Induction of protein kinase C activation and Ca^{2+} mobilization by fibroblast growth factor in Swiss 3T3 cells

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Addition of fibroblast growth factor (FGF) to quiescent cultures of Swiss 3T3 cells rapidly induced diacylglycerol formation, protein kinase C activation and Ca^{2+} mobilization. Protein kinase C-activating agents such as 12-O-tetradecanoylphorbol-13-acetate (TPA) and 1-oleoyl-2-acetylglycerol (OAG) mimicked the action of FGF and stimulated DNA synthesis in the presence of insulin. Prolonged treatment of the cells with phorbol-12,13-dibutyrate (PDBu) led to the down-regulation and complete disappearance of protein kinase C. In these cells, TPA and OAG did not induce DNA synthesis any more. FGF still elicited Ca^{2+} mobilization and DNA synthesis, but the magnitude of DNA synthesis was reduced to almost half as compared with that in the control cells. These results clearly indicate that both diacylglycerol and Ca^{2+} may serve as second messengers for FGF and suggest that these messengers may be involved in the mitogenic action of this growth factor.

Fibroblast growth factor   *Phosphoinositide*   *Calcium*   *Protein kinase*

1. INTRODUCTION

FGF was first isolated by Gospodarowicz from bovine pituitary as a factor which stimulates the division of BALB/c 3T3 cells in tissue culture [1,2]. Since then, this growth factor has been shown to stimulate the proliferation of a wide variety of mesoderm-derived cells in addition to fibroblasts [3]. Moreover, FGF has been demonstrated to serve as a 'competence factor' for BALB/c 3T3 cells as described for PDGF [4]. Although FGF has been purified to homogeneity and its physical properties well characterized [5–7], its mode of action has not yet been clarified.

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Abbreviations: FGF, fibroblast growth factor; PDGF, platelet-derived growth factor; OAG, 1-oleoyl-2-acetylglycerol; TPA, 12-O-tetradecanoylphorbol-13-acetate; PDBu, phorbol-12,13-dibutyrate; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; [Ca^{2+}]$_i$, cytoplasmic free Ca^{2+} concentration

In contrast, the mode of action of PDGF has been extensively investigated and its second messengers identified. In Swiss 3T3 cells, PDGF has been shown to stimulate the formation of diacylglycerol and inositol 1,4,5-trisphosphate which are derived from the initial reaction of phosphoinositide turnover [8,9]. These 2 substances are considered to serve as second messengers for the activation of protein kinase C and intracellular translocation of Ca^{2+}, respectively [10,11]. To clarify the mode of action of FGF, it is very important to identify the second messengers for this growth factor. Therefore, we have examined whether diacylglycerol and Ca^{2+} may serve as second messengers for FGF.

2. MATERIALS AND METHODS

2.1. Materials

Swiss 3T3 cells and pure human PDGF were kindly supplied by Dr E. Rozengurt (Imperial Cancer Research Fund, England) and Dr T.F. Deuel (Washington University School of Medicine,

USA), respectively. FGF purified to homogeneity from bovine pituitary was a generous gift from Dr D. Gospodarowicz (University of California, San Francisco). Protein kinase C was purified to homogeneity from rat brain as described [12]. OAG was synthesized as in [13]. TPA and PDBu were purchased from CCR. Quin2 acetoxymethyl ester and Cytodex 1 microcarrier beads were obtained from Dojindo Laboratories and Pharmacia, respectively. [3H]Arachidonic acid, [3H]inositol and [γ-32P]ATP were purchased from Amersham. [3H]Thymidine and carrier-free $^{32}P_i$ were from New England Nuclear and Japan Radioisotope Association, respectively. Other materials and chemicals were obtained from commercial sources.

2.2. Cell culture

Stock cultures of Swiss 3T3 cells were maintained in DMEM supplemented with 10% FCS, penicillin (100 units/ml) and streptomycin (100 μg/ml) in a humidified atmosphere of 10% CO_2:90% air at 37°C as described [14]. For experimental purposes, the cells were seeded at a density of 5.7×10^4/35-mm dish in 2.5 ml DMEM containing 10% FCS, refed with the same medium after 2 days and used at least 5 days after the last change of medium.

2.3. Assays for phosphoinositide turnover, protein phosphorylation, Ca^{2+} mobilization and DNA synthesis

For analysis of diacylglycerol formation, the cells were prelabeled with 1.5 μCi [3H]arachidonic acid for 24 h and stimulated by various agonists for 15 min as described by Habenicht et al. [8]. For analysis of ^{32}P incorporation into phosphatidylinositol, the cells were stimulated by various agonists for 1 h and labeled with 50 μCi $^{32}P_i$ for 30 min before the termination of the reaction as described [15]. The incubation was terminated by the addition of 0.1% SDS. Radioactive lipids were extracted by the method of Bligh and Dyer [16]. Neutral lipids were separated as in [17] and phospholipids according to Skipski et al. [18]. The areas corresponding to diacylglycerol and phosphatidylinositol were scraped into vials and the radioactivity determined. For analysis of protein phosphorylation in intact cells, the cells prelabeled with 200 μCi $^{32}P_i$ for 1 h were stimulated for 5 min by each agonist under the

conditions described in [19]. For analysis of protein phosphorylation in a cell-free system, a Triton X-100 extract of the cells was phosphorylated by protein kinase C under the conditions in [20]. The reaction mixture (0.1 ml) contained 2 μmol Tris/HCl at pH 7.5, 0.5 μmol magnesium acetate, 2 nmol [γ-32P]ATP (5×10^6 cpm/nmol), 150 nmol $CaCl_2$, 5 μg phosphatidylserine, 0.2 μg diolein, 3T3 cell extract (equivalent to 3×10^5 cells) and 0.1 μg pure protein kinase C. After incubation for 2 min at 30°C, the reaction was terminated by the addition of 62 mM Tris-HCl at pH 6.7 containing 3% SDS, 2% 2-mercaptoethanol, 5% glycerol and 0.001% bromophenol blue. The radioactive proteins were subjected to SDS-polyacrylamide gel electrophoresis and an autoradiograph prepared. To prepare the Triton X-100 extract, the cells scraped from the dishes were disrupted at 4°C by sonication for 20 s in 20 mM Tris-HCl at pH 7.5 containing 5 mM EGTA, 2 mM phenylmethylsulfonyl fluoride, 10 mM 2-mercaptoethanol, 0.25 M sucrose and 0.1% Triton X-100. The suspension was centrifuged for 60 min at $100000 \times g$, and the supernatant used for the experiments. For analysis of $[Ca^{2+}]_i$, cells were grown on Cytodex 1 microcarrier beads and $[Ca^{2+}]_i$ measured by means of the fluorescent Ca^{2+} indicator quin2 as described in [21]. DNA synthesis was assayed by measuring the incorporation of [3H]thymidine into DNA as described by Dicker and Rozengurt [22] except that the cells were stimulated for 24 h by each agonist and exposed to 2 μCi [3H]thymidine for 4 h before the termination of the reaction.

3. RESULTS AND DISCUSSION

Incubation of quiescent cultures of Swiss 3T3 cells with various concentrations of PDGF and FGF elicited diacylglycerol formation as well as ^{32}P incorporation into phosphatidylinositol in a dose-dependent manner as shown in fig.1. Diacylglycerol might be derived from the hydrolysis of phosphoinositides including phosphatidylinositol, phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate by the action of phospholipase C as described for other agonists in other tissues [11]. Under comparable conditions, PDGF and FGF stimulated the phosphorylation of an 80 kDa protein as shown in fig.2. Phosphorylation of this protein was also
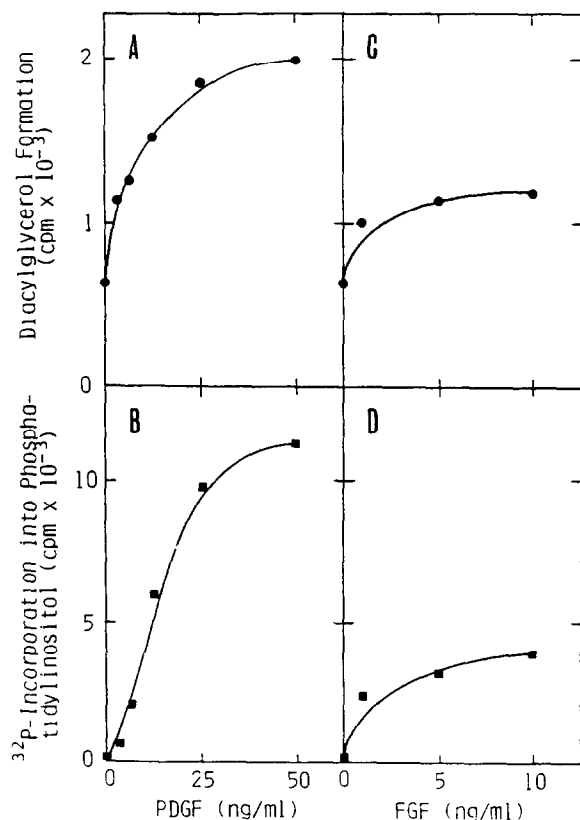
Fig.1. Induction of phosphoinositide turnover by PDGF and FGF. (A,C) [³H]Diacylglycerol formation, (B,D) ^{32}P incorporation into phosphatidylinositol, (A,B) with PDGF, (C,D) with FGF. Each value is the mean of triplicate determinations.
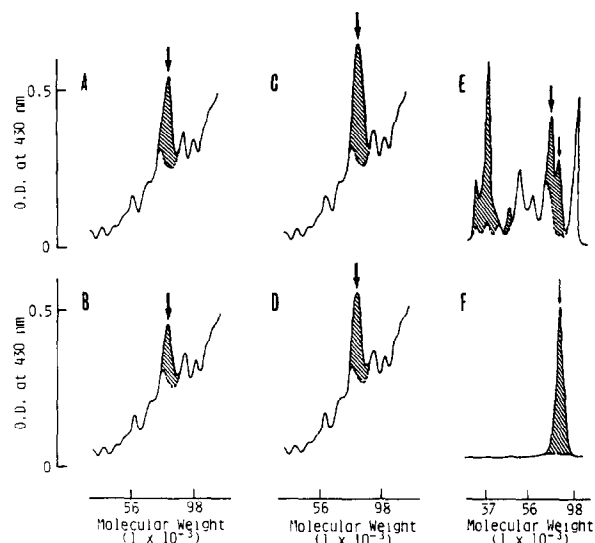

Fig.2. Densitometer tracings of autoradiograph of protein phosphorylation in intact cell and cell-free systems. (A,D) Protein phosphorylation in intact cells. (A) With 50 ng/ml of PDGF, (B) with 10 ng/ml of FGF, (C) with 100 ng/ml of TPA, (D) with 200 μg/ml of OAG. Solid and dotted lines indicate protein phosphorylation in stimulated and unstimulated cells, respectively. (E) Phosphorylation of Triton X-100 extract by protein kinase C in a cell-free system. Solid and dotted lines indicate protein phosphorylation in the presence and absence of protein kinase C, respectively. (F) Autophosphorylation of protein kinase C without the Triton X-100 extract. The large and small arrows indicate 80 kDa protein and protein kinase C, respectively. The results are from an experiment typical of 3 different experiments.

elicited by TPA and OAG, which are known as specific activators for protein kinase C [17,23]. Moreover, this protein was phosphorylated in a cell-free system by pure protein kinase C. Protein kinase C has been shown to be activated by diacylglycerol or phorbol ester in the presence of Ca^{2+} and phospholipid [23,24]. Phosphorylation of the 80 kDa protein by protein kinase C was dependent on these 3 activators (not shown). Other proteins besides the 80 kDa protein were also phosphorylated by protein kinase C in a cell-free system, but the phosphorylation of these proteins was not detected in intact cells after incubation with the agonists described above. The reason for these different patterns of protein phosphorylation in intact cell and cell-free systems is not clear, but

the difference may be due to the compartmentalization of the enzyme and substrates. The identity of the 80 kDa protein is unknown, but this protein was different from autophosphorylated protein kinase C. These results strongly suggest that FGF as well as PDGF induces the activation of protein kinase C through the formation of diacylglycerol and that the enzyme activated in this way then phosphorylates the 80 kDa protein.

It has been described that PDGF stimulates the generation of inositol 1,4,5-trisphosphate from the hydrolysis of phosphatidylinositol 4,5-bisphosphate [9]. However, in our experiments, it was difficult to measure directly the generation of inositol trisphosphate after stimulation of the cells with FGF, because phosphoinositides were poorly

labeled with [^{3}H]inositol in intact cells and the radioactivity recovered in inositol trisphosphate was too small to obtain reproducible results. Nevertheless, incubation of the cells with FGF significantly caused a rapid increase of [Ca^{2+}]$_i$ as shown in fig.3. PDGF also showed a similar effect. TPA and OAG did not elevate [Ca^{2+}]$_i$ under comparable conditions (not shown).

After protein kinase C activation and Ca^{2+} mobilization, PDGF and FGF markedly stimulated DNA synthesis only in the presence of insulin as shown in fig.4. Protein kinase C-activating agents such as TPA and OAG also stimulated DNA synthesis in the presence of insulin. The concentrations of these agonists necessary for DNA synthesis were nearly the same as those needed for protein kinase C activation as shown in the same figure. On the other hand, Ca^{2+}-mobilizing agents such as A23187 and ionomycin showed toxic effects on the cells after incubation for more than several hours, and the effect of these substances on DNA synthesis could not be examined.
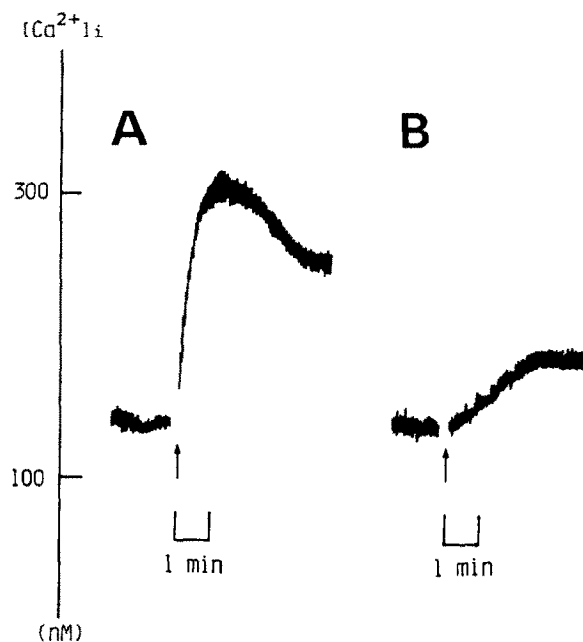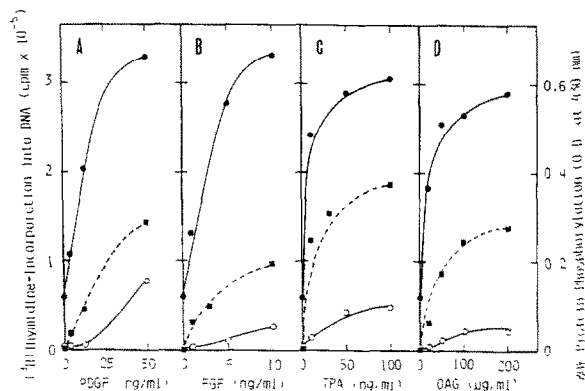

Fig.4. Dose-response curves for 80 kDa protein phosphorylation and DNA synthesis induced by PDGF, FGF, TPA and OAG. (■---■) 80 kDa protein phosphorylation, (●—●) DNA synthesis in the presence of insulin (1 μg/ml), (○—○) DNA synthesis in the absence of insulin. (A) With PDGF, (B) with FGF, (C) with TPA, (D) with OAG. Values of 80 kDa protein phosphorylation are expressed as the relative increase of A at 430 nm compared with unstimulated control cells. Each value is the mean of triplicate determinations.

In another set of experiments, quiescent cultures of the cells were pretreated with 200 nM PDBu for 24 h under the conditions employed by Rodriguez-Pena and Rozengurt [25]. By this pretreatment, protein kinase C was down-regulated and the enzymatic as well as [^{3}H]PDBu-binding activities disappeared completely (not shown). In these protein kinase C-deficient cells, PDGF and FGF still induced Ca^{2+} mobilization to the same extent as those in the control cells as shown in table 1. Moreover, these growth factors induced DNA synthesis in the presence of insulin, although the magnitude of DNA synthesis was reduced to almost half compared with that in control cells. In contrast, TPA and OAG no longer induced DNA synthesis in the protein kinase C-deficient cells.

This paper has demonstrated that FGF induces both protein kinase C activation and Ca^{2+} mobilization as described for PDGF [9,19]. Moreover, we have shown that TPA and OAG, specific activators for protein kinase C, substitute for FGF and induce DNA synthesis, and that the down-regulation of protein kinase C is accompanied by a decrease in FGF-induced DNA synthesis. Although these results do not provide convincing evidence, they do strongly suggest that at

Fig.3. Increase of [Ca^{2+}]$_i$ by PDGF and FGF. (A) With 50 ng/ml of PDGF, (B) with 25 ng/ml of FGF. The arrows indicate the time of the addition of PDGF and FGF. The results are from an experiment typical of 3 different experiments.

Table 1

Effects of PDBu pretreatment on PDGF-, FGF-, TPA- and OAG-induced changes of $[Ca^{2+}]_i$ and DNA synthesis

	PDBu treatment	None	PDGF (50 ng/ml)	FGF (10 ng/ml)	TPA (100 ng/ml)	OAG (200 μg/ml)
$[Ca^{2+}]_i$ (nM)	(−)	130	334	177[a]	130	130
	(+)	132	337	174[a]	ND	ND
DNA synthesis (cpm × 10^{-4})	(−)	3.6	20.8	20.5	19.2	15.9
	(+)	2.0	11.8	11.2	4.0	3.1

Swiss 3T3 cells, which were preincubated for 24 h in the absence and presence of 200 nM PDBu, were stimulated by each agonist, and then $[Ca^{2+}]_i$ and DNA synthesis were assayed as described in section 2. DNA synthesis was assayed in the presence of 1 μg/ml of insulin. [a] FGF (25 ng/ml) was employed. Each value is the mean of triplicate determinations. ND, not determined

least protein kinase C may be involved in the mitogenic action of FGF as well as PDGF. It may be noted that TPA and OAG induce protein kinase C activation and subsequently DNA synthesis without Ca^{2+} mobilization. This result implies that an increase in $[Ca^{2+}]_i$ elicited by FGF and PDGF at a very early stage may not be absolutely necessary for DNA synthesis. The role of Ca^{2+} in the action of FGF and PDGF remains to be clarified, but since these growth factors still induce DNA synthesis in the protein kinase C-deficient cells, it is most likely that Ca^{2+} may also be responsible for DNA synthesis as suggested in several other types of cells [26].

Another point to be discussed here is that the maximum levels of FGF-induced diacylglycerol formation, protein kinase C activation and Ca^{2+} mobilization are less than those induced by PDGF (figs 1–3). The exact reason for this difference is not known, but it is conceivable that the number of receptors for these growth factors may be different in Swiss 3T3 cells. In spite of the different efficiency of FGF and PDGF for protein kinase C activation and Ca^{2+} mobilization, both growth factors induce DNA synthesis to the same extent (fig.4). This may presumably be due to the cascade system from protein kinase C and Ca^{2+} to DNA synthesis in which the signals of these effectors may be amplified and the initial difference diminished.

In conclusion, the mode of action of FGF is very similar to that of PDGF, and both growth factors induce protein kinase C activation and Ca^{2+}

mobilization which appear to be involved in DNA synthesis.

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