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Early changes in inositol lipids and their metabolites induced by platelet-derived growth factor in quiescent Swiss mouse 3T3 cells

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Inositol lipid turnover was studied in quiescent Swiss mouse 3T3 cells stimulated by platelet-derived growth factor (PDGF). Stimulation of the cells by PDGF for 10 min at 37 °C induced the following changes in lipids: (1) in cells prelabelled with [^{32}P]P$_i$, a 28% decrease in [^{32}P]phosphatidylinositol 4,5-bisphosphate, a 41% decrease in [^{32}P]phosphatidylinositol 4-phosphate and a 1.7-fold increase in the ^{32}P-labelling of phosphatidic acid; (2) in cells prelabelled with [^{3}H$_8$]arachidonic acid, a 17.9-fold increase in [^{3}H]phosphatidic acid, a 20% decrease in [^{3}H]phosphatidylinositol (PtdIns), an 8.6-fold increase in [^{3}H]arachidonic acid released into the medium, a 57-fold increase in [^{3}H]prostaglandin E$_2$ in the medium, and a 5.3-fold increase in [^{3}H]monoacylglycerol released into the medium (the last was identified as the 2-acyl derivative); (3) in cells prelabelled with [2-^{3}H]glycerol, a 1.7-fold increase in [^{3}H]diacylglycerol, a 6.7-fold increase in [^{3}H]phosphatidic acid, a 1.6-fold increase in [^{3}H]lysophosphatidylcholine (lysoPtdCho), a 9% decrease in [^{3}H]PtdIns, and a 1.6-fold increase in [^{3}H]monoacylglycerol released into the medium. PDGF stimulated the formation of inositol tris-, bis- and mono-phosphates in the cells prelabelled with *myo*-[2-^{3}H]inositol. These results indicate that, in Swiss 3T3 cells stimulated by PDGF, diacylglycerol produced by the hydrolysis of inositol lipids is partly degraded to 2-acylglycerol and partly converted into phosphatidic acid. The increase in lysoPtdCho indicates that a portion of arachidonic acid released from the stimulated cells is formed by the hydrolysis of PtdCho with a phospholipase A$_2$. Different values of half-maximal doses of the partially purified PDGF used in this study were found for the various responses of quiescent Swiss 3T3 cells to PDGF. The values for half-maximal doses suggest that activation of a fraction of the cell-surface receptor for PDGF is sufficient for mitogenesis and for an increase in the cytoplasmic free Ca^{2+} concentration, and that the PGDF-stimulated lipid metabolism is probably proportional to the number of receptor sites activated by PDGF.

INTRODUCTION

Activation of one family of cell-surface receptors for intercellular messages induces hydrolysis of PtdIns(4,5)P_2, activation of Ca^{2+}-activated phospholipid-dependent protein kinase, and a rise in [Ca^{2+}]$_i$ (for reviews, see Berridge, 1984; Berridge & Irvine, 1984). Many of the intercellular messages activating this family of receptors bring about rapid and immediate cell responses, such as secretion, contraction, shape change and alterations in metabolic flow. However, some of these messages trigger a cascade of cellular reactions leading to proliferation and differentiation (Habenicht *et al.*, 1981; Sasaki & Hasegawa-Sasaki, 1985; for reviews, see Michell, 1982; Berridge, 1984).

PDGF stimulates the growth of connective-tissue cells in culture, such as Swiss mouse 3T3 cells (Vogel *et al.*, 1980), vascular smooth-muscle cells (Habenicht *et al.*, 1980) and glial cells (Westermark & Wasteson, 1976). PDGF induces the hydrolysis of inositol lipids and a rise in [Ca^{2+}]$_i$ in Swiss 3T3 cells and in human fibroblasts (Habenicht *et al.*, 1981; Berridge *et al.*, 1984; Moolenaar *et al.*, 1984). The relation of this transmembrane signalling to the mitogenic action of PDGF is not known at present. PDGF stimulates a tyrosine-specific protein phosphorylation (Ek & Heldin, 1982, 1984). Evidence has been presented that the tyrosine kinase is an integral part of the PDGF receptor molecule (Heldin *et al.*, 1983). The receptors for three other growth factors, epidermal growth factor (Ushiro & Cohen, 1980), insulin (Kasuga *et al.*, 1982) and insulin-like growth factor I (Jacobs *et al.*, 1983), have also been found to be associated with tyrosine kinase activity. Although the link between the tyrosine-specific protein phosphorylation and mitogenesis by these growth factors remains to be established, researches on oncogenes indicate a close association of tyrosine kinases with the control of cell proliferation (Heldin & Westermark, 1984). It has been suggested that co-operation of multiple transmembrane signallings are important in the initiation of proliferative response of cells (Jimenez de Asua *et al.*, 1981).

An extensive study by Habenicht *et al.* (1981) on the effect of PDGF on lipid metabolism in Swiss 3T3 cells does not contain an analysis of the changes in PtdIns(4,5)P_2 and PtdIns4P. Berridge *et al.* (1984) provided evidence for PDGF-stimulated hydrolysis of PtdIns(4,5)P_2 in Swiss 3T3 cells by showing the accumulation of InsP_3 and InsP_2 in [^{3}H]inositol-prelabelled

Abbreviations used: PDGF, platelet-derived growth factor; [Ca^{2+}]$_i$, cytoplasmic free Ca^{2+} concentration; DME medium, Dulbecco's modified Eagle's medium; PDS, plasma-derived serum; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; PtdIns4P, phosphatidylinositol 4-phosphate; PtdIns, phosphatidylinositol; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; lysoPtdCho, lysophosphatidylcholine; lysoPtdEtn, lysophosphatidylethanolamine; lysoPtdIns, lysophosphatidylinositol; InsP_3, inositol trisphosphate; InsP_2, inositol bisphosphate; Ins$P$, inositol phosphate.

cells. In the present paper, I tried to characterize the inositol lipid turnover in PDGF-stimulated Swiss 3T3 cells. Breakdown of PtdIns(4,5)P_2 and PtdIns4P has been directly shown in PDGF-stimulated cells prelabelled with [^{32}P]P_i. Moreover, the results in the present paper contain several new findings on the early changes in lipid metabolism induced by PDGF in Swiss 3T3 cells. I found an accumulation of phosphatidic acid in the PDGF-stimulated cells, which indicates that a portion of diacylglycerol formed by the receptor-stimulated hydrolysis of inositol lipids is phosphorylated to phosphatidic acid. Monoacylglycerol released into the medium from the PDGF-stimulated cells was found to be heavily labelled with [^{3}H]arachidonic acid and was identified as the 2-acyl derivative. An accumulation of lysoPtdCho was found in the PDGF-stimulated cells, which indicates that a portion of arachidonic acid is released from PtdCho by the action of a phospholipase A_2.

MATERIALS AND METHODS

Cell culture

Swiss 3T3 cells were routinely grown in DME medium supplemented with 10% (v/v) calf serum, 100 units of penicillin/ml and 100 μg of streptomycin/ml. Cells were plated at 5×10^3–10×10^3 cells/cm^2 in 9 cm-diam. plastic culture dishes (Nunc) containing 12 ml of the culture medium, in 35 mm-diam. plastic culture dishes (Nunc) containing 2 ml of the culture medium, in 12-well multidishes (22.5 mm diam.) (Costar) containing 1.5 ml of the culture medium, or in 24-well multidishes (15.5 mm diam.) (Nunc) containing 1 ml of the culture medium. On day 3 after plating, the medium was changed to DME medium containing 5% (v/v) PDS. The cells were used for experiments 1 or 2 days after the medium change, by which time they were confluent and quiescent. When cells were to be biosynthetically prelabelled with various precursors, the radioactive precursors were added to the PDS-containing medium and cells were labelled for 4 h (arachidonic acid) or 24 h (*myo*-inositol, P_i or glycerol) at the end of the culture described above.

Preparation of PDS

PDS was prepared from human anticoagulated blood by the method of Vogel *et al.* (1978).

Preparation of partially purified PDGF

Clinically outdated human platelets (2 days old) were concentrated by centrifugation at 3200 *g* for 30 min and stored at −20 °C. Platelets from 46 litres of blood were thawed and suspended in 300 ml of 10 mM-sodium phosphate (pH 7.4)/1 mM-phenylmethanesulphonyl fluoride/1 mM-β-hydroxymercuribenzoate/5 mM-EDTA. The platelets were homogenized with a Polytron (Kinematica G.m.b.H), followed by sonication. The homogenate was heated at 100 °C for 10 min and centrifuged (12000 *g*, 30 min), and the supernatant fluid was collected. The precipitate was extracted once with 200 ml of the buffer supplemented with 1 M-NaCl by homogenization, followed by stirring at 4 °C overnight. Supernatant fluid after centrifugation at 12000 *g* for 30 min was collected and the combined supernatant was dialysed for 2 days against 30 vol. of 0.1 M-NaCl/10 mM-sodium phosphate, pH 7.4. Insoluble material was removed by centrifugation at 33000 *g* for 2 h. The supernatant was subjected to ion-exchange chromatography on CM-Sephadex C-50 by the method of Antoniades *et al.* (1979). After washing of the column, PDGF activity was eluted with 3 bed vol. of 0.5 M-NaCl/10 mM-sodium phosphate, pH 7.4. The active fraction was subjected to Blue Sepharose chromatography by the method of Heldin *et al.* (1979). The fraction eluted with ethylene glycol-containing buffer was dialysed against 1 M-acetic acid and then concentrated by ultrafiltration with an Amicon PM-10 membrane. The partially purified PDGF was stored at −20 °C in 0.5 ml portions. Protein concentration of the preparation was 216 μg/ml. The stock solution of PDGF was diluted with 1% (w/v) fatty acid-free bovine serum albumin/25 mM-Tris/HCl (pH 7.4)/136 mM-NaCl/5 mM-KCl (PDGF-dilution buffer), sterilized by membrane filtration, and stored at 4 °C. The diluted solution was used in each experiment.

Analysis of accumulation of inositol phosphates in PDGF-stimulated Swiss 3T3 cells

Cells were plated in either 12-well or 24-well multidishes. On day 3 after plating, the medium was changed to either 1.5 ml (12-well multidishes) or 0.7 ml (24-well multidishes) of inositol-free DME medium containing 5% PDS and 0.5 μCi of *myo*-[2-^{3}H]inositol (15.8 Ci/mmol; New England Nuclear)/ml. After labelling for 1 day, the radioisotope-containing medium was removed and the cell monolayers were rinsed twice with Tris-buffered saline (136 mM-NaCl/5 mM-KCl/0.5 mM-$MgCl_2$/0.9 mM-$CaCl_2$/0.7 mM-Na_2HPO_4/25 mM-Tris/HCl, pH 7.4) containing 2% PDS and 0.1% glucose, in which some (10 mM) of NaCl had been replaced with 10 mM-LiCl. After preincubation of the cell monolayers at 37 °C for 15 min in either 0.9 ml (12-well multidishes) or 0.5 ml (24-well multidishes) of the same saline containing LiCl, PDS and glucose, the incubation in the absence or in the presence of partially purified PDGF was started by adding either PDGF solution or the PDGF-dilution buffer. After the indicated time at 37 °C, the incubations were terminated and inositol phosphates were extracted and analysed either by method I or by method II described below.

In method I, the dishes were placed on ice, the medium was removed and 1 ml of methanol was added. The cells were scraped from the dish and transferred to test tubes. Dishes were rinsed with 0.4 ml of water and the washing was combined with the methanol extract. To the combined extract, 0.5 ml of chloroform was added. The mixture was partitioned into two phases by addition of 0.5 ml each of chloroform and water. Upper phase was taken and diluted with 5 ml of water. Total inositol phosphate fraction in this solution was assayed by the method of Berridge *et al.* (1982), by using 0.5 ml of a 50% (w/v) slurry of Dowex-1. In method II, the incubations were terminated by the addition of 0.2 ml of 10% (w/v) $HClO_4$. The cells were scraped from the dish, transferred to test tubes, and centrifuged (500 *g*, 10 min) to obtain the supernatant. The acid extract was neutralized with 1 M-$KHCO_3$. $KClO_4$ was precipitated at 0 °C for 60 min on ice and removed by brief centrifugation (500 *g*, 10 min). The neutralized extracts were diluted to 5 ml with 5 mM-sodium tetraborate/0.5 mM-EDTA. Ins$P$, InsP_2 and InsP_3 were separated by the method of Bone *et al.* (1984). A 5 ml portion of each fraction was assayed for radioactivity as previously described (Sasaki & Hasegawa-Sasaki, 1985).

Effect of PDGF on the phospholipids of Swiss 3T3 cells prelabelled with [^{32}P]P_i

Cells were plated in 12-well multidishes. On day 3 after plating, the medium was changed to 2 ml of a modified DME medium containing 5% PDS and 10 μCi of [^{32}P]P_i (Japan Atomic Energy Research Institute)/ml. The modified medium contained NaH_2PO_4 at 40 μM and Hepes at 25 mM. After labelling for 1 day, the radioisotope-containing medium was removed and the cell monolayers were rinsed twice with P_i-free Tris-buffered saline (Hasegawa-Sasaki & Sasaki, 1981) containing 2% PDS and 0.1% glucose. After preincubation of the cell monolayers at 37 °C for 15 min in 0.45 ml of the saline containing PDS and glucose, the incubation in the presence or in the absence of partially purified PDGF was started by adding 50 μl of either PDGF solution (7.2 μg of protein of partially purified PDGF/ml) or the PDGF-dilution buffer. After the indicated time at 37 °C, the incubation was terminated by removal of the solution covering the cells, followed by addition of 0.5 ml of ice-cold 5% (w/v) trichloroacetic acid. The fixed cells were scraped from the dish and collected by centrifugation (500 **g**, 10 min). Extraction of lipids from the cells and separation of phospholipids by two-dimensional t.l.c. were carried out as described below. Phospholipids separated on t.l.c. plates were located by autoradiography.

Effect of PDGF on the lipid metabolism in Swiss 3T3 cells prelabelled with [$^3H_8$]arachidonic acid

Cells were plated in 35 mm dishes. On day 3 after plating, the medium was changed to 2 ml of DME medium containing 5% PDS. The culture medium was supplemented with 1 μCi of [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid (120 Ci/mmol; Amersham) in 20 μl on day 5. The radioisotope solution was prepared by mixing 250 μCi of [$^3H_8$]arachidonic acid in 250 μl of ethanol with 4.75 ml of 1% fatty acid-free bovine serum albumin. After labelling for 4 h, the radioisotope-containing medium was removed and the cell monolayers were rinsed twice with Tris-buffered saline containing 0.1% fatty acid-free bovine serum albumin and 0.1% glucose. After preincubation of the cell monolayers at 37 °C for 15 min in 0.72 ml of the saline containing albumin and glucose, incubation in the presence or in the absence of partially purified PDGF was started by adding 80 μl of either PDGF solution or the PDGF-dilution buffer. After the indicated time at 37 °C, the dishes were placed on ice, the medium was collected in tubes containing 3 ml of chloroform/methanol/conc. HCl (100:200:1, by vol.) and the cells were precipitated with 1 ml of 5% trichloroacetic acid. The cells were then scraped from the dish and the dishes were rinsed with 1 ml of water. The precipitated cells were collected by centrifugation (500 **g**, 10 min). Extraction of lipids from the precipitated cells and analysis of phospholipids by two-dimensional t.l.c. were performed as described below. PtdIns(4,5)P_2, PtdIns4P and PtdIns, all of which had been isolated from rabbit brain, and phosphatidic acid, which had been prepared from rat liver PtdCho, were added to each sample as carriers in the phospholipid analysis by t.l.c. Neutral lipids were separated by t.l.c. as described below.

The chloroform/methanol extract of the incubation medium was partitioned into two phases by adding 1 ml each of chloroform and water. Lower phase was washed twice with new upper phase. Fatty acid, monoacylglycerol and prostaglandins in the washed lower phase were separated by t.l.c. on precoated silica-gel plates (LK5D; Whatman Chemical Separation Inc.). Upper phase of ethyl acetate/iso-octane/acetic acid/water (9:5:2:10, by vol.) was used as a developing solvent. Fatty acids, monoacylglycerol, which had been prepared by hydrolysis of lysoPtdCho with phospholipase C from *Clostridium*, prostaglandin E_2, prostaglandin $F_{2\alpha}$ and 6-oxoprostaglandin $F_{1\alpha}$ were added to each sample as carriers.

Effect of PDGF on the lipid metabolism in Swiss 3T3 cells prelabelled with [2-^{3}H]glycerol

Cells were plated in 35 mm dishes. On day 3 after plating, the medium was changed to 2 ml of DME medium containing 5% PDS and 4 μCi of [2-^{3}H]glycerol (10 Ci/mmol; New England Nuclear). After labelling for 24 h, the radioisotope-containing medium was removed. The cell monolayers were rinsed twice and preincubated at 37 °C for 30 min in 0.72 ml of Tris-buffered saline containing 0.1% fatty acid-free bovine serum albumin and 0.1% glucose. Then the incubation in the absence or in the presence of partially purified PDGF (576 ng of protein) was started by adding 80 μl of either PDGF solution or the PDGF-dilution buffer. After the incubation at 37 °C for 10 min, the dishes were placed on ice, the medium was collected in tubes containing 3 ml of chloroform/methanol/conc. HCl (100:200:1, by vol.) and the cells were fixed in 0.8 ml of 0.1 M-HCl. The cells were then scraped from the dish and the dishes were rinsed with 0.3 ml of 0.1 M-HCl. Lipids in the cells collected in 0.1 M-HCl were extracted with 4 ml of chloroform/methanol/conc. HCl (100:200:1, by vol.). The mixture was partitioned into two phases by adding 1.2 ml each of chloroform and 2 M-KCl. Lower phase was washed once with 2 M-KCl/methanol (1:1, v/v) and dried under a stream of N_2. The dried lipid was dissolved in a small volume of chloroform/methanol (1:1, v/v) and divided into two equal portions for the analyses of phospholipids and neutral lipids. It was reported that 80–85% of lysoPtdIns and 85–90% of lysophosphatidic acid could be extracted from platelets by the method of lipid extraction described above (Billah & Lapetina, 1982*a*). Neutral lipids were separated by t.l.c. as described below. Phospholipids were separated two-dimensional t.l.c. as described below on precoated silica-gel plates (Silica Gel 60, Merck) impregnated with 1% potassium oxalate/2 mM-EDTA. PtdCho, PtdEtn, phosphatidic acid, lysoPtdCho, lysoPtdEtn, lysoPtdIns, lysophosphatidic acid, PtdIns, PtdIns4P and PtdIns(4,5)P_2 were added to each sample as carriers. Carrier PtdCho and PtdEtn were isolated from rat liver. LysoPtdCho, lysoPtdEtn and lysoPtdIns used as carriers were prepared by the hydrolysis of corresponding diacyl phospholipids with snake venom. Carrier lysophosphatidic acid was prepared from lysoPtdCho by hydrolysis with phospholipase D. Lipids separated on t.l.c. plates were located with iodine vapour.

The chloroform/methanol extract of the incubation medium was partitioned into two phases by adding 1 ml each of chloroform and 2 M-KCl. Lower phase was washed once with 2 M-KCl/methanol (1:1, v/v). Neutral lipids in the washed lower phase were separated by t.l.c. as described below.

Lipid analysis

Lipids in the cells precipitated with trichloroacetic acid were extracted with chloroform/methanol/conc. HCl (200:100:1, by vol.). The extract was partitioned into two phases by addition of 0.2 vol. of 0.1 M-HCl. PtdIns(4,5)P_2, PtdIns4P, PtdIns, phosphatidic acid and other phospholipids in the washed lower phase were separated by two-dimensional t.l.c. on silica-gel H plates impregnated with 1% potassium oxalate/2 mM-EDTA (Sasaki & Hasegawa-Sasaki, 1985). Chloroform/methanol/aq. 4.3 M-NH_3 (9:7:2, by vol.) and butanol/acetic acid/water (6:1:1, by vol.) were used as developing solvents in the first and second dimensions respectively. Lipids separated on t.l.c. plates were located either by autoradiography or by iodine vapour as indicated in each experiment. Each located area was scraped into a vial. The sample was mixed with 5 ml of ACS II (Amersham) and 0.3 ml of water. The radioactivity was determined with a Beckman LS-9000 liquid-scintillation spectrometer.

Neutral lipids were separated by t.l.c. on silica-gel H plates with either light petroleum (b.p. 30–60 °C)/diethyl ether/acetic acid (70:30:1, by vol.) or hexane/diethyl ether/methanol/acetic acid (90:20:3:2, by vol.) as a developing solvent. In the analysis by t.l.c., triacylglycerol, fatty acids and diacylglycerol, all of which had been prepared from egg-yolk lipids, and monoacylglycerol were added to each sample as carriers. Lipids separated on t.l.c. plates were located by iodine vapour.

Monoacylglycerol was separated into the 1- and 2-acyl derivatives by t.l.c. on plates impregnated with 0.4 M-boric acid, by the method of Thomas *et al.* (1965).

Quin-2 loading and fluorescence measurements

Cells were plated in 9 cm plastic culture dishes. On day 3 after plating, the medium was changed to 15 ml of DME medium containing 5% PDS; 1 day after the medium change, the medium was removed and 10 ml of EDTA solution (Sasaki, 1981) was added to each dish. After 3 min at room temperature, the cells were detached from each dish by giving its side a sharp rap with the hand. The cells were collected by centrifugation (30 *g*, 10 min) and suspended at 2×10^7 cells/ml in DME medium. Quin-2 tetra-acetoxymethyl ester (Dojin Chemicals, Kumamoto, Japan) was added at 100 μM to the cell suspension and incubated for 20 min at 37 °C. The suspension was then diluted 10-fold with DME medium and incubated for a further 40 min. After loading, the cells were washed once with Tris-buffered saline containing 0.1% bovine serum albumin and 0.1% glucose and then suspended in the same saline containing albumin and glucose at 1.5×10^6 cells/ml. For measurement of fluorescence, 1 ml of the cell suspension was transferred to a cuvette. Quin-2 fluorescence was recorded at 37 °C with a Hitachi 650-10S fluorescence spectrophotometer fitted with a magnetic stirrer and a thermostatically controlled cell holder. Excitation and emission wavelengths were 339 and 492 nm with 2 and 10 nm slits respectively. Fluorescence was calibrated in terms of $[Ca^{2+}]_i$ from the fluorescence value at 0.9 mM-Ca^{2+} and at 0.5 mM-$MnCl_2$, both in the presence of 0.1% Triton X-100 (Hesketh *et al.*, 1983; Tsien *et al.*, 1982). Triton X-100 was added from 10% (v/v) solution.

RESULTS AND DISCUSSION

Breakdown of PtdIns(4,5)P_2 and accumulation of phosphatidic acid induced by PDGF

Swiss 3T3 cells were labelled for 1 day with $[^{32}P]P_i$. Stimulation of the labelled cells with PDGF caused a decrease in $[^{32}P]$PtdIns(4,5)P_2 to about 72% of the control value and a decrease of $[^{32}P]$PtdIns4P to about 59% of the control value at 10 min after the stimulation (Table 1). The decreases were also found at 30 s, 1 min, 2 min, 5 min and 15 min after PDGF addition. A 1.7-fold increase in the ^{32}P labelling of phosphatidic acid was found at 10 min after PDGF addition (Table 1). No significant change was induced by PDGF in $[^{32}P]$PtdIns, $[^{32}P]$PtdCho, $[^{32}P]$lysoPtdCho and $[^{32}P]$PtdEtn.

PDGF-stimulated accumulation of InsP_3, InsP_2 and Ins$P$ in cells prelabelled with myo-[2-^{3}H]inositol

Formation of InsP_3, InsP_2 and Ins$P$ by the hydrolysis of inositol lipids is shown in Fig. 1. In this experiment, *myo*-[2-^{3}H]inositol-prelabelled cells were stimulated by PDGF in the presence of 10 mM-LiCl to inhibit inositol-1-phosphatase (Hallcher & Sherman, 1980; Berridge *et al.*, 1982). Accumulation of Ins$P$ was not detectable until after a 2 min lag period (Fig. 1*a*). Gradual accumulation of Ins$P$ started after this lag period. There was a significant accumulation of InsP_2 and InsP_3 at 1 min and at 2 min after the PDGF addition (Figs. 1*b* and 1*c*). It seems that the InsP_3 accumulation reached a plateau earlier than the InsP_2 accumulation. The radioactivity of accumulated InsP_2 was 3–5 times greater than that of accumulated InsP_3 (Fig. 1). In the Swiss 3T3 cells prelabelled with *myo*-[2-^{3}H]inositol, the distribution of radioactivity among the three inositol

Table 1. Effect of PDGF on the breakdown of PtdIns(4,5)P_2 and PtdIns4P and on the accumulation of phosphatidic acid in Swiss 3T3 cells prelabelled with $[^{32}P]P_i$

Each incubation in the presence or in the absence of partially purified PDGF (720 ng of protein/ml) was performed for 10 min at 37 °C by the use of cell monolayers in 12-well multidishes as described in the Materials and methods section. Results are means ± S.E.M. for four parallel cultures. P values versus control were determined by Student's t test: *$P < 0.01$; **$P < 0.001$.

Phospholipid	10^{-2} × Radioactivity (c.p.m./dish)	
	Control	PDGF
PtdIns(4,5)P_2	402 ± 10.0	289 ± 24.1*
PtdIns4P	170 ± 3.7	100 ± 5.5**
PtdIns	5214 ± 33.1	5326 ± 38.2
Phosphatidic acid	67.2 ± 2.4	116 ± 6.1**

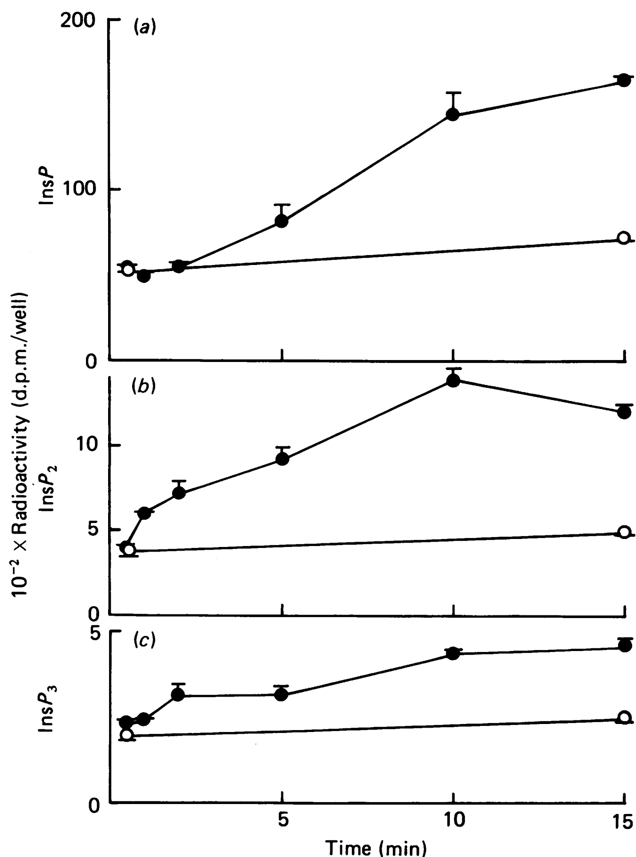

Fig. 1. Time courses of PDGF-induced accumulation of Ins$P$, InsP_2 and InsP_3 in Li^+-treated Swiss 3T3 cells prelabelled with *myo*-[2-^{3}H]inositol

Cells were cultured and labelled with *myo*-[2-^{3}H]inositol for 24 h in 12-well multidishes (see the Materials and methods section). At the end of the labelling, the cells were confluent and quiescent. The cell monolayers were rinsed twice and 0.9 ml of Tris-buffered saline containing 2% PDS and 0.1% glucose, in which some (10 mM) of the NaCl had been replaced with 10 mM-LiCl, was added to each monolayer. After preincubation at 37 °C for 15 min., the cultures were divided into two groups and either 100 μl of PDGF solution (720 ng of partially purified PDGF; ●) or 100 μl of saline containing bovine serum albumin (○) was added to each cell monolayer. After the indicated time at 37 °C, the incubation was terminated by addition of 0.2 ml of 10% (w/v) $HClO_4$. [^{3}H]Ins$P$, [^{3}H]InsP_2 and [^{3}H]InsP_3 in the acid-soluble fraction were separated from each other by anion-exchange chromatography as described in the Materials and methods section. The symbols and bars represent means ±S.E.M. for three parallel cultures (*a*), [^{3}H]Ins$P$; (*b*), [^{3}H]InsP_2; (*c*), [^{3}H]InsP_3.

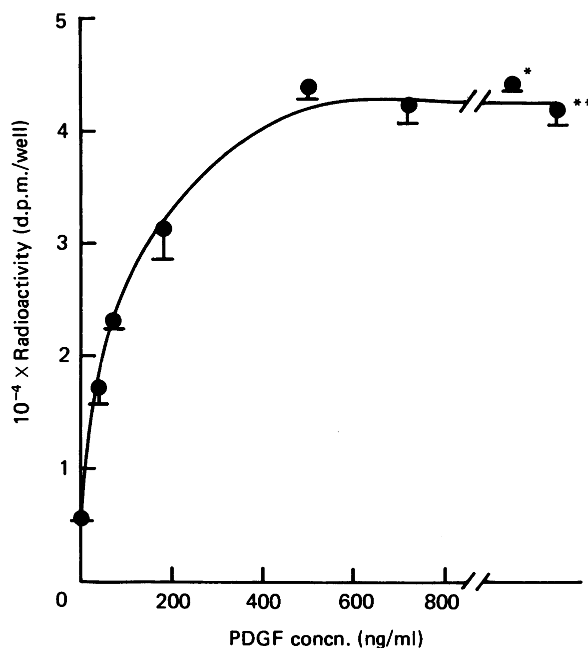

Fig. 2. Effect of PDGF concentration on inositol phosphate accumulation in Li^+-treated Swiss 3T3 cells prelabelled with *myo*-[2-^{3}H]inositol

Cells were cultured and labelled with *myo*-[2-^{3}H]inositol for 24 h in 24-well multidishes (see the Materials and methods section). At the end of the labelling, the cells were confluent and quiescent. The cell monolayers were rinsed twice, and 0.5 ml of Tris-buffered saline containing 2% PDS and 0.1% glucose, in which some (10 mM) of the NaCl had been replaced with 10 mM-LiCl, was added to each cell monolayer. After preincubation at 37 °C for 15 min, 50 μl of PDGF solution was added to each cell monolayer. The concentrations of the partially purified PDGF in the incubation mixtures are expressed in ng of protein/ml. The symbols * and ** correspond to 1.44 and 2.88 μg respectively of the PDGF preparation/ml. After incubation at 37 °C for 15 min, the dishes were placed on ice, the medium was removed and 1 ml of methanol was added. Total [^{3}H]inositol phosphates (sum of Ins$P$, InsP_2 and InsP_3) accumulated in the cells were determined as described in the Materials and methods section. The symbols and bars represent means ±S.E.M. for three parallel cultures.

lipids was 94.9% in PtdIns, 1.8% in PtdIns4P and 3.3% in PtdIns(4,5)P_2.

The time course of Ins$P$ accumulation suggests that Ins$P$ was not produced directly from PtdIns, but was formed by the dephosphorylation of InsP_2 and InsP_3 at least within 2 min after the PDGF addition. This interpretation is consistent with the results shown in Table 1. The results shown in Fig. 1 also suggest that much of the InsP_2 is formed by the hydrolysis of InsP_3. Storey *et al.* (1984) showed that rat liver homogenate hydrolyses Ins(1,4,5)P_3 about 8 times faster than Ins(1,4)P_2, and Ins(1,4)P_2 about 2.6 times faster than Ins(1)P. Even if all the inositol phosphates are formed from PtdIns(4,5)P_2 by the action of a phospholipase C, large difference in the rates of intracellular hydrolysis of inositol phosphates will result in the accumulation of InsP_3, InsP_2 and Ins$P$ in the proportions and with time courses just like those shown in Fig. 1. However, it is not possible from the results shown in Table 1 and Fig. 1 to exclude the possibility that PDGF induces in Swiss 3T3 cells the hydrolysis of PtdIns4P by a phospholipase C, in addition to the phosphorylation of PtdIns4P to PtdIns(4,5)P_2.

Fig. 2 shows the effect of PDGF concentration on the accumulation of total inositol phosphates in the presence of 10 mM-LiCl. The accumulation was dose-dependent up to 500 ng of partially purified PDGF/ml, with a dose of 80 ng/ml giving half-maximal effect.

Table 2. Radioactivity of various phospholipids, triacylglycerol, diacylglycerol, monoacylglycerol, non-esterified fatty acids and prostaglandin E_2 at 10 min after addition of PDGF in Swiss 3T3 cells biosynthetically prelabelled with [$^3H_8$]arachidonic acid

The experiment was performed as described in the Materials and methods section. Tris-buffered saline (0.72 ml) containing 0.1% fatty acid-free bovine serum albumin and 0.1% glucose was added to confluent or quiescent cell monolayers, in 35 mm dishes, which had been labelled with [$^3H_8$]arachidonic acid for 4 h. After preincubation at 37 °C for 15 min, the cultures were divided into two groups, and 80 µl of either partially purified PDGF (576 ng of protein) or buffered saline containing bovine serum albumin was added to each dish. The cell monolayers were incubated at 37 °C for 10 min. Lipids were extracted from both incubation medium and the trichloroacetic acid-precipitated cells. Non-esterified fatty acids,, monoacylglycerol and prostaglandins in the medium, and phospholipids, neutral lipids and non-esterified fatty acid in the cells, were analysed by t.l.c. as described in the Materials and methods section. Each [^{3}H]lipid was located on t.l.c. plates from the positions of carrier lipids added to each sample. Each value is the mean ± S.E.M. for five parallel cultures. *P* values were determined by Student's *t* test. Abbreviation: N.S., not significant.

Lipid	10^{-2} × Radioactivity (d.p.m./dish)		*P*
	Control	PDGF	
Medium			
Non-esterified fatty acid	17.1 ± 0.6	147 ± 4.7	< 0.0001
Prostaglandin E_2	0.6 ± 0.1	35.7 ± 2.4	< 0.0001
Monoacylglycerol	4.2 ± 0.9	22.3 ± 2.8	< 0.0003
Cell			
Triacylglycerol	110 ± 3.8	113 ± 1.9	N.S.
Diacylglycerol	69.9 ± 3.3	64.0 ± 3.6	N.S.
Monoacylglycerol	10.1 ± 0.5	19.7 ± 1.5	< 0.0003
Non-esterified fatty acid	69.8 ± 6.8	70.0 ± 1.4	N.S.
PtdIns(4,5)P_2	55.0 ± 1.1	51.9 ± 1.4	N.S.
PtdIns4*P*	37.9 ± 0.8	36.7 ± 1.8	N.S.
Phosphatidic acid	4.8 ± 0.5	86.1 ± 0.8	< 0.0001
PtdIns	1087 ± 21.7	865 ± 17.0	< 0.0001
PtdEtn	848 ± 18.4	835 ± 11.4	N.S.
PtdCho	2357 ± 40.6	2270 ± 37.8	N.S.

Accumulation of phosphatidic acid and releases of arachidonic acid, prostaglandin E_2 and monoacylglycerol induced by PDGF, measured in cells prelabelled with [$^3H_8$]arachidonic acid

The accumulation of phosphatidic acid in the PDGF-stimulated cells could be demonstrated most clearly by the use of cells prelabelled with [$^3H_8$]arachidonic acid. In the prelabelled cells, stimulation by PDGF for 10 min at 37 °C induced a 17.9-fold increase in [^{3}H]phosphatidic acid, a 20% decrease in [^{3}H]PtdIns, a 2-fold increase in [^{3}H]monoacylglycerol, an 8.6-fold increase in [^{3}H]arachidonic acid released into the medium, a 57-fold increase in prostaglandin E_2 in the medium, and a 5.3-fold increase in [^{3}H]monoacylglycerol released into the medium (Table 2). Habenicht *et al.* (1981) found about 2-fold increase in diacylglycerol at 10 min after stimulation by PDGF in Swiss 3T3 cells prelabelled with [$^3H_8$]arachidonic acid for 24 h. No significant accumulation of [^{3}H]diacylglycerol was found in the experiment shown in Table 2. The increase in phosphatidic acid in the PDGF-stimulated cells was not previously described. Induction of synthesis and release of prostaglandin E_2 by PDGF in Swiss 3T3 cells (Table 2) confirms the results reported by Shier & Durkin (1982) and indicates that a portion of arachidonic acid formed in the PDGF-stimulated cells is metabolized to prostaglandin E_2. Prostaglandin $F_{2\alpha}$ and 6-oxoprostaglandin $F_{1\alpha}$ were not released in any significant amount (results not shown). The decrease in PtdIns in the PDGF-stimulated cells was previously reported by Habenicht *et al.* (1981) and was probably due to the metabolic transformation of PtdIns to PtdIns(4,5)P_2.

The accumulation of [$^3H_8$]arachidonate-labelled monoacylglycerol both in the medium and in the cells suggests a possibility that this compound is the 2-acyl derivative. This was proved as follows. [^{3}H]Monoacylglycerol released into the medium from the stimulated cells was purified by t.l.c. Alkaline hydrolysis of the purified [^{3}H]monoacylglycerol in 0.11 M-NaOH in chloroform/methanol (1:4, v/v) at 37 °C for 15 min converted more than 80% of the radioactivity into [^{3}H]fatty acid methyl ester. Separation of the isolated [^{3}H]monoacylglycerol into the 1- and 2-acyl derivatives by t.l.c. on a borate-impregnated plate resulted in the location of 78% of the radioactivity at the position of the 2-acyl derivative and 22% at the position of the 1-acyl derivative. The result suggests that the monoacylglycerol lipase pathway may be one of the sources of arachidonic acid in PDGF-stimulated Swiss 3T3 cells, as is the case in thrombin-stimulated platelets (Prescott & Majerus, 1983).

Fig. 3 shows time courses of the accumulation of [^{3}H]phosphatidic acid in the cells and the release of [^{3}H]arachidonic acid into the medium in the [$^3H_8$]-arachidonate-prelabelled cells. The accumulation and release were evident as early as 1 min after PDGF addition and were almost complete by 10 min after the stimulation.

The effects of PDGF concentration on the accumulation of [^{3}H]phosphatidic acid and on the releases of [^{3}H]arachidonic acid and [^{3}H]prostaglandin E_2 are

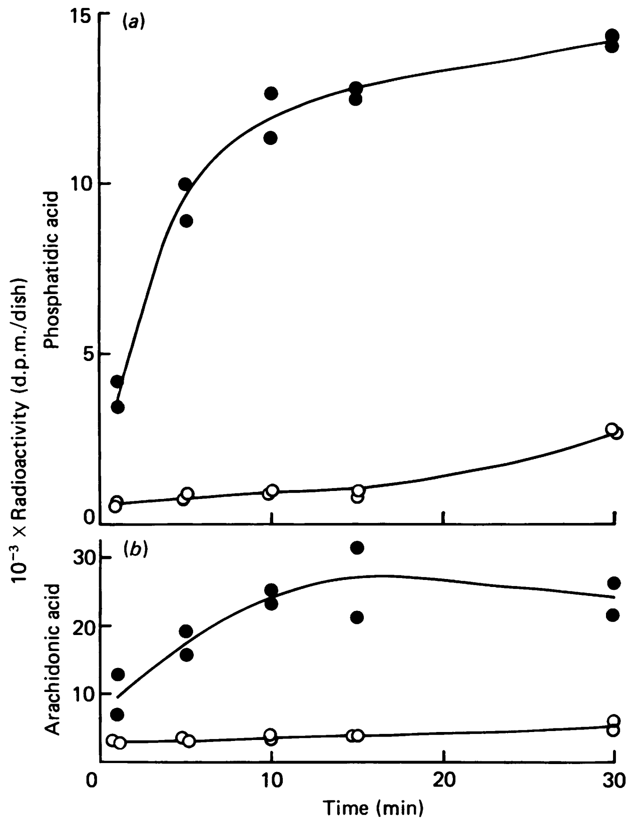

Fig. 3. Time courses of phosphatidic acid accumulation and arachidonic acid release induced by PDGF in Swiss 3T3 cells prelabelled with [$^3H_8$]archidonic acid

Cells were cultured and labelled with [$^3H_8$]arachidonic acid for 4 h in 35 mm dishes (see the Materials and methods section). At the end of the labelling, the cells were confluent and quiescent. The cell monolayers were rinsed twice, and 0.72 ml of Tris-buffered saline containing 0.1% fatty acid-free bovine serum albumin and 0.1% glucose was added to each cell monolayer. After preincubation at 37 °C for 15 min, the cultures were divided into two groups and either 80 μl of PDGF solution (576 ng of partially purified PDGF; ●) or 80 μl of saline containing bovine serum albumin (○) was added to each cell monolayer. After incubation for the indicated time at 37 °C, the dishes were placed on ice, the medium was subjected to lipid extraction and the cells were fixed by trichloroacetic acid. [^{3}H]Phosphatidic acid in the acid-precipitated cells (*a*) and [^{3}H]arachidonic acid in the medium (*b*) were determined as described in the Materials and methods section. The experiment was performed in duplicate, and the two values obtained from two parallel cultures were plotted.

shown in Fig. 4. These data represent measurements made over 10 min after stimulation by PDGF. The half-maximal dose of PDGF for the accumulation of [^{3}H]phosphatidic acid was 65 ng of partially purified PDGF/ml, that for the release of [^{3}H]arachidonic acid was 180 ng of the PDGF preparation/ml, and that for the release of [^{3}H]prostaglandin E_2 was 235 ng of the PDGF preparation/ml (Fig. 4).

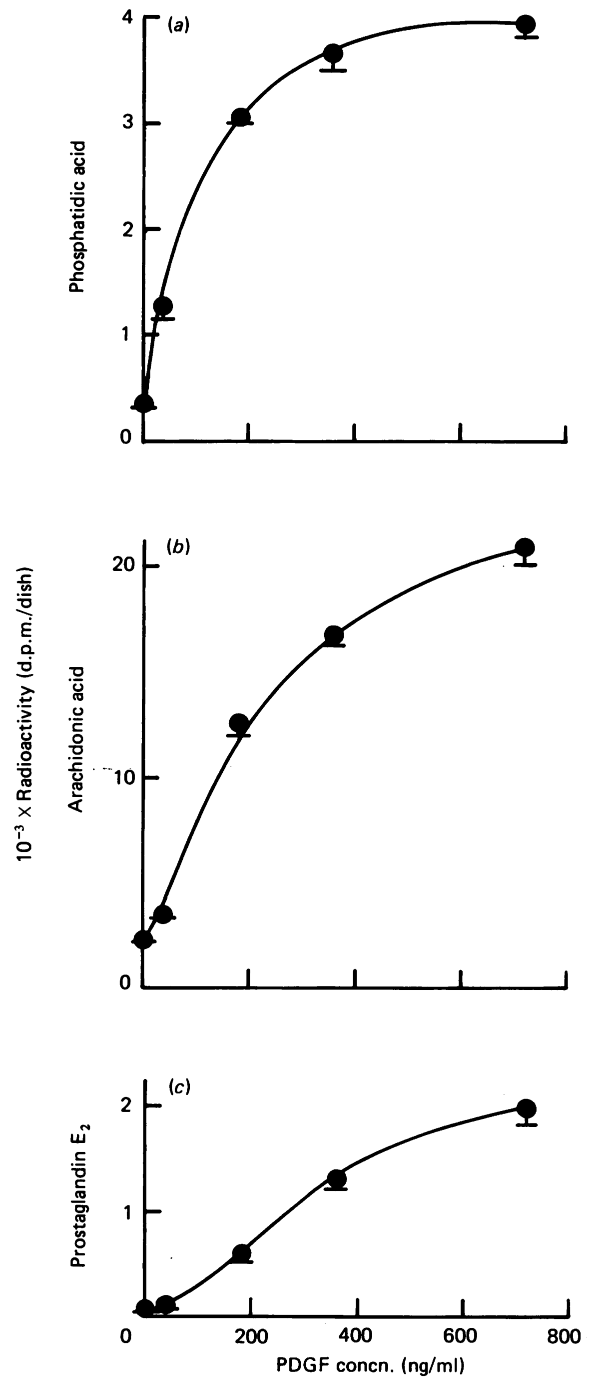

Fig. 4. Effect of PDGF concentration on accumulation of phosphatidic acid and releases of arachidonic acid and prostaglandin E_2 in Swiss 3T3 cells prelabelled with [$^3H_8$]arachidonic acid

The experiment was performed in the same manner as described in the legend to Fig. 3, except that the incubation was started by adding 80 μl of PDGF solution containing various amounts of the PDGF preparation, and terminated after 10 min at 37 °C. The concentrations of the partially purified PDGF in the incubation mixtures are expressed in ng of protein/ml. [^{3}H]Phosphatidic acid in the acid-precipitated cells (*a*) and [^{3}H]arachidonic acid (*b*) and [^{3}H]prostaglandin E_2 (*c*) in the medium were determined as described in the Materials and methods section. The symbols and bars represent means ± S.E.M. for four parallel cultures.

Table 3. Radioactivity of various phospholipids, triacylglycerol, diacylglycerol and monoacylglycerol at 10 min after addition of PDGF in Swiss 3T3 cells biosynthetically prelabelled with [2-^{3}H]glycerol

The experiment was performed as described in the Materials and methods section. Tris-buffered saline (0.72 ml) containing 0.1% fatty acid-free bovine serum albumin and 0.1% glucose was added to confluent or quiescent cell monolayers, in 35 mm dishes, which had been labelled with [2-^{3}H]glycerol for 24 h. After preincubation at 37 °C for 30 min, the cultures were divided into two groups, and 80 μl of either partially purified PDGF (576 ng of protein) or buffered saline containing bovine serum albumin was added to each dish. The cell monolayers were incubated at 37 °C for 10 min. Lipids were extracted from both incubation medium and cells. Monoacylglycerol in the medium, and phospholipids and neutral lipids in the cells, were analysed by t.l.c. as described in the Materials and methods section. Each value is the mean ± S.E.M. for five parallel cultures. *P* values were determined by Student's *t* test. Abbreviations: N.S., not significant; PtdSer, phosphatidylserine.

Lipid	10^{-2} × Radioactivity (d.p.m./dish)		*P*
	Control	PDGF	
Medium			
Monoacylglycerol	5.0±0.2	8.1±0.6	< 0.01
Cell			
Triacylglycerol	108±0.9	101±2.8	N.S.
Diacylglycerol	11.9±0.4	20.0±0.7	< 0.0001
Monoacylglycerol	7.5±0.7	10.7±0.8	N.S.
PtdIns(4,5)P_2	13.5±1.2	14.7±1.3	N.S.
PtdIns4P	8.4±0.6	8.7±0.7	N.S.
Phosphatidic acid	2.2±0.2	15.1±0.6	< 0.0001
PtdIns	142±4.0	128±2.0	< 0.02
PtdEtn	175±1.8	174±1.9	N.S.
PtdCho	972±3.7	970±2.7	N.S.
LysoPtdCho	17.8±0.8	28.1±1.9	< 0.01
LysoPtdEtn + PtdSer	107±3.9	100±2.3	N.S.
LysoPtdIns	8.5±0.5	10.5±1.0	N.S.
Lysophosphatidic acid	0.9±0.2	1.5±0.3	N.S.

Accumulation of phosphatidic acid, diacylglycerol and lysophosphatidylcholine and release of monoacylglycerol induced by PDGF, measured in cells prelabelled with [2-^{3}H]glycerol

In cells prelabelled with [2-^{3}H]glycerol for 24 h, stimulation by PDGF for 10 min at 37 °C induced a 1.7-fold increase in [^{3}H]diacylglycerol, a 6.7-fold increase in [^{3}H]phosphatidic acid, a 1.6-fold increase in [^{3}H]lysoPtdCho, a 9% decrease in [^{3}H]PtdIns, and a 1.6-fold increase in [^{3}H]monoacylglycerol released into the medium (Table 3). No obvious change was found in other cellular ^{3}H-labelled lipids (Table 3). The accumulation of diacylglycerol, the decrease in PtdIns and the release of monoacylglycerol into the medium agree with the results on [^{3}H]glycerol-prelabelled cells reported by Habenicht *et al.* (1981). Phosphatidic acid was not analysed in the study by Habenicht *et al.* (1981). The results in Table 2 and 3 suggest that, in Swiss 3T3 cells stimulted by PDGF, diacylglycerol formed by the hydrolysis of inositol lipids is partly degraded to monoacylglycerol and partly converted into phosphatidic acid. The formation of phosphatidic acid from the diacylglycerol produced by the receptor-stimulated hydrolysis of inositol lipids has been found in many cells stimulated by Ca^{2+}-mobilizing agonists since the first observation of this by Hokin & Hokin (1955) (Lapetina & Cuatrecasas, 1979; Billah & Lapetina, 1982*b*; Thomas *et al.*, 1983; Hasegawa-Sasaki & Sasaki, 1983; Orchard *et al.*, 1984; Sasaki & Hasegawa-Sasaki, 1985; for review, see Michell, 1975). The accumulation of lysoPtdCho in the PDGF-stimulated Swiss 3T3 cells (Table 3) indicates that a portion, at least, of the arachidonic acid released from the stimulated cells is derived from PtdCho as a result of the activation of a phospholipase A_2.

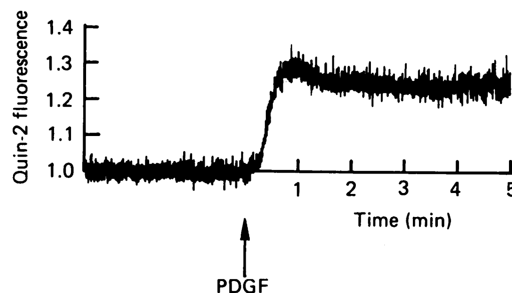

Fig. 5. Fluorescence response of quin-2-loaded Swiss 3T3 cells to PDGF

Quin-2 loading of quiescent Swiss 3T3 cells and fluorescence monitoring were done as described in the Materials and methods section. The trace represents the quin-2 fluorescence response of 1 ml of the cell suspension to addition (at zero time) of 20 μl (360 ng of protein) of partially purified PDGF dissolved in saline containing bovine serum albumin. Saline alone had no effect on resting fluorescence values.

Effect of PDGE on $[Ca^{2+}]_i$ in quin-2-loaded Swiss 3T3 cells

Fig. 5 illustrates the effect of PDGF on $[Ca^{2+}]_i$ of quin-2-loaded Swiss 3T3 cells. On addition of 360 ng of partially purified PDGF/ml, there was a 29% increase in quin-2 fluorescence after a lag period of about 15 s. The rise in fluorescence was complete by 40 s and represents

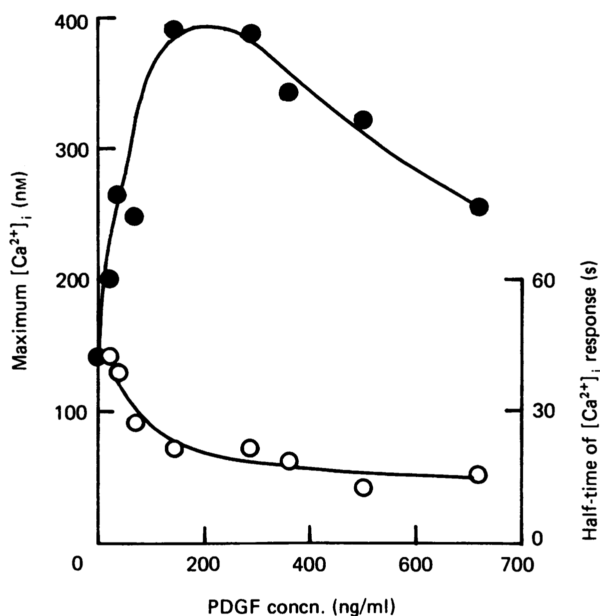

Fig. 6. Effect of PDGF concentration on elevation of $[Ca^{2+}]_i$ and half-time of $[Ca^{2+}]_i$ response in quin-2-loaded Swiss 3T3 cells

Quin-2 loading of quiescent Swiss 3T3 cells and measurements of $[Ca^{2+}]_i$ changes after addition of the indicated concentration (ng of protein/ml) of the partially purified PDGF were done as described in the Materials and methods section. The cells were preincubated at 37 °C in the cuvette chamber of the spectrofluorimeter for 7 min before addition of PDGF. ●, Maximal $[Ca^{2+}]_i$ achieved for each dose of PDGF; ○, half-time between the PDGF addition and the peak $[Ca^{2+}]_i$.

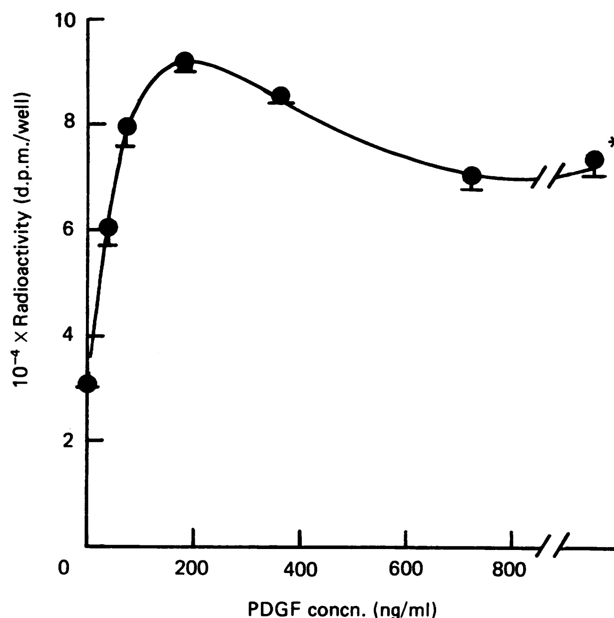

Fig. 7. Effect of PDGF concentration on [^{3}H]thymidine incorporation into DNA in quiescent Swiss 3T3 cells

Cells were plated at a density of $1 \times 10^4/cm^2$ in 24-well multidishes in medium containing 10% calf serum. On day 3 after plating, by which time the cells were confluent, the medium was changed to 1 ml of DME medium containing 5% PDS. The partially purified PDGF was added 4.5 h after the medium change at the indicated concentrations (ng of protein/ml). Then 2 μCi of [*methyl*-^{3}H]thymidine (2 Ci/mmol; New England Nuclear) was added to each well 18 h after the PDGF addition. The cells were labelled for 6 h, and incorporation of radioactivity into trichloroacetic acid-insoluble materials was determined. The symbol * corresponds to 1.44 μg of the PDGF preparation/ml. The symbols and bars represent means ± S.E.M. for three parallel cultures.

a 2.4-fold increase in $[Ca^{2+}]_i$. During the next 40 s, the PDGF-induced $[Ca^{2+}]_i$ response slightly declined to a new steady value, which was maintained for at least 15 min.

Fig. 6 shows the effect of PDGF concentration on maximum $[C^{2+}]_i$. Both peak $[Ca^{2+}]_i$ and the half-time between the PDGF addition and the peak $[Ca^{2+}]_i$ (an index of lag period and rate of $[Ca^{2+}]_i$ increase) are dependent on the PDGF concentration. A half-maximal dose of 45 ng of partially purified PDGF/ml is calculated for the $[Ca^{2+}]_i$ response (Fig. 6). For unknown reason(s), the response becomes smaller at higher doses of the PDGF preparation (Fig. 6).

Effect of the partially purified PDGF on DNA synthesis

Fig. 7 shows the dose–response curve for PDGF-stimulated [^{3}H]thymidine incorporation into DNA in quiescent Swiss 3T3 cells. A half-maximal dose of 35 ng of the partially purified PDGF/ml is calculated for this response. At higher doses of the PDGF preparation, stimulation of DNA synthesis becomes smaller, for unknown reason(s).

Dose–response relationships

Different values of half-maximal doses of the partially purified PDGF preparation were found for the various responses of quiescent Swiss 3T3 cells to PDGF. The half-maximal doses (ng of PDGF preparation/ml) were 35 for the [^{3}H]thymidine incorporation into DNA, 45 for the $[Ca^{2+}]_i$ response, 65 for the accumulation of phosphatidic acid, 80 for the inositol phosphate accumulation, 180 for the arachidonate release and 235 for the prostaglandin E_2 release (Figs. 2, 4, 6 and 7). These results suggest that activation of a fraction of the cell-surface receptor for PDGF is sufficient for the mitogenesis and for the $[Ca^{2+}]_i$ response, and that the PDGF-stimulated lipid metabolism is probably proportional to the number of receptor sites activated by PDGF.

Possible sources of variation in PDGF-induced lipid changes found in cells labelled by different methods

The PDGF-induced changes of labelled lipids at 10 min after the stimulation are partly different, depending on the methods of labelling of the Swiss 3T3 cells used in each experiment (Tables 1, 2 and 3). In the [^{32}P]P_i-prelabelled cells, decreases in [^{32}P]PtdIns(4,5)P_2 and [^{32}P]PtdIns4P were found, but no significant change was observed in [^{32}P]PtdIns (Table 1). A decrease of [^{3}H]PtdIns was found in cells prelabelled with either [$^3H_8$]arachidonic acid or [2-^{3}H]glycerol. However, no significant change in [^{3}H]PtdIns(4,5)P_2 or [^{3}H]PtdIns4P was observed in these ^{3}H-labelled cells (Tables 2 and 3). A PDGF-induced increase in [^{3}H]diacylglycerol was found in the [2-^{3}H]glycerol-prelabelled cells, but not in

the [$^3H_8$]arachidonate-prelabelled cells (Tables 2 and 3). The most plausible cause of these apparent inconsistencies is preferential labelling of certain metabolic pools or certain molecular species of each inositol lipid under the labelling conditions used in each experiment. Cells used in these experiments have not been labelled with each of these labelled lipid precursors up to complete isotope equilibrium in all cellular lipids. The radioactivity proportions of PtdIns(4,5)P_2:PtdIns4P:PtdIns in the control cells are 7.0:2.9:90.1 for cells prelabelled with [^{32}P]P_i for 24 h (Table 1), 4.7:3.2:92.1 for cells prelabelled with [$^3H_8$]arachidonic acid for 4 h (Table 2), 8.2:5.2:86.6 for cells prelabelled with [2-^{3}H]glycerol for 24 h (Table 3), and 3.3:1.8:94.9 for cells prelabelled with *myo*-[2-^{3}H]inositol for 24 h. The different values of the ratio suggest that each labelled precursor might have unevenly labelled different fractions of each inositol lipid pool under the conditions used in this study.

Relationship of inositol lipid turnover to the initiation of cell proliferation by PDGF

There is now convincing evidence which links the PDGF-induced hydrolysis of PtdIns(4,5)P_2 to two key ionic events implicated in cell proliferation (Rozengurt, 1983; Berridge, 1984; Berridge & Irvine, 1984). One product of the hydrolysis, InsP_3, functions in the increase in [Ca^{2+}]$_i$ (Berridge *et al.*, 1984), and the other product, diacylglycerol, activates protein kinase C, which either directly or indirectly enhances Na^+/H^+ exchange, resulting in increases in cytoplasmic Na^+ and pH (Dicker & Rozengurt, 1981; Schuldiner & Rozengurt, 1982; Cassel *et al.*, 1983; Moolenaar *et al.*, 1983; Rosoff *et al.*, 1984). The link between these two major ionic events and the onset of cell proliferation has not been understood. However, it has been shown that expression of the c-*fos* proto-oncogene is induced within a few minutes after exposure of fibroblasts to either PDGF or the tumour-promoting phorbol ester, which is an activator of protein kinase C (Kruijer *et al.*, 1984; Müller *et al.*, 1984).

The two second messengers, InsP_3 and diacylglycerol, initiate a cascade of protein phosphorylation. However, only limited information is available on the phosphoproteins and their activity involved in this cascade and also on those phosphorylated by PDGF-dependent tyrosine kinase (Cooper *et al.*, 1982; Rozengurt *et al.*, 1983; Ek & Heldin, 1984; Kohno, 1985). Therefore, it remains unknown how the inositol lipid turnover and the tyrosine kinase are connected to the induction of cell proliferation by PDGF. It has been shown that prostaglandin $F_{2\alpha}$ and insulin have synergistic effects on cell proliferation in Swiss 3T3 cells (Jimenez de Asua *et al.*, 1981). Prostaglandin $F_{2\alpha}$ induces an increase in [Ca^{2+}]$_i$ and in inositol lipid turnover in Swiss 3T3 cells (Morris *et al.*, 1984; Sasaki, 1985). Insulin activates tyrosine kinase (Kasuga *et al.*, 1982) but does not induce inositol lipid turnover (Farese *et al.*, 1984). Therefore it is possible that inositol lipid turnover and tyrosine kinase act synergistically in the initiation of cell proliferation. One possible mechanism of this synergism is an enhancement of the supply of PtdIns(4,5)P_2, which seems to occur in cells transformed by Rous sarcoma virus (Sugimoto *et al.*, 1984) and avian sarcoma virus UR2 (Macara *et al.*, 1984).

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