Quantitative Changes in Polyphosphoinositides 1,2-Diacylglycerol and Inositol 1,4,5-Trisphosphate by Platelet-derived Growth Factor and Prostaglandin F₂₅

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We developed a novel method to quantify trace amounts of phosphatidylinositol 4-phosphate (PIP) and phosphatidylinositol 4,5-bisphosphate (PIP₂) using antibodies against PIP and PIP₂. With this method, polyphosphoinositides can be measured in the range from 20 to 500 pmol. We applied the method to quantify changes in PIP and PIP₂ levels in Balb/c/3T3 cells stimulated by platelet-derived growth factor (PDGF) and prostaglandin F₂₅ (PGF₂₅), growth factors that stimulate the hydrolysis of PIP and PIP₂. PIP₂ content decreased rapidly to about 60% of control within 1 min while PIP content decreased gradually but significantly to 60% (PDGF) or 70% (PGF₂₅) of control. Simultaneously we measured the mass levels of inositol 1,4,5-trisphosphate and 1,2-diacylglycerol (DG). Inositol 1,4,5-trisphosphate levels rapidly increased and reached a maximum at 30 s after PDGF or PGF₂₅ stimulation and then decreased to the control level within 2 min. On the other hand, DG formation showed biphasic changes. In the first phase, DG rapidly accumulated and reached a maximum at 30 s after PDGF or PGF₂₅ stimulation and then quickly decreased. In the second phase, DG accumulated gradually, but very markedly, 2 min after PDGF or PGF₂₅ stimulation. Considering the changes in PIP₂, DG in the first phase seems to be derived mainly from PIP₂ while most of the DG in the second phase derived from other lipids.

Numerous mitogens stimulate the phospholipase C-mediated cleavage of phosphatidylinositol 4,5-bisphosphate (PIP₂), resulting in the formation of two second messengers, inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (DG). Growth factors that enhance PIP₂ hydrolysis in a number of different fibroblasts include PDGF, bombesin, thrombin, vasoressin, and PGF₂₅ (1-10).

On the other hand, epidermal growth factor, insulin, and fibroblast growth factor have been considered to cause mitogenic action without PIP₂ hydrolysis in these cells (11, 12). These results suggest that there are at least two signal transduction systems for cell proliferation. In most studies investigating the stimulation of inositol phospholipid metabolism only metabolic changes have been measured due to the inability to detect low mass amounts of polyphosphoinositides, IP₃, and DG. These studies used the radiolabeling techniques, which provide useful qualitative information, but show only metabolic changes rather than quantitative changes in these materials (3, 13, 14). Recently, new methods to measure trace amounts of IP₃ and DG have been developed (15, 16). Determination of polyphosphoinositide levels in tissue culture also requires a new method capable of detecting extremely low levels of these lipids.

Recently, we developed a monoclonal antibody against PIP₂ and demonstrated that the antibody abolishes the mitogenic effect of PDGF and bombesin, suggesting the essential role of PIP₂ in the action of these mitogens (17, 18).

Now we have also developed a monoclonal antibody against phosphatidylinositol 4-phosphate (PIP) in addition to the anti-PIP₂ antibody. Using these antibodies, we established a sensitive assay method capable of measuring mass amounts of PIP₂ and PIP. Next, we have used these assays to examine the kinetic changes in PIP₂ and PIP when Balb/c/3T3 cells are stimulated by PDGF and PGF₂₅. In addition, we have measured the quantitative changes in IP₃ and DG following PDGF or PGF₂₅ stimulation.

EXPERIMENTAL PROCEDURES

Materials—PDGF was obtained from Takara (Tokyo, Japan). PGF₂₅ was from Funakoshi (Osaka, Japan). IP₃ and DG assay kits were from Amersham Corp. Dimyristoyl phosphatidylincholine, phosphatidylinositol (porcine liver), phosphatidylethanolamine (bovine liver), phosphatidylserine (porcine brain), dioloyl phosphatidic acid, and cardiolipin (bovine heart) were from Sedy Research Laboratory (Ontario, Canada). Lipid A was from List Biological Laboratories (Campbell, CA). Peroxidase-conjugated IgG (goat) anti-mouse immunoglobulin was from Cappel Laboratories. PIP₂ and PIP were prepared from bovine spinal cords by the method of Schacht (19). [3H]PIP (1 Ci/mmol) was obtained from Amersham Japan (Tokyo). Anti-PIP antibody (IgG 2b) was prepared as described before (18).

Cell Culture—Balb/c/3T3 cells were maintained at 37°C in Dulbecco’s modified Eagle’s medium supplemented with 5% calf serum. The cells were plated at 6.4 × 10⁴ cells in 9-cm dishes. Two days after plating, subconfluent cells were serum-starved by washing twice with Dulbecco’s modified Eagle’s medium 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-PIP Antibody—Anti-PIP antibody was made by a method similar to that described before (18). Briefly, BALB/c mice were immunized with liposomes containing dimyristoyl phosphatidylincholine, cholesterol, PIP (0.5 mg), and lipid A (molar ratio, 1:1:5:1:3:0:08) every 2 weeks for 4 months. The immune spleen cells were fused with P3-X63-Ag8 cells using polyethylene glycol. Two weeks after cell hybridization, hybridoma supernatants were evaluated by ELISA. The hybridoma cells secreting anti-PIP antibody were doubly cloned by limited dilution. The cells were inoculated into pristane-primed mice, and the resulting ascites fluid was used to measure PIP content. The antibody, named KD2, was found to be...
IgM by subclass analysis. The specificity of the antibody was evaluated by ELISA and TLC immunostaining as described before (18).

Mass Analysis of PIP and PIP2—Cells treated with growth factors were incubated at 30 °C for the indicated time, and then the incubations were terminated by aspirating the medium, washing with phosphate-buffered saline, and immediately adding 1.5 ml of ice-cold methanol containing 1 N HCl. The cells were scraped, and the dishes were further washed with 1.5 ml of chloroform/methanol (1:2, v/v). After adding 1.5 ml of chloroform, lipids were then extracted and washed with 1 N HCl as described before (20). The lipids were spotted on a TLC polygram (Macherey-Nagel) and then developed with chloroform/methanol/aqueous ammonia/water (90:65:12:8). The TLC plate was soaked overnight in phosphate-buffered saline containing 3% bovine serum albumin, 1% polyvinylpyrrolidone 40. The plate was then treated for 2 h at room temperature with ascites of anti-PIP and anti-PIP2 antibodies diluted 100-fold with PBS. Treated plates were washed 4–5 times with 50 mM Tris/HCl buffer (pH 7.4) containing 0.15 M NaCl and 0.05% Tween 20, treated with peroxidase-conjugated anti-mouse immunoglobulins, and stained with a Konica staining kit (Konica, Tokyo). The contents of PIP and PIP2 were measured by an image processor system (ADS Co., Nara, Japan).

Mass Analysis of DG—Cells were incubated with growth factors at 30 °C for the indicated times. The reactions were terminated by aspirating the culture medium, washing with PBS, and adding 1.5 ml of ice-cold methanol. The cells were scraped off, and the dishes were washed with 1.5 ml of chloroform/methanol (1:2, v/v). Lipids were extracted with 1.5 ml of chloroform and washed with 1 M KCl. An aliquot of the extracts (corresponding to 5 × 10^6 cells) was used for DG analysis according to the method of Preiß et al. (16) using a DG assay kit.

RESULTS

Specificity of Anti-PIP and Anti-PIP2 Antibodies and Application of the Antibodies to the Measurement of PIP and PIP2—Antibody KD2 bound to PIP very specifically. On ELISA, KD2 had little cross-reactivity with PIP2, PI, or other lipids (Fig. 1A). Moreover, TLC immunostaining (Fig. 1B) showed that the antibody reacted only with PIP, even when rat brain total phospholipids (15 μg of phosphorus/spot) were used as antigens. The anti-PIP2 antibody has already been demonstrated to react very specifically with PIP2 (18). We tried to measure PIP and PIP2 content by a TLC immunostaining assay method using the anti-PIP and anti-PIP2 antibodies (Fig. 2). PIP and PIP2 contents could be measured in the range from 20 to 500 pmol. To ascertain the reliability of this assay, a determined amount (20, 50, 100, or 200 pmol of PIP and PIP2) was added to cells (1 × 10^6), and then PIP and PIP2 levels were extracted and quantitated. PIP and PIP2 levels were shifted in proportion to the amounts added to the original cells. In this system, recovery of PIP and PIP2 was found to be more than 95%. This assay system enables us to measure trace amounts of PIP and PIP2.

Time Course of PIP and PIP2 Decreases by PDGF and PGF2α—Serum-starved quiescent cultures of Balb/c/3T3 cells contain PIP (130 pmol/10^6 cells) and PIP2 (110 pmol/10^6 cells). Stimulation of these cells by 2 units/ml PDGF or 3.3 μM PGF2α caused a rapid decrease in PIP and PIP2, as shown in Fig. 3. Especially, the decrease in PIP was very marked, with levels decreasing very rapidly to about 60% of the pre-stimulation level within 1 min after stimulation and remaining almost the same for up to 20 min (PGF2α) or with a continuing slight decrease (PDGF). PIP levels decreased gradually over 20 min to 60% of the original level following PDGF stimulation while in PGF2α-stimulated cells, the decrease in PIP reached a maximum 7 min after stimulation, and then the level gradually increased up to 20 min. The results suggest two possibilities. The first is that PIP2 breakdown continues for a long time, while the resynthesis of PIP2 is enhanced simultaneously. The second possibility is that PIP2 breakdown is only transiently enhanced but that compensatory resynthesis is very slow. To examine these possibilities, the effect of PDGF or PGF2α on the synthesis of PIP and PIP2 was studied. As shown in Fig. 4, PDGF and PGF2α caused enhanced incorporation of ^32P into PA and PI. However, ^32P incorporation into PIP and PIP2 was not significantly increased by PDGF and only very slightly increased by PGF2α. These results suggest that the compensatory resynthesis of PIP and PIP2 is not so active in Balb/c/3T3 cells. The dose-response curve for PDGF and PGF2α on ^32P incorporation was also examined. As shown in Fig. 5, even high doses of PDGF
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FIG. 2. Measurement of PIP$_2$ and PIP content by TLC immunostaining. A, TLC plates loaded with various amounts of PIP$_2$ and PIP were developed and then immunostained with a mixture of anti-PIP$_2$ and anti-PIP antibodies. B, the standard curves for PIP$_2$ and PIP content are shown. The immunostained plate was scanned by an image processor system and integrated optical density (IOD) was measured.

FIG. 3. Time course of PIP$_2$ and PIP decrease by PDGF and PGF$_2\alpha$. Quiescent cells ($2 \times 10^6$) were incubated with $[^{32}P]$PiO$_4^-$ (0.5 mCi/ml) at 30°C for 20 min in phosphate-free Dulbecco’s modified Eagle’s medium and then stimulated without (□) or with 2 units/ml PDGF (●) or 3.3 μM PGF$_2\alpha$ (▲) for the indicated times. After separating PIP$_2$, PIP, PI, and PA, $^{32}$P incorporated into each lipid was measured.

FIG. 4. Synthesis of PIP and PIP$_2$. Quiescent cells ($2 \times 10^6$) were incubated with $[^{32}P]$PiO$_4^-$ (0.5 mCi/ml) at 30°C for 20 min in phosphate-free Dulbecco’s modified Eagle’s medium and then stimulated without (□) or with 2 units/ml PDGF (●) or 3.3 μM PGF$_2\alpha$ (▲) for the indicated times. After separating PIP$_2$, PIP, PI, and PA, $^{32}$P incorporated into each lipid was measured.

and PGF$_2\alpha$ did not enhance the $^{32}$P incorporation into PIP and PIP$_2$, although the incorporation into PI and PA was dramatically increased at doses of PDGF greater than 0.5 unit/ml or of PGF$_2\alpha$ greater than 0.033 μM. On the other hand, mass levels of PIP$_2$ and PIP were markedly decreased at doses higher than 0.5 unit/ml PDGF or 0.033 μM PGF$_2\alpha$ (Fig. 6).

FIG. 7. IP$_3$ levels reached a maximum at 30 s after stimulation and then decreased very rapidly to control level. At 30 s after stimulation, the amounts of accumulated IP$_3$ were about 13 pmol/10^6 cells in PDGF-stimulated cells and 16 pmol/10^6 cells in PGF$_2\alpha$-stimulated cells. On the other hand, these growth factors induced a biphasic accumulation of DG (Fig. 8). The early phase showed a transient peak of DG at 30 s with a rapid decline between 30 and 60 s. In the second phase, DG accumulated gradually up to 7 min (PGF$_2\alpha$) or up to 20 min (PDGF). The increase in DG 30 s after PDGF stimulation was approximately 40 pmol/10^6 cells; at 20 min after stimulation the increase was 140 pmol/10^6 cells. DG levels 30 s after PGF$_2\alpha$ stimulation were increased by about 40 pmol/10^6 cells and maximally increased by 120 pmol/10^6 cells after 7 min.

DISCUSSION

The novel methods we developed enabled us to measure trace amounts of polyphosphoinositides. These novel methods also produce different results from the $[^{3}H]$inositol-labeling methods so far used (3, 13, 14). Several attempts to quantify polyphosphoinositides have been made using steady-state labeling techniques. However, the labeling efficiency of polyphosphoinositide with $[^{3}H]$myoinositol is very low, perhaps due to the metabolically inactive pools (21). Therefore, it has been difficult to determine the precise levels of polyphosphoinositides. We have measured the mass levels of signal-producing lipids and two second messengers, IP$_3$ and DG, since
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**Fig. 5.** Effect of various concentrations of PDGF and PGF\(_2\alpha\) on \(^{32}\text{P}\) incorporation into PIP\(_2\), PIP, PI, and PA. Quiescent cells (2 x 10\(^6\)) were treated without or with various concentrations of PDGF (units/ml) or PGF\(_2\alpha\) (\(\mu\text{M}\)) for 20 min at 30 °C. O, nontreatment; ●, PDGF; ▲, PGF\(_2\alpha\).

**Fig. 6.** Dose-response decrease in PIP\(_2\) and PIP by PDGF and PGF\(_2\alpha\). Quiescent cells (2 x 10\(^6\)) were treated without or with various concentrations of PDGF (units/ml) or PGF\(_2\alpha\) (\(\mu\text{M}\)) for 20 min at 30 °C. PIP\(_2\) and PIP contents were determined by the method described under "Experimental Procedures." O, nontreatment; ●, PDGF; ▲, PGF\(_2\alpha\).

**Fig. 7.** Enhanced formation of IP\(_3\) by PDGF and PGF\(_2\alpha\). Quiescent cells (2 x 10\(^6\)) were treated without (O) or with 2 units/ml PDGF (●) or 3.3 \(\mu\text{M}\) PGF\(_2\alpha\) (▲) at 30 °C for the indicated times. IP\(_3\) content was measured by an IP\(_3\) assay kit. Data are the mean ± S.E. of duplicate determinations from two separate experiments.

**Fig. 8.** Enhanced formation of DG by PDGF and PGF\(_2\alpha\). Quiescent cells (2 x 10\(^6\)) were treated without (O) or with 2 units/ml PDGF (●) or 3.3 \(\mu\text{M}\) PGF\(_2\alpha\) (▲) at 30 °C for the indicated times. DG content was measured by a DG assay kit. Data were the mean ± S.E. of duplicate determinations from two separate experiments.

It is thought that the absolute content is the most important factor in deciding the strength of the signals. Our novel methods show that the decrease in PIP and PIP\(_2\) content by PDGF or PGF\(_2\alpha\) stimulation is more marked than that previously found by steady-state labeling methods (3, 12, 13). PIP\(_2\) content decreased by almost 40% within 1 min following PDGF or PGF\(_2\alpha\) stimulation, demonstrating that 45 pmol/10\(^6\) cells of PIP\(_2\) was hydrolyzed. On the other hand, IP\(_3\) accumulation was transient, although DG accumulation continued for a long time. In addition, biphasic accumulation of DG was observed following PDGF and PGF\(_2\alpha\) stimulation. The first phase of accumulation peaked just about where IP\(_3\) levels were maximal. Wright et al. (22) also reported the biphasic accumulation of DG following \(\alpha\)-thrombin stimulation in Chinese hamster embryo fibroblast, suggesting that DG is produced through a pathway independent of PIP\(_2\) hydrolysis in the second phase. Considering the changes in PIP\(_2\) and PIP levels, DG formation in the first phase, which is finished within 1 min, is caused by PIP\(_2\) hydrolysis, since the decrease in PIP\(_2\) is almost equivalent to the increase in DG in both PDGF- and PGF\(_2\alpha\)-stimulated cells. Time course studies of the PIP and PIP\(_2\) decrease show different patterns between PDGF and PGF\(_2\alpha\), stimulation. PDGF causes a rapid decrease in PIP\(_2\) during the first minute and then a gradual decrease thereafter. The decrease in PIP occurs much more slowly than that of PIP\(_2\) and then continues for 20 min. On the other hand, PGF\(_2\alpha\) causes a rapid decrease in PIP\(_2\) within 1 min, but a further decline is not observed thereafter. PIP levels decrease gradually up to 7 min, but then the levels begin to recover. However, compensatory resynthesis of polyphosphoinositides is not so active in Balb/c/3T3 cells (Fig. 4). Most of the DG formed in the second phase, therefore, may be derived from other lipids. But in cells stimulated by PDGF, there is the possibility that a part of the DG still arises from PIP\(_2\).
difference might be due to the different mechanisms of action between PDGF and PGF. We previously demonstrated that the mitogenic effect of PDGF or bombesin in NIH/3T3 cells is abolished by anti-PIP2 antibody. As well, in Balb/c/3T3 cells the antibody inhibits PDGF-induced cell proliferation (data not shown) showing that PIP2 plays a key role in PDGF-induced mitogenesis. However, there have been several reports suggesting that PIP2 hydrolysis is not essential for cell growth

(22–24). Although it is not clear whether the discrepancy depends only on the type of cells examined, our novel method for quantifying PIP2 and PIP may afford a key to explaining the discrepancy. IP3 accumulation is very transient even in the presence of LiCl, suggesting rapid metabolism of IP3, while PIP2 hydrolysis continues (especially in the case of PDGF stimulation) after IP3 levels return to basal levels (Fig. 3). Therefore, it is difficult to know whether PIP2 hydrolysis occurs solely through the measurement of IP3 formation. Also in ras-transformed cells, PIP2 content is significantly low compared with that in normal cells, although IP3 levels do not differ. To measure PIP2 levels may be one of the best ways to solve the problem. Besides the role as signal-producing lipids, polyphosphoinositides have been implicated in cell motility through modulation of profilactin and gelsolin which regulate actin polymerization (25, 26). Therefore, the development of assay methods for polyphosphoinositides may also supply a powerful tool to study the involvement of these lipids in cell motility.

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