Analysis of Platelet-derived Growth Factor-induced Phospholipase D Activation in Mouse Embryo Fibroblasts Lacking Phospholipase C-γ1

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Platelet-derived growth factor (PDGF) activates phospholipase D (PLD) in mouse embryo fibroblasts (MEFs). In order to investigate a role for phospholipase C-γ1 (PLC-γ1), we used targeted disruption of the Plcg1 gene in the mouse to develop Pleg1+/− and Pleg1−/− cell lines. Pleg1+/− MEFs treated with PDGF showed a time- and dose-dependent increase in the production of total inositol phosphates that was substantially reduced in Pleg1−/− cells. Pleg1+/− cells also showed a PDGF-induced increase in PLD activity that had a similar dose dependence to the PLC response but was down-regulated after 15 min. Phospholipase D activity, however, was markedly reduced in Pleg1−/− cells. The PDGF-induced inositol phosphate formation and the PLD activity that remained in the Pleg1−/− cells could be attributed to the presence of phospholipase C-γ2 (PLC-γ2) in the Pleg1+/− cells. The PLC-γ2 expressed in the Pleg1−/− cells was phosphorylated on tyrosine in response to PDGF treatment, and a small but significant fraction of the Pleg1−/− cells showed Ca2+ mobilization in response to PDGF, suggesting that the PLC-γ2 expressed in the Pleg1+/− cells was activated in response to PDGF. The inhibition of PDGF-induced phospholipid hydrolysis in Pleg1−/− cells was not due to differences in the level of PDGF receptor or in the ability of PDGF to cause autophosphorylation of the receptor. Upon treatment of the Pleg1−/− cells with oleoylacetylglycerol and the Ca2+ ionophore ionomycin to mimic the effect of PLC-γ1, PLD activity was restored. The targeted disruption of Pleg1 did not result in universal changes in the cell signaling pathways of Pleg1+/− cells, because the phosphorylation of mitogen-activated protein kinase was similar in Pleg1+/− and Pleg1−/− cells. Because increased plasma membrane ruffles occurred in both Pleg1+/− and Pleg1−/− cells following PDGF treatment, it is possible neither PLC nor PLD are necessary for this growth factor response. In summary, these data indicate that PLC-γ1 is required for growth factor-induced activation of PLD in MEFs.

Platelet-derived growth factor (PDGF)1 binds receptors (PDGFs) located on the cell surface. Upon ligand binding, these receptors undergo dimerization and activation of the intrinsic tyrosine kinase, which results in autophosphorylation of the receptor (1–3). The phosphorylated tyrosine residues on the PDGFRA act as docking sites for the SH2 domain of cytosolic signaling molecules, including phosphoinositide phospholipase C-γ (PLC-γ), the phosphotyrosine phosphatase syp, the regulatory subunit of phosphatidylinositol 3-kinase, the Ras GTPase activating protein, the cytosolic tyrosine kinase Src, and adapter proteins, such as Shc, Grb2, and Nck (4, 5). Upon binding to phosphorylated Tyr-1021, PLC-γ is phosphorylated and activated, resulting in the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) to diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP3). Diacylglycerol activates protein kinase C (PKC), whereas IP3 liberates Ca2+ from stores in the endoplasmic reticulum (6, 7).

Phospholipase D hydrolyzes phosphatidylcholine, generating choline and phosphatic acid (PA) (8, 9). Phosphatic acid exerts many effects in vitro, including the stimulation of PLC-γ, phosphatidylinositol-4-phosphate kinase, and protein kinases (10). In addition, through the actions of PA phosphohydrolase and a specific phospholipase A2, PA can be converted to DAG and the signaling molecule lysophosphatic acid, respectively (11).

There is conflicting evidence about whether the activation of PLC-γ and the subsequent activation of PKC are necessary for agonist stimulation of PLD. Although there are many studies reporting the involvement of PKC in the activation of PLD by agonists (12–15), there also are reports that PKC is not involved (16–19). Furthermore, some studies have indicated that PLD can be activated by certain agonists in the absence of detectable PIP2 hydrolysis. For example, in Madin-Darby canine kidney cells, studies with neomycin indicate that activation of PLD by purinergic agonists is independent of PLC-γ activity (20). In certain fibroblasts, PLD activation by epidermal growth factor (EGF) has been reported to occur in the absence of measurable PIP2 breakdown (21). However, in Swiss 3T3 fibroblasts and TRMP cells, activation of PLC-γ1 is necessary for stimulation of PLD activity by FDOF (22). Previously, we used homologous recombination to selectively disrupt the Pleg1 gene encoding PLC-γ1 in mice (23).

Although this mutation was lethal, immortal mouse embryo fibroblast (MEF) cell lines were produced from Pleg1+/− and Pleg1−/− embryos. We have now used these cells to study PDGF-induced PLD activity. The results indicate that PLC-γ1 activity is required for PDGF-induced PLD activation.

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† The abbreviations used are: PDGF, platelet-derived growth factor; PDGFR, PDGF receptor; PLD, phospholipase D; MEF, mouse embryo fibroblast; PLC-γ1, phospholipase C; PIP2, phosphatidylinositol 4,5-bisphosphate; DAG, diacylglycerol; IP3, inositol-1,4,5-trisphosphate; PKC, protein kinase C; PA, phosphatic acid; DMEM, Dulbecco’s modified Eagle’s medium; OAG, 1-oleoyl-2-acetyl-sn-glycerol; PBS, phosphate-buffered saline; Ins, inositol; InsPn, inositol phosphates; P-Tyr, phosphotyrosine; EGF, epidermal growth factor; MAP kinase, mitogen-activated protein kinase; PtdBut, phosphatidylbutanol.
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EXPERIMENTAL PROCEDURES

Materials—Dulbecco’s modified Eagle’s medium (DMEM) with 1-glutamine and high glucose and Earle’s modified Eagle’s medium were purchased from Life Technologies, Inc. EGF was from G. Carpenter, PDGF B/B (human recombinant) was from Boehringer Mannheim, and ionomycin was from Sigma. 1-Oleoyl-2-acetyl-sn-glycerol (OAG) and phosphatidyldiethylene (PtdEtn) standards were from Avanti Polar Lipids. 

90-% glycerol and myo-2-hydroxilinositol were purchased from NEN Life Science Products. Antibodies to the PDGF type B receptor (rabbit anti-human) and phosphotyrosine (P-Tyr) (4G10) were from Upstate Biotechnology, and antibodies to p44/42 mitogen-activated protein (MAP) kinase and phospho-specific p44/42 MAP kinase (Thr-202/Tyr-204) were purchased from New England Biolabs. The antibody to PLC-γ2 was purchased from Santa Cruz. SDS-polyacrylamide gels were from NOVEX. Dithiobispyridine (DTMP) 558/568 phalloidin and fluo-3 AM were from Molecular Probes.

Cell Culture—MEFs were prepared from embryonic day 9.5 embryos with (Plcg1−/−) and without (Plcg1+/+) targeted disruption of the Plcg1 gene, using the targeting vectors TVI and TVII (23). Wild type and null cells from the same litter were established in culture according to standard methods and maintained as immortalized non-transformed cell lines. MEFs grown in serum-free DMEM mobilized PLC-γ1 at a concentration of 1 × 104 EGF receptors /100 mm dish.

Mobilization of Intracellular Ca2+-—Plcg1−/− and Plcg1+/+ cells were plated on coverslips and grown to 80–90% confluence before the addition of Earle’s modified Eagle’s medium plus 0.5% fetal calf serum overnight. The coverslips were then washed twice with wash buffer (10 mM Hepes, pH 7.4, 140 mM NaCl, 5 mM KCl, 1 mM MgCl2, 0.55 mM Na2SO4, 1 μg/ml insulin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin). A protein assay using the BCA method (Pierce) was performed on the samples, and cells on a duplicate 100-mm dish were counted using a hemocytometer.

Western Blotting—Subconfluent cells in 100-mm dishes that were serum-starved in serum-free DMEM containing 0.5% bovine serum albumin for 24 h were washed once with 5 ml of PBS and scraped directly in 300 ml of PBS containing 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 mM NaN3, 1 μg/ml actinomycin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin. A protein assay using the BCA method (Pierce) was performed on the samples, and cells on a duplicate 100-mm dish were counted using a hemocytometer.

Proteins were separated by SDS-PAGE on a 6% gel (PDGFR and phospho-tyrosine) or a 4–20% gradient gel (MAP kinase and phospho-MAP kinase), transferred to Immobilon-P, blocked in 3% dry milk (MAP kinase) or 3% dry milk/PBS (P-Tyr), and probed with the antibody to PDGF receptor, p44/42 MAP kinase, or phosphorylated p44/42 MAP kinase. The cells were incubated with 25 ng/ml PDGF or 1% fresh fetal calf serum for 10 min at 37 °C.

In order to test for a role of PLC-γ1 in growth factor-induced PLD activity, we treated TVI- and TVII-targeted Plcg1−/− and Plcg1+/+ MEFs with EGF or PDGF. TVI-targeted Plcg1−/− MEFs have approximately 2 × 104 EGF receptors/cell, whereas the TVI-targeted Plcg1−/− MEFs that were greatly enriched in TVI-targeted Plcg1−/− MEFs. Most strikingly, PDGF produced a robust PLD response in TVII-targeted Plcg1−/− MEFs that was greatly enriched in TVI-targeted Plcg1−/− MEFs (Fig. 1B). Thus, both the TVI- and TVII-targeted Plcg1−/− MEFs showed PDGF-induced PLD activity that was inhibited in Plcg1−/− MEFs. Because the response to PDGF was so much greater in the TVII-targeted Plcg1−/− MEFs, we selected this cell line to

of protein A/G PLUS-agarose (Santa Cruz) at 4 °C with rocking. A protein assay was performed on the precleared cell lysate, which was then diluted to 1 mg/ml in 500 μl and immunoprecipitated with 3 μg of rabbit polyclonal PLC-γ2 antibody for 1 h at 4 °C. Twenty μl of protein A/G PLUS-agarose was added overnight at 4 °C with rocking. In the presence of EGF, the protein A/G PLUS-agarose beads were collected with centrifugation, washed three times with 1 ml of radioimmune precipitation buffer, and resuspended in 20 μl of 2× SDS sample buffer. The samples were boiled, and the immunoprecipitated proteins were separated on a 6% SDS-PAGE gel. The proteins were transferred to Immobilon-P, blocked in 3% dry milk in PBS, and probed with an antibody to P-Tyr at a concentration of 1 μg/ml.
further investigate the role of PLC-γ1 in the PDGF-induced PLD response.

InsP₆ Formation and PtdBut Formation in Plcg1¹/² and Plcg1⁻/⁻ MEFs—In order to investigate the extent to which disruption of PLC-γ1 eliminated PDGF-induced inositol phosphate formation, we measured total InsP₆ in the Plcg1¹/² and Plcg1⁻/⁻ MEFs in response to PDGF. Platelet-derived growth factor increased the production of InsP₆ in Plcg1¹/² cells in a dose- and time-dependent fashion (Fig. 2). In Plcg1⁻/⁻ MEFs, PDGF caused a smaller increase in InsP₆ formation. As shown in Fig. 3, PDGF treatment of Plcg1¹/² MEFs resulted in a dose- and time-dependent increase in PtdBut, an unambiguous marker of PLD activity when cells are treated with agonist in the presence of butan-1-ol (27). However, PDGF-induced PLD activity was inhibited in Plcg1⁻/⁻ MEFs. Whereas the dose-response curves for InsP₆ were similar to those for PtdBut (Fig. 3A; cf. Fig. 2A), the time course for PtdBut indicated no further production after 15 min despite further increases in InsP₆ (Fig. 3B; cf. Fig. 2B). These results are consistent with PLC-γ1 acting upstream of PLD in growth factor-induced activation, but they do not explain the cessation of PLD activation at 15 min.

Expression of PLC-γ2 and Ca²⁺ Mobilization in Plcg1⁻/⁻ Cells—In Plcg1⁻/⁻ cells, PDGF caused a small increase in both inositol phosphate production (Fig. 2) and phospholipase D activity (Fig. 3). Furthermore, a small fraction (13.2%) of the Plcg1⁻/⁻ cells showed intracellular Ca²⁺ mobilization upon
treatment with 25 ng/ml PDGF, whereas a large majority (94%) of the Plcg1−/− cells showed intracellular Ca2+ mobilization upon treatment with 1% fetal calf serum. It has been reported that cells of hematopoietic origin express PLC-γ2, a PLC isoform that is closely related to PLC-γ1 (28). Treatment of Rat 2 cells overexpressing PLC-γ2 with PDGF causes an increase in the tyrosine phosphorylation and activation of PLC-γ2 (29). Thus, we investigated whether Plcg1−/− cells expressed PLC-γ2. Western blot analysis of Plcg1+/+ and Plcg1−/− cells with a PLC-γ2 antibody showed a large expression of PLC-γ2 in Plcg1−/− cells when compared with Plcg1+/+ cells (Fig. 4A). Furthermore, upon treatment of Plcg1−/− cells with 25 ng/ml PDGF, PLC-γ2 was phosphorylated on tyrosine and co-immunoprecipitated with the PDGF receptor. In Plcg1+/+ cells treated with PDGF, there was no apparent tyrosine phosphorylation of PLC-γ2 and very little co-immunoprecipitation with the PDGF receptor (Fig. 4B). These data suggest that the Plcg1−/− cells may compensate for the disruption of Plcg1 by up-regulating PLC-γ2 and that the activation of PLC-γ2 by PDGF accounts for the small increase in inositol phosphate formation, PLD activation, and the Ca2+ mobilization seen in the Plcg1−/− cells.

**PDGF Receptor Level and Autophosphorylation**—To establish that the decrease in PDGF-induced production of InsP₃ and PtdBut in Plcg1−/− cells was not due to a decrease in the number of PDGF receptors, Western blotting of the receptors was performed. This showed no difference in the level of PDGF receptors in Plcg1−/− and Plcg1+/+ cells treated with 25 ng/ml PDGF for the indicated times. The data are represented as fold increases over basal InsP₃ production and are plotted as the means ± S.E. of three separate experiments.

**FIG. 2.** Platelet-derived growth factor-induced accumulation of InsP₃. A, total InsP₃ levels were measured in TVII-targeted Plcg1+/+ and Plcg1−/− cells labeled overnight with [3H]inositol and treated with the indicated concentrations of PDGF for 10 min as described under “Experimental Procedures.” The results were normalized to the total amount of [3H]inositol incorporated into the lipids of each cell line. The data are from a single experiment representative of two separate experiments. B, total InsP₃ were measured in TVII-targeted Plcg1+/+ and Plcg1−/− cells treated with 25 ng/ml PDGF for the indicated times. The data are represented as fold increases over basal InsP₃ production and are plotted as the means ± S.E. of three separate experiments.

**FIG. 3.** Activation of PLD by PDGF. A, TVII-targeted Plcg1+/+ and Plcg1−/− cells labeled as described in Fig. 1 were treated with the indicated concentrations of PDGF for 10 min, and the radioactivity incorporated into PtdBut was measured. The results are presented as the radioactivity incorporated into PtdBut as a percentage of the total label incorporated into phospholipids. The results are plotted as the means of two separate experiments. B, TVII-targeted Plcg1+/+ and Plcg1−/− cells were labeled as described in Fig. 1 and treated with 25 ng/ml PDGF for the times indicated. The data are from a single experiment representative of two.
Reconstitution of PDGF Induced PLD Activation in Plcg1−/− Cells—In cells treated with PDGFR, PLC-γ1 is recruited via its SH2 domain to the receptor, where it is activated (6). The immunoprecipitated proteins were separated on a 6% SDS-PAGE gel and probed with an antibody to PLC-γ2 as described under “Experimental Procedures.” The results are representative of three separate experiments.

FIG. 4. PLC-γ2 Western blot and PLC-γ2 immunoprecipitation and Western blot with P-Tyr. A, TVII-targeted Plcg1−/− and Plcg1−/+ cells were lysed in 300 μl of PBS containing 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin. Ten μg of each cell lysate was separated by SDS-PAGE on a 6% gel, and the proteins were transferred to Immobilon P and probed with an antibody to PLC-γ2 as described under “Experimental Procedures.” HL-60 cells were used as the source of the PLC-γ2 standard. The results are representative of three separate experiments. B, TVII-targeted Plcg1−/− and Plcg1−/+ cells were treated with 25 ng/ml PDGF or serum-free medium for 3 min, lysed in radioimmune precipitation buffer, and immunoprecipitated overnight with an antibody to PLC-γ2. The immunoprecipitated proteins were separated on a 6% SDS-PAGE gel and probed with an antibody to P-Tyr (4G10) as described under “Experimental Procedures.” The results are representative of two separate experiments.

Reconstitution of PDGF Induced PLD Activation in Plcg1−/− Cells—In cells treated with PDGF, PLC-γ1 is recruited via its SH2 domain to the receptor, where it is activated (6). The activated PLC-γ1 hydrolyzes PIP2, resulting in the formation of DAG and IP3. Diacylglycerol activates most isozymes of PKC, whereas IP3 promotes the release of intracellular Ca2+. It has been proposed that these two second messengers, acting alone or in combination, mediate growth factor activation of PLD (30). If this is true, then replacing these PLC-γ1 products should reconstitute PDGF-induced PLD activation in Plcg1−/− MEFs. As shown in Fig. 6, the addition of the calcium ionophore ionomycin (5 μM) plus the cell-permeable DAG analog OAG (40 μM) resulted in an equal PLD response in the Plcg1+/− and Plcg1−/− cells. The response in the Plcg1−/− cells to OAG plus ionomycin was similar to that induced by 50 ng/ml PDGF in Plcg1+/− cells. These data show that the deficient PDGF-induced PLD response in Plcg1−/− cells can be entirely restored by addition of agents that mimic the activation of PLC-γ1.

PDGF-Induced Activation of MAP Kinase—Treatment of many cell types with growth factors results in activation of the MAP kinase pathway. This activation occurs through the activation of Ras, followed by the activation of MEK kinase, MEK, and finally, MAP kinase (31). There is evidence that phospholipase C-γ1 is not involved in the growth factor-induced activation of MAP kinase (26, 32). In order to confirm that the deletion of Plcg1 by targeted gene disruption did not result in secondary changes to other PDGF signaling pathways in the MEFs derived from the targeted embryos, we assessed the activation of MAP kinase in Plcg1+/− and Plcg1−/− cells using an antibody that recognizes the phosphorylated form of MAP kinase. We showed that PDGF treatment of Plcg1+/− and Plcg1−/− cells resulted in a rapid and transient increase in MAP kinase phosphorylation that was similar between the two cells (Fig. 7). This result suggests that only pathways that require the activation of PLC-γ1 are inhibited in Plcg1−/− MEFs.

Requirement of PLD for PDGF-induced Membrane Ruffling—Two recent reports suggest that the stimulation of actin stress fiber formation by lysophosphatidic acid or α-thrombin is mediated by the activation of PLD (33, 34). Platelet-derived growth factor has been shown to induce the formation of polymerized actin at the plasma membrane in Swiss 3T3 cells, forming membrane ruffles (35). Because PLD activation by PDGF is inhibited in Plcg1−− cells, we used these cells to test whether PLD activation was necessary for PDGF-induced membrane ruffles. Fig. 8 shows that PDGF activation in Plcg1−− cells, and this is not inhibited in Plcg1−/− MEFs. These data suggest that PLD activation may not be required for the increase of polymerized actin localized in ruffles at the plasma membrane induced by PDGF.

DISCUSSION

Growth factors stimulate the PLD-induced hydrolysis of phosphatidylincholine (PC) to PA and choline in a variety of cell types (30). The exact pathway by which this occurs is not clear. A number of mechanisms of activation have been proposed involving protein tyrosine kinases, PKC, Ca2+, and GTP-binding proteins (36). In an elegant study seeking to define a role for PLC-γ1 in PDGF-induced PLD activation, Yeo et al. (22) measured PLD activity in TRMP cells (a kidney epithelial cell line) expressing wild type PDGF receptors or various tyrosine mutated PDGF receptors. They reported that PDGF had no effect on PLD activity in PDGFR kinase-deficient TRMP cells, but the PDGF-induced PLD activity was restored in cells containing a mutant PDGFR that was able to bind PLC-γ1 but not other signaling proteins. Furthermore, they showed that a mutant PDGFR that could not activate PLC-γ1 was unable to activate PLD. These data suggest that PLC-γ1 is necessary and sufficient for PDGF-induced PLD activity. However, the experiments were conducted with a cell line that normally lacked the PDGFR (37). Further evidence for a role of PLC-γ in the activation of PLD came from a study in which PLC-γ1 was overexpressed in NIH3T3 cells. Lee et al. (38) found that PDGF-induced PLD activity was directly related to the level of PLC-γ1 expressed in the cells, and that down-regulation of PKC by PMA pretreatment completely blocked PLD activation. These data again suggest PLD lies downstream of PLC-γ1 and PKC.

On the other hand, there are data that show agonist-induced PC hydrolysis or PLD activation in the absence of detectable PI-P breakdown (16, 18, 20). Cook and Wakelam (21) showed EGF stimulation of PLD activity in Swiss 3T3 cells in the absence of measurable PI-P breakdown and in the presence of a PKC inhibitor, although it was later found that EGF induced a...
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small increase in InaP_3 in these cells and that a PKC inhibitor did decrease the PLD response (12). When these reports are coupled with the frequent finding that PKC inhibitors produce only partial inhibition of the actions of growth factors and other agonists on PLD (10, 30), questions remain about the extent of the contribution of PIP_2 hydrolysis and PKC to the regulation of PLD.

The data presented strongly suggest that PLC-γ1 is required for PLD activation by PDGF. The PLD response of TVI-targeted Plec1<sup>+/+</sup> cells to EGF and PDGF is small, and the response is inhibited in TVI-targeted Plec1<sup>−/−</sup> cells (Fig. 1). Furthermore, the PDGF-induced PLD response of TVI-targeted Plec1<sup>+/+</sup> cells is robust and is greatly inhibited in TVI-targeted Plec1<sup>−/−</sup> cells (Fig. 1). A small but reproducible, increase in PLD activity with EGF and a contrasting robust PLD response to PDGF have been seen in a variety of cell types, including Rat1 and Swiss 3T3 fibroblasts (12, 39). The present study thus adds to earlier data reporting differences in the signal transduction pathways for the two growth factors (40–42).

Phospholipase C is responsible for the hydrolysis of PIP_2 to IP_3 and DAG (6). In Plec1<sup>+/+</sup> cells, PDGF elicits an increase in the production of total inositol phosphates in a dose- and time-dependent manner (Fig. 2). In Plec1<sup>−/−</sup> cells, PDGF caused a small increase in inositol phosphate production (Fig. 2). Our data suggest that the small increase in inositol phosphate production in Plec1<sup>−/−</sup> cells is due to the expression of PLC-γ2 in the Plec1<sup>−/−</sup> cells (Fig. 4A). Treatment of Plec1<sup>−/−</sup> cells with PDGF resulted in tyrosine phosphorylation of PLC-γ2 (Fig. 4B). PDGF treatment of rat-2 cells overexpressing PLC-γ2 increases the tyrosine phosphorylation and the in vivo activity of PLC-γ2 (29). Moreover, treatment of Plec1<sup>−/−</sup> cells with PDGF resulted in the mobilization of intracellular Ca<sup>2+</sup> in a small population of the cells. Over-expression of PLC-γ2 in NIH3T3 cells also enhances PDGF-induced mobilization of intracellular Ca<sup>2+</sup> (43). Thus, our data suggest that the disruption of Plec1 resulted in a compensatory up-regulation of PLC-γ2 and that this isoform, which is closely related to PLC-γ1, is responsible for the increase in InaP_3 production (Fig. 2), PLD activity (Fig. 3), and intracellular Ca<sup>2+</sup> mobilization seen in Plec1<sup>−/−</sup> cells upon treatment with PDGF.

If PLC-γ1 acts upstream of PLD in the PDGF-induced PLD-activation pathway, then PLD activity should be inhibited in Plec1<sup>+/+</sup> cells. Furthermore, there should be a correlation between PLC and PLD activities. Treatment of Plec1<sup>+/+</sup> cells with PDGF results in a dose-dependent increase in PtdBut formation that mirrors the dose-response curve for PDGF-induced InaP_3 production in Plec1<sup>−/−</sup> cells (Fig. 3A; cf. Fig. 2A).

The PDGF response in Plec1<sup>−/−</sup> cells is inhibited in parallel with the decrease in the PLC response (Fig. 3A; cf. Fig. 2A). However, the PDGF-induced PLD response reached a maximum at 15 min in the Plec1<sup>+/+</sup> or the Plec1<sup>−/−</sup> cells, at which time the production of PtdBut ceased (Fig. 3B). This is in contrast to the PDGF-induced InaP_3 production, which was still increasing at 60 min. This same pattern of phosphatidylinositol and inositol phosphate production was reported in NIH3T3 cells, which overexpress PLC-γ1 and in which phosphatidylyethanol production reached a maximum at 10 min in response to PDGF, whereas InaP_3 production was still increasing at 30 min (44). Exploration of the reasons for the cessation of PtdBut formation is outside the scope of the present study, but it is possible that activation of PLC and the consequent acti-
Fig. 8. PDGF-induced membrane ruffling. TVII-targeted Plcg1−/− and Plcg1+/− cells plated on glass coverslips were treated with 5 ng/ml PDGF for 10 min. The cells were fixed and permeabilized, and the actin was stained with BODIPY phallolidin as described under “Experimental Procedures.” Membrane ruffles were visualized with a ×40 objective using a Leica DMRB microscope. A, Plcg1+/− cells treated with vehicle for 10 min. B, Plcg1+/− cells treated with 5 ng/ml PDGF for 10 min. C, Plcg1−/− cells treated with vehicle for 10 min. D, Plcg1−/− cells treated with 5 ng/ml PDGF for 10 min.

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Phosphorylation of PLD by PKC has recently been reported to perhaps to phosphorylation of PLD or an inhibitory protein.

The inhibition of PDGF-induced PLD activity in Plcg1−/− cannot be attributed to a decreased level of PDGF receptors in the Plcg1−/− cells (data not shown) or to a defect in PDGF-induced autophosphorylation (Fig. 5). In fact, the autophosphorylation of the receptor occurred more rapidly in the Plcg1−/− cells as compared with the Plcg1+/− cells, reaching a maximum level at 5 min and decreasing toward basal level by 60 min, but this difference in autophosphorylation cannot account for the decreased PDGF-induced PLD activity seen in the Plcg1−/− cells. Another possible explanation is that PKC is deficient in the Plcg1−/− cells. However, this does not seem to be the case because treatment of Plcg1+/− and Plcg1−/− cells with phorbol ester, an activator of PKC, results in a similar dose-dependent activation of PLD (data not shown).

In cells treated with growth factors, activated PLC-γ hydrolyzes PIP₂ to form IP₃ and DAG, resulting in an increase in intracellular Ca²⁺ and the activation of PKC. Treatment of Plcg1−/− cells with the Ca²⁺ ionophore ionomycin and the cell-permeable DAG analogue OAG resulted in a PLD response that was similar to that in Plcg1+/− cells and slightly greater than the PLD response induced by PDGF (Fig. 6). Thus, the addition of PLC-γ activation products to the Plcg1−/− cells reconstituted the PDGF-induced PLD response in the Plcg1−/− cells to the level seen in the wild type cells. These data suggest that the PLC-γ activation products are sufficient to completely restore the PDGF-induced PLD response lost in the Plcg1−/− cells on disruption of PLC-γ in these cells. Furthermore, these results and those with phorbol ester prove that PLD is not deficient in the Plcg1−/− cells.

Growth factor treatment of cells results in a mitogenic response that is mediated by the MAP kinase. The growth factor-induced activation of MAP kinase involves the sequential activation of Ras, MEK kinase, and MEK (31). In a 3T3 cell line derivative, NR6 cells, EGF-stimulated MAP kinase activity was not affected by the inhibition of PLC with U73122, and data from Ji et al. (26) showed that EGF-induced activation of MAP kinase in the TVI-targeted Plcg1+/− and Plcg1−/− cells was similar. Thus, these findings indicate that PLC-γ is not involved in the phosphorylation and activation of MAP kinase by growth factors. We also observed PDGF-induced phosphorylation of MAP kinase in Plcg1+/− and Plcg1−/− cells (Fig. 7), indicating that the targeted gene disruption of PLC-γ did not result in global changes to PDGF-signaling pathways.

Data from two recent reports suggest a role for PA in the polymerization of actin stress fibers (33, 34). Actin stress fibers are a major component of the cytoskeleton in fibroblasts, where actin filaments can exist in three types of structures, including actin stress fibers, the cortical actin network, and cell surface protrusions, such as membrane ruffles and filopodia (35). Ha and Exton (34) reported that treatment of IEC9 fibroblasts with thrombin, PLD from Streptomyces chromofuscus, or exogenous PA resulted in actin stress fiber formation. In porcine aortic endothelial cells, lysophosphatidic acid treatment activated PLD, resulting in the formation of PA, in the apparent absence of the formation of other lipid second messengers (33). Lyso phosphatidic acid, like exogenously added PA, also stimulated the formation of actin stress fibers (33). Although these observations generally support a role for PLD in stress fiber formation, it is possible that signals evoked by the exogenous PLD and PA are different from those elicited by activation of endogenous PLD (46). For example, they could generate lysophosphatidic acid, which could induce actin polymerization by a different mechanism.

In Swiss 3T3 cells, PDGF has been shown to induce the formation of membrane ruffles (35), and we therefore utilized the Plcg1−/− cells to examine the role of PLD in this effect. In Plcg1−/− cells, 5 ng/ml PDGF induced membrane ruffles similar to those induced in Plcg1+/− cells (Fig. 8), even though activation of PLD was significantly inhibited (Fig. 2B). Thus, it appears that PLD and PLC activity may not be necessary for the PDGF-induced formation of membrane ruffles. It seems unlikely that the small level of PLC-γ2, inositol phosphate formation, and Ca²⁺ mobilization would be sufficient to provoke maximal ruffling response.

In summary, the present data suggest that in mouse embryo fibroblasts, PLC-γ1 activation is necessary for the PDGF-induced activation of PLD. However, caution should be exercised in extrapolating the findings to other agonists or cell types. We are currently investigating a role for PLC-γ1 in the activation of PLD by various other agonists, including those that activate heterotrimeric G-proteins.

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