Phosphatidic Acid That Accumulates in Platelet-derived Growth Factor-stimulated Balb/c 3T3 Cells Is a Potential Mitogenic Signal*

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We developed a monoclonal antibody specific to phosphatidic acid (PA). Using this antibody, a novel method to quantify trace amounts of PA was achieved. With the method, PA can be measured in the range of 20–500 pmol. We applied this method to quantify changes in PA levels in Balb/c 3T3 cells stimulated by platelet-derived growth factor. PA contents were very low in quiescent cells and dramatically increased with time up to 15 min. On the other hand, a biphasic diacylglycerol (DG) increase was found. The early phase showed a transient small peak of DG at 30 s followed by a decrease to 1 min. In the second phase, DG accumulated gradually but very markedly up to 15 min. Treatment with propranolol, a PA phosphohydrolase inhibitor, enhanced the accumulation of PA and inhibited the formation of DG in the second phase. However, R59022, a DG kinase inhibitor, did not influence the accumulation of DG or PA, suggesting that platelet-derived growth factor stimulates mainly phospholipase D-catalyzed hydrolysis of phospholipids rather than phospholipase C-catalyzed hydrolysis in the second phase. PA, even after contaminating lyso-PA was removed, could stimulate DNA synthesis, although lyso-PA was 25 times more potent. Moreover, phospholipase D was found to be a much stronger mitogen than phospholipase C. Phospholipase D treatment caused a biphasic accumulation of PA. PA levels reached a maximum at 1 h, and then decreased between 1 and 2 h; finally, there was a gradual elevation up to 10 h. In this case, there was no significant DG accumulation. On the other hand, phospholipase C treatment induced only DG accumulation without any significant change in PA. These results indicate that PA accumulation, rather than an increase in DG, correlates well with mitogenesis.

Many mitogens produce their signals by stimulating the hydrolysis of cellular phospholipids. A stimulation of the breakdown of phosphatidylinositol 4,5-bisphosphate (PIP₂) to two second messengers, inositol 1,4,5-trisphosphate (IP₃) and DG, has been demonstrated to occur in response to growth factors (reviewed in Ref. 1). IP₃ mobilizes Ca²⁺ from intracellular stores (2), and DG activates protein kinase C (3), a kinase shown to be a key enzyme in mitogenic signaling (4, 5). Therefore, DG is considered to be an important second messenger regulating cell growth and transformation. More recently, it has been found that many agonists stimulate the hydrolysis of phosphatidylincholine (PC) in addition to PIP₂ hydrolysis (reviewed in Ref. 6). Moreover, the hydrolysis of PC rather than PIP₂ has been shown to be the major source of DG formed in stimulated cells (7) (reviewed in Ref. 8). In this case, hydrolysis of PC by either or both phospholipase C and D could lead to DG production, the latter through PA. PA also accumulates to a level comparable with that of DG in response to growth factors (9–12). This lipid is not only a crucial intermediate in de novo lipid synthesis, it also appears to play an important role in cell proliferation (13, 14). PA has also been shown to evoke various cellular responses such as the promotion of Ca²⁺ entry into cells and the mobilization of intracellular Ca²⁺ (15–17), and also inhibits adenylate cyclase, apparently through interaction with Gi (18). Recently, PA has been found to increase the activity of the ras GTPase-inhibiting protein (19) and to inhibit the activity of ras GTPase-activating protein (GAP) (20). These results suggest that PA may link growth factor receptor signaling to a cellular Ras that is critical in the control of proliferation (14).

We have been developing antibodies to phospholipids related to signal transduction, such as PIP₂ and PIP₃, in order to evaluate the role of these lipids (21, 22).

We report here a very sensitive method based on specific anti-PA antibodies to quantify levels of PA and its variants upon mitogenic stimulation. Our results point out that PA accumulation, rather than DG increase, correlates well with mitogenesis.

**EXPERIMENTAL PROCEDURES**

Materials—PDGF was purchased from Takara (Tokyo, Japan). Various kinds of lipids used were: PA (egg), dipalmitoyl PA, diolein PA, dioleyl PA, tetraoleoyl bis-PA, cardiolipin, phosphatidylinositol (PI, soybean), 1,2-diolein, and lyso-PA (prepared from egg lecithin) (Sedary Research Laboratory Inc., Ontario, Canada); 1-palmitoyl-2-oleoyl PA, dilinoleoyl PA, dinyristoyl PA, distearoyl PA, dioleyl phosphatidylglycerol, and 1,α₁-oleoyl-2-acetoyl-sn-3-glycero (Avanti Polar Lipid Inc.); 1-stearoyl-2-arachidonoyl PA, phospholipase C (Bacillus cereus, type XIII), and phospholipase D (Streptomyces chromofuscus, type VI) were from Sigma. PIP₂ and PIP₂ were prepared from spinal cords by the methods of Schacht (23). Propranolol was from Wako Pure Chemical Ind. (Osaka, Japan). R59022 was from Boehringer Mannheim, Japan. IP₃ and DG assay kits were from Amersham Japan. Lipid A was from List Biological Laboratories, Inc. (Campbell, CA). Peroxidase-conjugated IgG (goat) anti-mouse immunoglobulin was from List Biological Laboratories, Inc.
Reactivities of anti-PA antibody KA-1 with various PA species on ELISA

Various PA species (0.5 μg) were coated on ELISA plates as antigens. Reactivities are shown as absorbance at A_{450}.

| Species               | A_{450} |
|-----------------------|---------|
| Dimyristoyl PA        | 0.149   |
| Dipalmitoyl PA        | 0.083   |
| Distearoyl PA         | 0.048   |
| Didecanoyl PA         | 0.038   |
| Dioleoyl PA           | 0.780   |
| Dilinoleoyl PA        | 1.003   |
| 1-Stearyl-2-arachidonoyl PA | 0.842 |
| 1-Palmitoyl-2-oleoyl PA | 0.801 |
| Egg PA                | 0.881   |

**FIG. 1.** Specificity of anti-PA antibody, KA-1. The specificity of the antibody was examined by ELISA (A) and TLC immunostaining (B and C). A, microtiter wells were coated with 0.5 μg of PA (egg, ●), cardiolipin (▲), tetraoleoyl bis-PA (■), 1,2-diolein (○), lyso-PA (△), and PI (□), and then reacted with KA-1. B, TLC plates loaded with rat brain total lipid (30 μg of phosphorus) (left) or 10 μg of authentic egg PA (right) were developed with chloroform/methanol/ammonia/water (90/65/12/8, v/v) in the first dimension and n-butyl alcohol/acetic acid/water (95/15/15, v/v) in the second dimension and immunostained with KA-1 (left) or visualized with 3% CuSO_{4} solution (right). C, 1 μg of various PA species were spotted on TLC-polgram, developed with chloroform/methanol/water (60/25/4, v/v), and immunostained with KA-1. Lane 1, dimyristoyl PA; 2, dipalmitoyl PA; 3, distearoyl PA; 4, didecanoyl PA; 5, dioleoyl PA; 6, dilinoleoyl PA; 7, 1-oleoyl-2-arachidonoyl PA; 8, 1-palmitoyl-2-oleoyl PA; 9, egg PA.

**FIG. 2.** Measurement of PA content by immunostaining. Various concentrations of PA were spotted on TLC plates. The plates were developed in chloroform/methanol/water (60/25/4, v/v) and reacted with the antibody, KA-1. The density of the immunostaining was measured by a Scanning Imager by Molecular Dynamics. Data are shown as mean ± S.D. in three separate experiments. IOD, integrated optical density.

calf serum. The cells were plated at 6 × 10^5 cells/9-cm dish. Two days after plating, subconfluent cells were serum-starved by washing 2 times with DMEM containing 5 μg/ml transferrin and 100 μg/ml bovine serum albumin and then incubated for 24 h at 37°C in the same medium.

**Production of Anti-PA Monoclonal Antibody**—Anti-PA antibody was made by a method similar to that described before (21, 22). Briefly, BALB/c mice were immunized with liposomes containing dimyristoyl phosphatidylcholine, cholesterol, PA (egg, 0.5 mg), and lipid A (molar ratio, 1:1.5:1:0.08) every 2 weeks for 9 months. The immune spleens were fused with P3-X63-Ag8 cells using polyethylene glycol (M, 3,350). Two weeks after cell hybridization, hybridoma supernatants were evaluated by ELISA. The hybridoma cells secreting anti-PA antibody were doubly cloned by limiting dilution. The cells were inoculated into pristane-primed mice, and the resulting ascites fluid was used to measure PA content. The antibody was found to be IgG2b by subclass analysis. The specificity of the antibody was evaluated by ELISA and TLC immunostaining as described before (21, 22).

**Mass Analysis of PA**—Cells treated with growth factors, phospholipase C or D, were incubated at 37°C for the indicated time. The incubations were terminated by aspirating the medium, washing with phosphate-buffered saline (PBS), and immediately adding 1.5 ml of ice-cold methanol. The cells were scraped and transferred to tube and the dishes were further washed with 1.5 ml of chloroform/methanol (1/2, v/v). Then chloroform (1.5 ml) was added to the tube, and the lipids were extracted with 1 N HCl as described before (22). The lipids were spotted on thin layer chromatography (TLC) plates (Polygram, Macherey-Nagel) and developed in chloroform/methanol/water (60/25/4, v/v). The plates were soaked overnight in PBS containing 3% bovine serum albumin and 1% polyvinylpyrrolidone 25, and then treated for 2 h at room temperature with anti-PA antibody ascites diluted 500-fold with PBS. The plates were washed 4-5 times with PBS containing 0.05% Tween 20, reacted with peroxidase-conjugated anti-mouse immunoglobulins, and stained with a Konica staining kit (Konica, Tokyo). The content of PA was measured by an area
**RESULTS**

**Specificity of Anti-PA Antibody**—Anti-PA antibody KA-1 binds to PA very specifically. On ELISA, KA-1 showed no cross-reactivity with lyso-PA, phosphatidylglycerol, PI, PIP, PIP₂, or 1,2-diolein, and very little with cardiolipin (7%) and tetraoleoyl bis-PA (5%) (Fig. 1A). TLC immunostaining showed that antibody KA-1 reacted only with PA when rat brain total phospholipids (30 μg of phosphorus/spot) were used as antigens (Fig. 1B, left). Furthermore, KA-1 reacted with dioleoyl PA, dilinoleoyl PA, 1-palmitoyl-2-oleoyl PA, or 1-stearoyl-2-arachidonoyl PA, but not with dimyristoyl PA, dipalmitoyl PA, distearoyl PA, or didecanoyl PA on both ELISA (Table I) and TLC (Fig. 1C). These results suggest that the antibody reacts with PA molecules that contain an unsaturated fatty acid at the A₂ position. Next, we tried to establish a method for measuring PA content by TLC immunostaining. By this method, PA content could be measured in the range of 20–500 pmol (Fig. 2). To ascertain the reliability of this assay, a fixed amount of PA (10, 20, 50, or 100 pmol) was added to cells (1 x 10⁶ cells) and the PA was extracted and quantified. PA levels shifted in proportion to the amount of PA added to the original cells with a recovery of greater than 90%. Using this assay system, we were able to measure trace amounts of PA without the use of a radioactive precursor.

**DG and PA Formation in Response to PDGF**—To determine how DG and PA were formed in response to PDGF-stimulation, the time course for the changes in DG and PA levels was examined. As shown in Fig. 3, increase of DG was biphasic, which is consistent with previous results (22). The early phase showed a transient small peak at 30 s followed by a decrease to 1 min. In the second phase, which occurred after 1 min, DG accumulated gradually but very markedly to about 600 pmol/10⁶ cells up to 15 min. In the previous report, we showed that DG in the early peak was derived from PIP₂, while most of the DG in the second phase came from other lipids (22). On the other hand, PA content increased steadily
PA was found to be much stronger than that of DG. We used purified PA to examine whether PA by itself has any lyso-PA during incubation. However, the mitogenic effect of lyso-PA. There is still the possibility that PA is degraded to DNA synthesis, but its potency was about 25 times less than ever. Recently Jalink et al. (27) reported that commercial sources of PA were contaminated with lyso-PA. Therefore, phospholipase C.

Mitogenic Effect of PA, Lyso-PA, DG, Phospholipase C, Phospholipase D, and PDGF—Next, we examined the time course of changes in DG and PA levels when cells were treated with PDGF, phospholipase C, or phospholipase D in order to see the correlation between PA levels and cell proliferation (Fig. 6). In this case, a long term time course experiment was planned. In PDGF-stimulated cells, both DG in the second phase of PDGF-induced formation of DG and PA. As shown in Fig. 4, propranolol treatment enhanced accumulation of DG and inhibited the formation of DG in the second phase (15 min after stimulation). However, R59022 treatment did not influence the accumulation of DG and PA even at 15 min after stimulation. These results suggest that DG in the second phase is derived from PA rather than formed directly by phospholipase C.

Phosphatidylinositol (PI) 4-phosphate (PI(4)P) and 5-phosphate (PI(5)P) are important signaling intermediates in many cell processes, including cell growth and differentiation. PI(4)P is hydrolyzed by phosphatidylinositol 4-phosphate 5-kinase (PI(4)P5K) to PI(5)P, which is then hydrolyzed by PI(5)P phosphatase (PI(5)Pase). PI(5)P can also be produced by PI(4)P phosphatase (PI(4)Pase) and PI(5)P 5-kinase (PI(5)P5K). PI(5)P regulates the activity of PI(3,4,5)P3 phosphatase (PI(3,4,5)P3P), which dephosphorylates PI(3,4,5)P3 to PI(3,4)P2, PI(3,4)P2, and PI(3)P.

Mitogenic effect of lipids (A), phospholipase C, phospholipase D, or PDGF (B). PA (egg) purified by TLC was used as a mitogen. Briefly, commercial sources of PA were applied on TLC plates and developed in chloroform/methanol/water (60/25/4, v/v/v). After the authentic PA and lyso-PA were visualized with iodine, the spots were scraped off, and the radioactivities were measured by a liquid scintillation counter.

| PA | Lyso-PA |
|----|--------|
| 16,524 ± 43 (100.0 ± 0.3) | 1,189 ± 78 (100.0 ± 6.6) |
| 63,232 ± 971 (382.7 ± 5.9) | 2,137 ± 7 (179.5 ± 0.5) |

with time to about 250 pmol/10⁶ cells from 1 to 15 min. In addition, PA content was very low (less than 20 pmol/10⁶ cells) in growth-arrested cells, while that of DG was fairly high (about 320 pmol/10⁶ cells).

Effect of Propranolol and R59022 on PDGF-induced DG and PA Formation—Propranolol is known to be an inhibitor of PA phosphohydrolase and has been used to examine the pathway of DG (25). R59022 has been used as an inhibitor of DG kinase (26). Here, we used these drugs to investigate the route of PDGF-induced formation of DG and PA. As shown in Fig. 4, propranolol treatment enhanced accumulation of DG and inhibited the formation of DG in the second phase (15 min after stimulation). However, R59022 treatment did not influence the accumulation of DG and PA even at 15 min after stimulation. These results suggest that DG in the second phase is derived from PA rather than formed directly by phospholipase C.
phase, PA levels reached a maximum at 1 h followed by a decrease to 2 h. In the second phase, PA increased gradually up to 10 h. In this case, there was no significant DG accumulation. On the other hand, when cells were treated with phospholipase C, DG accumulation occurred without any marked increase in PA. DG accumulated rapidly up to 1 h and then remained stable for a long time.

Lyso-PA Formation by PDGF Treatment—In case of PDGF-stimulation of cells prelabeled with $^{32}$PPO$_4^-$, incorporation into lyso-PA increased by 1.8-fold, while incorporation into PA increased 3.8-fold. Radioactivity incorporated into lyso-PA was 2,137 cpm, which was 3.4% of the radioactivity incorporated into PA (Table II). In this isotope labeling method, PDGF-induced enhancement of PA synthesis was only 3.8-fold, while 10–15-fold enhancement was detected with the mass level-determining method using anti-PA antibody (Fig. 3). This discrepancy may be accounted for by the difference of PA species formed in response to PDGF. Indeed Fig. 7A showed that two distinct PAs (arrows 3 and 4) and lyso-PAs (arrows 1 and 2) were detected on autoradiography and the spots which could be visualized by iodine vapor (arrows 2 and 4) and be stained with KA-1 (arrow 4) were increased by PDGF stimulation. Arrows 3 and 4 were confirmed to be saturated and unsaturated fatty acid-containing PAs by using authentic PAs (Fig. 7B). These results suggest that most PA synthesized in response to PDGF are PA species having unsaturated fatty acid at the $A_2$ position. Therefore it is very important to measure unsaturated fatty acid PA mass levels to know the change in PA.

**DISCUSSION**

Many growth factors have been shown to enhance the hydrolysis of PIP$_2$ to IP$_3$ and DG (1). However, Hill et al. (31) reported that PDGF-induced activation of PIP$_2$-phospholipase C is not required for the induction of DNA synthesis in C3H10T1/2 cells. On the other hand, it is becoming clear that PDGF-stimulation of cells prelabeled with $^{32}$PPO$_4^-$ were stimulated with (right) or without (left) 5 units/ml PDGF for 20 min. Total lipids were extracted with chloroform/methanol/concentrated HCl (200/100/1, v/v/v), and then two-dimensional TLC was carried out as described under “Experimental Procedures.” At least two distinct PAs (arrows 3 and 4) and lyso-PAs (arrows 1 and 2) were detected. B, separation of saturated and unsaturated fatty acid-containing PAs. Distearoyl PA (30 $\mu$g) and 1-stearoyl-2-arachidonoyl PA (6 $\mu$g) were applied to a TLC plate, and a two-dimensional TLC was carried out. The plate was first immunostained with KA-1 (closed arrow) and then visualized with phosphorous-detection reagent (open arrow).

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**Fig. 7.** A, TLC patterns of $[^{32}\text{P}]\text{PA}$ and $[^{32}\text{P}]\text{lyso-PA}$ formed in response to PDGF. Balb/c 3T3 cells prelabeled with $[^{32}\text{P}]\text{PO}_4^-$ were stimulated with (right) or without (left) 5 units/ml PDGF for 20 min. Total lipids were extracted with chloroform/methanol/concentrated HCl (200/100/1, v/v/v), and then two-dimensional TLC was carried out as described under “Experimental Procedures.” At least two distinct PAs (arrows 3 and 4) and lyso-PAs (arrows 1 and 2) were detected. B, separation of saturated and unsaturated fatty acid-containing PAs. Distearoyl PA (30 $\mu$g) and 1-stearoyl-2-arachidonoyl PA (6 $\mu$g) were applied to a TLC plate, and a two-dimensional TLC was carried out. The plate was first immunostained with KA-1 (closed arrow) and then visualized with phosphorous-detection reagent (open arrow).
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phospholipase D treatment, significant DG accumulation was not observed. Similarly, phospholipase C treatment caused only DG accumulation. These data suggest that conversion of PA to DG is very low in phospholipase D-treated cells in contrast with PDGF-stimulated cells. As shown in Fig. 5, phospholipase D treatment causes a stronger mitogenic response than phospholipase C treatment, suggesting that PA by itself can induce DNA synthesis without DG formation. In this case, lyso-PA, rather than PA, may play the major role in cell proliferation since lyso-PA seems to be formed in PDGF-stimulated cells (Table II, Fig. 7). However, we could not detect a mitogenic effect of PA when PA was injected into cells (29). This discrepancy may be explained by the fact that injected PA forms micelles which are not effectively incorporated into membranes. Recently, PA has been demonstrated to have an inhibitory effect on Ras GAP and to increase the activity of the GTPase-inhibiting protein (20, 19). The interaction of PA with these two regulatory proteins may increase a Ras activity by keeping Ras as GTP complex and stimulate cell proliferation. Indeed, Ras activation has been demonstrated in PDGF-stimulated cells (30). Therefore, a dramatic increase in PA in PDGF-stimulated cells may cause Ras activation leading to cell proliferation. However, it is not clear whether PA induces cell growth through direct interaction with Ras or not, although PA-induced mitogenesis is inhibited by the injection of anti-Ras antibody into cells (14). This problem remains to be resolved in future.

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