Involvement of Phosphatidylcholine-specific Phospholipase C in Platelet-derived Growth Factor-induced Activation of the Mitogen-activated Protein Kinase Pathway in Rat-1 Fibroblasts*

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Marc C. M. van Dijk, Francisco J. G. Muriana†, John de Widt, Henk Hilkmann, and Wim J. van Blitterswijk§
From the Division of Cellular Biochemistry, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands

The role of phosphatidylcholine (PC) hydrolysis in activation of the mitogen-activated protein kinase (MAPK) pathway by platelet-derived growth factor (PDGF) was studied in Rat-1 fibroblasts. PDGF induced the transient formation of phosphatidic acid, choline, diacylglycerol (DG), and phosphocholine, the respective products of phospholipase D (PLD) and phospholipase C (PC-PLC) activity, with peak levels at 5–10 min. PLD-catalyzed transphosphatidylation (with n-butyl alcohol) diminished DG formation at 5 min but not at later stages of PDGF stimulation. Phorbol ester-induced down-regulation of protein kinase C (PKC) completely blocked PLD activation but not the formation of DG and phosphocholine at 10 min of PDGF stimulation. Collectively, these data indicate that PDGF activates both PLD and PC-PLC. In contrast, epidermal growth factor did not activate PC-PLC in these cells, and it activated PLD only weakly. DG formation by itself, through Bacillus cereus PC-PLC treatment of cells, was sufficient to mimic PDGF in activation of MAPK independent of phorbol ester-sensitive PKC. Since PKC down-regulation blocked PDGF-induced PLD but not MAPK activation, we conclude that PLD is not involved in MAPK signaling. In contrast, MAPK activation by exogenous (bacterial) PLD was not affected by PKC down-regulation, indicating that signals evoked by exogenous PLD differ from endogenous PLD. D609 (2–10 μg/ml), an inhibitor of PC-PLC, blocked PDGF- but not epidermal growth factor-induced MAPK activation. However, D609 should be used with caution since it also affects PLD activity. The results suggest that PC-PLC rather than PLD plays a critical role in the PDGF-activated MAPK pathway.

Activation of receptor tyrosine kinases leads to receptor autophosphorylation and subsequent recruitment and activation of many signaling proteins that interact in cascades of enzymatic reactions (for review, see Refs. 1–3). A major signaling pathway through Ras, Raf-1, and mitogen-activated protein kinase (MAPK)† may lead to DNA synthesis in fibroblasts (2, 3). Most mitogens such as platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) acting through such type of receptors also elicit phospholipid breakdown, particularly of phosphoinositides and phosphatidylcholine (PC) (for review, see Refs. 4–6). Hydrolysis of PC, initiated through as yet unknown mechanisms, may occur by phospholipase C (PC-PLC) and/or phospholipase D (PLD) activity, resulting in (presumed) second messengers diacylglycerol (DG) and phosphatidic acid (PA), respectively. It has been suggested that PDGF-induced DNA synthesis in Swiss 3T3 fibroblasts requires long-term PC-PLC activation (7) and that in NIH 3T3 cells, PC-PLC mediates EGF- and serum-induced Raf-1 activation in minutes (8). However, besides delivering DG for translocation and activation of “new” protein kinase C (PKC) isoforms (9, 10), no essential function of these phospholipase activities in the mitogenic process has as yet been firmly established.

We recently showed that MAPK activation by PDGF in Rat-1 fibroblasts could be mimicked to some extent by bacterial PC-PLC (11). Contrary to PDGF treatment, however, MAPK activation by the exogenous PC-PLC was only transient and, at variance with the above-mentioned study in Swiss 3T3 cells (7), did not result in DNA synthesis (11).

In this paper, we have investigated receptor-stimulated PC hydrolysis in Rat-1 fibroblasts. We demonstrate that PDGF transiently activates both PLD and PC-PLC and present evidence that the latter phospholipase activity is necessary for PDGF-induced MAPK activation. This evidence is based on the use of tricyclodecan-9-yl-xanthogenate (compound D609), an inhibitor of PC-PLC (12, 13).

EXPERIMENTAL PROCEDURES

Materials and Cells—[14C]Choline and [3H]myristic acid were obtained from DuPont NEN. 32P, and the enhanced chemiluminescence (ECL) system were from Amersham. EGF was from Collaborative Research. Human recombinant PDGF-B/B and PC-specific PLC (from Bacillus cereus) were from Boehringer Mannheim. Phospholipase D (type VI; from Streptomyces chromofuscus) and lipid standards were obtained from Sigma. 12-Tetracosanoylphorbol-13-acetate and Phorbol-12,13-dibutyrate (PDBu) were obtained from LC Services Corp., Woburn, MA. Ro 31-8220 (compound 3 from Ref. 14) was kindly provided by Roche Research Center (Welwyn Garden City; U. K.). D609, an inhibitor of PC-PLC, was kindly provided by Dr. G. Quack of Merz & Co., Germany. Dulbecco’s modified Eagle’s Medium (DMEM) was obtained from Life Technologies, Inc. Modified Eagle’s Medium (MEM) was obtained from Life Technologies, Inc. Minimal essential medium (without phosphate) was obtained from Flow. All chemicals were of analytical grade. Rat-1 fibroblasts were routinely grown in DMEM containing 7.5% fetal calf serum (from Life Technologies, Inc.) and serum starved for 48 h prior to stimulation.

Antibodies—Polyclonal anti-p42 MAPK (ERK2) and anti-PKC anti-
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**RESULTS**

**PC Hydrolysis Induced by PDGF and EGF**—To examine the effect of growth factor addition to quiescent Rat-1 fibroblasts on PC hydrolysis, cells were labeled with $[^{14}C]$cholesterol and $[^{3}H]$myristate under conditions where the label is predominantly incorporated in PC (20, 21). Fig. 1, A and B, shows that PDGF induces the formation of the radiolabeled PC breakdown products, DG, PA, phosphocholine, and choline almost simultaneously, due to PA phosphohydrolase activity by the following two approaches. First, in the presence of 0.2% n-butyl alcohol (transphosphatidylation; Figs. 2 and 5). PLD-catalyzed formation of phosphatidylbutanol (PBut) in the presence of 0.2% n-butyl alcohol was analyzed using the last mentioned thin layer chromatography system, as described (19, 20). Water-soluble choline metabolites were recovered as described (20) and analyzed by thin layer chromatography with the solvent system 0.9% NaCl (in H$_2$O)/methanol/ammonia (10:10:1, v/v/v) in two runs with intermittent drying (20). Commercial standards (from Sigma), choline, and phosphocholine were co-chromatographed and visualized in iodine vapor. Spots were scraped off, and radioactivity was determined by liquid scintillation counting.

**MAP Kinase Activation**—Stimulated cells were washed with cold phosphate-buffered saline and lysed in hot sodium dodecyl sulfate-sample buffer. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% acrylamide, 0.4% bisacrylamide) and transferred to nitrocellulose membranes. These were blocked with 5% milk powder in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20), incubated with anti-p42 MAPK (ERK2) antibodies, washed with TBST, and subsequently incubated with peroxidase-conjugated second antibody. Immunostained antibodies were visualized by enhanced chemiluminescence. A reduced electrophoretic mobility (gel shift) is indicative for MAPK phosphorylation and activation (15).

This composite thin layer chromatography system allows separation of triacylglycerol, 1,3-diacylglycerol (1,3-DG), 1,2-DG, free fatty acids, monoacylglycerol, PA and the remainder of the phospholipids, including PC (in decreasing order of R$_f$). PLD-catalyzed formation of phosphatidylcholine (PC) was from Life Technologies, Inc. The antisera against protein kinase C isotypes -a, -b, -g (17), and -e (18) were kindly provided by Dr. P. Parker (London). Anti-PKC-δ was from Life Technologies, Inc. The specificity of these antibodies was verified by immunoblotting of the individual PKC isotypes overexpressed by transfection in COS cells.

**Determination of Lipid Metabolites**—Rat-1 cells were seeded in 5-cm glass dishes and grown until 80% confluence, serum starved for 48 h, and radiolabeled with 1.0 μCi of $[^{14}C]$cholesterol for 48 h and with 2.0 μCi of $[^{3}H]$myristic acid for 3 h. The $[^{3}H]$label incorporated mainly (85%) in the PC fraction of the phospholipids. Prior to stimulation, cells were washed twice with DMEM and incubated for 30 min at 37 °C. Cells were stimulated, followed by washing off the medium, lipid extraction with chloroform:methanol (2:1, v/v), and phase separation (19). Lipids were separated by thin layer chromatography (Silica Gel 60 plates; Merck) in one dimension, in four runs with intermittent drying. First were two runs in hexane:diethyl ether:methanol (4:1:1; v/v) for the full-length of the plate. Then, the plate was sprayed with primuline to visualize lipid markers. The third and the fourth run were performed with the upper phase of ethyl acetate:isooctane:acetic acid:water (15:2.3:10; v/v) up to the position of the free fatty acids and monoacylglycerol, respectively.

**FIG. 1.** PC hydrolysis induced by PDGF and EGF in Rat-1 fibroblasts. Cells were labeled for 48 h with $[^{14}C]$cholesterol and for 3 h with $[^{3}H]$myristic acid. Cells were stimulated in DMEM with (solid symbols) or without (open symbols) PDGF (25 ng/ml) (panels A and B) or EGF (50 ng/ml) (panel C) for the indicated time periods. Lipids were extracted, and levels of radioactive DG (circles) and PA (triangles) (panels A and C) and phosphocholine (squares) and choline (inverted triangles) (panel B) were determined in triplicate. Values are means ± S.D. Similar results were obtained in three separate experiments.

**FIG. 2.** Effect of n-butyl alcohol on PDGF-induced formation of DG and PBut from PC. Cells were labeled with $[^{3}H]$myristic acid for 3 h, then preincubated with (dashed lines) or without (circles, solid lines) n-butyl alcohol (0.2%) for 5 min and stimulated with PDGF (25 ng/ml) (solid symbols) or control DMEM (open symbols) for the times indicated. Lipids were extracted, and levels of radioactive DG (circles and triangles) and PBut (squares) were determined in triplicate. Values are means ± S.D. Similar results were obtained in three separate experiments.
were obtained in three separate experiments. To mimic receptor-mediated PC hydrolysis, cells were stimulated for 10 min with PDGF (25 ng/ml) or DMEM medium (Control), as indicated. Lipids were extracted, and levels of radioactive PA, DG, choline, and phosphocholine (PCholine) (indicated) were determined in triplicate. Values are means \( \pm \) S.D. Similar results were obtained in three separate experiments.

To investigate whether PKC would mediate this formation of these lipids by themselves can cause MAPK activation, we down-regulated PKC by prolonged exposure to PDBu. PKC-\( \alpha \) activation, we down-regulated PKC by prolonged exposure to PDBu for 24 h (middle panel) or with 10 \( \mu \)M Ro 31-8220 for 10 min (lower panel; Control). Cells were stimulated for 10 min with EGF (50 ng/ml), PDGF (25 ng/ml), B. cereus PC-PLC (0.5 unit/ml), or S. chromofuscus PLD (5 units/ml). Total cell lysates were immunoblotted with anti-MAPK antibodies. The electrophoretic mobility shift of the p42 and p44 isoforms of MAPK, shown here, is indicative of their phosphorylation and activation (16). Similar results were obtained in four separate experiments.

**Role of PKC—**

As previously presented evidence (11) that atypical PKC-\( \zeta \) mediates MAPK activation by PDGF and PC-PLC. We note here the similarity of these stimuli in inducing MAPK activation with respect to the PKC sensitivity of this process. We also note that DG formation is no longer decreased by n-butyl alcohol, supporting the notion that at least part of the DG formation is due to a PC-PLC. In the second approach, we down-regulated PKC by prolonged PDBu treatment of cells. This completely blocked PDGF-induced PLD activation, as demonstrated by the lack of PA and choline formation and the absence of PBut formation in the presence of \( n \)-butyl alcohol (Figs. 3 and 5). Yet, under these conditions, PDGF is still capable of inducing DG and phosphocholine formation (Fig. 3).

Taken together, these results indicate that PDGF activates both PLD and PC-PLC in Rat-1 cells. EGF, however, induces only modest activation of PLD but no PC-PLC.

**Exogenous PC-PLC Mimics PDGF in Activation of MAPK; Role of PKC—**

To mimic receptor-mediated PC hydrolysis, cells were treated with B. cereus PC-PLC and S. chromofuscus PLD. We verified that the respective PC breakdown products, DG and PA, are properly generated (for PC-PLC treatment, see Ref. 11; data not shown for PLD treatment). Fig. 4 shows that the formation of these lipids by themselves can cause MAPK activation. To investigate whether PKC would mediate this activation, we down-regulated PKC by prolonged exposure to PDBu. PKC-\( \alpha \), -\( \beta \), and -\( \epsilon \), the only conventional and new PKC isoforms expressed in these cells, respectively, were completely down-regulated in this way (11). Fig. 4 shows that such PKC down-regulation does not affect MAPK activation by the bacterial phospholipases, nor by EGF and PDGF, indicating that phorbol ester-sensitive PKCs are not involved in MAPK activation. On the other hand, Ro 31-8220, a highly selective inhibitor of all PKC isoforms (14, 22), including the atypical ones (11, 23), inhibits MAPK activation by PDGF and exogenous PC-PLC but not by EGF or exogenous PLD (Fig. 4). We have previously presented evidence (11) that atypical PKC-\( \zeta \) mediates MAPK activation by PDGF and PC-PLC. We note here the similarity of these stimuli in inducing MAPK activation with respect to the PKC sensitivity of this process. We also note that DG formation by itself, through PDGF stimulation and PC-PLC activation, apparently is sufficient to activate the MAPK pathway. As described previously (11), however, PDGF and bacterial PC-PLC differ with respect to duration of MAPK activation, lasting 4 h and 45 min, respectively. Bacterial PC-PLC, in contrast to PDGF, does not elicit a mitogenic response in Rat-1 cells, which likely relates to the relatively short MAPK activation by this stimulus (11, 24). PC-PLC mimics PDGF only in early MAPK activation (10 min), where we focus in the present paper.

**Activation of PLD Is Dispensable for MAPK Activation—**

Fig. 3 has already shown that down-regulation of PKC by prolonged PDBu treatment blocks PDGF-induced (endogenous) PLD activation. This is confirmed, also for EGF as an agonist, in a different type of experiment where PLD activity is measured by its transphosphatidylation product PBut, in the presence of \( n \)-butyl alcohol (Fig. 5). Since PKC down-regulation does not affect PDGF- or EGF-induced MAPK activation (Fig. 4), we conclude that PLD is not involved in agonist-induced MAPK activation, at least in this Rat-1 cell system.

In contrast, depletion of PKC activity does not affect bacterial PLD, either in its capability to generate FA or the transphosphatidylation product PBut (Fig. 5), or to activate MAPK (Fig. 4). We therefore also conclude that the exogenous bacterial PLD activates a signaling route different from that activated by endogenous PLD. This conclusion is corroborated further by our finding that MAPK activation by exogenous PLD and PDGF (activates endogenous PLD) shows a different sensitivity toward Ro 31-8220 (Fig. 4).

**PDGF Activation of MAP Kinase Depends on PC-PLC Activity—**

Unlike PLD or EGF, activation of endogenous PC-PLC by PDGF fully correlates with MAPK activation under all conditions tested. The question now is whether PC-PLC activation is necessary for PDGF-induced MAPK activation. Since PC-PLC has not been cloned yet, so that no dominant-negative derivatives are available, we utilized D609, a presumed selective inhibitor of the enzyme (8, 12, 13, 25; see, however, Ref. 26 and below). We confirmed that D609 (15–50 \( \mu \)g/ml) inhibited B. cereus PC-PLC but not S. chromofuscus PLD (results not
shown). Fig. 6A shows that D609, at the commonly used dose of 50 μg/ml (12, 13, 25, 26), does not affect electrophoretic mobility of MAPK in control cells and in EGF-stimulated cells but blocks MAPK activation in PC-PLC-treated cells. Since in other experiments (not shown) this high concentration of D609 by itself tended to induce a MAPK electrophoretic mobility shift, we reduced the D609 concentration in subsequent experiments on PDGF-activated MAPK to the lowest effective dose.

Fig. 6B shows that D609 inhibits PDGF-induced MAPK activation in a dose-dependent fashion. Significant inhibition is already achieved at 2 μg/ml. At the 2–50 μg of D609/ml concentration range tested, EGF-activated MAPK remained unaffected (Fig. 6, A and B). Taking some control experiments into account (see below), these results suggest that PDGF but not EGF activation of MAPK requires a PC-PLC activation step.

Effect of D609 on Lipid Metabolism—Fig. 7 (upper panel) shows that D609 dose dependently inhibits PDGF-induced DG formation, whereas DG levels in control or EGF-stimulated cells remain unaffected. The lowest effective concentration of D609, 2–5 μg/ml, correlates with that inhibiting PDGF-activated MAPK but is at least a factor 10 lower than used by others (13, 25). Concentrations of D609 higher than 15 μg/ml often caused adverse effects in Rat-1 cells, i.e. raised DG levels by an unknown mechanism (data not shown).

Side effects of D609 were investigated. At a concentration of 15 μg/ml, i.e. 3–5-fold higher than necessary to block PC-PLC, D609 also inhibited PDGF-activated PLD, as measured by PA and choline generation (Fig. 7, lower panels). However, D609 by itself tended to increase cellular levels of radiolabeled PA and choline independently of receptor stimulation (Fig. 7). These “nonspecific” effects are partly due to D609-induced PLD activation, since in the presence of n-butyl alcohol, we found a small amount of D609-induced [3H]PBut formation (PLD transphosphatidylation). At the concentrations of 2, 5, and 15 μg/ml D609 used, it represented respectively 1, 11, and 23% of the [3H]PBut generated by PDGF (not shown in figure). D609 did not affect PDGF-induced receptor autophosphorylation (data not shown).

We conclude that D609 inhibits endogenous PC-PLC and MAPK activation by PDGF. However, D609 may also affect other lipid interconversions, particularly those mediated by PLD. D609 seems, therefore, not a very reliable “specific” inhibitor of PC-PLC in general. Yet, D609 at the low concentrations used does by itself not affect the electrophoretic mobility of MAPK and, in fact, does not affect EGF-induced MAPK activation at all (Fig. 6). Since PLD activation is irrelevant for the MAPK pathway in Rat-1 cells (as reasoned above), it seems fair to conclude that D609 inhibits PDGF activation of the MAPK pathway at the level of PC-PLC.

DISCUSSION

In this paper we have shown that PDGF activates PLD and PC-PLC in a time-dependent fashion. Initial DG formation is caused by sequential PLD/PA phosphohydrolase activity. At later time points, DG results mainly from PC-PLC activity. We conclude this from three sets of experimental data. First, PDGF concomitantly induces formation of PA and choline (products of PLD) and, on the other hand, DG and phosphocholine (products of PC-PLC), slightly lagging behind. Second, n-butyl alcohol, which preferentially induces PLD transphosphatidylation (PBut formation) at the cost of formation of PA and its subsequent phosphohydrolase product DG, indeed decreases initial DG formation, indicative of PLD activity. No n-butyl alcohol effect is seen at later stages of PDGF stimulation, indicative of PLD-independent DG formation (by PC-PLC). Third, down-regulation of PKC blocks PDGF-induced PLD activation (formation of PA, choline or, in the presence of n-butyl alcohol, PBut) but leaves formation of the PC-PLC products, DG and phosphocholine, for a significant part unaffected (at 10 min).

Having concluded that both PLD and PC-PLC are activated by PDGF, we mimicked these endogenous activities by treating the cells with the corresponding bacterial phospholipases, and found that, like PDGF, both exogenous PLD and PC-PLC were capable of activating the MAPK pathway. We then modulated activation of the endogenous PLD and PC-PLC and looked for the effect on MAPK activity. PDGF-induced PLD activity could be blocked by PKC down-regulation, whereas PC-PLC activity was blocked by D609 in a dose-dependent fashion. In this way, it appears that endogenous PC-PLC plays an essential role in PDGF activation of MAPK but that PLD does not. The latter conclusion is in agreement with our previous finding that 12-tetradecanoylphorbol-13-acetate-activated PLD (via PKC) does not lead to MAPK activation in these cells (11). Our finding that PLD plays no role in agonist-stimulated MAPK activation in Rat-1 cells does exclude that in other cell systems and/or with other agonists, PLD might be more relevant.
PLD activation by stimulation of receptor tyrosine kinases seems to be a ubiquitous phenomenon (4, 5, 9, 25, 27–30). In most cases, including the present study, this activation is PKC-dependent (25, 27–29). In contrast, we have shown that hydrolysis of cell surface PC by bacterial PLD does not require PKC, nor does the subsequent activation of MAPK. We therefore conclude that this exogenous PLD has a different mode of action on cells than (agonist-activated) endogenous PLD. This conclusion is supported further by our observation that MAPK activation by exogenous PLD and PDGF (activates endogenous PLD) shows a completely different sensitivity toward Ro 31-8220 (Fig. 4).

Difficulties in assessing PC-PLC activity in cells have been mentioned comprehensively by Dinh and Kennerly (31) and described comprehensively by van Blitterswijk and Hilkmann (1993) (9, 27, 28). In contrast, we have shown that hydrolysis of cell surface PC by bacterial PLD does not require PKC, nor does the subsequent activation of MAPK. We therefore conclude that this exogenous PLD has a different mode of action on cells than (agonist-activated) endogenous PLD. This conclusion is supported further by our observation that MAPK activation by exogenous PLD and PDGF (activates endogenous PLD) shows a completely different sensitivity toward Ro 31-8220 (Fig. 4).

Difficulties in assessing PC-PLC activity in cells have been described comprehensively by Dinh and Kennerly (31) and Cook and Wakelam (4). In addition, it should be noted that growth factors not only elicit hydrolysis of PC, producing DG, but also PC (re)synthesis, which consumes DG, resulting in a "PC cycle" (32, 33). Thus, the effect of PDGF receptor triggering is to accelerate PC turnover and the flux of DG rather than to enhance DG levels to large extents. This makes reproducible detection and quantitation of receptor-induced DG sometimes difficult and dependent on subtle conditions of the cells. This may also be a reason why in different studies (7, 9, 27, 30, 34, 35), including the present one, the same growth factor (PDGF) on similar cells (fibroblasts) yields variable results with regard to extent and time dependence of the increase of DG and/or phosphocholine levels. It is therefore not surprising that for assessing the possible role of PC-PLC in a signaling pathway, investigators prefer to use an inhibitor, such as D609, provided that its action is specific. Unfortunately, we have to conclude from our data and those of Kiss and Tomono (26) that D609 cannot be considered a specific inhibitor of PC-PLC since it also affects other lipid conversions including the activity of PLD. However, since we found that the PDGF activation of the MAPK signaling route in Rat-1 does not require PLD activity, the D609 data still allow us to conclude that the MAPK pathway requires a PC-PLC activation step.

In conclusion, we have shown that DG generated by PC-PLC plays an essential role in PDGF-, but not EGFR-induced MAPK activation. It does so independently of phorbol ester-sensitive PKC but, instead, via PKC-dependent (25, 27–29). In contrast, we have shown that hydrolysis of cell surface PC by bacterial PLD does not require PKC, nor does the subsequent activation of MAPK. We therefore conclude that this exogenous PLD has a different mode of action on cells than (agonist-activated) endogenous PLD. This conclusion is supported further by our observation that MAPK activation by exogenous PLD and PDGF (activates endogenous PLD) shows a completely different sensitivity toward Ro 31-8220 (Fig. 4).

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**FIG. 7.** D609 inhibits PDGF-activated PC-PLC but also affects PLD. Rat-1 cells were labeled with [3H]choline for 48 h and with [3H]myristate for 3 h and preincubated without or with the indicated concentrations of D609 for 1 h at pH 6.8. Cells were stimulated for 10 min with PDGF (25 ng/ml), EGF (50 ng/ml), or DMEM (control). Levels of [3H]DG (upper panel), [3H]PA (middle panel), and [14C]choline (lower panel) were determined. Data are means of triplicates ± S.D., representative of three separate experiments.
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