Platelet-derived growth factor stimulates synthesis of 1,2-diacylglycerol from monoacylglycerol in Balb/c 3T3 cells

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1,2-Diacylglycerol (1,2-DAG) plays an important role in the protein kinase C-mediated signal-transduction system. Several reports have shown that 1,2-DAG is generated through various pathways other than classical phospholipid hydrolysis. We observed a rapid incorporation of [³H]myristate into 1,2-DAG in platelet-derived-growth-factor (PDGF)-treated Balb/c 3T3 cells. [¹⁴C]Palmitate was similarly incorporated into 1,2-DAG. The effect of PDGF, which was inhibited by cycloheximide, became maximal after 60 min treatment with PDGF, and disappeared 300 min after removal of PDGF. Treatment with triacylglycerol lipase revealed that labelled saturated fatty acid was incorporated into the *sn*-1 position. PDGF barely stimulated incorporation of [³H]glycerol or [¹⁴C]glucose into 1,2-DAG. Incorporation of [³H]myristate into 1,2-DAG preceded that into triacylglycerol and phospholipids. These results suggest that synthesis of 1,2-DAG from monoacylglycerol is enhanced in PDGF-treated cells. However, there is no significant difference in the activity of monoacylglycerol acyltransferase measured *in vitro* in quiescent and PDGF-treated cells. The reason for this discrepancy is discussed.

INTRODUCTION

Protein kinase C is a key enzyme in membrane signal transduction and mediates a variety of biological functions, such as cell proliferation, cell differentiation and secretion (for review, see [1]). The activation of protein kinase C requires Ca²⁺ and acidic phospholipid. In addition, DAG enhances Ca²⁺-sensitivity of the enzyme and renders it fully active in the presence of physiological concentrations of Ca²⁺. Thus DAG plays an important role in the protein kinase C-mediated system. A classical pathway for DAG generation is via an activation of phosphoinositide-specific phospholipase C. Several reports have shown that DAG is produced by other mechanisms: hydrolysis of PC [2,3], deacylation of TAG [4], synthesis *de novo* [5] and glycolipid hydrolysis [6].

In the present study, we attempted to investigate whether another mechanism of DAG generation is involved during cell proliferation in Balb/c 3T3 cells. Quiescent Balb/c 3T3 cells require two types of growth factors for initiation of DNA replication: competence factor and progression factor [7]. PDGF and other known competence factors act initially to render quiescent cells competent to respond to progression factors in platelet-poor plasma. Biochemical mechanisms of the action of these competence factors are of great interest. PDGF is believed to exert its mitogenic action through breakdown of inositol phospholipids and resultant production of DAG and inositol phosphates [8]. Moreover, PDGF is shown to elicit various other effects on lipid metabolism: PDGF stimulates synthesis of TAG and phospholipids other than phosphoinositides and production of arachidonate and prostaglandins [9–11]; and PDGF

enhances production of DAG through hydrolysis of PC in 3T3-L₁ cells, as shown by Besterman *et al.* [2]. It is therefore possible that, in PDGF-treated cells, generation of DAG is augmented via several pathways. We examined this possibility by measuring incorporation of labelled fatty acids in quiescent and in competent cells. We found that generation of 1,2-DAG from MAG is increased in PDGF-treated cells.

EXPERIMENTAL

Materials

Partially purified PDGF was prepared from outdated platelet-rich plasma [12], obtained from Red Cross Hospital, Tokyo. [*methyl*-³H]Thymidine (42 Ci/mmol), [9,10-³H]myristate (22.4 Ci/mmol), [2-³H]glycerol (200 mCi/mmol), [1-¹⁴C]arachidonic acid (54.9 mCi/mmol), [1-¹⁴C]palmitic acid (8.9 mCi/mmol), [1-¹⁴C]acetic anhydride (10 mCi/mmol), D-[1-¹⁴C]glucose (5 mCi/mmol) and [³²P]P_i (carrier-free) were obtained from New England Nuclear. [¹⁴C]Myristoyl-CoA (54 mCi/mmol) was from Amersham Corp. *Rhizopus arrhizus* triacylglycerol lipase and lipids other than 2-mono-C_{18:1}-acylglycerol were purchased from Sigma. 2-Mono-C_{18:1}-acylglycerol was from Serdary Research Laboratories.

Cell culture

Balb/c 3T3 cells (clone A31) were grown in Dulbecco's modified Eagle's (DME) solution supplemented with 10% (v/v) calf serum as described previously [13]. For experimental purposes, cells were plated at a density of 2 × 10⁵ cells/35 mm Falcon plastic plate containing 2 ml

Abbreviations used: PDGF, platelet-derived growth factor; MAG, monoacylglycerol; DAG, diacylglycerol; TAG, triacylglycerol; PA, phosphatidic acid; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; DME medium, Dulbecco's modified Eagle's solution.

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of culture medium supplemented with 10% calf serum, and experiments were performed on day 6. For measurement of DNA synthesis, cells were plated in Corning 24-well plates at a density of 5×10^4 cells/ml.

Lipid extraction and t.l.c.

Neutral lipids were extracted from the cell lysate with chloroform/methanol/conc. HCl (10:20:1, by vol.) [14] and were separated on silica-gel G plates with benzene/chloroform/methanol (16:3:1, by vol.) as solvent. Phospholipids were extracted with chloroform/methanol (1:1, v/v), and were usually analysed in the solvent system chloroform/methanol/28 M-NH₃ (13:7:1, by vol.). In this solvent system, PI and PS cannot be separated. For PA analysis, chloroform/acetone/methanol/acetic acid/0.1 M-boric acid (125:50:25:25:3, by vol.) was used as solvent. The major lipids were identified by brief exposure to iodine, and the ³²P- or ¹⁴C-labelled lipids were identified by autoradiography. Ninhydrin and rhodamine 6G were used when necessary.

Assay of MAG acyltransferase activity

Cells plated at a density of 1×10^6 cells/Falcon dish were scraped off with a rubber policeman and homogenized in solution containing 10 mM-Tris/HCl (pH 7.4), 10 mM-EDTA and 2 mM-phenylmethanesulphonyl fluoride with an ultrasonic cell disruptor at 4 °C to obtain whole-cell lysate. Cell homogenization was done just before the enzyme assay. MAG acyltransferase activity was determined by the method of Coleman & Haynes [15]. The reaction mixture contained 175 mM-Tris/HCl (pH 7.0), 1 mg of bovine serum albumin/ml, 15 mg of 1:1 (w/w) mixture of PC and PS, 10 nCi of [¹⁴C]myristoyl-CoA and 75 nM-*sn*-mono-C_{18:1}-acylglycerol in a final volume of 200 µl. The reaction was stopped by adding 3.1 ml of methanol/chloroform/HCl (20:10:1, by vol.). The generated 1,2-DAG was extracted and radioactivity was quantified.

Treatment with TAG lipase

PDGF-treated cells were prelabelled with 1 µCi of [³H]myristate/ml and 0.1 µCi of [¹⁴C]arachidonate/ml for 10 min. Neutral lipids from these cells were applied on to t.l.c., and the radiolabelled 1,2-DAG was extracted with diethyl ether, dried down under N₂ gas and stored at -20 °C. The TAG lipase reaction was carried out in 0.02 M-acetate buffer (pH 5.6) containing 1 mg of bovine serum albumin/ml and 2 mM-CaCl₂. Reactions were started by adding 40 units of *Rhizopus arrhizus* lipase/ml. Samples were incubated for 60 min at 30 °C. Liberated fatty acids and remaining MAG were analysed by t.l.c. with hexane/diethyl ether/acetic acid (80:40:1, by vol.) as solvent.

Assay of DNA synthesis

Cells were cultured in 24-well plates for 6 days. Density-arrested cells were treated with DME medium containing either 10 units of PDGF/ml dissolved in 10 µl of 100 mM-acetic acid or 10 µl of 100 mM-acetic acid as vehicle for 3 h to obtain competent and quiescent cells. Cells were then washed three times with DME medium and were incubated with DME medium alone for times indicated in Fig. 2(b). Then the medium was changed again in DME medium containing 1 µCi of [³H]-thymidine/ml and 5% platelet-poor plasma. Cells were

further incubated for 24 h, and [³H]thymidine incorporation into trichloroacetic acid-precipitable material was measured as described by McNiel *et al.* [16].

Measurement of DAG mass

This was performed by the method of Bansbach *et al.* [17]. Cells were cultured in 100 mm-diam. Falcon dishes, and quiescent and competent cells were obtained as described above. Neutral lipids extracted from these cells were applied on to t.l.c. 1,2-DAG was extracted with diethyl ether and transferred into a small glass tube. After the diethyl ether was dried, 25 µl of pyridine, 6 µl of [¹⁴C]acetic anhydride and 0.3 µl of HClO₄ were added. DAG was completely acetylated by incubation for 30 min. Acetylated DAG produced was separated by t.l.c. with heptane/ether/acetic acid (40:10:1, by vol.) as solvent, and the radioactivity was counted. To plot a standard curve, we acetylated a known amount of 1,2-dipalmitoylglycerol. The reaction was linear in terms of DAG mass from 0.02 to 0.4 µmol per assay.

RESULTS

Incorporation of myristate into 1,2-DAG in quiescent and PDGF-treated cells

To determine whether production of 1,2-DAG was augmented in PDGF-treated cells, we measured incorporation of fatty acids into 1,2-DAG in quiescent and PDGF-treated cells. Balb/c 3T3 cells were cultured as described in the Experimental section. On day 6, the spent medium was replaced with the medium containing vehicle or 10 units of PDGF/ml. After incubation for 3 h, the medium was removed and cells were washed three times with DME medium. Then, 1 ml of new medium containing 1 µCi of [³H]myristate/ml, 0.05 µCi of [¹⁴C]arachidonate/ml and 1 mg of fatty acid-free albumin/ml was added to each culture dish. At the time points indicated, the reaction was stopped by adding ice-cold saline, and radioactivity in the 1,2-DAG fraction was measured.

In quiescent cells, incorporation of labelled myristate increased biphasically, with a peak value of $(1.50 \pm 0.42) \times 10^3$ c.p.m. at 5 min. The radioactivity then decreased slightly and remained elevated for 120 min. In PDGF-treated cells, incorporation of labelled myristate into 1,2-DAG was also biphasic, and the peak value was increased approx. 2-fold (Fig. 1a). The radioactivity of labelled myristate accumulated in the 1,2-DAG fraction at 1 min was $(9.60 \pm 0.83) \times 10^2$ c.p.m., and the peak value of $(3.40 \pm 0.48) \times 10^3$ c.p.m. was obtained at 5 min. The radioactivity then decreased to a steady-state value, which was approx. 150% of that observed in quiescent cells.

Although incorporation of labelled arachidonate into 1,2-DAG was also biphasic, there was no initial peak of arachidonate incorporation (Fig. 1b). In quiescent cells, radioactivity in 1,2-DAG increased rapidly to a first plateau within 5 min, and then further increased gradually. In PDGF-treated cells, radioactivity in DAG was nearly twice that in quiescent cells.

When incorporation of radioactive palmitate, another saturated fatty acid, into 1,2-DAG was examined, the incorporation was biphasic, with an initial peak followed by a plateau phase. The initial peak of incorporation in PDGF-treated cells was approx. 150% of that in quiescent cells (Fig. 1c).

Fig. 1. Incorporation of $[^3\text{H}]$ myristate, $[^{14}\text{C}]$ arachidonate and $[^{14}\text{C}]$ palmitate into 1,2-DAG

Cells were cultured as described in the Experimental section. On day 6, the cultures were divided into groups. Quiescent ($\bullet$) and PDGF-treated ($\circ$) cells were incubated with medium containing $1 \mu\text{Ci}$ of $[^3\text{H}]$ myristate/ml (a), $0.05 \mu\text{Ci}$ of $[^{14}\text{C}]$ arachidonate/ml (b) and $0.05 \mu\text{Ci}$ of $[^{14}\text{C}]$ palmitate/ml (c) for the time indicated, and the radioactivity accumulated in 1,2-DAG was counted. Data represent means $\pm$ S.E.M. for three parallel cultures.

In contrast with incorporation into DAG, incorporation of labelled fatty acid into TAG was slower, and reached a maximum at 60 min. Incorporation of both arachidonate and myristate into TAG was greater in PDGF-treated cells than in quiescent cells (results not shown). Radioactivities in labelled free myristate were identical in both quiescent and competent cells.

Time course of effect of PDGF on myristate incorporation into DAG

Fig. 2(a) shows the time course of the action of PDGF on the rate of incorporation of myristate into 1,2-DAG. After incubation for 60 min with PDGF, incorporation was fully activated. When PDGF was removed after 180 min treatment, the enhanced rate reverted to a value similar to that in quiescent cells after 5 h.

PDGF-induced activation of myristate incorporation into 1,2-DAG decreased within 3 h after the removal of PDGF (Fig. 2a), whereas PDGF-induced activation of incorporation of $[^3\text{H}]$ thymidine remained fully activated for as long as 5 h (Fig. 2b). This fact indicates that the

Fig. 2. Time course of effect of PDGF on $[^3\text{H}]$ myristate incorporation into 1,2-DAG and on DNA synthesis

(a) Quiescent Balb/c 3T3 cells were incubated in medium containing 10 units of PDGF/ml. After incubation for 20, 60 or 180 min, the medium was changed to one containing $1.0 \mu\text{Ci}$ of $[^3\text{H}]$ myristate/ml, and radioactivity incorporated into 1,2-DAG during 10 min was counted. In some experiments, cells were incubated for 180 min with PDGF, and the medium was changed to that without PDGF. After incubation for 1, 3 or 5 h with PDGF-free medium, the medium was changed to one containing $[^3\text{H}]$ myristate, and radioactivity incorporated into 1,2-DAG during 10 min was counted. (b) Quiescent cells were incubated in medium containing 10 units of PDGF/ml. After 180 min, cells were washed and the PDGF-treated cells were incubated in medium alone for 1, 2, 3 or 5 h. Then the medium was changed to one containing 5% plasma and $1 \mu\text{Ci}$ of $[^3\text{H}]$ thymidine/ml and incubated further for 24 h. DNA synthesis was assayed as described in the Experimental section. The data are means $\pm$ S.E.M. for three parallel cultures.

PDGF-induced competence state persists even 5 h after the removal of PDGF.

Effect of cycloheximide on PDGF-induced enhancement of myristate incorporation into DAG

It is known that PDGF-induced competence is not affected by treatment with cycloheximide, whereas actinomycin D abolishes the competence-inducing activity of PDGF [7]. To determine whether PDGF-induced enhancement of myristate incorporation into DAG shares the same characteristics, we examined the effect of cycloheximide. Cycloheximide ($100 \mu\text{g/ml}$) abolished the effect of PDGF on the incorporation of myristate into 1,2-DAG, whereas actinomycin D ($10 \mu\text{g/ml}$) had essentially no effect (results not shown).

Table 1. Effect of *Rhizopus arrhizus* TAG lipase

PDGF-treated cells were incubated for 10 min in medium containing 1 μ Ci of [3 H]myristate/ml and 0.1 μ Ci of [14 C]arachidonate/ml. Lipids were extracted and were applied on to t.l.c. Labelled DAG was obtained by scraping plates and extraction with diethyl ether, and then incubated with *Rhizopus arrhizus* TAG lipase for 60 min. The radioactivities of 3 H and 14 C in DAG, MAG and fatty acids before and after the treatment were counted. Values are means of three parallel cultures.

	Radioactivity (c.p.m.)					
	Fatty acid		MAG		DAG	
	3 H	14 C	3 H	14 C	3 H	14 C
Before	—	—	—	—	12312	1344
After	9398	94	8906	198	1173	1054

Incorporation of myristate into the *sn*-1 position

To determine whether myristate was incorporated into the *sn*-1 or *sn*-2 position of DAG, we employed *Rhizopus arrhizus* TAG lipase. This lipase does not liberate fatty acid from the *sn*-2 position. We labelled PDGF-treated cells with [3 H]myristate and [14 C]arachidonate, giving double-labelled 1,2-DAG. When the double-labelled 1,2-DAG was incubated with TAG lipase, about 80 % of labelled myristate was liberated from 1,2-DAG, whereas labelled arachidonic acid was barely liberated (Table 1). When radioactivity in the remaining MAG was measured, radioactivity of [14 C]arachidonate was greatly increased after the treatment with the lipase, whereas that of [3 H]myristate was barely changed.

Incorporation of glycerol and glucose into DAG in quiescent and PDGF-treated cells

We measured the rate of [3 H]glycerol incorporation into 1,2-DAG to test whether PDGF stimulated synthesis of 1,2-DAG *de novo*. When quiescent and PDGF-treated cells were incubated for 60 min with [3 H]glycerol, incorporation of [3 H]glycerol into 1,2-DAG was almost identical in quiescent and PDGF-treated cells. We also measured the rate of incorporation of [14 C]glucose into 1,2-DAG in quiescent and PDGF-treated cells. Quiescent and PDGF-treated cells were incubated with [14 C]glucose (16 mCi/mmol) for 10 and 30 min, and radioactivity in 1,2-DAG was measured. Rates of incorporation of [14 C]glucose were not different in quiescent and PDGF-treated cells.

Incorporation of myristate into phospholipids

It is believed that a major origin of 1,2-DAG is the hydrolysis of phospholipids. It is possible that labelled myristate, which is first taken up into phospholipid, appears in the 1,2-DAG fraction after hydrolysis of phospholipids. Fig. 3 shows the time course of incorporation of labelled myristate into major phospholipids. Incorporation into PC and PI+PS was increased in PDGF-treated cells, but the time course was dissociated from that into 1,2-DAG. Incorporation into PE was rapid. However, there was no difference in quiescent and PDGF-treated cells. Also, myristate incorporation into

Fig. 3. Incorporation of [3 H]myristate into phospholipids

Quiescent (●, ■, ▲) and PDGF-treated (○, □, △) cells were incubated in medium containing 1.0 μ Ci of [3 H]myristate/ml for the indicated time. Phospholipid labelling was measured by scraping and counting of plates after t.l.c. The data represent means $\pm$ S.E.M. for three parallel cultures. ○, ●, PE; □, ■, PC; △, ▲, PI+PS.

PA was slightly faster than that into PI+PS, but there was no difference in quiescent and PDGF-treated cells (results not shown).

Incorporation of [32 P]P_i into PA

One route of diacylglycerol metabolism is the phosphoinositide cycle via PA. To check the temporal relationship of the metabolism of DAG to that of PA in PDGF-treated cells, we measured incorporation of [32 P]P_i into PA in an experiment identical in design with that shown in Fig. 2. Incorporation of [32 P]P_i into PA occurred rapidly, and was fully stimulated in 20 min after addition of PDGF (Fig. 4a). When the effect of PDGF on 32 P labelling of PA was studied, the augmentation was maintained for at least 5 h after the removal of PDGF (Fig. 4b).

Assay of MAG acyltransferase activity *in vitro*

The above results imply that in PDGF-treated cells synthesis of 1,2-DAG catalysed by MAG acyltransferase is stimulated. We tested whether or not the activity of this acyltransferase was enhanced in PDGF-treated cells. Cells were quickly homogenized and the activity of the enzyme was measured *in vitro* with [14 C]myristoyl-CoA as substrate. The activity of the enzyme was linear in the protein range 4–20 μ g. With 10 μ g of protein, the reaction was linear for up to 15 min. The enzyme activities, expressed as pmol/min per mg of protein, in quiescent and competent cells were 33.4 ± 4.0 and 33.9 ± 3.9 respectively.

Fig. 4. Incorporation of [³²P]P_i into PA

(a) Quiescent (●) and PDGF-treated (○) cells were incubated in buffer A (10 mM-Tris/HCl, pH 7.4, 5.6 mM-glucose, 0.5 mM-MgCl₂, 1.5 mM-CaCl₂, 4.5 mM-KCl, 135 mM-NaCl) containing 1.0 μCi of [³²P]P_i for the time indicated, and radioactivity incorporated into PA was counted. (b) Treatment with and removal of PDGF were performed as described in the legend for Fig. 2(a). At the time point indicated, the medium was changed to buffer A containing 1.0 μCi of [³²P]P_i, and radioactivity incorporated into PA for 15 min was counted. Data represent means ± S.E.M. for three parallel cultures.

Mass of 1,2-DAG

Finally, we measured the mass of 1,2-DAG in both quiescent and competent cells. It was 1.95 ± 0.53 nmol/100 mm dish in quiescent cells and 1.27 ± 0.25 nmol/100 mm dish in PDGF-treated cells.

DISCUSSION

The major findings obtained in the present study are: (1) saturated fatty acids are rapidly incorporated into the *sn*-1 position of 1,2-DAG in competent cells; (2) 60 min treatment with PDGF is necessary for the enhancement of incorporation of saturated fatty acids into 1,2-DAG, and protein synthesis may be involved in this process; (3) activation of the synthesis of DAG through this pathway remains elevated for 3 h after the removal of PDGF; and (4) MAG acyltransferase activity is detected in Balb/c

3T3 cells, but the activity of this enzyme measured *in vitro* is not affected by the treatment with PDGF.

DAG is considered to be generated through four pathways: hydrolysis of phospholipids [2,3,8]; synthesis *de novo* from glycerol via PA [5]; deacylation of TAG [4]; and finally, acylation of MAG. PDGF is believed to generate 1,2-DAG mainly through the first pathway, particularly breakdown of phospholipids, namely PI and PC. We have found, however, that incorporation of saturated fatty acids into 1,2-DAG in PDGF-treated cells precedes that into phospholipids. Labelled saturated fatty acids were incorporated into 1,2-DAG earlier than into PC and PI. This time course suggests that saturated fatty acids are incorporated directly into 1,2-DAG. Incorporation into PE is rapid, but there is no significant difference in PDGF-treated and untreated cells. There remains, however, a possibility that saturated fatty acids are first incorporated into PE, and PE-specific phospholipase C is activated in PDGF-treated cells. We have tested this possibility by measuring PE breakdown after PDGF treatment. The PE breakdown is negligible, and the activity of PE-specific phospholipase C cannot be demonstrated (results not shown). Moreover, incorporation of labelled glycerol or glucose is not enhanced in PDGF-treated cells, and incorporation of fatty acids into TG is delayed. Based on these results, we conclude that labelled saturated fatty acids found in 1,2-DAG are not derived from any of the following pathways: hydrolysis of phospholipids, synthesis *de novo* or TAG deacylation. We show that labelled saturated fatty acids incorporated into 1,2-DAG in competent Balb/c 3T3 cells are situated in the *sn*-1 position, which is sensitive to *Rhizopus arrhizus* TAG lipase. It is therefore conceivable that saturated fatty acids are incorporated directly into the *sn*-1 position of 2-MAG in PDGF-treated cells. The enzyme which acts as a catalyst in this process is MAG acyltransferase, which has been demonstrated in intestinal cells and in liver cells [15]. As a next step, we have done an assay *in vitro* for this enzyme. Whole-cell lysate of Balb/c 3T3 cells contains MAG acyltransferase activity. We found no difference in the specific activity of the enzyme in quiescent cells and in PDGF-treated cells. There are at least three possibilities that explain this discrepancy: (1) labelled myristate is accumulated more actively in PDGF-treated cells, and intracellular specific radioactivity is increased; (2) MAG acyltransferase is not the rate-limiting enzyme in the metabolism of 1,2-DAG, and the activity of another enzyme, which, for example, catabolizes 1,2-DAG, is changed by treatment with PDGF; and (3) activity of MAG acyltransferase is indeed increased in PDGF-treated cells, but its activation requires an intact cell structure or its activity is lost during our preparation of cell lysate. The first possibility is excluded by the measurement of labelled non-esterified fatty acids in the cells (results not shown). To test the second possibility, it is necessary to assay the enzymes that catalyse DAG: DAG lipase, DAG acyltransferase and DAG kinase. The activity of DAG lipase has been demonstrated in platelets [18]. The DAG lipase hydrolyses fatty acid at the *sn*-1 position faster than at the *sn*-2 position. If this enzyme is more active in PDGF-treated cells, our observation that saturated fatty acids are rapidly incorporated into the *sn*-1 position can be explained. DAG kinase catalyses conversion of DAG into PA. Inhibition of this enzyme in platelets elevates DAG content and leads to the stimula-

tion of protein kinase C activity [19]. DAG kinase is presumed to play a central role in the regulation of DAG content, but our attempts to assay its activity in Balb/c 3T3 cells are unsatisfactory because the activity is too low (our preliminary results suggest 0.02–0.3 pmol/min per mg of protein). Results of the ^{32}P -incorporation study indicate that ^{32}P incorporation into PA is enhanced in 20 min when PDGF is added to quiescent cells. This result indicates that the enhancement of ^{32}P incorporation into PA occurs earlier than that of incorporation of saturated fatty acids into 1,2-DAG after PDGF treatment. This fact is consistent with the assumption that DAG kinase phosphorylates DAG, and the synthesis of DAG from MAG is subsequently activated. DAG acyltransferase was assayed in liver cells, intestinal cells and fat-cells. The time course of fatty acid incorporation into DAG and TAG supports the idea that 1,2-DAG is further metabolized to TAG through acylation. We have not checked the activity of DAG acyltransferase in Balb/c 3T3 cells.

In our experiments, the activity of MAG acyltransferase in PDGF-treated cells measured *in vitro* is 35.1 ± 4.4 pmol/min per mg of protein. Given the protein content of 193.3 ± 5.8 $\mu\text{g}/35$ mm Falcon dish, the activity of MAG acyltransferase per 35 mm Falcon dish is calculated to be about 6.78 pmol/min. As shown in Fig. 1a, 960 c.p.m. of labelled myristate is incorporated into 1,2-DAG in the first 1 min and 2.32×10^4 c.p.m. of labelled myristate is accumulated into the cells in the first 1 min (results not shown). The amount of non-esterified fatty acid in Balb/c 3T3 cells is estimated to be 1.65 times as much as 1,2-DAG, based on the distribution of radioactivity when cells are cultured in medium containing labelled myristate for 4 days (1.13% of the label is associated with 1,2-DAG, whereas 1.87% is found in non-esterified fatty acids). Considering the mass of 1,2-DAG measured, the quantity of non-esterified fatty acid per 35 mm Falcon dish is estimated to be 0.79 nmol. Given the specific radioactivity of labelled myristate, we estimate that 33 pmol of myristate is incorporated into 1,2-DAG (4% of total 1,2-DAG) in 1 min. Therefore the activity of MAG acyltransferase in our assay *in vitro* represents only 20% of the activity measured *in vivo*. These conjectures support the third possibility.

In conclusion, generation of 1,2-DAG from 2-MAG is increased in PDGF-treated Balb/c 3T3 cells. The significance of this stimulation is not known at present. Although the mass of 1,2-DAG generated by this pathway is small, it is possible that DAG thus generated is localized in a specific portion of the cell. It is of great

interest to test whether DAG thus generated plays any role in regulating the activity of C-kinase.

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