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Phospholipase C-γ1 can induce DNA synthesis by a mechanism independent of its lipase activity

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ABSTRACT **Inositol phospholipid-specific phospholipase C (PLC) is involved in several signaling pathways leading to cellular growth and differentiation. Our previous studies reported the induction of DNA synthesis in quiescent NIH 3T3 cells after microinjection of PLC and the inhibition of serum- or Ras-stimulated DNA synthesis by a mixture of monoclonal antibodies to PLC-γ1. In the course of our investigation of anti-PLC-γ1 monoclonal antibodies, we found that each antibody exerts different inhibitory effects on the phosphatidylinositol-hydrolyzing activity of PLC-γ1 and that the inhibition of enzymatic activity does not correlate with the inhibition of DNA synthesis observed in the microinjection assay. PLC-γ1 with defective enzymatic activity was synthesized by substituting phenylalanine for histidine within the PLC-γ1 catalytic domain at amino acids 335 and 380, and mutant enzymes were expressed using a vaccinia expression system. The mutant enzymes were purified and microinjected into quiescent NIH 3T3 cells to evaluate their mitogenic activity. A moderate induction of DNA synthesis occurred after injection of mutant PLC-γ1. This mitogenic activity was inhibited by an antibody (αE 8-4) that does not significantly inhibit PLC-γ1 enzyme activity, which indicates that something else has to be inhibited. Furthermore, the partial induction of DNA synthesis observed with mutant PLC-γ1 was increased to levels seen with wild-type PLC-γ1 by coinjection of mutant PLC-γ1 with two second messengers, diacylglycerol and inositol trisphosphate. These results suggest that the mitogenic activity of PLC-γ1 does not exclusively result from the enzymatic activity of the lipase and that another activity inherent to the PLC-γ1 molecule can also induce DNA synthesis in quiescent cells.**

Inositol phospholipid-specific phospholipase C (PLC) catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate to generate inositol trisphosphate (IP_3) and diacylglycerol (DAG) in response to several receptor-binding hormones and neurotransmitters (1–3). The hydrolysis products, IP_3 and DAG, serve as intracellular second messenger molecules that amplify the initial signaling events leading to cellular calcium mobilization and protein kinase C activation (1, 4). The PLC enzyme family is composed of several distinct proteins, which can be divided into three types: PLC-β, PLC-γ, and PLC-δ. Each type contains more than one subtype—e.g., PLC-β1, PLC-β2, PLC-γ1, PLC-γ2, etc. PLC-γ enzymes are in general activated by receptors that are protein-tyrosine kinases or through interactions with nonreceptor protein-tyrosine kinase, whereas PLC-β enzymes are regulated by guanine nucleotide-binding proteins (5).

PLC-γ1 contains stretches of amino acids that are homologous to regulatory regions of c-Src (SH2 and SH3 domains) (6, 7). In the case of Src, these regions are involved in regulation of its tyrosine kinase and transforming activities (8, 9). Several reports have shown that the SH2 regions of PLC-γ1, GTPase-activating protein, the 85-kDa subunit of phosphatidylinositol 3′-kinase, and Crk all associate specifically with other phosphotyrosine proteins (10, 11). The SH2 domains of PLC-γ1 are responsible for its association with platelet-derived growth factor, epidermal growth factor, and fibroblast growth factor receptors in a ligand-dependent manner (12). Elevation of PLC enzyme activity has been reported in growth factor-treated cells as well as many transformed cells (13, 14). In our earlier studies, overexpressed levels of PLC-γ1 were achieved by direct microinjection of the protein, resulting in S-phase induction and transformation of quiescent NIH 3T3 cells (15). The PLC-γ1-induced DNA synthesis was inhibited by coinjection with a mixture of monoclonal antibodies (mAbs) to PLC-γ1 (16). In the course of these studies with anti-PLC-γ1 antibodies, we observed that injection of some single mAbs did not block PLC-γ1-induced DNA synthesis, even though PLC enzyme activity was markedly inhibited by the mAb (e.g., antibody αB 2-5) in *in vitro* lipase assays (16, 17). To investigate whether the catalytic activity of PLC-γ1 is essential for its mitogenic activity, we substituted phenylalanine for histidine at the catalytic active sites and characterized the properties of the resulting mutant enzymes for their enzymatic and mitogenic activities.

MATERIALS AND METHODS

Cell Culture. Murine NIH 3T3 fibroblasts were maintained in Dulbecco's modified essential medium (DMEM; GIBCO/BRL) supplemented with 10% calf serum and nonessential amino acids (GIBCO/BRL). Cells were plated on etched glass coverslips in 35-mm plates at a concentration of ≈4 × 10^4 cells per ml. After 1–2 days, the contact-inhibited monolayers were made quiescent by replacing the medium with DMEM containing 0.5% (vol/vol) fetal calf serum for a period of 20–36 hr before microinjection.

Reagents, Enzymes, and Antibodies. DAG and IP_3 were purchased from Sigma. Wild-type PLC-γ1 and anti-PLC-γ1 antibodies were prepared as described (15–17).

Point Mutants of PLC-γ1. pSC11-PLC4-γ1, a vaccinia expression vector harboring the entire coding sequence of rat brain PLC-γ1, and the construction of mutant pSC11-PLC-γ1 with the substitution of phenylalanine for tyrosine-783 have been described (14). Point mutations were introduced into pSC11-PLC-γ1 by oligonucleotide-directed mutagenesis with the oligonucleotides 5′-ATACGTATTAAACGAGGAAGA (H335F) and 5′-GGTGTGCCCAAAGTAAATGAC (H380F), resulting in substitution of phenylalanine for histidine at

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Abbreviations: PLC, phospholipase C; SH, Src homology; IP_3, inositol triphosphate; DAG, diacylglycerol; mAb, monoclonal antibody.

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amino acid residues 335 and 380 according to the method of Kunkel et al. (18).

To express PLC-γ1, HeLa cells were cultured in minimal essential medium containing 5% (vol/vol) horse serum in a spinning culture jar at 37°C. Cell concentrations were maintained between 2×10^5 and 5×10^5/ml. One liter of cells was harvested by centrifugation, the cell pellet was suspended in 25 ml of prewarmed medium in a culture dish (Falcon 175), and the cells were infected with the trypsinized recombinant virus (≈8 virus particles per cell) and incubated for 30 min at 37°C. The cells were diluted into 1 liter of prewarmed medium and incubated further for 36 hr. Cells were harvested by centrifugation, and the pellet was washed three times with 10 volumes of ice-cold, phosphate-buffered saline (pH 7.4; PBS). Cells were suspended in two volumes of homogenization buffer [20 mM Tris·HCl, pH 7.6/1 mM EGTA/1 mM dithiothreitol/1 mM phenylmethylsulfonyl fluoride/leupeptin (4 μg/ml)], disrupted by sonication (10 sec, five times), and centrifuged at 100,000 × g for 10 min. The resulting supernatant was applied to a preparative HPLC TSK DEAE-5PW column (21.5 × 150 cm) that had been equilibrated with 50 mM Tris·HCl (pH 7.4) containing 0.1 mM dithiothreitol and 1 mM EGTA. Proteins were eluted at a flow rate of 5 ml/min by an increasing KCl gradient from 0 to 0.3 M for 35 min. PLC-γ1 fractions were pooled, diluted with an equal volume of 4 M KCl, and applied to an HPLC TSK phenyl-5PW column (7.5 × 75 mm) that had been equilibrated with 20 mM Hepes, pH 7.0/1 mM dithiothreitol/3 M KCl. Consecutive applications of decreasing KCl gradients from 3.0 to 1.2 M for 10 min and from 1.2 M to 0 M for 25 min resulted in a single PLC-γ1 peak, which closely resembled one protein peak, monitored by absorbance at 280 nm. Elution profiles were nearly identical for wild-type and mutant PLC-γ1 enzymes. Purified enzymes were pooled, concentrated, and stored frozen at −70°C. SDS/PAGE analysis indicated >95% purity for the purified enzymes.

Microinjection Assay. NIH 3T3 fibroblasts were grown to ≈80% confluence; the normal growth medium was replaced with medium containing 0.5% fetal calf serum. Cultures were starved for 24–36 hr before microinjection of $\approx 1 \times 10^{-11}$ ml of PLC-γ1, mutant enzymes, antibodies, or chemicals into each cell. Injected cultures were maintained in low serum media for 18 hr. In some experiments (see Table 1), anti-PLC-γ1 mAbs were injected and 12% fetal calf serum was added to the injected cultures. Microinjection assays were performed with double-blind samples. A 4-hr pulse of [^{3}H]thymidine (0.5 μCi/ml; 1 Ci = 37 GBq; Amersham) was performed 18–24 hr after injection. Cultures were washed with isotonic PBS and fixed with 2.5% (vol/vol) glutaraldehyde in PBS.

Autoradiographic Procedure for the Determination of [^{3}H]Thymidine Incorporation into DNA. The fixed cells on the coverslips were mounted onto glass slides, coated with nuclear tracking emulsion (NTB2; Eastman Kodak), and exposed for 36–48 hr. The cells were stained with Geimsa, photographed, and counted. The number of cells that incorporated [^{3}H]thymidine into nuclei were monitored by microscopic observation.

Fold induction of DNA synthesis is defined as the ratio of injected cells that incorporated [^{3}H]thymidine into DNA divided by the ratio of uninjected cells near the injected area that incorporated [^{3}H]thymidine into DNA. Labeling efficiency is a measure of the ability of an injected antibody to inhibit serum-stimulated DNA synthesis of quiescent cells. After injection, the culture is stimulated with serum growth factors. The definition of labeling efficiency is the same as for fold induction of DNA synthesis.

RESULTS

Inhibition of Serum- and PLC-γ1-Induced DNA Synthesis by Antibodies to PLC-γ1. Previously, a mixture of mAbs against PLC-γ1 was identified that neutralizes the biological activity of both endogenous and injected PLC-γ1 (16). The effect of individual anti-PLC-γ1 antibodies on PLC-γ1- and serum-stimulated DNA synthesis was investigated in quiescent NIH 3T3 fibroblasts (Table 1). All of the antibodies formed soluble complexes with PLC-γ1 and inhibited PLC-γ1 enzyme activity. A wide range of inhibition was observed. Among the five antibodies, maximum inhibition was observed with antibody αB 2-5 (81%) followed by αB 16-5 (64%), αF 7-2 (64%), αE 8-4 (24%), and αE 9-4 (13%). Although antibodies αB 2-5 and αB 16-5 inhibited PLC enzymatic activity 81% and 64%, respectively, they exerted almost no effect on the inhibition of serum-induced mitogenic activity of endogenous PLC-γ1. In contrast, serum-induced DNA synthesis was markedly reduced by microinjection of antibody αE 8-4, whereas the PLC enzymatic activity was only slightly inhibited by the antibody (24%). In addition to serum-induced DNA synthesis, induction was also observed after microinjection of PLC-γ1 into quiescent NIH 3T3 cells (15). The effect of individual mAbs on the PLC-γ1-induced DNA synthesis was compared with the effects observed after serum-induced DNA synthesis. The results are summarized in Table 1. Both serum- and PLC-γ1-induced DNA synthesis were not affected by the injection of antibody αB 2-5. Antibody αE 8-4 markedly inhibited serum-induced DNA synthesis but not PLC-γ1-induced DNA syn-

Table 1. mAbs to PLC-γ1 respond differently to inhibit PLC-γ1 lipase activity compared to their effects on DNA synthesis in the microinjection assay

Injected anti-PLC-γ1 antibody*	% inhibition of PLC-γ1 enzyme activity*	Serum-induced DNA synthesis, % labeling efficiency†	PLC-γ1-induced DNA synthesis, fold induction‡
PBS	—	108 (12)	23.2 (6.8)
αE 9-4	13	88 (11)	10.9 (5.2)
αE 8-4	24	15 (5)	20.3 (6.1)
αF 7-2	64	20 (7)	7.9 (3.1)
αB 16-5	64	112 (21)	12.7 (4.6)
αB 2-5	81	85 (14)	25.1 (6.5)
αPLC-γ1 mix	89	12 (3)	1.3 (0.8)

*The designations of anti-PLC-γ1 antibodies and the percent inhibition of PLC-γ1 enzyme activity by these antibodies were as described in a previous paper (17). All anti-PLC-γ1 antibodies belong to the IgG_2 subclass. The antibodies were listed in the order of percent inhibition of PLC-γ1 enzyme activity. The antibodies were injected at 2 mg/ml. The PLC-γ1 mix contained equal amounts of αE 9-4, αE 8-4, αF 7-2, αB 16-5, and αB 2-5 antibodies. PLC activity was measured with [^{3}H]phosphatidylinositol 4,5-bisphosphate as the substrate as described (17).

†After injection, 12% fetal calf serum was added to the injected cultures, and they were incubated for 18 hr, labeled with [^{3}H]thymidine (0.5 μCi/ml; Amersham) for 4 hr, fixed in 2.5% glutaraldehyde in PBS, and subjected to autoradiography for 48 hr. The number of cells that had incorporated [^{3}H]thymidine into nuclei were monitored by microscopic observation. Percent labeling efficiency is defined as the ratio of injected cells that incorporated [^{3}H]thymidine into DNA divided by the ratio of cells that incorporate label into DNA near the injected area times 100%. Numbers in parentheses are standard deviations from at least four experiments.

‡Wild-type PLC-γ1 was injected at 250 μg/ml. Individual antibodies and mixtures of antibodies were included in the samples as indicated. Injected cultures were maintained in low serum medium for 18 hr, labeled with [^{3}H]thymidine for 4 hr, fixed in 2.5% glutaraldehyde in PBS, and subjected to autoradiography for 48 hr. After autoradiography, the number of injected cells that incorporated [^{3}H]thymidine was divided by the total number of injected cells (percent of thymidine incorporation). Fold induction was calculated by dividing the percent of thymidine incorporation of the injected cells by the background (percent of thymidine incorporation). Numbers in parentheses are standard deviations from at least four experiments.

thesis. Almost no inhibition of serum-induced DNA synthesis was observed after injection of antibodies αE 9-4 and αB 16-5, whereas the PLC-γ1-induced DNA synthesis was partially inhibited. Partial inhibition of PLC-γ1-induced DNA synthesis was also seen after injection of antibody αF 7-2; this antibody significantly inhibited serum-induced DNA synthesis. These results indicate that the inhibition of lipase activity by various antibodies does not correlate with the inhibition of serum- and PLC-γ1-induced DNA synthesis. It is possible that mitogenic signals other than the generation of second messengers (DAG and IP_3) may be responsible for the mitogenic activity of PLC-γ1 (e.g., the interaction of PLC-γ1 with other intracellular proteins).

Recent reports (19, 20) demonstrate that certain anti-PLC-γ1 mAbs specifically immunoprecipitate not only PLC-γ1 and growth factor receptors but also three other phosphoproteins of ≈100, 84, and 47 kDa from ^{32}P-labeled lysates of NIH 3T3 cells, demonstrating that PLC-γ1 exists as a complex (20). The 47-kDa protein was identified as the SH2/SH3 domain-containing protein, Nck, and its phosphorylation was shown to be stimulated in response to a variety of growth factors, phorbol myristate acetate, and cAMP (19, 20). Nck consists mainly of three SH3 domains and one SH2 domain and like the Crk oncoprotein has no demonstrable catalytic function. The protein, Nck, is recognized by certain anti-PLC-γ1 mAbs; therefore, the effect of these antibodies on the serum- and PLC-γ1-induced DNA synthesis could be due to their interaction with Nck or interaction with other proteins that contain similar domains. Although the mixture of anti-PLC-γ1 antibodies immunoprecipitate two other phosphoproteins (100 and 84 kDa) complexed with PLC-γ1 (19, 20), the effect of the complex formation on PLC-γ1 enzymatic and mitogenic activity is not known. To determine whether the enzyme activity of PLC-γ1 is essential for its mitogenic activity, mutant enzymes with defective lipase activity were prepared.

S-Phase Induction of Quiescent NIH 3T3 Cells After Microinjection of Mutant PLC-γ1 Enzymes. The two regions of PLC named X and Y, of ≈170 and 260 amino acids, respectively, have similar sequences in the three types of mammalian PLC (β, γ, and δ). Deletion in either the X or Y domain of PLC-γ1 led to a complete loss of lipase activity. It appears, therefore, that domains X and Y, but not SH, are essential for PLC enzymatic activity. To investigate the relevance of the X region of PLC-γ1 for its mitogenic activity, we substituted phenylalanine for histidine at amino acid positions 335 and 380 and expressed the mutant enzymes in a vaccinia expression system. The mutant enzymes were purified as described in *Materials and Methods*. Recent reports demonstrate that platelet-derived growth factor stimulation of inositol phospholipid hydrolysis requires PLC-γ1 phosphorylation on tyrosine residues 783 and 1254 (14) and that tyrosine phosphorylation increases PLC-γ1 catalytic activity (13). We have, therefore, prepared and purified a mutant PLC-γ1 by substituting phenylalanine for tyrosine at position 783. The purities of the wild-type and mutant PLC-γ1 enzymes were determined by SDS/PAGE in the presence of 2-mercaptoethanol. The enzymes were highly purified with one major protein band of ≈148 kDa (>95% purity). These preparations were used for the subsequent experiments.

As shown in Table 2, mutant PLC-γ1 enzymes with substitution of phenylalanine for histidine at positions 335 and 380 (H335F and H380F) had markedly reduced lipase activity (<10% of PLC catalytic activity compared with wild-type PLC-γ1). In contrast, the lipase activity was not affected by substituting phenylalanine for tyrosine at position 783 (PLC-γ1 Y783F, Table 2), indicating that tyrosine phosphorylation at amino acid residue 783 is not important for the catalytic activity of PLC-γ1. Although PLC-γ1 H335F and H380F enzymes were defective in PLC enzyme activity, they still induced intermediate levels of DNA synthesis when microinjected into quiescent NIH 3T3 cells (≈50% of mitogenic activity compared with the wild-type PLC-γ1 or mutant PLC-γ1 Y783F; Table 2). These results suggest that the lipase-defective PLC-γ1 mutants exert their mitogenic activity through a mechanism other than the generation of second messenger molecules (DAG and IP_3). Since several mAbs with different epitopic specificities did not completely block DNA synthesis induced by microinjected wild-type PLC-γ1 (Table 1), we examined the effect of these antibodies on the inhibition of DNA synthesis induced by the microinjected lipase-defective PLC-γ1 mutants (Table 3). DNA synthesis was partially inhibited by coinjection of PLC-γ1 H335F with mAb αE 9-4 or αB 16-5. No inhibition was seen with mAb αB 2-5, although this antibody had the highest inhibitory effect on the lipase activity of PLC-γ1. Furthermore, the mitogenic activity of PLC-γ1 H335F was significantly blocked by coinjection of the mutant enzyme together with antibody αF 7-2 or αE 8-4 (antibodies αF 7-2 and αE 8-4 inhibited PLC-γ1 lipase activity by 64% and 24%, respectively). Fig. 1 illustrates some of the biological effects summarized in Tables 1–3—i.e., the effects of coinjection with antibody αF 7-2 or αE 8-4 on the inhibition of DNA synthesis induced by mutant PLC-γ1 H335F. Wild-type PLC-γ1 induced ≈2-fold more DNA synthesis than the lipase-deficient PLC-γ1 mutant H335F (Fig. 1 *A* and *B*, Table 2). Although the DNA synthesis induced by wild-type PLC-γ1 was not inhibited by antibody αE 8-4 (Table 1), the mitogenic activity of mutant PLC-γ1 defective in lipase activity was completely inhibited after injection of antibody αE 8-4 (Fig. 1*C*, Table 3). These results also support the observation that a domain within PLC-γ1, distinct from the catalytic site, may be involved in mitogenesis.

Table 2. PLC-γ1 mutants defective in lipase activity induce intermediate levels of DNA synthesis in quiescent NIH 3T3 cells

Protein	Fold induction of DNA synthesis	Lipase activity, % wild type PLC-γ1
BSA	1.7 (0.8)	—
PLC-γ1	21.6 (5.9)	100
PLC-γ1 H335F	10.4 (4.1)	≈5
PLC-γ1 H380F	9.1 (3.6)	≈9
PLC-γ1 Y783F	22.1 (6.4)	96

Experimental details for PLC-γ1-induced DNA synthesis were as described in footnote ‡ for Table 1. Proteins were injected at 250 μg/ml with an injection volume of $\approx 10^{-11}$ ml per cell. PLC lipase activity was measured with [^3H]phosphatidylinositol 4,5-bisphosphate as the substrate as described (17). BSA, bovine serum albumin.

Table 3. mAbs to PLC-γ1 block the DNA synthesis induced by lipase-defective PLC-γ1 mutants

Sample	Fold induction of DNA synthesis
PLC-γ1 H335F	13.2 (4.8)
PLC-γ1 H335F + αE 9-4	5.3 (0.3)
PLC-γ1 H335F + αE 8-4	1.2 (0.5)
PLC-γ1 H335F + αF 7-2	2.9 (2.1)
PLC-γ1 H335F + αB 16-5	4.9 (3.1)
PLC-γ1 H335F + αB 2-5	15.5 (6.5)
BSA	1.7 (0.8)

Experimental details for PLC-γ1-induced DNA synthesis were as described in footnote ‡ of Table 1. Mutant PLC-γ1 was injected at 250 μg/ml alone or in the presence of various antibodies as indicated. Similar results were obtained with PLC-γ1 H380F mutant enzyme. BSA, bovine serum albumin.

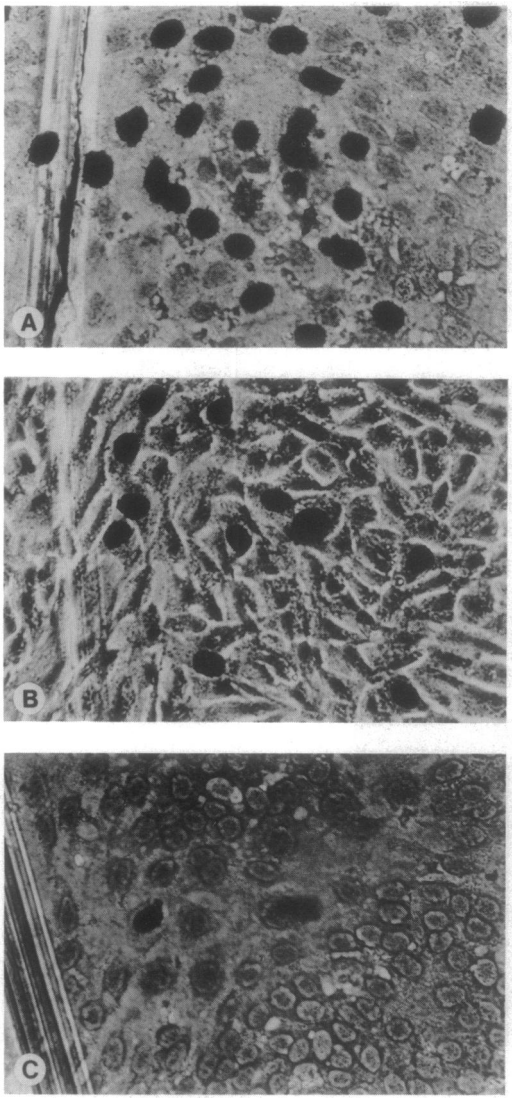

FIG. 1. Microinjection of wild-type PLC-γ1 or mutant PLC-γ1 H335F induces DNA synthesis in quiescent NIH 3T3 cells, and coinjection of mutant PLC-γ1 H335F with mAb αE 8-4 completely inhibited the mutant's activity. Approximately 80 cells in the area of the photomicrographs were injected with wild-type PLC-γ1 (*A*), mutant PLC-γ1 H335F (*B*), or mutant PLC-γ1 H335F plus αE 8-4 (*C*). After injection, cultures were maintained in serum-free medium for 18 hr, pulsed with [^{3}H]thymidine for 4–6 hr, washed with PBS, and fixed with 2.5% glutaraldehyde in PBS. Coverslips were mounted to microscope slides and dipped into nuclear tracking emulsion. Autoradiography was carried out for 48 hr. The cells were stained with Giemsa and microinjected areas were then photographed. Similar results were obtained with antibody αF 7-2.

Restoration of Mutant PLC-γ1 Mitogenic Activity by Coinjection with DAG and IP_3. Microinjection of PLC-γ1 mutant enzymes defective in lipase activity induced intermediate levels of DNA synthesis in quiescent NIH 3T3 cells. Similar induction levels were attained after microinjection of second messenger molecules (DAG plus IP_3; Table 4). The effect of DAG and IP_3 on the DNA synthesis-inducing activity of mutant PLC-γ1 H380F was investigated. The partial induction of DNA synthesis seen after microinjection of the catalytic defective PLC-γ1 mutant H380F was completely restored to levels seen with wild-type PLC-γ1 by coinjection of the mutant with DAG and IP_3 (Table 4). Fig. 2 illustrates the restoration of mitogenic activity of mutant PLC-γ1 (H380F) by coinjection with DAG and IP_3. These results suggest that the generation of second messenger molecules by PLC-γ1 and the interaction of PLC-γ1 with other signaling molecules are both responsible for the DNA synthesis-inducing activity.

Table 4. Restoration of wild-type PLC-γ1 levels of DNA synthesis after coinjection of a catalytic defective mutant with the products of the lipase reaction

Sample	Fold induction of DNA synthesis
IP_3	1.9 (0.9)
DAG	4.2 (2.1)
IP_3 + DAG	11.1 (3.5)
PLC-γ1 H380F	14.3 (4.9)
IP_3 + DAG + PLC-γ1 H380F	33.7 (13.7)
PLC-γ1	28.4 (9.6)
PBS	1.2 (0.6)

Experimental details for PLC-γ1-induced DNA synthesis were as described in footnote ‡ of Table 1. IP_3 and DAG were injected at 200 μg/ml and 4 mM, respectively. Similar results were obtained with PLC-γ1 H335F mutant enzyme.

DISCUSSION

The mechanism by which PLC-γ1 induces DNA synthesis in quiescent NIH 3T3 cells is not clear. Microinjection of excess amounts of PLC directly into the cells may increase the production of second messengers (DAG and IP_3), leading to the induction of DNA synthesis. In support of this view, injection of DAG plus IP_3 induced intermediate levels of DNA synthesis (Table 4).

In this report, we presented evidence that the mitogenic activity of PLC-γ1 does not exclusively result from its lipase activity to generate DAG and IP_3. On the basis of the ability of an antibody to inhibit the *in vitro* enzyme activity of PLC-γ1, there was no correlation between the inhibition of lipase activity and PLC-γ1-induced DNA synthesis by coinjection with different mAbs against PLC-γ1 (Table 1). An excess of mAb was mixed with PLC-γ1 and then injected into quiescent NIH 3T3 cells (antibody at 2 mg/ml vs. PLC-γ1 at 250 μg/ml); the injection is not expected to affect the interaction between antibody and PLC-γ1. Furthermore, we demonstrate that mutant PLC-γ1 enzymes with defective lipase activity induce intermediate levels of DNA synthesis after microinjection into quiescent NIH 3T3 cells (Table 2). These results strengthen our hypothesis that mitogenic signals other than the generation of DAG and IP_3 are involved in PLC-γ1-induced DNA synthesis.

Finally, we show the restoration of mitogenic activity of the mutant PLC-γ1 enzymes by coinjection with DAG and IP_3 (Table 4). Taken together, these data indicate that the mitogenic activity of PLC-γ1 results from both the generation of second messengers and the interaction of PLC-γ1 with other mitogenic signaling intermediates. Although the SH domains of PLC-γ1 are not essential for its catalytic activity, they are responsible for the interaction of PLC-γ1 with certain growth factor receptors, and the receptors must be autophosphorylated for PLC-γ1 to bind. The importance of SH domains to PLC-γ1 mitogenic activity can be investigated by using lipase-defective PLC-γ1 (H338F or H380F) with mutations in the SH domains. In addition to growth factor receptors, PLC-γ1 interacts with other intracellular proteins [e.g., two phosphoproteins of ≈100 kDa and 84 kDa (19, 20)]. The nature of the interactions and the function(s) of these interacting proteins, with respect to PLC-γ1 biological activities, is not known. Once all the functional domains of PLC-γ1 (responsible for its mitogenic activity) are identified, the interaction of these domains with other intracellular signaling components should be investigated. These studies should provide us with a better understanding of the signal-transduction pathways mediated by PLC-γ1.

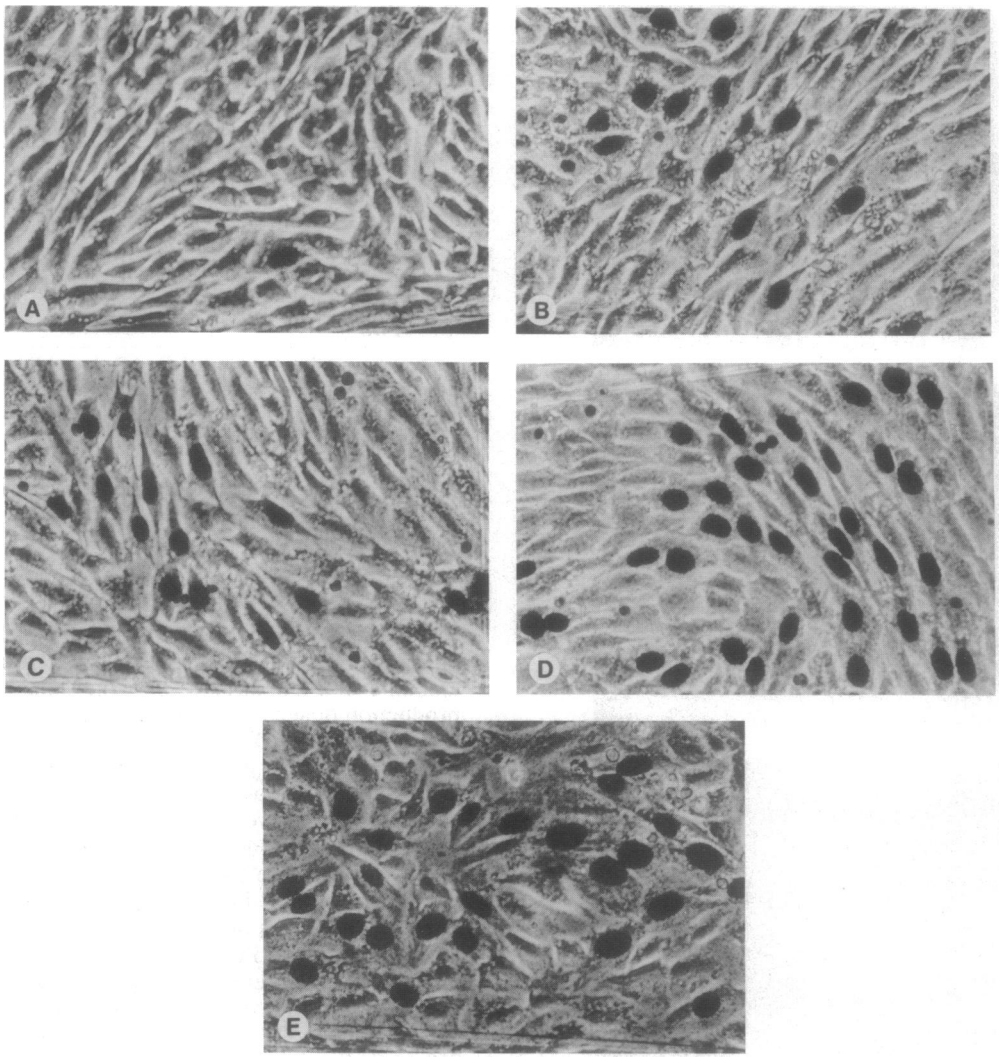

FIG. 2. Microinjection of DAG and IP_3 restores the DNA synthesis-inducing activity of the mutant enzyme to levels seen after injection of wild-type PLC-γ1. Approximately 80 cells in the area of the photomicrographs were injected with IP_3 (*A*), IP_3 plus DAG (*B*), mutant PLC-γ1 H380F (*C*), mutant PLC-γ1 H380F plus IP_3 and DAG (*D*), and wild-type PLC-γ1 (*E*). Experimental details were as described in Table 4 and the legend to Fig. 1.

In summary, phosphoinositol metabolism mediated by inositol phospholipid-specific PLC-γ1 plays a role in regulating the metabolic pathways that control growth and differentiation and may contribute to the process of neoplastic transformation. We have characterized two separate functional domains of PLC-γ1, a domain with catalytic lipase activity and a noncatalytic structural domain suspected to interact with other intracellular signaling components. Both domains are required for maximum induction of DNA synthesis in the microinjection assay. Catalytic-defective mutants of PLC-γ1 induce an intermediate level of DNA synthesis that is inhibited by antibodies thought to interact with the PLC-γ1 noncatalytic domains. The DNA synthesis-inducing activity of the lipase-defective mutants can be restored to wild-type PLC-γ1 levels by coinjection with the products of the lipase reaction, DAG and IP_3. These results suggest that protein–protein interaction via the noncatalytic structural domain (possibly SH2/SH3 regions) of PLC-γ1 is a critical aspect of PLC signaling.

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