Involvement of three intracellular messenger systems, protein kinase C, calcium ion and cyclic AMP, in the regulation of c-*fos* gene expression in Swiss 3T3 cells

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In quiescent cultures of Swiss 3T3 cells, platelet-derived growth factor or fibroblast growth factor known to induce both protein kinase C activation and Ca^{2+} mobilization raised c-*fos* mRNA. This action of the growth factors was mimicked by the specific activators for protein kinase C, such as phorbol esters and a membrane-permeable synthetic diacylglycerol, and also by the Ca^{2+} ionophores, such as A23187 and ionomycin. Prostaglandin E_1 known to elevate cyclic AMP also raised c-*fos* mRNA, and this action was mimicked by 8-bromo-cyclic AMP, dibutyryl cyclic AMP and forskolin. These results suggest that expression of the c-*fos* gene is regulated by three different intracellular messenger systems, protein kinase C, Ca^{2+} and cyclic AMP, in Swiss 3T3 cells.

Oncogene *Growth substance* *Protein kinase C* *Ca^{2+}* *cAMP* *(Fibroblast)*

1. INTRODUCTION

Various growth factors including PDGF, FGF and PGE_1 stimulate DNA synthesis and cell division in the presence of insulin in Swiss 3T3 cells [1–4]. In this cell line, PDGF and FGF have been shown to induce phosphoinositide turnover resulting in both diacylglycerol formation and Ca^{2+} mobilization [5–8] whereas PGE_1 has been shown to elevate cyclic AMP [4]. It is well established that the diverse actions of diacylglycerol, Ca^{2+} and cyclic AMP are mediated through the activation of protein kinase C, calmodulin and protein kinase A, respectively [9–11]. We have recently obtained the evidence that PGE_1 elevates $[Ca^{2+}]_i$ as well as cyclic AMP in Swiss 3T3 cells (Yamashita et al., submitted). Moreover, we have demonstrated that these three intracellular messengers, diacylglycerol, Ca^{2+} and cyclic AMP, are responsible for the c-*myc* gene expression stimulated by the respective growth factors in this cell line (Yamashita et al., submitted) [8,12], and that the action of diacylglycerol is mediated through the activation of protein kinase C [8].

Stiles and his colleagues [13] have proposed that the competence gene family activated directly by PDGF plays essential roles in the regulation of cell division in BALB/c-3T3 cells. They have demonstrated that 10–30 genes may belong to this gene family [13,14], and that the c-*myc* gene is one of them [13,15]. The c-*fos* gene has also been shown to belong to this gene family and to be activated by the action of PDGF and FGF in

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Abbreviations: PDGF, platelet-derived growth factor; FGF, fibroblast growth factor; PGE_1, prostaglandin E_1; protein kinase A, cyclic AMP-dependent protein kinase; $[Ca^{2+}]_i$, cytoplasmic free Ca^{2+} concentration; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; PDBu, phorbol-12,13-dibutyrate; 4α-PDD, 4α-phorbol-12,13-didecanoate; OAG, 1-oleoyl-2-acetylglycerol; 8-Br-cAMP, 8-bromo-cyclic AMP; Bt_2cAMP, dibutyryl cyclic AMP; DMEM, Dulbecco's modified Eagle's medium

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BALB/c-3T3 cells [13,16–18]. However, it has not been clarified whether expression of this gene is controlled by protein kinase C, Ca^{2+} or cyclic AMP in Swiss 3T3 cells. The present communication describes that expression of the c-*fos* gene is regulated by these three intracellular messenger systems in Swiss 3T3 cells as described for the c-*myc* gene (Yamashita et al., submitted) [8,12].

2. MATERIALS AND METHODS

2.1. *Materials*

pBR322 plasmid containing the v-*fos* gene (p*fos*-1) was a generous gift from Drs T. Sugiyama and R. Takahashi (Kobe University School of Medicine, Kobe, Japan) who originally obtained it from Dr I.M. Verma (Molecular Biology and Virology Laboratory, The Salk Institute, San Diego, USA). Swiss 3T3 cells, bovine pituitary FGF and human PDGF were kindly donated by Dr E. Rozengurt (Imperial Cancer Research Fund, London, England), Dr D. Gospodarowicz (University of California, San Francisco, USA) and Dr T.F. Deuel (Washington University, St. Louis, USA), respectively. PGE_1 was purchased from Funakoshi Pharmaceutical Co. TPA and PDBu were from CCR, 4α-PDD from Sigma and A23187 and ionomycin from Calbiochem. OAG was synthesized as described [19]. 8-Br-cAMP and Bt_2cAMP were from Yamasa Shoyu Co. Forskolin was a gift from Nihonkayaku Co. [α-^{32}P]dCTP (3000 Ci/mmol) was from Amersham and other materials and chemicals were obtained from commercial sources.

2.2. *Cell culture*

Stock cultures of Swiss 3T3 cells were maintained under the conditions described [20]. For experiments, the cells were plated at a density of 1.5×10^5 cells/100-mm dish with DMEM containing 10% fetal calf serum and refed with the same medium after 2 days. Such cultures were used at least 5 days after the last change of medium. These cells were confluent and quiescent. The cells were washed twice with DMEM and employed for each experiment.

2.3. *Measurement of* c-fos *mRNA*

The washed Swiss 3T3 cells were stimulated by various agents in 5 ml of DMEM containing 0.1% bovine serum albumin for 60 min at 37°C. Each reaction was terminated by chilling the cells on ice and washed twice with ice-cold phosphate-buffered saline. Total RNA was extracted from the cells by the procedure described by Chirgwin et al. [21] using CsCl gradient centrifugation. For dot blot analysis, total RNA samples were dotted onto nitrocellulose filters under a slow vacuum. After baking, the filters were hybridized for 48 h at 42°C with ^{32}P-labeled DNA probe and the unbound fraction was removed by extensive washing. The filters were autoradiographed using intensifying screens for 24 h at −70°C and the dots were excised and counted in a scintillation counter. The amount of the probe hybridized was proportional to the amount of RNA on the filter at a range from 1 to 10 μg. The levels of c-*fos* mRNA are expressed as cpm/6 μg of total RNA. The ^{32}P-labeled DNA probe was a nick-translated 1.0-kb v-*fos*-specific *Pst*I fragment from p*fos*-1 of cloned FBJ murine osteosarcoma virus proviral DNA [22].

3. RESULTS

Incubation of quiescent cultures of Swiss 3T3 cells with PDGF, FGF or PGE_1 caused the dose-dependent elevation of c-*fos* mRNA as shown in fig.1. The maximal levels obtained by PDGF and

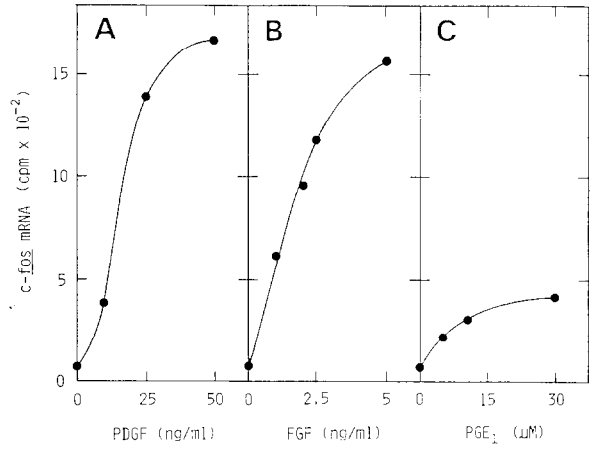

Fig.1. Increases of c-*fos* mRNA by PDGF, FGF and PGE_1. Quiescent cultures of Swiss 3T3 cells were incubated with various doses of either PDGF (A), FGF (B) or PGE_1 (C) for 60 min at 37°C. Other details are described in section 2. Each value is the mean of three independent experiments.

FGF were nearly the same, but the maximal level obtained by PGE_1 was about one fourth of that obtained by PDGF or FGF. c-*fos* mRNA increased within 15 min and reached maximal levels 45–60 min after the stimulation by each growth factor, followed by gradual declines (not shown).

The effect of protein kinase C-activating, Ca^{2+}-mobilizing and cyclic AMP-elevating agents on the levels of c-*fos* mRNA was examined in the next experiments. As shown in fig.2, TPA, a specific activator for protein kinase C, A23187, a Ca^{2+} ionophore, and 8-Br-cAMP, a membrane-permeable derivative of cyclic AMP, raised c-*fos* mRNA in a dose-dependent manner. The maximal levels obtained by A23187 and 8-Br-cAMP were about two thirds and one third of that obtained by TPA, respectively. The time courses for increases of c-*fos* mRNA by the action of TPA, A23187 and 8-Br-cAMP were similar to those obtained by the growth factors described above; this mRNA increased within 15 min and reached maximal levels within 60 min after the stimulation by the respective agents, followed by gradual declines (not shown).

Table 1 shows the effect of other protein kinase C-activating, Ca^{2+}-mobilizing and cyclic AMP-elevating agents on the levels of c-*fos* mRNA. PDBu and OAG known to be as active as TPA for protein kinase C also elevated c-*fos* mRNA, but 4α-PDD known to be inactive for protein kinase C was ineffective in this capacity. Ionomycin known to be a specific Ca^{2+} ionophore was also active. Bt_2cAMP known to be another membrane-permeable derivative of cyclic AMP and forskolin known to activate adenylate cyclase increased c-*fos* mRNA to the level obtained by 8-Br-cAMP.

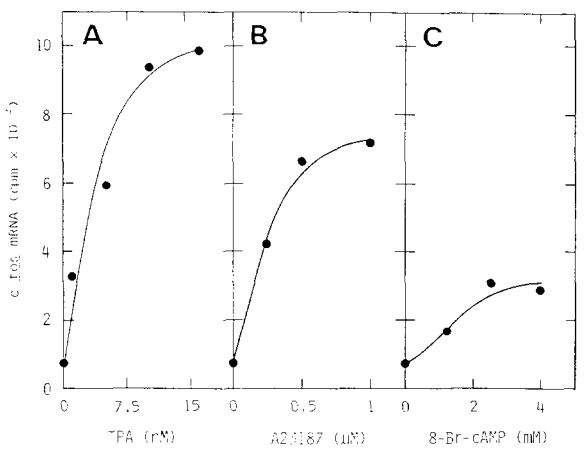

Fig.2. Increases of c-*fos* mRNA by TPA, A23187 and 8-Br-cAMP. Quiescent cultures of Swiss 3T3 cells were incubated with various doses of either TPA (A), A23187 (B) or 8-Br-cAMP (C) for 60 min at 37°C. Other details are described in section 2. Each value is the mean of three independent experiments.

Table 1

Effect of protein kinase C-activating, Ca^{2+}-mobilizing and cyclic AMP-elevating agents on the increases of c-*fos* mRNA

Addition	c-*fos* mRNA
	(cpm)
None	80
TPA (16 nM)	990
PDBu (16 nM)	420
4α-PDD (16 nM)	90
OAG (100 μg/ml)	320
A23187 (1 μM)	720
Ionomycin (1 μM)	1000
8-Br-cAMP (2.5 mM)	310
Bt_2cAMP (2.5 mM)	280
Forskolin (30 μM)	270
PGE_1 (30 μM)	400
PDGF (50 ng/ml)	1670
FGF (5 ng/ml)	1580

Quiescent cultures of Swiss 3T3 cells were incubated with various agents for 60 min at 37°C. Other details are described in section 2. Each value is the mean of three independent experiments

4. DISCUSSION

It has been described that PDGF and FGF induce phosphoinositide turnover resulting in both protein kinase C activation and Ca^{2+} mobilization in Swiss 3T3 cells [5–8,23] whereas PGE_1 induces both cyclic AMP elevation [4] and Ca^{2+} mobilization (Yamashita et al., submitted). This paper has demonstrated that PDGF and FGF elevate c-*fos* mRNA and that the specific activators for protein kinase C and the Ca^{2+} ionophores substitute for these growth factors in this action. Evidence has also been presented that PGE_1 elevates c-*fos* mRNA and that the derivatives of cyclic AMP and

the activator for adenylate cyclase substitute for this growth factor in this action. These results suggest that both protein kinase C and Ca^{2+} are responsible for the PDGF- and FGF-induced increase of c-*fos* mRNA whereas both cyclic AMP and Ca^{2+} are responsible for the PGE_1-induced increase of this mRNA in Swiss 3T3 cells. Although it has not been examined whether Ca^{2+} and cyclic AMP elevate c-*fos* mRNA through the activation of calmodulin and protein kinase A, respectively, it is conceivable from the modes of action of these messengers that the actions of Ca^{2+} and cyclic AMP are mediated through the activation of the respective effectors.

The c-*fos* gene as well as the c-*myc* gene has been shown to belong to the competence gene family in BALB/c-3T3 cells [13,15,17]. We have previously described that the c-*myc* gene expression is regulated also by protein kinase C, Ca^{2+} and cyclic AMP in Swiss 3T3 cells (Yamashita et al., submitted) [8,12]. Although it is unknown how many genes belong to the competence gene family in Swiss 3T3 cells, our results suggest that the three intracellular messenger systems, protein kinase C, Ca^{2+} and cyclic AMP, are involved in the expression of at least these two competence genes which are directly regulated by growth factors in this cell line.

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