MOLECULAR AND CELLULAR BIOLOGY, July 1989, p. 2934–2943
0270-7306/89/072934-10$02.00/0

Vol. 9, No. 7

Platelet-Derived Growth Factor Induces Rapid and Sustained Tyrosine Phosphorylation of Phospholipase C-γ in Quiescent BALB/c 3T3 Cells

MATTHEW I. WAHL,[1] NANCY E. OLASHAW,[2] SHUNZO NISHIBE,[3] SUE GOO RHEE,[4] W. J. PLEDGER,[2] AND GRAHAM CARPENTER[1,3]*

Departments of Biochemistry,[1] Cell Biology,[2] and Medicine,[3] Vanderbilt University School of Medicine, Nashville, Tennessee 37232-0146, and Laboratory of Biochemistry, National Heart, Lung, and Blood Institute, Bethesda, Maryland 20892[4]

Received 8 March 1989/Accepted 13 April 1989

Platelet-derived growth factor (PDGF) stimulates the proliferation of quiescent fibroblasts through a series of events initiated by activation of tyrosine kinase activity of the PDGF receptor at the cell surface. Physiologically significant substrates for this or other growth factor receptor or oncogene tyrosine kinases have been difficult to identify. Phospholipase C (PLC), a key enzyme of the phosphoinositide pathway, is believed to be an important site for hormonal regulation of the hydrolysis of phosphatidylinositol 4,5-bisphosphate, which produces the intracellular second-messenger molecules inositol 1,4,5-trisphosphate and 1,2-diacylglycerol. Treatment of BALB/c 3T3 cells with PDGF led to a rapid (within 1 min) and significant (greater than 50-fold) increase in PLC activity, as detected in eluates of proteins from a phosphotyrosine immunoaffinity matrix. This PDGF-stimulated increase in phosphotyrosine-immunopurified PLC activity occurred for up to 12 h after addition of growth factor to quiescent cells. Interestingly, the PDGF stimulation occurred at 3 as well as 37°C and in the absence or presence of extracellular Ca^{2+}. Immunoprecipitation of cellular proteins with monoclonal antibodies specific for three distinct cytosolic PLC isozymes demonstrated the presence of a 145-kilodalton isozyme, PLC-γ (formerly PLC-II), in BALB/c 3T3 cells. Furthermore, these immunoprecipitation studies showed that PLC-γ is rapidly phosphorylated on tyrosine residues after PDGF stimulation. The results suggest that mitogenic signaling by PDGF is coincident with tyrosine phosphorylation of PLC-γ.

Platelet-derived growth factor (PDGF) initiates a proliferative response in fibroblasts through a complex mechanism that includes the induction of specific gene products (12, 50). The biochemical mechanisms involved in transduction of the mitogenic signal from the cell surface to the nucleus are not well understood. PDGF binds to a specific cell surface glycoprotein receptor that mediates the mitogenic signal (7, 27, 32). The initial biochemical response after binding of PDGF to its receptor is stimulation of the receptor tyrosine kinase activity (15, 65). Several other growth factor receptors and oncogene products demonstrate this enzymatic activity (reviewed in references 8, 33, and 66), suggesting that this biochemical property is a fundamental property of growth regulatory systems. Furthermore, expression in intact cells of mutated PDGF receptors lacking tyrosine kinase activity revealed that such receptors are incapable of transducing a mitogenic signal after binding PDGF (16). At present, key tyrosine kinase substrates in the mitogenic process have not been identified in either the growth factor-regulated or oncogene system. The detection of substrates having a known biochemical function may provide clues to the transduction signals involved in growth factor modulation of cell proliferation.

In addition to the stimulation of tyrosine kinase activity, other intracellular signaling pathways are rapidly modulated by PDGF binding. One of these is the stimulation of inositol phosphate (InsP) formation and Ca^{2+} mobilization (6, 11, 23, 25). Although the mechanisms by which extracellular ligands modulate the turnover of inositol phospholipids have not been thoroughly elucidated, many hormones are believed to stimulate phospholipase C (PLC), the key enzyme of the phosphoinositide cycle (reviewed by Abdel-Latif [1]). The biochemical characteristics of the family of PLC isozymes purified from mammalian tissues and a revised system of nomenclature have been reported by S. G. Rhee, P. G. Suh, and S. H. Ryu (Science, in press). PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate (PtdIns 4,5-P_2) to produce two important intracellular second messengers, inositol 1,4,5-triphosphate (Ins 1,4,5-P_3) and diacylglycerol. These compounds mediate, respectively, the liberation of stored intracellular Ca^{2+} and stimulation of protein kinase C (reviewed in references 5 and 46). Knowledge of the biological processes regulated by Ins 1,4,5-P_3 and diacylglycerol as well as of the processes regulating their formation is fundamental to a mechanistic understanding of cell proliferation.

Treatment of fibroblasts with PDGF induces a variety of rapid responses, including cellular alkalinization (10, 41), redistribution of the cytoskeletal components vinculin and actin (29), and induction of numerous gene products, such as c-*myc* and c-*fos* (12, 22, 37, 39). It is plausible that all of these events are mediated, at least in part, by Ins 1,4,5-P_3 and diacylglycerol (28, 34, 41, 44, 62). These responses are induced rapidly by PDGF and thus may be involved in PDGF-induced competence formation, the process by which PDGF renders quiescent BALB/c 3T3 cells responsive to plasma factors that mediate subsequent traversal of the cell cycle (51). Although the roles of Ins 1,4,5-P_3 and diacylglycerol in growth factor-initiated early responses have been studied extensively, comparatively less attention has focused on the potential involvement of these second-mes-

* Corresponding author.

senger molecules in events that occur at later times after mitogenic stimulation. The importance of this signaling system in growth factor-mediated cell proliferation is supported by the observation that when antibody to PtdIns 4,5-P_2 was microinjected into NIH 3T3 fibroblasts 2 h before or after PDGF addition, PDGF-induced DNA synthesis was blocked (42).

Our studies have focused on the mechanisms by which the tyrosine kinase activity of the PDGF receptor may communicate with the phosphoinositide signaling system. The results show that PDGF induces the rapid and sustained tyrosine phosphorylation of PLC-γ and that this event coincides with PDGF-stimulated formation of InsPs.

MATERIALS AND METHODS

Cell culture and metabolic labeling. BALB/c 3T3 cells were grown in 100-mm-diameter tissue culture plates in Dulbecco-Vogt modified Eagle medium (DMEM) plus 10% calf serum, 4 mM L-glutamine, 50 U of penicillin per ml, and 50 μg of streptomycin per ml. Cells were grown for 5 days to confluence, and the medium was changed to inositol-free DMEM plus 2% platelet-poor plasma (PPP) 20 h before an experiment. [^{3}H]inositol (2 μCi/ml) was included in this medium for cultures in which the levels of [^{3}H]InsPs were determined.

Extraction and separation of [^{3}H]InsPs. BALB/c 3T3 cells labeled with [^{3}H]inositol in DMEM plus 2% PPP were treated for 10 min with 20 mM LiCl; PDGF was then added, and cells were incubated as indicated in the figure legends. Cells were then rapidly washed three times with ice-cold Ca^{2+}-Mg^{2+}-free phosphate-buffered saline (CMF-PBS), and cellular metabolism was stopped by addition of 1.0 ml of 10% perchloric acid. After 15 to 30 min, the acidic supernatants were recovered. [^{3}H]InsPs were then separated and quantitated as described previously (47). Data represent the average amounts of [^{3}H]InsPs recovered from duplicate plates per treatment condition.

Antiphosphotyrosine recovery of PLC activity. BALB/c 3T3 cells in DMEM plus 2% PPP were treated for 10 min with 100 μM Na_3VO_4; PDGF was then added, and cells were incubated as indicated in the figure legends. Cells were then rapidly washed three times with ice-cold CMF-PBS, and cellular proteins were extracted with 1.3 ml of solubilization buffer [1.0% octylglucoside, 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.2), 30 mM sodium pyrophosphate, 50 mM sodium chloride, 5 mM β-glycerophosphate, 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*,-tetraacetic acid (EGTA), 1 mM phenylmethylsulfonyl fluoride, 100 μM Na_3VO_4, 10 μg of leupeptin per ml, 10 μg of aprotinin per ml) per plate for 15 min on ice. The supernatant was recovered and clarified by centrifugation for 15 min at 12,200 rpm in a microfuge. The crude protein extracts (1.0 to 2.0 mg/ml; 1,400 μl) were added to 100 μl of Sepharose-linked antiphosphotyrosine (1G2)-packed bead matrix (18). After adsorption for 2 h at 4°C, the matrix was washed three times with 1,000 μl of solubilization buffer. Specifically bound proteins were then recovered by elution with 150 μl of 5 mM phenylphosphate in solubilization buffer.

PLC assay. Samples were assayed by addition of 5 μl of the 150-μl eluate (containing 1% octylglucoside) to a reaction buffer composed of 15 μl of solubilization buffer without octylglucoside, 20 μl of 50 mM sodium phosphate (pH 6.8)–1 mM EGTA–100 mM potassium chloride, 5 μl of 2 mM [^{3}H]PtdIns 4,5-P_2 (25,000 cpm) in 2.5% octylglucoside, and 5 μl of 8 mM calcium chloride. This yields final concentrations in 50 μl of 200 μM [^{3}H]PtdIns 4,5-P_2, 1 μM free Ca^{2+}, and 0.35% octylglucoside. The reaction mixture was incubated for 15 min at 37°C, and the reaction was stopped by transfer to an ice bath and addition of 100 μl of 1% bovine serum albumin and 500 μl of 10% trichloroacetic acid. The precipitate was removed by centrifugation, and the release of [^{3}H]Ins 1,4,5-P_3 was measured by combining the acidic supernatant with aqueous counting scintillant and determination of radioactivity by fluorimetry. The radioactivity present in a buffer-only control (approximately 250 cpm) was subtracted from the experimental values. A control experiment demonstrated that the only water-soluble product formed in the PLC assay was [^{3}H]Ins 1,4,5-P_3 (data not shown). Data presented in the figures represent average PLC activity (picomoles of $InsP_3$ per min) present in the antiphosphotyrosine eluate derived from a 100-mm-diameter plate.

Immunoprecipitation of PLC. BALB/c 3T3 cell proteins were radioactively labeled by treatment with 80 μCi of [^{35}S]methionine-cysteine *trans* label per ml in methionine-free DMEM plus 2% PPP for 20 h or with 0.75 mCi of $^{32}P_i$ in phosphate-free DMEM plus 2% PPP for 4 h. After PDGF treatment, the cells were washed four times with CMF-PBS and then extracted with 1.0 ml of 1.0% Triton X-100–20 mM HEPES (pH 7.2)–10% glycerol–50 mM NaF–1 mM phenylmethylsulfonyl fluoride–1 mM Na_3VO_4 per plate. The soluble extracts (1,000 μl) were treated first with 20 μl of 10% heat-inactivated *Staphylococcus aureus* cells precoated with rabbit anti-mouse immunoglobulin G for 30 min at 4°C. The precoated *S. aureus* cells were removed by centrifugation. The extracts were next treated with 3 μg of monoclonal antibodies to PLC-β1, -γ, or -δ plus 20 μl of 10% heat-inactivated precoated *S. aureus* cells. The mixtures of monoclonal antibodies were PLC-β1 (K-32-3, K-82-3, and K-92-3), PLC-γ (F-7-2, B-20-3, B-6-4, D-7-4, E-8-4, and E-9-4), and PLC-δ (S-11-2, R-29-1, R-39-2, and Z-78-5), whose properties are described elsewhere (57).

The mixtures of radiolabeled cell proteins plus antibodies were incubated at 4°C overnight. The immunoprecipitates were recovered by centrifugation in a microfuge for 15 s and then washed four times with a buffer containing 1% Triton X-100, 1% deoxycholate, 0.1% sodium dodecyl sulfate, 50 mM Tris (pH 8.5), 150 mM NaCl, and 0.02% sodium azide. The immunoprecipitated proteins were released by addition of 120 μl of Laemmli buffer and heating for 5 min at 60°C. The preparations were centrifuged for 15 s in a microfuge, and the proteins present in the clarified supernatants were separated by 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The ^{32}P-radiolabeled PLC-γ bands from control and PDGF-treated cells were excised from the gels, and the ^{32}P-phosphoamino acids were determined as described previcusly (14).

Materials. PDGF (recombinant BB homodimer) was obtained from Amgen Biologicals (Thousand Oaks, Calif.). PtdIns 4,5-P_2 was obtained from Boehringer Mannheim Biochemicals (Indianapolis, Ind.). [^{3}H]PtdIns 4,5-P_2 was obtained from Dupont, NEN Research Products (Boston, Mass.). Octylglucoside and *S. aureus* cells were obtained from Calbiochem-Behring (La Jolla, Calif.). Rabbit anti-mouse immunoglobulin G was obtained from Accurate Chemical and Scientific Corp. (Westbury, N.Y.). [^{35}S]methionine-cysteine *trans* label and $^{32}P_i$ were obtained from ICN Pharmaceuticals Inc. (Irvine, Calif.). [^{3}H]inositol was obtained from American Radiolabeled Chemicals (St. Louis, Mo.).

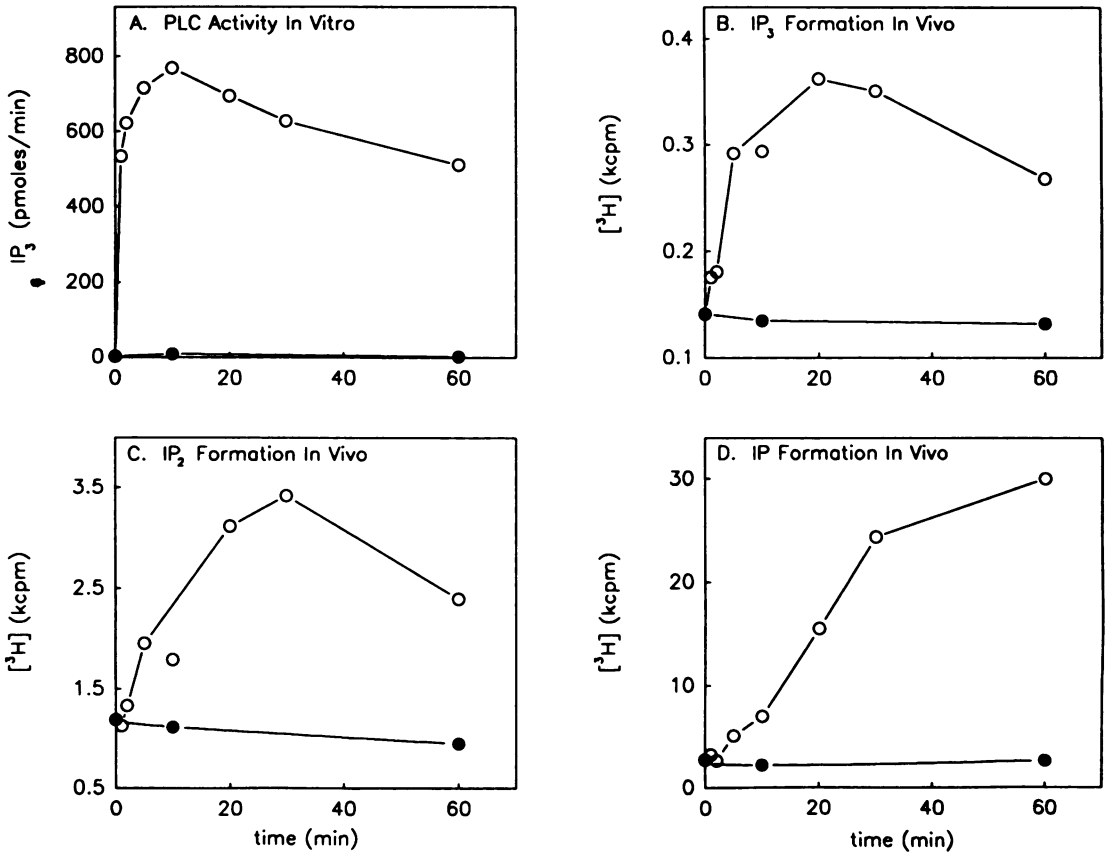

FIG. 1. Comparison of the time course of PDGF stimulation of PLC activity in antiphosphotyrosine eluates (A) with formation of $[^3H]InsP_3$ (B), $[^3H]InsP_2$ (C), and $[^3H]InsP_1$ (D) in intact cells. BALB/c 3T3 cells were prepared as described in the text, treated with 100 μM Na_3VO_4 (A) or 20 mM LiCl (B to D) for 10 min, and then treated with 100 ng of PDGF (○) or carrier (●) per ml at 37°C for the indicated periods of time. The cells were extracted with solubilization buffer (A) or 10% $HClO_4$ (B to D), and the levels of PLC activity and $[^3H]InsPs$ were determined as described in the text.

RESULTS

Rapid and sustained PDGF effect. To demonstrate the occurrence of growth factor-sensitive PLC activity in cells that are nontransformed and mitogenically stimulated by growth factor, density-arrested BALB/c 3T3 cells were treated with PDGF for 0 to 60 min. Control and PDGF-treated cells were solubilized in buffer containing 1% octylglucoside, and phosphotyrosine-containing proteins in the extracts were separated by adsorption to a phosphotyrosine antibody (1G2) coupled to Sepharose. Phosphotyrosine-containing proteins were eluted from the antiphosphotyrosine matrix with the phosphotyrosine analog phenylphosphate and then assayed for PLC activity in vitro. There was a substantial and rapid (60-fold at 1 min) increase in the level of phosphotyrosine-immunoisolated PLC activity from cells treated with PDGF (Fig. 1A). The level of PLC activity continued to increase for 10 min after addition of PDGF to a maximal level of 80-fold higher than the level of untreated cells. At later times (20 to 60 min after PDGF treatment), there was a slow decrease in the level of PLC activity recovered by phosphotyrosine immunoisolation even though the cells were treated during the experiment with sodium orthovanadate, a tyrosine phosphatase inhibitor (61).

The relationship between the appearance of the PDGF-sensitive PLC activity, as judged by antiphosphotyrosine isolation and enzyme assay in vitro, and PDGF-stimulated formation of InsPs in [^{3}H]inositol-labeled BALB/c 3T3 cells is demonstrated in Fig. 1B to D. The rise in PDGF-sensitive PLC activity (Fig. 1A) occurred at least as rapidly as did the increase in formation of $[^3H]InsP_3$ (Fig. 1B). Furthermore, the increase in $[^3H]InsP_3$ preceded increases in $[^3H]InsP_2$ (Fig. 1C) and $[^3H]InsP_1$ (Fig. 1D), consistent with the direct formation of $InsP_3$ from PtdIns 4,5-P_2.

In this experiment and those described below, cells were pretreated with Na_3VO_4 or LiCl, respectively, in the assay of PDGF-stimulated antiphosphotyrosine recovery of PLC activity or the cellular formation of InsPs. These compounds are useful as pharmacologic inhibitors of cellular phosphatases for tyrosine phosphate and $InsP_1$, respectively. The data presented may reflect subtle differences in the magnitude or duration of the PDGF-stimulated PLC responses in intact cells or extracts that resulted from inclusion of these different inhibitors.

Many of the rapid cellular responses to PDGF are transient. Therefore, we investigated whether interaction of the ligand-stimulated PDGF receptor with PLC, resulting in enhanced antiphosphotyrosine recovery of PLC activity, was a transient or prolonged response. Quiescent BALB/c 3T3 cells were stimulated with PDGF (in the absence of Na_3VO_4) for increasing periods of time from 10 min to 12 h. A maximal increase in antiphosphotyrosine recovery of PLC activity (100-fold) from PDGF-treated cells occurred after a 10-min treatment (Fig. 2A). Subsequently, the amount of immunoisolated PLC activity from PDGF-treated cells

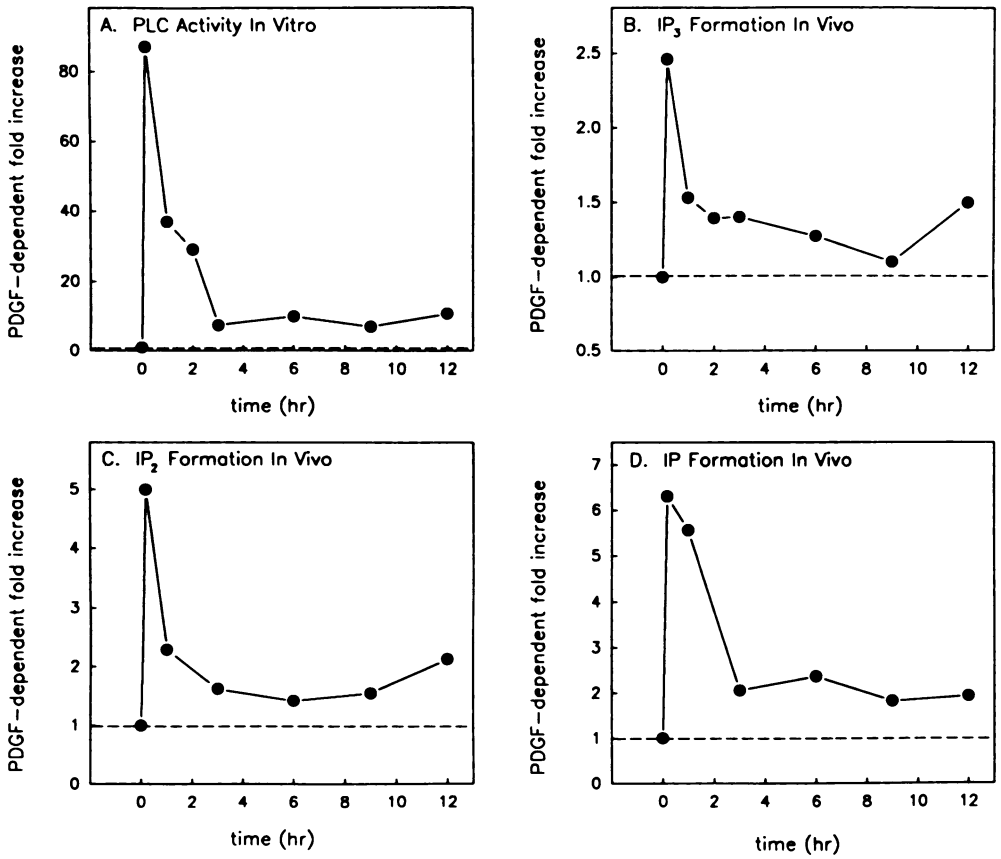

FIG. 2. Comparison of the long-term PDGF stimulation of PLC activity in antiphosphotyrosine eluates (A) with the rates of formation of [^{3}H]InsP$_3$ (B), [^{3}H]InsP$_2$ (C), and [^{3}H]InsP$_1$ (D) in intact cells. BALB/c 3T3 cells were prepared as described in the text except that Na_3VO_4 and LiCl were omitted. Then the cells were treated with 100 ng of PDGF or carrier per ml at 37°C for the indicated periods of time. For determination of the rate of [^{3}H]InsP formation (B to D), LiCl was added 20 min before extraction with 10% $HClO_4$. For determination of PLC activity (A), cells (not exposed to Na_3VO_4) were extracted with solubilization buffer. Levels of PDGF-sensitive PLC activity and [^{3}H]InsPs were determined as described in the text and are expressed as fold increase over control levels.

slowly decreased until 3 h after mitogen addition. Thereafter and for up to 12 h of PDGF exposure, the PLC activity recovered from PDGF-treated cells remained approximately 10-fold above the level of activity recovered from control cells. During this prolonged exposure to PDGF, the rate of formation of InsPs was assessed in [^{3}H]inositol-labeled cells by adding LiCl 20 min before acid extraction of the cells. Increased rates of formation of InsP$_3$, InsP$_2$, and InsP$_1$ could be detected throughout the course of the experiment (Fig. 2B to D). This result demonstrates a prolonged coincidence between PDGF-stimulated antiphosphotyrosine recovery of PLC activity and enhanced turnover of inositol phospholipids.

Tyrosine phosphorylation of PLC-γ induced by PDGF. The antiphosphotyrosine recovery of PLC activity suggested that PLC, or a protein to which PLC is tightly coupled, is phosphorylated by the PDGF receptor tyrosine kinase. To distinguish between these two possibilities, we first determined which PLC isozyme was present in BALB/c 3T3 cells. BALB/c 3T3 cells were metabolically labeled with [^{35}S]methionine-cysteine for approximately 18 h, and a soluble extract of cell proteins was prepared. The extract was treated with specific monoclonal antibodies to three distinct cytosolic PLC isozymes, PLC-β1 (150 kilodaltons [kDa]), -γ (145 kDa), and -δ (85 kDa) (57; Rhee et al., in press). The immunoprecipitated proteins were separated by gel electrophoresis, and the proteins were visualized by radiofluorography (Fig. 3A). Of these three isozymes, only the 145-kDa (PLC-γ) isozyme was detected in BALB/c 3T3 cells (Fig. 3, lane 2), consistent with the more ubiquitous distribution of this isozyme in tissue and cell lines (S. G. Rhee, unpublished data). It is likely that other PLC isozymes were present also (see below). To determine whether PLC-γ is phosphorylated after growth factor treatment, BALB/c 3T3 cells were metabolically labeled with $^{32}P_i$ for 4 h and treated with PDGF for 10 min. Detergent-solubilized extracts of cell proteins were prepared and treated with monoclonal antibodies to PLC-γ. The immunoprecipitated proteins were separated by gel electrophoresis and visualized by autoradiography (Fig. 3B). A phosphoprotein corresponding to the apparent molecular size, 145 kDa, of PLC-γ was present. PDGF treatment increased the phosphorylation of PLC-γ by approximately sevenfold above the level of control cells, as determined by measuring Cerenkov radioactivity present in excised bands from the gel. A second phosphoprotein, whose ^{32}P content was increased by PDGF and which had a molecular size of approximately 180 kDa, was also visible in the immunoprecipitate from PDGF-stimulated cells (lane 5). The identities of the other phosphoproteins (with apparent molecular sizes of 44, 76, and 83 kDa) immunoprecipitated from both the control and PDGF-stimulated preparations are unknown. The coprecipitating bands were not detected in similar immunoprecipitates in assays using antibodies to PLC-β1 or PLC-δ (data not shown).

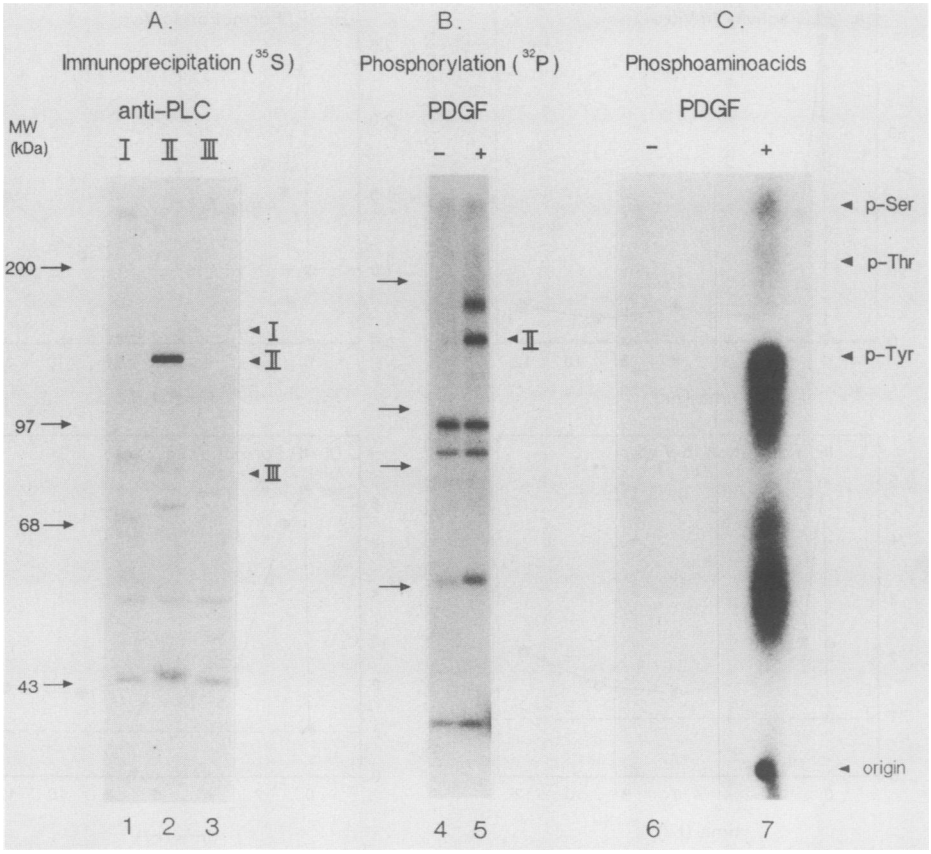

FIG. 3. Influence of PDGF on phosphorylation of PLC-γ in BALB/c 3T3 cells. BALB/c 3T3 cells were radiolabeled with [^{35}S]methionine (A) or $^{32}P_i$ (B and C). (A) Extracts containing ^{35}S-radiolabeled BALB/c 3T3 cell proteins were treated with antibodies to PLC-β1 (lane 1), PLC-γ (lane 2), or PLC-δ (lane 3), and the immunoprecipitated proteins were separated by gel electrophoresis. The radiofluorograph was exposed to Enlightening-treated gel for 24 h at −70°C. (B) Extracts containing ^{32}P-radiolabeled proteins from BALB/c 3T3 cells treated without (lane 4) or with (lane 5) PDGF (100 ng/ml) for 10 min were treated with antibodies to PLC-γ, and the immunoprecipitated proteins were separated by gel electrophoresis. The autoradiograph was exposed to the gel for 24 h at −70°C. (C) Gel slices containing the ^{32}P-labeled PLC-γ bands from several experiments were excised, and radioactivity (Cerenkov counts) was determined: without (−) PDGF, 260 cpm; with (+) PDGF, 1,625 cpm. The ^{32}P-labeled PLC-γ was extracted from the gel slices and acid hydrolyzed, and the ^{32}P-phosphoamino acids were separated by high-voltage thin-layer electrophoresis. The autoradiograph was exposed to the plate for 5 days at −70°C. After exposure, the radioactive spots corresponding to the locations of the phosphoamino acid standards were scraped and eluted with water, and radioactivity was determined by using aqueous counting scintillant.

The identities of the PLC-γ amino acid residues phosphorylated after PDGF treatment were determined by acid hydrolysis of the ^{32}P-labeled PLC-γ protein (Fig. 3C). A sevenfold PDGF-induced increase was found in the radioactive content of phosphotyrosine, and a smaller increase (twofold) was detected in the contents of phosphoserine and phosphothreonine. This result demonstrates that the antiphosphotyrosine recovery of PLC activity resulted from the growth factor-stimulated phosphorylation of PLC-γ on tyrosine residues. In addition, Western blot (immunoblot) analysis with antibodies to PLC-γ of the phosphotyrosine-immunoisolated PLC activity from PDGF-treated cells recognized a protein of 145 kDa, corresponding to PLC-γ (data not shown). These results are convincing evidence that PDGF increased the recovery of PLC activity in eluates from the antiphosphotyrosine matrix as a result of the presence of phosphotyrosine on PLC-γ.

Stoichiometry of PLC-γ phosphorylation in vivo. To determine the percentage of total cellular PLC-γ that was phosphorylated, we investigated more thoroughly PDGF-stimulated phosphorylation of PLC-γ by using a combination of antiphosphotyrosine adsorption and immunoprecipitation with PLC-γ antibodies (data not shown). Cells radiolabeled with $^{32}P_i$ or [^{35}S]methionine were treated with or without PDGF and then adsorbed to antiphosphotyrosine. In control and PDGF-treated cells, respectively, approximately 0.1 and 0.5% of total acid-precipitable ^{32}P radioactivity and 1.0 and 1.5% of total ^{35}S radioactivity were recovered in the antiphosphotyrosine eluates. Next, the ^{32}P- or ^{35}S-radiolabeled nonadsorbed and specific eluate fractions from the antiphosphotyrosine matrix were treated with excess PLC-γ antibody. After gel electrophoresis of PLC-γ immunoprecipitates and quantification of radioactivity in corresponding gel slices, the specific eluate was observed to contain 70 to 100% of the PDGF-stimulated increase in [^{32}P]PLC-γ radioactivity. Furthermore, the specific eluates from ^{35}S-radiolabeled preparations contained less than 10% of total [^{35}S]PLC-γ from control cells, but specific eluates from PDGF-treated cells contained 70% of total [^{35}S]PLC-γ. Taken together, these data reveal that a large portion (approximately 70%) of the PLC-γ pool was rapidly phosphorylated on tyrosine residues after PDGF treatment of BALB/c 3T3 cells and that essentially all of the tyrosine-phosphorylated PLC-γ was recovered by using phosphotyrosine immunoisolation.

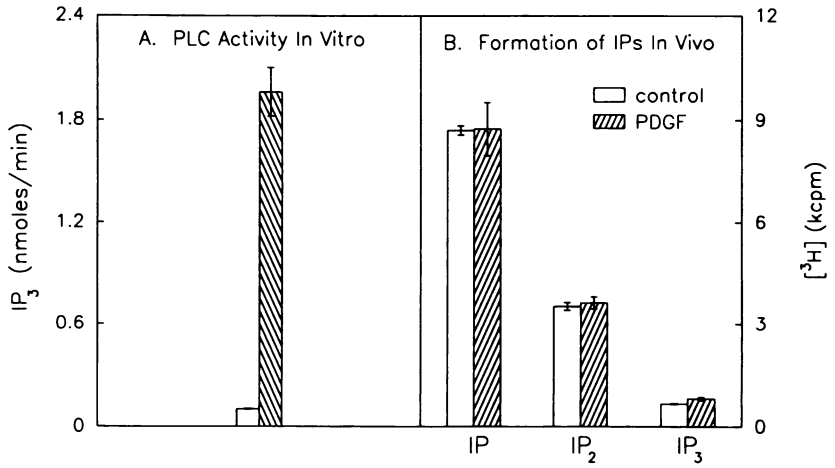

FIG. 4. Comparison of 3°C PDGF stimulation of PLC activity in antiphosphotyrosine eluates (A) with formation of [^{3}H]InsP$_3$, [^{3}H]InsP$_2$, and [^{3}H]InsP$_1$ in intact cells (B). BALB/c 3T3 cells were prepared as described in the text except that they were first cooled to 3°C, Na_3VO_4 (A) or LiCl (B) was added, and 10 min later cells were treated with 100 ng of PDGF (▨) or carrier (□) for an additional 10 min. The cells were extracted with solubilization buffer (A) or 10% $HClO_4$ (B), and the levels of PLC activity and [^{3}H]InsPs were determined as described in the text.

Additional evidence suggested that the PLC-γ pool comprised a minority of the total detergent-extractable PLC activity (data not shown). When detergent extracts of either control or PDGF-stimulated cells were treated with excess PLC-γ antibody mixture, approximately 70 to 75% of the PLC activity remained in the soluble, nonprecipitated fraction. Antiphosphotyrosine eluates from control and PDGF-treated (10 min at 37°C) cells routinely contained approximately 0.06 and 6%, respectively, of the total extract PLC activity. Furthermore, PLC-γ antibody immunoprecipitated 100% of the PLC-γ activity present in the specific eluate from PDGF-treated cells. These results suggest that the PLC-γ isozyme was selectively phosphorylated after PDGF treatment. We consistently observed that the PLC activity recovered in the antiphosphotyrosine eluates or immunoprecipitates was lower than the amount of PLC activity that had been removed from the corresponding nonadsorbed soluble extract. This discrepancy may reflect the influence of other components present in these preparations. Comparison of the relative levels of PLC-γ protein (based on ^{35}S radiolabeling) and PLC activities present in the total cell extracts, antiphosphotyrosine eluates, and PLC-γ immunoprecipitates suggests that no tyrosine phosphorylation-associated increase in PLC-γ enzymatic activity was detected. However, the relationship between PLC activities measured in soluble or precipitate preparations and PLC activities within intact cells is unclear.

PLC-γ phosphorylation at low temperature. We performed several experiments to compare mechanistic aspects of PDGF-stimulated phosphorylation of PLC-γ and formation of InsPs. The experiments compared these events upon PDGF stimulation at a low temperature, upon stimulation in the presence or absence of extracellular Ca^{2+}, and upon stimulation with a G-protein agonist. BALB/c 3T3 cells were incubated at 3°C in the absence or presence of PDGF for 10 min, and the levels of phosphotyrosine-immunoisolated PLC activity and [^{3}H]InsPs were measured. PDGF activation of the PDGF receptor tyrosine kinase has been shown to occur at 3°C (19). PDGF stimulation at 3°C led to phosphotyrosine recovery of PLC activity (Fig. 4A), but there was no effect of PDGF treatment on the levels of InsP$_3$, InsP$_2$, and InsP$_1$ (Fig. 4B). The amount of PLC activity recovered by antiphosphotyrosine from cells treated with PDGF at 3°C was approximately 70% of the amount recovered upon treatment at 37°C. This experiment demonstrates that PDGF-stimulated phosphorylation of PLC-γ occurred in the absence of PLC-mediated formation of Ins 1,4,5-P$_3$, consequent Ca^{2+} mobilization, and other events distal to PLC stimulation in intact cells. This finding suggests that PDGF-stimulated tyrosine phosphorylation of PLC-γ is an early event in the stimulation of phosphoinositide metabolism by PDGF and shows that internalization of PDGF-receptor complexes, which is blocked by low temperature (7), is not necessary for tyrosine kinase phosphorylation of PLC-γ.

PLC-γ phosphorylation independent of extracellular Ca^{2+}. All mammalian phosphoinositide-specific PLC isozymes display Ca^{2+}-dependent stimulation of activity. Within a cell, the level of free Ca^{2+} is regulated by a variety of influences, including the presence of extracellular hormones. PDGF is capable of stimulating an increase in the intracellular free Ca^{2+} level due in part to the influx of extracellular Ca^{2+} (25). We investigated whether the PDGF stimulation of antiphosphotyrosine recovery of PLC activity or the formation of InsPs in intact cells was affected by the presence of extracellular Ca^{2+}. Increases in the antiphosphotyrosine recovery of PLC activity at 2 and 30 min after PDGF treatment were independent of extracellular Ca^{2+} (Fig. 5A). In contrast, the PDGF-stimulated formation of InsP$_3$ (Fig. 5B) was independent of extracellular Ca^{2+} when measured at 5 min after treatment, whereas extracellular Ca^{2+} strongly potentiated the PDGF-stimulated formation of InsPs measured at 30 min. These data suggest that PDGF stimulates phosphorylation of PLC-γ and that PLC-mediated formation of InsP$_3$ is initially independent of extracellular Ca^{2+}; however, growth factor-stimulated InsP$_3$ formation within the cells was enhanced at later times (after 5 min) by a mechanism dependent on extracellular Ca^{2+}.

No PLC-γ phosphorylation induced by AlF_4^-. A common finding in the investigation of hormone-stimulated formation of InsP$_3$ is the modulation of this effect by reagents that modify G proteins (reviewed in references 13 and 17). The identity of such phosphoinositide pathway G proteins and their relationship to previously characterized G proteins is uncertain. Formation of InsPs in BALB/c 3T3 cells can be

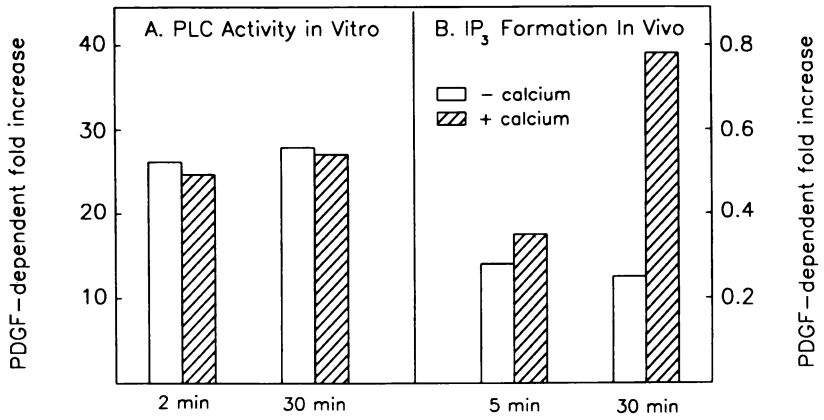

FIG. 5. Comparison of the requirement for extracellular Ca^{2+} of PDGF stimulation of PLC activity in antiphosphotyrosine eluates (A) with formation of [^{3}H]$InsP_3$ in intact cells (B). BALB/c 3T3 cells were prepared as described in the text except that they were changed to Ca^{2+}-free DMEM containing 0.5 mM EGTA before addition of Na_3VO_4 (A) or LiCl (B). The cells were then treated for 2 (A) or 5 (B) min and for 30 min at 37°C with 100 ng of PDGF or carrier per ml in the absence or presence of 2.0 mM free Ca^{2+}. The cells were extracted with solubilization buffer (A) or 10% $HClO_4$ (B), and the levels of PLC activity and [^{3}H]InsPs were determined as described in the text.

stimulated by a variety of G-protein reagents. To assess whether tyrosine phosphorylation of PLC-γ was detectable in cells treated with any agent that increases cellular PLC activity, we compared the stimulation of antiphosphotyrosine recovery of PLC activity and the cellular formation of InsPs after treatment of BALB/c 3T3 cells with PDGF or AlF_4^-, which is believed to interact with and activate a G-protein-regulating PLC activity. Both PDGF and AlF_4^- stimulated formation of $InsP_3$ in BALB/c 3T3 cells (Fig. 6B); however, an increase in phosphotyrosine-immunoisolated PLC activity was found only in the PDGF-treated cells (Fig. 6A). Furthermore, the immunoprecipitation of PLC-γ from ^{32}P-labeled cells treated with either PDGF or AlF_4^- demonstrated that only PDGF stimulated the phosphorylation of PLC-γ (data not shown). Thus, not all stimulators of cellular $InsP_3$ formation led to enhanced tyrosine phosphorylation of PLC-γ.

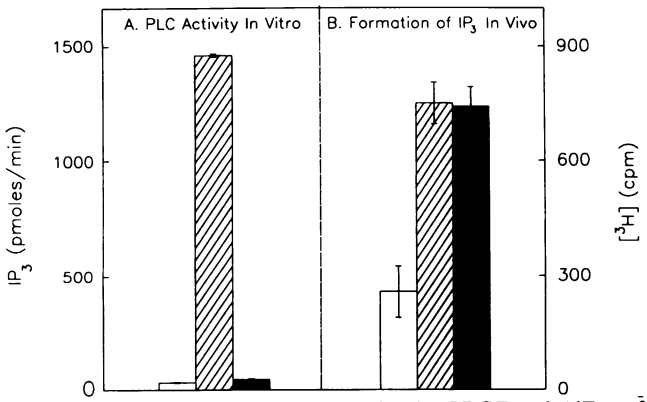

FIG. 6. Comparison of the stimulation by PDGF and AlF_4^- of PLC activity in antiphosphotyrosine eluates (A) with the formation of [^{3}H]$InsP_3$ in intact cells (B). BALB/c 3T3 cells were prepared as described in the text, were treated with Na_3VO_4 (A) or LiCl (B) for 10 min, and then received either no addition (□) 100 ng of PDGF per ml (▨), or 10 mM NaF plus 10 μM $AlCl_3$ (■). After 10 min at 37°C, the cells were extracted with solubilization buffer (A) or 10% $HClO_4$ (B), and the levels of PLC activity and [^{3}H]$InsP_3$ were determined as described in the text.

DISCUSSION

Numerous forms of PLC have been isolated from several sources, including rat brain (31), bovine brain (53–55), human platelets (2, 3), guinea pig uterus (4), sheep seminal vesicular glands (30), and rat liver (20, 60). Ryu et al. (54, 55), for example, purified three PLC enzymes from bovine brain, initially designated PLC-I, -II, and -III and recently named PLC-β1, -γ, and -δ (Rhee et al., in press). Isolation and sequencing of cDNA encoding PLC-β1, -γ, and -δ revealed two domains conserved among all three isozymes but low overall sequence homology (36, 59). In addition, a striking sequence similarity between regions of PLC-γ and the amino-terminal, noncatalytic domain of the *src* tyrosine kinase has been found (43, 56, 58). The biological significance of multiple PLC enzymes is obscure, and the expression of the different PLC enzymes in cultured lines has not been previously addressed.

In our studies, extracts prepared from cells metabolically labeled with [^{35}S]methionine were immunoprecipitated with monoclonal antibodies (59) specific for three distinct cytosolic PLC isozymes (PLC-β1, 150 kDa; PLC-γ, 145 kDa; and PLC-δ, 85 kDa). Density-arrested BALB/c 3T3 cells expressed detectable levels of PLC-γ but not β1 or δ (Fig. 3). Whether this selective expression of PLC-γ reflects proliferative status, cell type, or other factors is not known. Furthermore, antibodies to PLC-γ did not precipitate all of the detergent-extracted PLC activity.

We have demonstrated the rapid tyrosine phosphorylation of the PLC-γ isozyme in BALB/c 3T3 cells in response to PDGF (Fig. 3). BALB/c 3T3 cells labeled with $^{32}P_i$ were immunoprecipitated with antibodies to PLC-γ, and the immunoprecipitated proteins were separated by gel electrophoresis. Increased ^{32}P radiolabeling of PLC-γ was clearly evident in material prepared from PDGF-treated as compared with control cells (Fig. 3). Amino acid analysis of the PLC-γ gel slice showed that PDGF-induced PLC-γ phosphorylation occurred predominantly on tyrosine residues. Additional experiments revealed that the majority of the cellular PLC-γ pool was rapidly tyrosine phosphorylated upon PDGF treatment. Furthermore, all of the PLC activity recovered by phosphotyrosine immunoisolation from

PDGF-treated cells was immunoprecipitated with PLC-γ antibody.

Our data are consistent with a direct phosphorylation of PLC-γ by the PDGF receptor kinase. PLC-γ phosphorylation, as assessed by antiphosphotyrosine recovery of PLC activity, occurred rapidly in response to PDGF; a significant increase in recovery of PLC activity was evident within 1 min of addition of PDGF to cells at 37°C (Fig. 1A). The kinetics of PLC-γ phosphorylation are similar to those previously described for PDGF receptor autophosphorylation. Studies of BALB/c 3T3 cells have shown that receptor autophosphorylation is rapidly induced by PDGF, is maximal at 10 to 15 min, and then diminishes by 30 to 60 min (24). Furthermore, PDGF-induced PLC-γ phosphorylation occurred at 3°C (Fig. 4), suggesting that PLC-γ is a readily accessible substrate. An indication of an interaction of the PDGF receptor with PLC-γ is the coprecipitation of a phosphoprotein of M_r ~180,000 in the PLC-γ immunoprecipitates from ^{32}P-radiolabeled cells (Fig. 3B). The apparent molecular mass and the PDGF-dependent enhancement of phosphorylation both suggest that this 180-kDa protein represents the PDGF receptor. The existence of a complex between PLC and the PDGF receptor in PDGF-treated cells is an intriguing possibility. Whether the PDGF receptor kinase directly phosphorylates PLC-γ or activates an intervening tyrosine kinase cannot be ascertained from studies in intact cells. However, we have observed that purified PLC-γ is an excellent substrate for purified EGF receptor tyrosine kinase (S. Nishibe, M. I. Wahl, S. G. Rhee, and G. Carpenter, J. Biol. Chem., in press).

Phosphoamino acid analysis of PLC-γ immunoprecipitated from PDGF-treated, ^{32}P-labeled cells also revealed small increases in serine and threonine phosphorylation (Fig. 3). Thus, PLC-γ appears to be a substrate for an additional and, at present, unidentified PDGF-activated serine-threonine kinase(s).

Our data support, but do not conclusively prove, a relationship between PLC-γ tyrosine phosphorylation and the stimulation of InsP formation. PDGF-induced PLC-γ phosphorylation and $InsP_3$ formation were closely correlated in both short-term (Fig. 1) and long-term (Fig. 2) time courses and with respect to PDGF concentration dependence (data not shown). In the short-term time course, antiphosphotyrosine recovery of PLC activity increased at least as rapidly as did $InsP_3$ formation. The initiation of a mitogenic response required only a relatively brief period of PDGF treatment, during which ligand binding, receptor tyrosine kinase activity, recovery of PLC activity, and InsP turnover attain maximally stimulated rates. We found a rapid, 100-fold increase after PDGF addition in phosphotyrosine-immunopurified PLC activity (Fig. 2). The level of PLC activity isolated from PDGF-treated cells then slowly declined to 10-fold above the basal level and remained at that level for 12 h. This decline in PLC activity recovered does not reflect PDGF receptor down-regulation, since chloroquine, which blocks receptor degradation, did not affect the PLC recovery measured 4 h after PDGF stimulation (data not shown). It is possible, however, that another factor present in the plasma-containing culture medium, such as insulinlike growth factor I, which also stimulates a receptor tyrosine activity, may have contributed to the elevation of phosphotyrosine-associated PLC activity at later times. This is unlikely, since PDGF stimulated antiphosphotyrosine recovery of PLC activity for at least 4 h in the absence of plasma, whereas the addition of insulinlike growth factor type I to untreated cells or to cells treated with PDGF for 4 h had no detectable effect on either PLC recovery or InsP formation (data not shown).

The antiphosphotyrosine recovery of PLC activity, but not $InsP_3$ formation, at low temperature (Fig. 4) suggests that PLC-γ phosphorylation occurs before $InsP_3$ formation. Furthermore, whereas $InsP_3$ formation was enhanced by the presence of extracellular Ca^{2+}, antiphosphotyrosine recovery of PLC activity was independent of extracellular Ca^{2+} (Fig. 5). These data support a role for tyrosine phosphorylation in initiating the stimulation of PLC activity in BALB/c 3T3 cells, whereas the influx of extracellular Ca^{2+} maintains $InsP_3$ formation at a stimulated rate for a prolonged period of time.

Previous studies on pp60^{src} provide precedent for regulation of enzymatic and biological activity by tyrosine phosphorylation (9, 38, 49). Furthermore, studies by Ralston and Bishop (52) and Gould and Hunter (21) showed that pp60^{src} was phosphorylated at tyrosine in response to PDGF and that the tyrosine kinase activity of pp60^{src} was consequently enhanced. The PDGF-modulated phosphotyrosines were localized to the amino-terminal region of pp60^{src}; as noted above, this region of *src* shares homology with PLC-γ. Thus, it is feasible that PLC-γ, like pp60^{src}, is activated by tyrosine phosphorylation. However, the high intrinsic activity of purified PLC isozymes in relation to the small amount of PtdIns 4,5-P_2 present in cell membranes suggests that in intact cells, PLC isozymes must be under the influence of proteins that are negative regulators of PLC activity (Rhee et al., in press). Thus, tyrosine phosphorylation-mediated alteration in PLC-γ activity may be undetectable in detergent extracts or immunoprecipitates because of dissociation of a putative negative regulatory protein(s). A more thorough understanding will require identification of the actual phosphorylation sites in the primary amino acid structure of PLC-γ and selective dephosphorylation of phosphotyrosine, phosphoserine, and phosphothreonine residues.

A number of ligands have been demonstrated to affect PLC activity by a mechanism sensitive to regents (AlF_4^-, cholera toxin, pertussis toxin, and GDP and GTP analogs) that modify the actions of certain guanine nucleotide-binding proteins (G proteins), suggesting that PLC activity is controlled in a manner analogous to the modulation of adenylate cyclase activity by regulatory proteins G_i and G_s (reviewed in references 13 and 17). Growth factor activation of PLC by an alternative or additional mechanism is supported by several recent findings. Paris et al. (48) reported that mitogens activating tyrosine kinases (e.g., PDGF, EGF, fibroblast growth factor, and insulin) potentiated thrombin and AlF_4^--stimulated, G-protein-mediated InsP production in Chinese hamster fibroblasts and suggested that tyrosine kinases promote InsP formation by phosphorylation of either PLC or a G protein. Hasegawa-Sasaki et al. (26) and Nanberg and Rozengurt (45) compared the kinetics of $InsP_3$ formation with Ca^{2+} mobilization in WFB rat fibroblasts and Swiss 3T3 fibroblasts stimulated by bombesin, vasopressin, or PDGF. Both groups determined that with PDGF, there was a distinct lag of at least 10 s before detection of Ca^{2+} mobilization, whereas the response to bombesin or vasopressin occurred without a detectable lag. Hasegawa-Sasaki et al. (26) also observed a differential sensitivity of the InsP response to G-protein-modifying reagents in agonist-stimulated permeabilized cells. Kamata and Kung (35) examined the possible role of G proteins by using membrane preparations from normal rat kidney fibroblasts and *ras*-transformed normal rat kidney cells. PDGF was reported to stimulate PtdIns 4,5-P_2 hydrolysis in membranes from nontransformed

normal rat kidney cells in the presence of GTP[γ-S], whereas no hydrolysis was observed in the absence of GTP[γ-S] or in membranes from transformed cells. The possibility of a direct interaction between receptor tyrosine kinases and G proteins was suggested by the results of Krupinski et al. (40), who demonstrated that the insulin receptor kinase was capable of phosphorylating in vitro G_i and G_o, primarily on the α subunits. Thus, clarification of the role of G proteins in growth factor-stimulated PtdIns 4,5-P_2 hydrolysis is required.

Our previous studies (63, 64) measuring PLC activity in solubilized cell extracts immunoadsorbed and eluted from an antiphosphotyrosine matrix demonstrated increased activity in material prepared from EGF-treated A-431 cells. The data presented here show that this effect is also observed in PDGF-treated BALB/c 3T3 cells and thus is not an anomalous EGF response in cell lines overexpressing EGF receptors. Furthermore, the mechanisms of growth factor-stimulated PLC-γ phosphorylation in relation to increased cellular formation of $InsP_3$ are similar for PDGF and EGF in three respects: (i) stimulation of PLC-γ tyrosine phosphorylation at low temperature without $InsP_3$ formation, (ii) PLC-γ phosphorylation independent of extracellular Ca^{2+} and biphasic Ca^{2+} dependence of InsP formation, and (iii) tyrosine phosphorylation of PLC-γ associated only with growth factor stimulation of receptor tyrosine kinase activity. Phosphorylation of PLC-γ at tyrosine residues may be a mechanism by which certain receptor tyrosine kinases interact with the phosphoinositide pathway upon stimulation by the corresponding growth factors.

ACKNOWLEDGMENTS

We thank Brenda Crews, Kelly Adair, and John Neff for technical assistance and Susan Heaver and David Sullins for typing the manuscript.

This research was supported by Public Health Service research grants CA 42713 and CA 43720 and training grants DK 07563 and GM 07347 from the National Institutes of Health.

ADDENDUM IN PROOF

Meisenhelder et al. (J. Meisenhelder, P.-G. Suh, S. G. Rhee, and T. Hunter, Cell, in press) have recently demonstrated PDGF-stimulated tyrosine and serine phosphorylation of PLC-γ in intact NIH 3T3 fibroblasts and PDGF receptor tyrosine phosphorylation of PLC-γ in vitro.

LITERATURE CITED

1. **Abdel-Latif, A. A.** 1986. Calcium-mobilizing receptors, polyphosphoinositides and the generation of second messengers. Pharmacol. Rev. **38**:227–272.
2. **Banno, Y., S. Nakashima, and Y. Nozawa.** 1986. Partial purification of phosphoinositide phospholipase C from human platelet cytosol; characterization of its three forms. Biochem. Biophys. Res. Commun. **136**:713–721.
3. **Banno, Y., Y. Yada, and Y. Nozawa.** 1988. Purification and characterization of membrane-bound phospholipase C specific for phosphoinositides from human platelets. J. Biol. Chem. **263**:11459–11465.
4. **Bennett, C. F., and S. T. Crooke.** 1987. Purification and characterization of a phosphoinositide-specific phospholipase C from guinea pig uterus. J. Biol. Chem. **262**:13789–13799.
5. **Berridge, M. J.** 1987. Inositol trisphosphate and diacylglycerol: two interacting second messengers. Annu. Rev. Biochem. **56**: 159–194.
6. **Berridge, M. J., J. P. Heslop, R. F. Irvine, and K. D. Brown.** 1984. Inositol trisphosphate formation and calcium mobilization in Swiss 3T3 cells in response to platelet-derived growth factor. Biochem. J. **222**:195–201.
7. **Bowen-Pope, D. F., and R. Ross.** 1982. Platelet-derived growth factor. II. Specific binding to cultured cells. J. Biol. Chem. **257**:5161–5171.
8. **Carpenter, G.** 1987. Receptors for epidermal growth factor and other polypeptide mitogens. Annu. Rev. Biochem. **56**:881–914.
9. **Cartwright, C. A., W. Eckhart, S. Simon, and P. L. Kaplan.** 1988. Cell transformation by pp $60^{c\text{-}src}$ mutated in the carboxy-terminal regulatory domain. Cell **49**:83–91.
10. **Cassel, D., P. Rothenberg, Y. X. Zhuang, T. Deuel, and L. Glaser.** 1983. Platelet-derived growth factor stimulates Na^+/H^+ exchange and induces cytoplasmic alkalinization in NR6 cells. Proc. Natl. Acad. Sci. USA **80**:6224–6228.
11. **Chu, S. H. W., C. J. Hoban, A. J. Owen, and R. P. Geyer.** 1985. Platelet-derived growth factor stimulates rapid polyphosphoinositide breakdown in fetal human fibroblasts. J. Cell. Physiol. **124**:391–396.
12. **Cochran, B. H., A. C. Reffel, and C. D. Stiles.** 1983. Molecular cloning of gene sequences regulated by platelet-derived growth factor. Cell **33**:939–944.
13. **Cockcroft, S.** 1987. Polyphosphoinositide phosphodiesterase: regulation by a novel guanine nucleotide binding protein, Gp. Trends Biol. Sci. **12**:75–78.
14. **Cooper, J. A., B. M. Sefton, and T. Hunter.** 1983. Detection and quantification of phosphotyrosine in proteins. Methods Enzymol. **99**:387–402.
15. **Ek, B., B. Westermark, H. Wasteson, and C. H. Heldin.** 1982. Stimulation of tyrosine-specific phosphorylation by platelet-derived growth factor. Nature (London) **295**:419–420.
16. **Escobedo, J. A., P. J. Barr, and L. T. Williams.** 1988. Role of tyrosine kinase and membrane-spanning domains in signal transduction by the platelet-derived growth factor receptor. Mol. Cell. Biol. **8**:5126–5131.
17. **Fain, J. N., M. A. Wallace, and K. J. H. Wojcikiewicz.** 1988. Evidence for involvement of guanine nucleotide-binding regulatory proteins in the activation of phospholipase C by hormones. FASEB J. **2**:2569–2574.
18. **Frackelton, A. R., Jr.** 1983. Characterization of phosphotyrosyl proteins in cells transformed by Abelson murine leukemia virus: use of a monoclonal antibody to phosphotyrosine. Cancer Cells **3**:339–345.
19. **Frackelton, A. R., Jr., P. M. Tremble, and L. T. Williams.** 1984. Evidence for the platelet-derived growth factor-stimulated tyrosine phosphorylation of the platelet-derived growth factor receptor *in vivo*. J. Biol. Chem. **259**:7909–7915.
20. **Fukui, T., R. J. Lutz, and J. M. Lowenstein.** 1988. Purification of a phospholipase C from rat liver cytosol that acts on phosphatidylinositol 4,5-bisphosphate and phosphatidylinositol 4-phosphate. J. Biol. Chem. **263**:17730–17737.
21. **Gould, K. L., and T. I. Hunter.** 1988. Platelet-derived growth factor induces multisite phosphorylation of pp $60^{c\text{-}src}$ and increases its protein-tyrosine kinase activity. Mol. Cell. Biol. **8**:3345–3356.
22. **Greenberg, M. E., and E. B. Ziff.** 1984. Stimulation of 3T3 cells induces transcription of the c-fos oncogene. Nature (London) **311**:433–438.
23. **Habenicht, A. J. R., J. A. Glomset, W. C. King, C. Nist, C. D. Mitchell, and R. Ross.** 1981. Early changes in phosphatidylinositol and arachidonic acid metabolism in quiescent Swiss 3T3 cells stimulated to divide by platelet-derived factor. J. Biol. Chem. **256**:12329–12335.
24. **Harrington, M. A., J. E. Estes, E. Leof, and W. J. Pledger.** 1985. PDGF stimulates transient phosphorylation of 180,000 dalton protein. J. Cell. Biochem. **27**:67–81.
25. **Hasegawa-Sasaki, H.** 1985. Early changes in inositol lipids and their metabolites induced by platelet-derived growth factor in quiescent Swiss mouse 3T3 cells. Biochem. J. **232**:99–109.
26. **Hasegawa-Sasaki, H., F. Lutz, and T. Sasaki.** 1988. Pathway of phospholipase C activation initiated with platelet-derived growth factor is different from that initiated with vasopressin and bombesin. J. Biol. Chem. **263**:12970–12976.
27. **Heldin, C. H., B. Westermark, and A. Wasteson.** 1981. Specific receptors for platelet-derived growth factor on cells derived

from connective tissue and glia. Proc. Natl. Acad. Sci. USA **78**:3664–3668.
28. **Herman, B., M. A. Harrington, N. E. Olashaw, and W. J. Pledger.** 1986. Identification of the cellular mechanisms responsible for platelet-derived growth factor induced alterations in cytoplasmic vinculin distribution. J. Cell. Physiol. **126**:115–125.
29. **Herman, B., and W. J. Pledger.** 1985. Platelet-derived growth factor-induced alterations in vinculin and actin distribution in BALB/c 3T3 cells. J. Cell. Biol. **100**:1031–1040.
30. **Hoffman, S. L., and P. W. Majerus.** 1982. Identification and properties of two distinct phosphatidylinositol-specific phospholipase C enzymes from sheep seminal vesicular glands. J. Biol. Chem. **257**:6461–6469.
31. **Homma, Y., J. Imaki, O. Nakanishi, and T. Takenawa.** 1988. Isolation and characterization of two different forms of inositol phospholipid-specific phospholipase C from rat brain. J. Biol. Chem. **263**:6592–6598.
32. **Huang, J. S., S. S. Huang, B. Kennedy, and T. F. Deuel.** 1982. Platelet-derived growth factor: Specific binding to target cells. J. Biol. Chem. **257**:8130–8136.
33. **Hunter, T., and J. A. Cooper.** 1985. Protein-tyrosine kinases. Annu. Rev. Biochem. **54**:897–930.
34. **Kaibuchi, K., T. Tsuda, A. Kikuchi, T. Tanimoto, T. Yamashita, and Y. Takai.** 1986. Possible involvement of protein kinase C and calcium ion in growth factor-induced expression of c-myc oncogene in Swiss 3T3 fibroblasts. J. Biol. Chem. **262**:1187–1192.
35. **Kamata, T., and H. F. Kung.** 1988. Effects of ras-encoded proteins and platelet-derived growth factor on inositolphospholipid turnover in NRK cells. Proc. Natl. Acad. Sci. USA **85**:5799–5803.
36. **Katan, M., R. W. Kriz, N. Totty, R. Philp, E. Meldrum, R. A. Aldape, J. L. Knopf, and P. J. Parker.** 1988. Determination of the primary structure of PLC-154 demonstrates diversity of phosphoinositide-specific phospholipase C activities. Cell **54**: 170–177.
37. **Kelly, K., B. H. Cochran, C. D. Stiles, and P. Leder.** 1983. Cell-specific regulation of the c-myc gene by lymphocyte mitogens and platelet-derived growth factor. Cell **35**:603–610.
38. **Kmiecik, T. E., and D. Shalloway.** 1988. Activation and suppression of pp $60^{c\text{-}src}$ transforming ability by mutation of its primary sites of tyrosine phosphorylation. Cell **49**:65–73.
39. **Kruijer, W., J. A. Cooper, T. Hunter, and I. M. Verma.** 1984. Platelet-derived growth factor induces rapid but transient expression of the c-fos gene and protein. Nature (London) **312**:711–715.
40. **Krupinski, J., R. Rajaram, M. Lakonishok, J. L. Benovic, and R. A. Cerione.** 1988. Insulin-dependent phosphorylation of GTP-binding proteins in phospholipid vesicles. J. Biol. Chem. **263**:12333–12341.
41. **Lopez-Rivas, A., P. Stroobant, M. D. Waterfield, and E. Rozengurt.** 1984. Ionic responses rapidly elicited by porcine platelet-derived growth factor in Swiss 3T3 cells. EMBO J. **3**:939–944.
42. **Matuoka, K., K. Fukami, O. Nakanishi, S. Kawai, and T. Takenawa.** 1988. Mitogenesis in response to PDGF and bombesin abolished by microinjection of antibody to PIP_2. Science **239**:640–643.
43. **Mayer, B. J., M. Hamaguchi, and H. Hanafusa.** 1988. A novel viral oncogene with structural similarity to phospholipase C. Nature (London) **322**:272–275.
44. **Moolenaar, W. H., L. G. J. Tertoolen, and S. W. de Laat.** 1984. Phorbol ester and diacylglycerol mimic growth factors in raising cytoplasmic pH. Nature (London) **312**:371–374.
45. **Nanberg, E., and E. Rozengurt.** 1988. Temporal relationship between inositol polyphosphate formation and increases in cytosolic Ca^{2+} in quiescent 3T3 cells stimulated by platelet-derived growth factor, bombesin, and vasopressin. EMBO J. **7**:2741–2748.
46. **Nishizuka, Y.** 1984. The role of protein kinase C in cell surface signal transduction and tumor promotion. Nature (London) **308**:693–698.
47. **Olashaw, N. E., and W. J. Pledger.** 1988. Epidermal growth factor stimulates formation of inositol phosphates in BALB/c 3T3 cells pretreated with cholera toxin and isobutylmethylxanthine. J. Biol. Chem. **263**:1111–1114.
48. **Paris, S., J. C. Chambard, and J. Pouyssegur.** 1988. Tyrosine kinase-activating growth factors potentiate thrombin- and AlF_4^--induced phosphoinositide breakdown in hamster fibroblasts. J. Biol. Chem. **263**:12893–12900.
49. **Piwnica-Worms, H., K. B. Saunders, T. M. Roberts, A. E. Smith, and S. H. Cheng.** 1988. Tyrosine phosphorylation regulates the biochemical and biological properties of pp $60^{c\text{-}src}$. Cell **49**:75–82.
50. **Pledger, W. J., C. A. Hart, K. L. Locatell, and C. D. Scher.** 1981. Platelet-derived growth factor-modulated proteins: constitutive synthesis by a transformed cell line. Proc. Natl. Acad. Sci. USA **78**:4358–4362.
51. **Pledger, W. J., C. D. Stiles, H. N. Antoniades, and C. D. Scher.** 1977. Induction of DNA synthesis in BALB/c 3T3 cells by serum components: Re-evaluation of the commitment process. Proc. Natl. Acad. Sci. USA **74**:4481–4485.
52. **Ralston, R., and J. M. Bishop.** 1985. The product of the protooncogene c-src is modified during the cellular response to platelet-derived growth factor. Proc. Natl. Acad. Sci. USA **82**:7845–7849.
53. **Rebecchi, M. J., and O. M. Rosen.** 1987. Purification of a phosphoinositide-specific phospholipase C from bovine brain. J. Biol. Chem. **262**:12526–12532.
54. **Ryu, S. H., K. S. Cho, K. Y. Lee, P. G. Suh, and S. G. Rhee.** 1987. Purification and characterization of two immunologically distinct phosphoinositide-specific phospholipase C from bovine brain. J. Biol. Chem. **262**:12511–12518.
55. **Ryu, S. H., P. G. Suh, K. S. Cho, K. Y. Lee, and S. G. Rhee.** 1987. Bovine brain cytosol contains three immunologically distinct forms of inositol-phospholipid-specific phospholipase C. Proc. Natl. Acad. Sci. USA **84**:6649–6653.
56. **Stahl, M. L., C. R. Ferenz, K. L. Kelleher, K. W. Kriz, and J. L. Knopf.** 1988. Sequence similarity of phospholipase C with the non-catalytic region of src. Nature (London) **322**:269–272.
57. **Suh, P. G., S. H. Ryu, W. C. Choi, K. Y. Lee, and S. G. Rhee.** 1988. Monoclonal antibodies to three phospholipase C isozymes from bovine brain. J. Biol. Chem. **263**:14497–14504.
58. **Suh, P. G., S. H. Ryu, K. H. Moon, H. W. Suh, and S. G. Rhee.** 1988. Inositol phospholipid-specific phospholipase C: complete cDNA and protein sequences and sequence homology to tyrosine kinase-related oncogene products. Proc. Natl. Acad. Sci. USA **85**:5419–5423.
59. **Suh, P. G., S. H. Ryu, K. H. Moon, H. W. Suh, and S. G. Rhee.** 1988. Cloning and sequence of multiple forms of phospholipase C. Cell **54**:161–169.
60. **Takenawa, T., and Y. Nagai.** 1981. Purification of phosphatidylinositol-specific phospholipase C from rat liver. J. Biol. Chem. **256**:6769–6775.
61. **Tonks, N. K., C. D. Diltz, and E. H. Fischer.** 1988. Characterization of the major protein-tyrosine-phosphatases of human placenta. J. Biol. Chem. **263**:6731–6737.
62. **Villereal, M. L., N. E. Owen, L. M. Vicenti, and L. L. Mix.** 1985. Regulation of Na^+/H^+ exchange in cultured human fibroblasts. Soc. Gen. Physiol. Ser. **39**:21–42.
63. **Wahl, M. I., T. O. Daniel, and G. C. Carpenter.** 1988. Antiphosphotyrosine recovery of phospholipase C activity after EGF treatment of A-431 cells. Science **241**:968–970.
64. **Wahl, M. I., S. Nishibe, P.-G. Suh, S. G. Rhee, and G. Carpenter.** 1989. Epidermal growth factor stimulates tyrosine phosphorylation of phospholipase C-γ independently of internalization and extracellular Ca^{2+}. Proc. Natl. Acad. Sci. USA **86**:1568–1572.
65. **Yarden, Y., J. A. Escobedo, W. J. Kuang, T. L. Yang-Feng, T. O. Daniel, P. M. Tremble, E. Y. Chen, M. E. Ando, R. N. Harkins, U. Francke, V. A. Fried, A. Ullrich, and L. T. Williams.** 1986. Structure of the receptor for platelet-derived growth factor helps define a family of closely related growth factor receptors. Nature (London) **323**:226–232.
66. **Yarden, Y., and A. Ullrich.** 1988. Growth factor receptor tyrosine kinases. Annu. Rev. Biochem. **57**:443–478.