Platelet-derived-growth-factor-stimulated heterogeneous polyphosphoinositide metabolism and phosphate uptake in C3H fibroblasts

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Pig platelet-derived growth factor (PDGF) increased the rate of [32 P]P_i uptake by murine fibroblasts, resulting in a 3–9-fold elevation of the specific radioactivity of ATP, PtdInsP, PtdInsP₂, PtdIns and phosphatidic acid. The specific radioactivity was 10–60-fold higher in ATP than in the four phospholipids. These substances are therefore not in metabolic equilibrium, which complicates determination of inositol phospholipid turnover.

INTRODUCTION

PDGF-induced mitosis is preceded by formation of diacylglycerol (Habenicht *et al.*, 1981; Hasegawa-Sasaki, 1985) and InsP₃ (Berridge *et al.*, 1984; Brown *et al.*, 1984; Hasegawa-Sasaki, 1985; Chu *et al.*, 1985; Mene *et al.*, 1987), strongly suggesting that occupancy of the PDGF receptor leads to phosphodiesteratic cleavage of PtdInsP₂, although not necessarily through the same mechanisms as for vasopressin and bombesin (Hasagawa-Sasaki *et al.*, 1988). In these studies the cells were preincubated with appropriate radioactive precursors, and variations in the total metabolite radioactivity were reported, which does not give information as to whether PDGF causes an increase in the flux through the polyphosphoinositide cycle (Berridge *et al.*, 1984).

In order to measure flux variations, MacDonald *et al.* (1987) stimulated cells with PDGF in the presence of excess [32 P]P_i, which caused increases in the radioactivity of PtdInsP₂, PtdInsP, PtdIns and PtdOH (phosphatidic acid), suggesting increased turnover. However, serum (containing PDGF) causes an immediate increase in the uptake of P_i in various cells (Cunningham & Pardee, 1969; Hilborn, 1975; Gray *et al.*, 1976; Greenberg *et al.*, 1976; Barsh *et al.*, 1977; Scheinman, 1988). Thus the increase in metabolite radioactivity found by MacDonald *et al.* (1987) could have been due to increased uptake of [32 P]P_i, as has been found during platelet stimulation in the presence of [32 P]P_i, causing increase in the specific radioactivity of ATP and the polyphosphoinositides (Verhoeven *et al.*, 1987a). We have therefore studied the effect of purified PDGF on uptake of [32 P]P_i and the specific radioactivity of 32 P in ATP, PtdOH and polyphosphoinositides in CH3/10T1/2 cells.

MATERIALS AND METHODS

Materials

PDGF (97–98.5 % pure, according to polyacrylamide-gel electrophoresis/silver stain) was from Sangvinor a/s (Bergen, Norway). Three batches of PDGF, with specific

activities of 10, 24 and 33 ng of PDGF per unit (1 unit is defined as the amount of PDGF giving 50 % of maximal DNA synthesis) were used in this study. [32 P]P_i (code PBS 11, carrier-free) and [*methyl*- 3 H]thymidine (code TRK 418, 40–60 Ci/mmol) were from Amersham International (Amersham, Bucks., U.K.). C3H/10T1/2 Cl 8 murine fibroblasts were seeded on 32 mm-diam. plastic dishes in 1.0 ml of Basal Medium Eagle (Gibco) and grown to quiescence 7–8 days before the experiments.

Incorporation experiments

Various amounts of PDGF were added in 0.28 M-NaCl/10 mM-Na₂HPO₄, pH 7.3, to quiescent fibroblasts (test cells) together with 33 μ Ci of [32 P]P_i. A corresponding volume of the same solution containing 33 μ Ci of [32 P]P_i but no PDGF was added to control cells. In some experiments the concentration of Na₂HPO₄ was varied. After incubation for various times at 37 °C as noted, the medium was carefully removed and cell extracts were made and analysed as described below.

Extractions and analyses

For determination of the mass and radioactivity of the inositides and PtdOH, the cells in the dishes were extracted with 1.5 ml of chloroform/methanol/conc. HCl (20:40:1, by vol.) and processed as described by Langeland *et al.* (1986). The concentrated extract was subjected to t.l.c. with chloroform/methanol/aq. 20 % (v/v) methylamine (12:7:2, by vol.), followed by overnight radioautography and determination of the 32 P radioactivity of the fractions as described by Tysnes *et al.* (1985). The content of P in the fractions obtained was determined by a Malachite Green method after digestion with HClO₄ (Verhoeven *et al.*, 1987b).

For determination of the specific radioactivity of ATP, the cells were extracted with 0.65 ml of ice-cold 0.6 M-HClO₄. The extracts were neutralized with K₂CO₃ and their contents of ATP determined by a firefly luciferin-luciferase assay (Steen & Holmsen, 1985), and the radioactivity of ATP was determined after two-dimensional paper chromatography (Holmsen *et al.*, 1983).

For determination of total phosphate uptake, the cells

Abbreviations used: PDGF, platelet-derived growth factor; PtdOH, phosphatidic acid.

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were washed three times with ice-cold phosphate-buffered saline (136.7 mM-NaCl/2.7 mM-KCl/13.1 mM- Na_2HPO_4 /1.5 mM- KH_2PO_4) and then dissolved in 0.3 ml of 0.3% Triton X-100. The content of ^{32}P in these extracts was determined by liquid-scintillation counting. When the cell media contained different known concentrations of phosphate, the P-radioactivity/P-mass ratio was established in a sample of the medium from each dish after incubation.

Determination of DNA synthesis

PDGF in different concentrations was added to dishes with quiescent C3H/10T1/2 cells, which were incubated at 37 °C for 17 h. [^3H]Thymidine (1–2 μCi) was then added to each dish; 4 h later DNA was collected as a trichloroacetic acid precipitate, and its ^3H radioactivity determined as described by Male *et al.* (1985).

Cell protein

Each dish with cells grown to confluence (2.5×10^5 cells) contained 0.22 mg of protein. Protein was determined by the method of Bradford (1976), with the Bio-Rad Protein Assay Kit. Bovine γ -globulin was used as protein standard.

RESULTS

Uptake of P_i by C3H/10T1/2 cells

With 0.2 mM- P_i in the incubation medium, the ac-

Fig. 1. Time course of uptake of [^{32}P]P $_i$ by C3H/10T1/2 cells in the absence and presence of PDGF

Dishes with confluent C3H/10T1/2 cells were incubated at 37 °C with 33 μCi of [^{32}P]P $_i$ per dish (0.2 mM- P_i) in the absence (○) or presence (●) of 2 units of PDGF. At the times indicated, the ^{32}P content in the cells was determined as described in the Materials and methods section. Each point represents the average of five experiments, and the vertical bars give the S.D. The broken line represents 'non-specific uptake' (see the text).

Fig. 2. Effect of PDGF concentration on the uptake of [^{32}P]P $_i$ and incorporation of [^3H]thymidine by C3/10T1/2 cells

Confluent C3H/10T1/2 cells were incubated with 33 μCi of [^{32}P]P $_i$ per dish (0.2 mM- P_i) at 37 °C with PDGF at the concentrations shown. After 60 min the ^{32}P contents of the cells were determined (○) as described in the Materials and methods section. Another set of cells was incubated in parallel without [^{32}P]P $_i$ for 17 h, after which [^3H]thymidine was added for determination of DNA synthesis (▲), which is expressed as a percentage of the ^3H incorporated with 3 units of PDGF (60000 c.p.m.). Without PDGF 4000 c.p.m. of [^3H]thymidine was incorporated.

cumulation of [^{32}P]P $_i$ by (control) CH3/10T1/2 cells was linear with time over the 60 min incubation period studied. However, the regression line intersected the ordinate at 286 ± 96 pmol of P_i /mg of protein ($n = 5$) (Fig. 1). When PDGF (20 ng/ml) was added together with [^{32}P]P $_i$, the accumulation of [^{32}P]P $_i$ was still linear with time, but at a rate more than twice that of control cells and with the same intersection on the ordinate as found in the absence of PDGF (Fig. 1). It is possible that this intersection on the ordinate (i.e. at zero time) represents the amount of P_i trapped within the cell monolayer ('non-specific uptake'), although it could represent an early, rapid, phase of P_i uptake. Fig. 1 thus shows that PDGF causes a 2.2-fold (range 0.4–4.1) increase in the entire or a second (slow) phase of P_i uptake by quiescent fibroblasts.

The rate of P_i uptake increased with increasing concentrations of P_i with a dose-response relationship giving linear double-reciprocal plots, with an apparent half-maximal rate at 9.3 ± 3.4 mM extracellular P_i and a V_{max} of 757 ± 123 pmol/min per mg of protein ($n = 4$) (results not shown). The presence of PDGF increased V_{max} to 1033 ± 106 pmol/min per mg ($n = 4$; range 0.1–2.3-fold), without altering the extracellular concentration of P_i giving half-maximal rate (' K_m '; results not shown).

Exactly the same results were obtained with 3T3 cells and calf serum as growth factor source, except that ' K_m ' was 0.33 mM (Hilborn, 1975) or 0.06 mM (Barsh *et al.*, 1977).

The PDGF-induced stimulation of P_i uptake at 1 mM- P_i increased with increasing concentrations of PDGF up to about 10–20 ng of PDGF/ml (i.e. per dish), above which concentration no further stimulation took place (Fig. 2). The stimulation of phosphate uptake by PDGF in these experiments was assessed by incubation for 60 min. A parallel series of dishes was incubated with the same concentrations of PDGF without [32 P] P_i for 17 h, after which [3 H]thymidine was added to measure DNA synthesis. As shown in Fig. 2, the dose-response relationship for DNA synthesis with respect to the PDGF concentration was shifted to higher concentrations of PDGF.

PDGF-induced increase in incorporation of [32 P] P_i into phosphoinositides and phosphatidic acid of C3H/10T1/2 cells

Addition of [32 P] P_i to the quiescent fibroblasts caused a linear increase in the labelling of the phospholipids, with relative rates in descending order from PtdIns P_2 , PtdIns P , PtdIns to PtdOH. In the presence of PDGF incorporation was still linear, except for PtdIns, but the rates increased 13-fold for PtdOH and 3-fold for PtdIns P and PtdIns P_2 (results not shown). PDGF caused a progressive increase in PtdIns labelling, which was 19 times that of control cells at 60 min. Our incorporation patterns for PtdIns P_2 , PtdIns P and PtdIns were the same as reported by Habenicht *et al.* (1981) and MacDonald *et al.* (1987), whereas the increase in PtdOH labelling in our experiments was 6 times that reported by these investigators. Our results for the PDGF-induced increase in the rate of [32 P] P_i incorporation into PtdIns and PtdInsOH are also in agreement with the results obtained by Chu *et al.* (1985).

PDGF-induced increase in the specific radioactivity of fibroblast ATP, phosphatidic acid and phosphoinositides

The specific radioactivity of ATP increased linearly with time during incubation (60 min) of C3H/10T1/2 cells with [32 P] P_i , but the rate was 2–3 times higher in the

presence than in the absence of PDGF (20 ng/ml; results not shown). The amount of ATP was the same and constant in the fibroblasts during incubation with or without PDGF (value at 30 min shown in Table 1), and thus similar to the observed maintenance of ATP contents despite increased P_i uptake in serum-stimulated 3T3 cells (Gray *et al.*, 1976).

Table 1 shows the specific radioactivity in ATP and the phospholipids after 60 min of incubation of the cells with [32 P] P_i in the absence and presence of PDGF. The specific radioactivity of ATP, PtdIns P and PtdIns P_2 was about 4 times higher after incubation with PDGF than without it. For PtdOH and PtdIns the corresponding increase was 6–7-fold. However, the specific radioactivity of ATP was 10–30 times that of PtdIns P and PtdIns P_2 (Table 1). This was unexpected, since in platelets the γ - and β -phosphoryl groups in ATP have the same specific radioactivity as that of the phosphomonoesters of PtdIns P and PtdIns P_2 (Verhoeven *et al.*, 1987b).

DISCUSSION

The present study shows that purified PDGF increases V_{max} for P_i uptake without altering the K_m in C3H/10T1/2 fibroblasts, exactly as was found for serum-induced growth of 3T3 cells (Hilborn, 1975; Barsh *et al.*, 1977). However, since intracellular accumulation of 32 P was measured, these observations do not distinguish between unidirectional P_i uptake or increased metabolic trapping of P_i intracellularly. In our experiments the 32 P accumulation reached maximal rates at lower PDGF concentrations than for stimulation of DNA synthesis. This could indicate different affinity for PDGF binding to one receptor in controlling both the increase in 32 P accumulation and DNA synthesis. However, human foreskin fibroblasts have two binding sites for PDGF, one of which is conveying the mitotic signal (Heldin *et al.*, 1988). It is therefore possible that the C3H/10T1/2 cells used possess high- and low-affinity PDGF receptors, controlling the increase in 32 P accumulation and DNA synthesis respectively. Either mechanism would fit with our demonstration that neomycin inhibits PDGF-induced DNA synthesis, but not PDGF-induced increase

Table 1. Specific radioactivity of ATP and phospholipids in C3H/10T1/2 cells incubated with [32 P] P_i in the absence and presence of PDGF

Confluent C3H/10T1/2 cells were incubated at 37 °C with 33 μ Ci of [32 P] P_i per dish (1 mM- P_i) for 60 min in the absence and presence of 2 units of PDGF. The cells were then extracted and processed for determination of specific radioactivity of phospholipids and ATP as described in the Materials and methods section. The experiment is representative of four identical experiments. Abbreviation: PtdCho, phosphatidylcholine.

	Content (nmol/mg of protein)		Sp. radioactivity			
	No PDGF	+ PDGF	(c.p.m./nmol)		(c.p.m./mol of P)	
			No PDGF	+ PDGF	No PDGF	+ PDGF
ATP	13.5	14.3	1306	6413	435	2137
PtdCho	577.3	583.2	3	9	3	9
PtdOH	103.1	107.0	5	36	5	36
PtdIns	110.8	163.9	6	38	6	38
PtdIns P	24.1	27.9	48	173	24	87
PtdIns P_2	15.9	20.8	132	405	45	135

in ^{32}P accumulation (F. S. Vassbotn, N. Langeland & H. Holmsen, unpublished work). A two-receptor system for one ligand, with each individual receptor coupled to neomycin-sensitive and -insensitive signalling systems, has been reported (Slivka & Insel, 1988).

A rapid increase in ^{32}P accumulation during cell-agonist interaction, causing an increase in the specific radioactivity of ATP, has been shown for platelets and thrombin, collagen or platelet-activating factor (Verhoeven *et al.*, 1987a), cultured mesangial cells and vasopressin (Troyer *et al.*, 1987), LLC-PK₁ cells and serum (Scheinman, 1988) and fibroblasts and PDGF (F. S. Vassbotn, N. Langeland & H. Holmsen, unpublished work; and the present work). We show here that this rapid ^{32}P accumulation is accompanied by the same relative increase in the specific radioactivity of ATP, PtdInsP and PtdInsP₂, which has three important implications. Firstly, the turnover rates of the β - and γ -phosphate groups of ATP and of the phosphomonoesters in the phosphatides must be much greater than the rate of phosphate uptake. Secondly, the 3-fold linear increase in the total radioactivity of PtdInsP and PtdInsP₂ in PDGF-treated fibroblasts could solely be due to increase in their specific radioactivity, and not in their masses. Our measurements also show that the masses of PtdInsP and PtdInsP₂ are practically unchanged, whereas their total radioactivity increases 3-fold after PDGF treatment for 60 min. Thirdly, the PDGF-induced increase in the total radioactivity of the polyphosphoinositides during treatment of fibroblasts with PDGF in the presence of extracellular [^{32}P]P_i may not mean increased turnover of these substances, as has previously been suggested (Chu *et al.*, 1985; MacDonald *et al.*, 1987).

In contrast, the specific radioactivity of PtdIns and PtdOH increases relatively more upon PDGF treatment than does that of ATP, PtdInsP and PtdInsP₂, and thus cannot be solely due to increase in the ^{32}P uptake. The additional increase suggests that PDGF stimulates turnover of the phosphate moiety in PtdOH and PtdIns. The similar increase in the specific radioactivity in PtdIns and PtdOH is to be expected, since the monoester in PtdOH becomes the diester phosphate in PtdIns in the polyphosphoinositide cycle (Berridge *et al.*, 1984). This increase in PtdIns and PtdOH turnover suggests that the turnover of the diester phosphate in PtdInsP and PtdInsP₂ is also increased by PDGF-receptor occupancy.

The ^{32}P specific radioactivity in ATP was 10–30 times that of the polyphosphoinositides and 60 times that of PtdIns and PtdOH in the C3H/10T1/2 cells. In erythrocytes the specific radioactivity in PtdInsP₂ and PtdInsP phosphomonoesters was much lower than in the ATP γ -phosphate during incubation of the cells with [^{32}P]P_i (Muller *et al.*, 1986; King *et al.*, 1987). However, in platelets the monoesters of the polyphosphoinositides have the same specific radioactivity as the ATP γ -phosphate during [^{32}P]P_i uptake (Verhoeven *et al.*, 1987b). Thus, although we did not distinguish between phosphomono- and phosphodi-esters of PtdInsP and PtdInsP₂ in the C3H/10T1/2 cells, our results strongly suggest that the polyphosphoinositides may be metabolically compartmentalized in these fibroblasts and that they are more like erythrocytes than platelets in this regard. We are not aware of previous reported values of the contents of PtdInsP and PtdInsP₂ in C3H/10T1/2

fibroblasts. Although the ATP to PtdInsP (or PtdInsP₂) mass ratio in platelets is about 22 (Verhoeven *et al.*, 1987b), this ratio is about 0.6 in C3H/10T1/2 cells, suggesting the presence of unusually high amounts of polyphosphoinositides that could well be compartmentalized in the fibroblasts.

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