- or γ-thrombin, but not DFP-α-thrombin (17, 39, 43, 44). The low-affinity pathway activates phospholipase C to a greater extent than the high-affinity process explaining the inability to saturate the formation of inositol phosphates in vivo at α-thrombin concentrations below 100 nM (Ref. 21 and Fig. 7). In some cell lines the two pathways operate synergistically to produce the optimum mitogenic response (35, 38); however, we failed to demonstrate synergism between DFP-α-thrombin and γ-thrombin nor did we observe significant stimulation of cell division by low concentrations (10 nM) of α-thrombin, suggesting that the high-affinity pathway is either inoperative or unrelated to mitogenesis in CHO-K1-Ins cells. The characteristics of α- and γ-thrombin enhancement of PtdIns catabolism in vivo, PtdIns-P2 phospholipase C activity in vitro, and mitogenesis reflect the properties of the low-affinity α- or γ-thrombin-dependent activation pathway. These results illustrate that PtdIns catabolism plays a determinant role in the ability of thrombins to initiate cell cycle progression in CHO cells. We, and others (45), have not observed PtdIns turnover associated with either serum- or insulin-dependent mitogenesis in CHO cells. The ability of serum to enhance the breakdown of polyphosphoinositides in other cell lines is due to their response to platelet-derived growth factor (46, 47). The inability of serum to increase the production of inositol phosphates in CHO-K1-Ins cells is attributed to the lack of platelet-derived growth factor receptors on CHO cells (48).

Therefore, it is apparent that insulin and serum factors utilize other phospholipase C-independent mitogenic pathways in CHO cells.

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