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Multiple sources of *sn*-1,2-diacylglycerol in platelet-derived-growth-factor-stimulated Swiss 3T3 fibroblasts

Evidence for activation of phosphoinositidase C and phosphatidylcholine-specific phospholipase D

Robin PLEVIN, Simon J. COOK, Susan PALMER and Michael J. O. WAKELAM*
Molecular Pharmacology Group, Department of Biochemistry, University of Glasgow, Glasgow G12 8QQ, Scotland, U.K.

Platelet-derived growth factor (PDGF) stimulated *sn*-1,2-diacylglycerol (DAG) mass formation in Swiss 3T3 fibroblasts with a lag time of some 30 s. The response was biphasic, with the second phase being sustained over time. PDGF also stimulated the formation of Ins(1,4,5)P_3 with a similar lag time to the DAG response, suggesting that DAG is derived from PtdIns(4,5)P_2 hydrolysis at this time point. PDGF-stimulated phosphatidylcholine (PtdCho) hydrolysis in Swiss 3T3 fibroblasts, as measured by the formation of water-soluble choline metabolites and phosphatidylbutanol (PtdBut) accumulation, was by a phospholipase D (PLD)-catalysed pathway which was kinetically downstream of initial PtdIns(4,5)P_2 hydrolysis. Accumulation of PtdBut increased up to 15 min, suggesting that PLD activity is not rapidly densitized in response to PDGF. The kinetics of PtdCho hydrolysis closely parallelled the second phase of DAG formation, strongly suggesting that during prolonged stimulation periods PtdCho is a major source of DAG in these cells. However, since PtdIns(4,5)P_2 breakdown was also prolonged, PDGF-stimulated DAG may be derived from both phospholipids. Down-regulation of protein kinase C (PKC), by pre-treatment with phorbol 12-myristate 13-acetate, abolished both [^{3}H]choline and [^{3}H]PtdBut formation, suggesting that PLD-catalysed PtdCho hydrolysis may be dependent on PKC activation, supporting its dependence on prior PtdIns(4,5)P_2 hydrolysis.

INTRODUCTION

Platelet-derived growth factor (PDGF), a potent mitogen for smooth-muscle cells and fibroblasts, is believed to play a role in responding to cell damage, repair and proliferation (see review by Ross *et al.*, 1986). The PDGF receptor, like those for epidermal growth factor, insulin and colony-stimulating factor-1, belongs to the class which exhibit intrinsic tyrosine kinase activity (reviewed by Ullrich & Schlessinger, 1990). Occupation of the PDGF receptor results in its autophosphorylation, and this is thought to be an obligatory event in the generation of diverse second-messenger signals (Escobedo & Williams, 1988; Westermark *et al.*, 1990). In fibroblast cell lines, PDGF stimulates the hydrolysis of polyphosphoinositides (Berridge *et al.*, 1984; Hasegawa-Sasaki, 1985), via the phosphorylation of phospholipase C (PLC) γ (Meisenhelder *et al.*, 1989; Wahl *et al.*, 1989), producing Ins(1,4,5)P_3 and *sn*-1,2-diacylglycerol (DAG) (Fukami & Takenawa, 1989). Ins(1,4,5)P_3 stimulates the release of Ca^{2+} from intracellular stores, whereas DAG is the physiological activator of protein kinase C (PKC) [see reviews by Berridge & Irvine (1984) and Nishizuka (1984)].

Although the role of Ins(1,4,5)P_3 in mitogenesis has recently been the focus of attention, it has now become clear that the DAG/PKC arm of the pathway may play a more crucial role. For example, down-regulation of PKC prevents agonist-induced cell proliferation (Lacal *et al.*, 1987), whereas over-expression of the PKC causes disordered growth patterns in fibroblasts (Krauss *et al.*, 1989) and enhanced growth under low-serum conditions in Swiss 3T3 cells (Eldar *et al.*, 1990). Furthermore, it has also been shown that micro-injection of DAG stimulates proliferation in Balb/c 3T3 cells, whereas that of Ins(1,4,5)P_3 is ineffective (Suzuki-Sekimori *et al.*, 1989). Agonist-stimulated formation of DAG and subsequent activation of PKC is therefore a crucial step in the generation of the mitogenic response.

Phosphatidylcholine (PtdCho) has now been recognized as an important long-term source of DAG in mitogen-stimulated cells (Loffelholz, 1989; Pelech & Vance, 1989; Billah & Anthes, 1990; Exton, 1990). We have recently shown that bombesin stimulates PtdCho hydrolysis in Swiss 3T3 cells through PKC-mediated activation of phospholipase D (PLD), an event which is downstream of initial Ins(1,4,5)P_3 and DAG formation (Cook & Wakelam, 1989; Cook *et al.*, 1990). However, it has been reported that some agonists, including noradrenaline (Slivka *et al.*, 1988), interleukin-3 (Whetton *et al.*, 1988) and epidermal growth factor (Wright *et al.*, 1990), may stimulate DAG formation or PtdCho hydrolysis in the absence of an inositol phosphate response, implying that DAG generation from PtdCho may, in part, be controlled directly at the receptor level. Although PDGF has been shown to stimulate PtdCho hydrolysis, DAG and InsP_3 formation in different tissues (Besterman *et al.*, 1986; Price *et al.*, 1989; Blakely *et al.*, 1989), it is unclear whether PtdCho metabolism is downstream of PtdIns(4,5)P_2 hydrolysis or occurs via a distinct mechanism of activation. To address this question, we have examined the kinetics of DAG formation in relation to both PtdIns(4,5)P_2 and PtdCho hydrolysis in PDGF-stimulated Swiss 3T3 fibroblasts.

MATERIALS AND METHODS

Materials

Tissue-culture media and supplies were from Gibco, Paisley, Scotland, U.K. Radiochemicals and PDGF (c-*sis*) were from Amersham International, Amersham, Bucks., U.K. DAG kinase (from *Escherichia coli*) was purchased from Lipidex, Westfield,

Abbreviations used: PDGF, platelet-derived growth factor; PKC, protein kinase C; PLD, phospholipase D; PLC, phospholipase C; DAG, *sn*-1,2-diacylglycerol; DMEM, Dulbecco's modified Eagle's medium; HBG, Hanks' buffered saline containing 1% (w/v) BSA and 10 mM-glucose; PMA, phorbol 12-myristate 13-acetate; PtdBut, phosphatidylbutanol; PtdCho, phosphatidylcholine.

* To whom correspondence should be addressed.

NJ, U.S.A. Ins(1,4,5)P_3 was obtained from Boehringer Mannheim. [^{3}H]Ins(1,3,4)P_3 was generously provided by Dr. R. F. Irvine (A.F.R.C., Babraham, Cambridge, U.K.). All other chemicals were of the highest grades commercially available.

Culture and isotopic labelling of Swiss 3T3 cells

Swiss 3T3 fibroblasts were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% (v/v) newborn-calf serum, 27 mg of glutamine/ml, 250 i.u. of penicillin and 250 μg of streptomycin/μl at 37 °C in air/CO_2 (19:1). Cells were labelled to isotopic equilibrium with: (i) *myo*-[2-^{3}H]inositol [2, 5 or 10 μCi/ml of inositol-free medium containing 1% (v/v) dialysed newborn-calf serum] in 24-well plates for 36–48 h; (ii) [*methyl*-^{3}H]choline (3 μCi/ml of medium containing 1% newborn-calf serum) in 6-well plates for 40–48 h; or (iii) [9,10(n)-^{3}H]palmitic acid (2 μCi/ml of medium containing 1% newborn-calf serum) in 6-well plates for 48 h. For DAG and Ins(1,4,5)P_3 mass experiments, cells were grown (on 6- and 24-well plates respectively) in medium containing 1% newborn-calf serum for 48 h before the experiment. In all cases, whatever labelling protocol was employed, the cells were confluent and quiescent at the time of the experiment.

Measurement of the inositol phospholipid hydrolysis

Cells labelled with *myo*-[2-^{3}H]inositol were washed twice in Hanks' buffered saline (pH 7.4), containing 1% (w/v) BSA (fraction V) and 10 mM-glucose (HBG), then preincubated for a further 20 min in HBG at 37 °C; 10 mM-LiCl was included in the latter incubation period in experiments where prolonged inositol phosphate accumulation was studied. Cells were then incubated with agonist or vehicle in a final volume of 150 μl, and the reaction was terminated by addition of 25 μl of ice-cold 10% (v/v) $HClO_4$. The samples were harvested, neutralized with 1.5 M-KOH/60 mM-Hepes, and the water-soluble [^{3}H]inositol phosphates separated. This involved both anion-exchange chromatography (batch elution of Dowex formate columns; Wakelam *et al.*, 1986) and h.p.l.c. [Partisil 5 WAX column with a gradient of 1–35% (v/v) 1 M-$(NH_4)_2HPO_4$, pH 3.7; 1 ml/min flow rate; fractions collected at 10 s intervals (Black & Wakelam, 1990)]. The resolution of [^{3}H]Ins(1,4,5)P_3 and [^{3}H]Ins(1,3,4)P_3 standards by h.p.l.c. was confirmed before and between experiments. Unlabelled cells were used for the assay of Ins(1,4,5)P_3 mass (Palmer *et al.*, 1989) by using competitive displacement of [^{3}H]Ins(1,4,5)P_3 binding to bovine adrenal-cortex microsomes and quantified by using an unlabelled Ins(1,4,5)P_3 standard curve.

Assay of total [^{3}H]PtdInsP_2 breakdown was by the method of Creba *et al.* (1983). Cells prelabelled with 5 μCi of *myo*-[2-^{3}H]inositol/ml were treated as outlined above. After termination of the reaction with 100 μl of ice-cold 10% $HClO_4$, the precipitate was washed with 500 μl of 5% (w/v) trichloroacetic acid/1 mM-EDTA, and the inositol-containing phospholipids were extracted with 500 μl of $CHCl_3$/CH_3OH/HCl (200:100:1, by vol.). After addition of $CHCl_3$ (500 μl) and 0.1 M-HCl (500 μl), the phases were separated by centrifugation and a sample of the organic layer was dried under vacuum. The samples were dissolved in 500 μl of $CHCl_3$ and treated with 100 μl of CH_3OH and 100 μl of 1 M-NaOH [dissolved in CH_3OH/water (19:1, v/v)] for 30 min. After addition of $CHCl_3$ (500 μl), CH_3OH (300 μl) and water (300 μl), the phases were separated by centrifugation, and a portion of the aqueous phase was neutralized with boric acid. The glycerophospholipids were then separated by Dowex formate anion-exchange chromatography. Glycerophosphoinositol monophosphate (from PtdIns$P$) was eluted with 20 ml of 0.3 M-ammonium formate/0.1 M-formic acid, and the glycerophosphoinositol bisphosphate (from PtdInsP_2) was eluted with 20 ml of 0.75 M-ammonium formate/0.1 M-formic acid.

Measurement of PtdCho hydrolysis

Cells labelled with [*methyl*-^{3}H]choline were washed and preincubated with HBG as described above. Cells were incubated with agonist or vehicle (150 μl) and the reaction was terminated with ice-cold CH_3OH (500 μl). The samples were harvested and transferred to vials together with 200 μl of CH_3OH, which was used to rinse the wells. $CHCl_3$ (500 μl) was added and the lipids were allowed to extract at room temperature for 30 min. $CHCl_3$ (310 μl) and water (310 μl) were added and the samples mixed before centrifugation (2 min at 3000 rev./min) to produce two phases. The water-soluble choline metabolites were separated by cation-exchange chromatography on Dowex (H^+ form) columns (Cook & Wakelam, 1989).

Measurement of DAG mass

Unlabelled cells were washed and preincubated with HBG as described above. Cells were incubated with agonist or vehicle (750 μl) and the reaction was terminated, after aspiration, with ice-cold CH_3OH (750 μl). The samples were harvested and transferred to vials together with 500 μl of CH_3OH, which was used to rinse the well. $CHCl_3$ (500 μl) was added and the lipids were allowed to extract for 60–90 min. Organic and aqueous phases were produced by addition of $CHCl_3$ and water. A sample of the lower organic phase (1 ml) was dried down and stored at −80 °C under N_2 before assay. Samples, or *sn*-1-stearoyl-2-arachidonoylglycerol standards (50–1000 pmol), were incubated with DAG kinase and [γ-^{32}P]ATP (1.25 μCi) in a mixed-micelle preparation [6 mol% PtdSer/0.3% (w/v) Triton X-100] in 50 mM-imidazole (pH 6.6) containing 50 mM-NaCl, 12.5 mM-$MgCl_2$ and 1.25 mM-EGTA at 30 °C for 30 min. The products were separated by t.l.c., and the radioactivity of the band co-migrating with pure 1-stearoyl-2-arachidonoyl-*sn*-glycerophosphate (R_F 0.41) was determined. DAG mass was quantified by using the *sn*-1-stearoyl-2-arachidonoylglycerol standard curve. Recoveries (in the range 70–80%) were linear over the standard curve and not affected by tissue extract.

Assay of PLD phosphatidyltransferase activity

Cells labelled with [9,10(n)-^{3}H]palmitate were washed in serum-free DMEM containing 20 mM-Hepes, pH 7.4, and 1% BSA (DMBH) for 20 min at 37 °C, followed by incubation for a further 5 min with DMBH containing 30 mM-butan-1-ol. Cells were incubated with agonist or vehicle (1 ml), and the reaction was terminated by removal of the medium and addition of ice-cold CH_3OH (500 μl). Samples were harvested and transferred to glass vials together with 200 μl of CH_3OH, which had been used to rinse the well. $CHCl_3$ (700 μl) was added and the lipids were extracted at room temperature for 15 min. Phases were resolved by addition of 1 M-NaCl (585 μl) and centrifugation (3000 rev./min, 5 min). The lower phase was dried *in vacuo*, dissolved in chloroform/methanol (100 μl; 19:1, v/v) and applied to Whatman LK5DF t.l.c. plates which were developed in the organic phase of 2,2,4-trimethylpentane/ethyl acetate/acetic acid/water (5:11:2:10, by vol). The radioactivity of [^{3}H]-phosphatidylbutanol ([^{3}H]PtdBut), identified by its co-migration with authentic [^{14}C]PtdBut, was determined. In separate experiments, the identity of [^{3}H]PtdBut was confirmed by its dependence on butan-1-ol concentration and by its formation in unlabelled Swiss 3T3 cells in the presence of carrier-free [^{3}H]butan-1-ol (results not shown).

RESULTS

The kinetics of PDGF-stimulated DAG mass formation is shown in Fig. 1. DAG production was biphasic and significant after a lag time of approx. 30 s. The first phase of the response was small, giving a 20–30% increase over basal values at 30 s. DAG formation then declined to basal values between 30 and 60 s, before rising again, between 1 and 5 min. DAG levels were

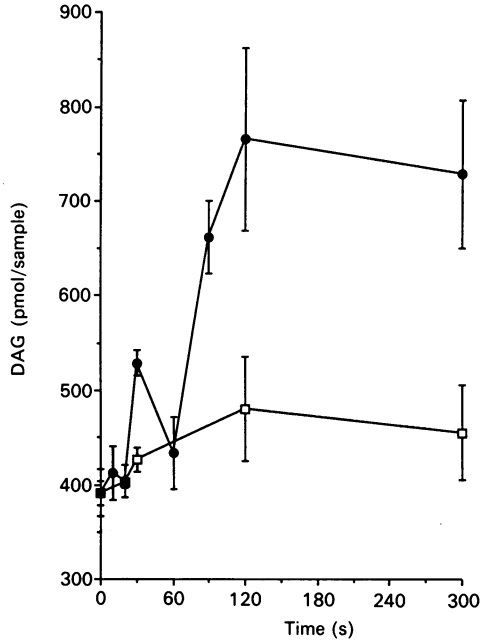

Fig. 1. Time course of PDGF-stimulated DAG mass formation in Swiss 3T3 fibroblasts

Cells, treated as outlined in the Materials and methods section, were incubated with vehicle (□) or PDGF (30 ng/ml) (●) for the times indicated. Each point represents the mean ± S.D. of triplicate determinations from a single experiment, typical of three.

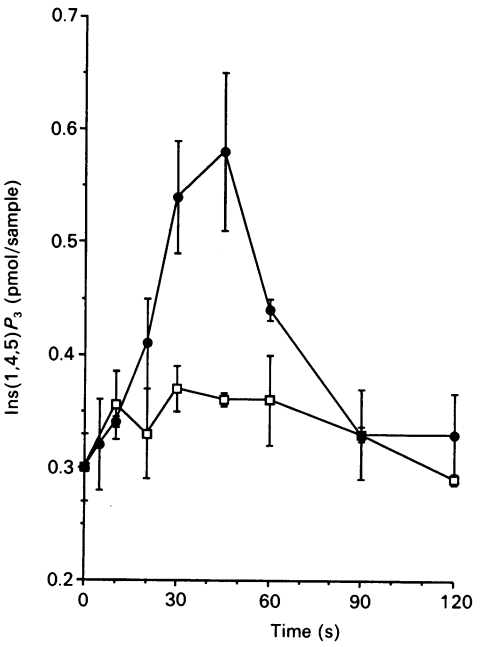

Fig. 2. Time course of PDGF-stimulated Ins(1,4,5)P_3 mass formation in Swiss 3T3 fibroblasts

Cells, treated as described in the Materials and methods section, were incubated with vehicle (□) or PDGF (30 ng/ml) (●) for the times indicated. Each point represents the mean ± S.D. of triplicate determinations from a single representative experiment ($n = 3$).

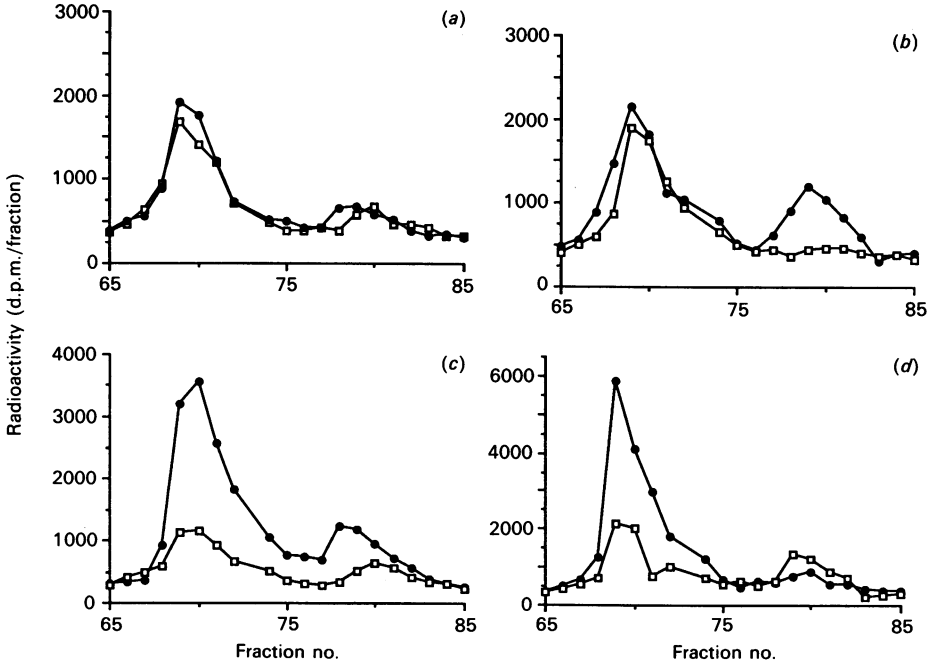

Fig. 3. H.p.l.c. analysis of [^{3}H]Ins(1,4,5)P_3 and [^{3}H]Ins(1,3,4)P_3 in control and PDGF-stimulated Swiss 3T3 fibroblasts

Cells, treated as outlined in the Materials and methods section, were incubated with either vehicle (□) or PDGF (30 ng/ml) (●) for 15 s (*a*), 30 s (*b*), 60 s (*c*) or 120 s (*d*). Standard [^{3}H]Ins(1,3,4)P_3 was eluted between fractions 66 and 74, and [^{3}H]Ins(1,4,5)P_3 was eluted between fractions 76 and 83. Each profile is representative of a single representative experiment, performed in triplicate.

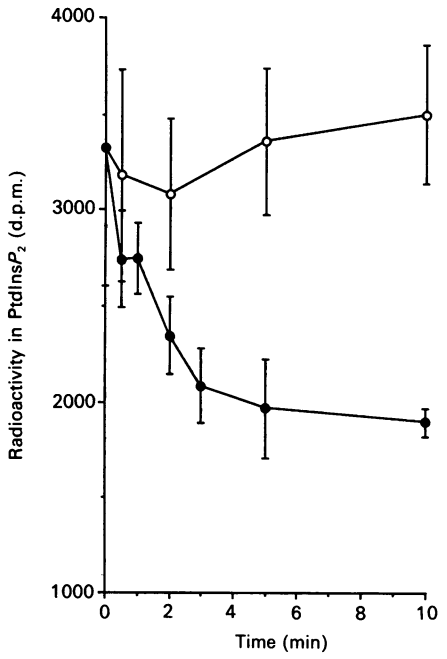

Fig. 4. Time course of PDGF-stimulated [^{3}H]PtdInsP_2 hydrolysis in Swiss 3T3 fibroblasts

Cells, treated as outlined in the Materials and methods section, were incubated with vehicle (□) or PDGF (30 ng/ml) (●) for the times indicated. Each point represents the mean ± S.D. of triplicate determinations from a single experiment, typical of three.

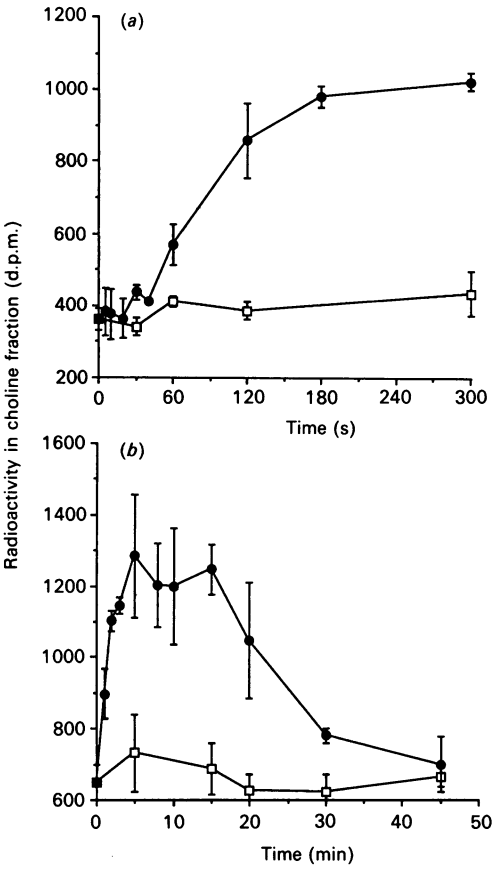

Fig. 5. Time course of PDGF-stimulated [^{3}H]choline formation in Swiss 3T3 fibroblasts

Cells, treated as outlined in the Materials and methods section, were incubated with vehicle (□) or PDGF (30 ng/ml) (●) for the times indicated. Each point represents the mean ± S.D. of triplicate determinations from a single experiment, typical of three.

maintained at stimulated values, approximately twice basal, for up to 15 min (results not shown).

The kinetics of the PDGF-stimulated Ins(1,4,5)P_3 formation were examined by using both the Ins(1,4,5)P_3 mass assay and h.p.l.c. analysis of [^{3}H]inositol-labelled cell extracts (Figs. 2 and 3). PDGF stimulation resulted in a 2–3-fold increase in Ins(1,4,5)P_3, mass which was observed as early as 20–30 s (Fig. 2). Although the lag time for the onset of the response varied between individual experiments (between 20 and 40 s), it was clear that Ins(1,4,5)P_3 production was detected earlier than has previously been reported from studies involving the use of isotopic techniques (Blakely *et al.*, 1989). H.p.l.c. analysis of [^{3}H]Ins(1,3,4)P_3 and [^{3}H]Ins(1,4,5)P_3 formation (Fig. 3) suggested that there was no significant production of either isomer at 15 s of stimulation. However, a clear 2–3-fold increase in [^{3}H]Ins(1,4,5)P_3 formation was observed at 30 s, which was decreased at 60 s and absent at 2 min. In contrast, [^{3}H]Ins(1,3,4)P_3 formation did not increase significantly until after 60 s of stimulation (2-fold increase), at which time it was the predominant product. [^{3}H]Ins(1,3,4)P_3 continued to be the major InsP_3 isomer present at 2 min stimulation (control: [^{3}H]Ins(1,4,5)P_3, 5149 ± 808; [^{3}H]Ins(1,3,4)P_3, 8945 ± 307; stimulated: [^{3}H]Ins(1,4,5)P_3, 5233 ± 675; [^{3}H]Ins(1,3,4)P_3, 19348 ± 900 d.p.m.; means ± S.D. of duplicate samples from a single typical experiment).

The time course of PDGF-stimulated [^{3}H]PtdInsP_2 hydrolysis (measured as glycerophosphoinositol bisphosphate) is shown in Fig. 4. After a lag period of 20–30 s, [^{3}H]PtdInsP_2 decreased rapidly to approx. 60% of control values after 3 min exposure of PDGF. Stimulated levels remained significantly decreased for the remainder of the time course. A similar result was obtained for PtdIns$P$ hydrolysis (results not shown), whereas PtdIns was not analysed.

PDGF-stimulated [^{3}H]PtdCho hydrolysis was examined by measuring the intracellular formation of water-soluble [^{3}H]choline-labelled metabolites over both short and prolonged periods (Fig. 5). PDGF stimulated an increase in intracellular [^{3}H]choline. The response was detected after a lag time of approx. 60 s and reached a peak at 2–5 min, at which time [^{3}H]choline formation was approx. 100% above basal values (Fig. 5*a*). [^{3}H]Choline levels remained elevated for 15–20 min, but then declined to basal values between 20 and 45 min (Fig. 5*b*). Formation of the other choline metabolites, [^{3}H]glycerophosphocholine and [^{3}H]phosphocholine, was not affected by PDGF at any time examined (results not shown), suggesting that PDGF stimulates [^{3}H]PtdCho breakdown, primarily through PLD activation.

To examine this hypothesis, PLD activity was assayed by the formation of [^{3}H]PtdBut in [^{3}H]palmitate-labelled cells, incubated in the presence of 30 mM-butan-1-ol (Fig. 6). PDGF stimulated [^{3}H]PtdBut accumulation in Swiss 3T3 fibroblasts, again with a lag time of approx. 60s (Fig. 6*a*). [^{3}H]PtdBut accumulation then increased in a linear manner before reaching a maximum between 15 and 20 min. After this time no further accumulation of [^{3}H]PtdBut was observed (Fig. 6*b*).

The dose–response relationship for PDGF-stimulated [^{3}H]inositol phosphate accumulation and [^{3}H]choline formation was also examined. Both responses occurred over a similar concentration range, with comparable EC_{50} values (concn. giving 50% of maximal response) ([^{3}H]Ins$P$ 4.13 ± 2.12 ng/ml, $n = 3$ experiments; [^{3}H]choline 2.02 ± 0.33 ng/ml, $n = 4$ experiments; data fitted to a logistic equation). These results suggest that both PLC-catalysed PtdInsP_2 hydrolysis and PLD-catalysed PtdCho

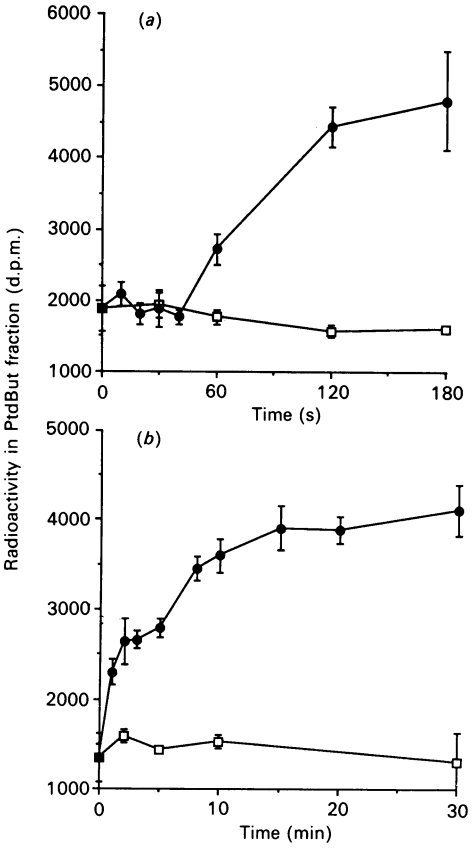

Fig. 6. Time course of PDGF-stimulated [^{3}H]PtdBut formation in Swiss 3T3 fibroblasts

Cells, treated as outlined in the Materials and methods section, were incubated with vehicle (□) or PDGF (30 ng/ml) (●) for the times indicated. Each point represents the mean ± S.D. of triplicate determinations from a single experiment, typical of three.

Table 1. Effect of PKC down-regulation on PDGF- and PMA-stimulated [^{3}H]choline and [^{3}H]PtdBut formation in Swiss 3T3 fibroblasts

Cells, treated as outlined in the Materials and methods section, were incubated with vehicle, PDGF (30 ng/ml) or PMA (400 nM) for 10 min (choline) or 20 min (PtdBut) and assayed for labelled [^{3}H]choline or [^{3}H]PtdBut. Each value represents the mean ± S.D. of triplicate determinations from a single representative experiment.

	Radioactivity in [^{3}H]choline (d.p.m.)		Radioactivity in [^{3}H]PtdBut (d.p.m.)	
Incubation	Control	Down-regulated	Control	Down-regulated
Vehicle	6518 ± 155	6292 ± 450	1654 ± 464	2866 ± 805
PMA	11196 ± 432	5932 ± 1042	21697 ± 953	2224 ± 757
PDGF	11649 ± 423	6796 ± 533	12605 ± 1029	2686 ± 211

hydrolysis may be controlled through activation of a single population of receptors.

To investigate the role of PKC in PDGF-stimulated [^{3}H]choline and [^{3}H]PtdBut formation, Swiss 3T3 cells were pre-treated with phorbol 12-myristate 13-acetate (PMA; 400 nM) for 48 h. Such treatment depletes these cells of PKC activity (Rodriguez-Pena & Rozengurt, 1984), PKC itself (by Western blotting; Brown *et al.*, 1990) and abolishes [^{3}H]phorbol 12,13-dibutyrate binding (S. Currie & M. J. O. Wakelam, unpublished work). Stimulation of control cells [pre-treated for 48 h with 0.1% (v/v) dimethyl sulphoxide] with either PMA or PDGF resulted in the formation of [^{3}H]choline and [^{3}H]PtdBut (Table 1). The response observed to both stimulants was completely attenuated in PKC-down-regulated cells.

DISCUSSION

The molecular mechanisms by which PDGF exerts its mitogenic effects remain unclear. However, the activation of PKC is believed to be necessary (Lacal *et al.*, 1987). Since DAG, produced in response to agonist stimulation, functions as the endogenous activator of PKC, the regulation of DAG production is critical to the mechanism by which PDGF stimulates cell proliferation. Two phospholipid sources from which DAG has been shown to be formed are PtdIns(4,5)P_2 and PtdCho (Pessin & Raben, 1989; Pessin *et al.*, 1990). By examining the kinetics of PDGF-induced Ins(1,4,5)P_3 and choline formation, we have sought to determine the phospholipid source of DAG at both early and late stimulation times.

In Swiss 3T3 cells. PDGF stimulated the biphasic production of DAG (Fig. 1). This finding is consistent with the observed responses in PDGF-stimulated 3T3-L1 and vascular-smooth-muscle cells (Hasegawa-Sasaki, 1985; Fukami & Takenawa, 1989; Sachinidis *et al.*, 1990). This biphasic increase in DAG is also characteristic of a number of G-protein-linked receptors (Griendling *et al.*, 1986; Cook *et al.*, 1990). The relatively slow onset of the response (lag time of 30 s) compared with receptor–G-protein-linked systems presumably reflects the delay between agonist occupation of the receptor, its autophosphorylation and subsequent phosphorylation of the effector(s) (Wahl *et al.*, 1989; Kazlauskas & Cooper, 1989).

PDGF-stimulated InsP_3 formation was also significant after 30 s, as measured by analysing both Ins(1,4,5)P_3 mass levels and by h.p.l.c. (Figs. 2 and 3). This increase coincided with the first phase of DAG production, suggesting that DAG is derived from the hydrolysis of PtdIns(4,5)P_2 at early time points. This suggestion is supported by the observed decrease in the levels of total radiolabelled PtdInsP_2 at 30 s, the first time point recorded. Using h.p.l.c. we have also confirmed that Ins(1,4,5)P_3 was formed before Ins(1,3,4)P_3. This suggested that initial PDGF-stimulated inositol phosphate generation was a result of PLCγ-catalysed PtdIns(4,5)P_2 hydrolysis rather than through the initial activation of the Type 1 PtdIns kinase pathway. The sustained decrease in total PtdInsP_2 levels also indicated that, although Ins(1,4,5)P_3 levels rapidly returned to basal values, stimulated PLC activity was maintained. This finding is in agreement with Fukami & Takenawa (1989), and suggests that the activities of the Ins(1,4,5)P_3 phosphatase and kinase prevent the sustained accumulation of the active InsP_3 isomer in this tissue. The relatively short lag time in the observed onset of Ins(1,4,5)P_3 formation was considerably smaller than that observed in some other recent studies in PDGF-stimulated Swiss 3T3 cells (Nanberg & Rozengurt, 1988; Blakely *et al.*, 1989); however, a similar onset (20–30s) has been observed both in Balb/c 3T3 cells (Fukami & Takenawa, 1989) and in smooth-muscle cells (Sachinidis *et al.*, 1990). The explanation for the differences observed is not clear, although the small increase in Ins(1,4,5)P_3 mass (approx. 2–3-fold) observed in our study may have previously been overlooked in preference to the later, but larger, accumulation of Ins(1,3,4)P_3 (Nanberg & Rosengurt, 1988).

PDGF stimulated PtdCho hydrolysis in Swiss 3T3 cells; this was demonstrated both by [^{3}H]choline formation (Fig. 5) and by PtdBut accumulation (Fig. 6). However, a lag time of some 60s was observed in the onset of both measures, indicating that

PtdCho hydrolysis was kinetically downstream of initial PtdIns(4,5)P_2 breakdown. Studies before the present one have not sought to assess closely the onset of PDGF-stimulated PtdCho hydrolysis in Swiss 3T3 fibroblasts (Price *et al.*, 1989; Larrodera *et al.*, 1990) and in other cell lines such as II C9 fibroblasts, PDGF can stimulate DAG generation from PtdCho hydrolysis apparently in the absence of PtdIns(4,5)P_2 breakdown (Pessin *et al.*, 1990). Therefore it is possible that PtdCho breakdown may be activated directly, rather than via a sequential pathway involving PtdIns(4,5)P_2 hydrolysis. If this was the case, then choline formation may be expected to precede, or be simultaneous with, Ins(1,4,5)P_3 formation. Consequently it would appear that the hydrolysis of the two lipids is indeed linked (see Figs. 2 and 6). This sequential nature of the pathway was also supported by the observation that in PKC-down-regulated cells, PtdBut accumulation and choline formation in response to both PDGF and PMA were abolished. This suggested that intermediate activation of PKC is required to activate PtdCho in PDGF-stimulated cells, a finding consistent with the results of Price *et al.* (1989). PDGF-stimulated PtdBut formation was also abolished by preincubation of the cells with the PKC inhibitor Ro-31-8220, a finding also consistent with this hypothesis (results not shown).

That an increase in [^{3}H]choline was detected before [^{3}H]phosphocholine suggested that a PLD-catalysed pathway was involved. This was confirmed by demonstrating that PDGF activated the transphosphatidylation reaction, assayed by [^{3}H]PtdBut formation (Fig. 6), a definitive marker for PLD stimulation (Billah *et al.*, 1989). This finding contrasts with the results of Larrodera *et al.* (1990), who suggested that PDGF-stimulated PtdCho hydrolysis involved a PtdCho-specific PLC. However, the earliest stimulation time in their study was after 2 h. Warden & Friedkin (1985) have shown that choline kinase activity is increased by some 3-fold after a 2 h incubation with PDGF containing serum. Thus any choline formed via PLD activation would be rapidly phosphorylated to phosphocholine, which could account for these observations. PDGF-stimulated [^{3}H]choline formation in Swiss 3T3 fibroblasts has been observed by others (Price *et al.*, 1989), again implicating PLD-catalysed PtdCho hydrolysis. However, only a single time point (20 min) was investigated by Price *et al.* (1989), which precludes a detailed mechanistic analysis of this hydrolytic pathway.

The second sustained phase of the PDGF-stimulated DAG response coincided with both PtdBut accumulation and choline formation in Swiss 3T3 cells. This strongly suggests that DAG may be derived, at least partly, from the sustained hydrolysis of PtdCho. This has been proposed for both G-protein-receptor linked agonists such as bombesin (Cook & Wakelam, 1989), endothelin-1 (MacNulty *et al.*, 1990) and gonadotropin-releasing hormone (Lavie & Liscovitch, 1990) and for tyrosine kinase receptor agonists such as epidermal growth factor (Pessin *et al.*, 1990). However, it is not clear in many of these studies if sustained activation of PLD is involved. It has recently been shown that in carbachol-stimulated 1321N1 cells (Martinson *et al.*, 1991), vasopressin-stimulated A10 smooth-muscle cells and bombesin-stimulated Swiss 3T3 fibroblasts (R. Plevin & M. J. O. Wakelam, unpublished work) PLD activation of PtdCho hydrolysis is a transient phenomenon. Indeed in many instances the accumulation of PtdBut or the formation of total labelled choline has not been observed beyond 2 min (Cook & Wakelam, 1989, 1991; Lavie & Liscovitch, 1990; Martin & Michaelis, 1989). In the present study, we have shown that PtdBut accumulated and that intracellular choline formation was maintained for up to 15 min. This suggests that PLD activity is maintained for a far longer period in response to PDGF than to most agonists.

In this study we have shown that PDGF stimulates the biphasic formation of DAG. At early times this is probably derived from PtdIns(4,5)P_2 hydrolysis. PDGF also stimulated PtdCho hydrolysis, through a sequential pathway, kinetically downstream of initial PtdIns(4,5)P_2 breakdown, involving the prior activation of PKC. However, in contrast with G-protein-linked agonists, both PDGF-stimulated PtdIns(4,5)P_2 hydrolysis and PLD-activated PtdCho breakdown were sustained for up to 15 min. Therefore, between 1 and 20 min of stimulation DAG may be derived from both PtdIns(4,5)P_2 and PtdCho.

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