Involvement of Ca^{2+} in platelet-derived growth factor-induced expression of c-*myc* oncogene in Swiss 3T3 fibroblasts

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Addition of platelet-derived growth factor (PDGF) to quiescent cultures of Swiss 3T3 fibroblasts rapidly increased the cytoplasmic free Ca^{2+} concentrations and afterwards the c-*myc* mRNA levels. The concentrations of PDGF needed for both reactions were roughly the same. Moreover, the Ca^{2+} ionophores, A23187 and ionomycin, mimicked the actions of PDGF and increased the c-*myc* mRNA levels. These results suggest that Ca^{2+} may be involved in the regulation of PDGF-induced expression of the c-*myc* gene.

Platelet-derived growth factor *Calcium* *Oncogene* *Fibroblast*

1. INTRODUCTION

PDGF is one of the major mitogens found in serum. This growth factor makes fibroblasts leave the G_0 state of the cell cycle, and stimulates the cells to initiate DNA synthesis and subsequently, to divide in the presence of other growth factors such as epidermal growth factor and insulin [1–3]. Recently, it has been clarified that PDGF stimulates expression of the c-*myc* gene in Balb/c 3T3 fibroblasts [4]. It has also been demonstrated that expression of c-*myc* from recombinant plasmids introduced into the cells by gene transfer eliminates partly the PDGF requirement for cell growth in this cell line [5]. These results strongly suggest that the c-*myc* gene may play a role of crucial importance in the mitogenic action of PDGF. However, the mode of action of PDGF in expression of this gene has not been clarified. It has been shown that PDGF rapidly increases the levels of $[Ca^{2+}]_i$ in Swiss 3T3 and human fibroblasts [6,7]. Ca^{2+} is generally considered to serve as a second messenger to various extracellular signals [8]. Therefore, we have investigated whether Ca^{2+} may be involved in PDGF-induced expression of the c-*myc* gene in Swiss 3T3 cells.

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Abbreviations: PDGF, platelet-derived growth factor; $[Ca^{2+}]_i$, cytoplasmic free Ca^{2+} concentration; FCS, fetal calf serum

2. MATERIALS AND METHODS

2.1. *Materials*

Swiss 3T3 cells, PDGF and pBR322 plasmids containing human c-*myc* gene were kindly supplied by Dr E. Rozengurt (Imperial Cancer Research Fund, England), Dr T.F. Deuel (Washington University School of Medicine, USA) and Dr J.M. Bishop (University of California, San Francisco), respectively. A23187 and ionomycin were obtained from Calbiochem-Behring. Quin2 acetoxymethyl ester and Cytodex 1 microcarrier beads were from Dojindo Laboratories and Pharmacia Fine Chemicals, respectively. [α-^{32}P]dATP was from Amersham. Other materials and chemicals were obtained from commercial sources.

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2.2. *Cell culture*

Stock cultures of Swiss 3T3 cells were maintained in Dulbecco's modified Eagle's medium (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 [9].

2.3. *Measurement of* $[Ca^{2+}]_i$

The levels of $[Ca^{2+}]_i$ were measured according to Morris et al. [10]. Briefly, cells were seeded at a density of 5×10^4 cells/ml onto Cytodex 1 microcarrier beads at 2 mg/ml, grown to confluence in DMEM containing 10% FCS stirred continuously at 25 rpm, refed with the same medium after 2 days, and used 5 days after the last change of medium. Cells attached to beads were washed by centrifugation in serum-free DMEM supplemented with 10 mM Hepes, 20 mM $NaHCO_3$ and gassed with 10% CO_2 at pH 7.2. Then, the cells were incubated with the same medium containing 10 μM quin2 acetoxymethyl ester for 45 min at 37°C in a humidified atmosphere of 10% CO_2:90% air. The cells were washed twice by centrifugation in Hepes-buffered saline (10 mM Hepes at pH 7.4 containing 145 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 0.5 mM Na_2HPO_4, 1 mM $CaCl_2$ and 5 mM glucose). Intracellular quin2 fluorescence (excitation 339 nm, emission 492 nm) was measured in a Shimadzu model RF-510LC fluorescence spectromonitor with constant stirring at 37°C. Values of $[Ca^{2+}]_i$ were calculated from fluorescence signals as described by Tsien et al. [11].

2.4. *Assay for* c-myc *mRNA*

For analysis of c-*myc* mRNA, the cells were seeded at a density of 1.5×10^5 cells in 100-mm dishes with 15 ml of DMEM containing 10% FCS, refed with the same medium after 2 days, and used at least 5 days after the last change of medium. The cultures were washed twice with DMEM at 37°C and then incubated for 3 h with various concentrations of stimuli. The reaction was stopped by chilling the dishes on ice and the cells were scraped off. Total RNA of the cells was extracted by the procedure of Chirgwin et al. [12] utilizing the CsCl gradient centrifugation. For dot blot analysis, RNA samples were dotted onto nitrocellulose filters under a low 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 an intensifying screen for 24 h at −70°C, and the dots were excised and counted in a scintillation counter. The ^{32}P-labeled DNA probe used was the *ClaI-EcoRI* fragment of the normal human c-*myc* gene (including most of the 3′-exon) labeled by the random-primer method [13,14].

3. RESULTS AND DISCUSSION

When quiescent cultures of Swiss 3T3 fibroblasts were incubated with PDGF, there was an immediate increase of $[Ca^{2+}]_i$. Typical increases of $[Ca^{2+}]_i$ in response to various amounts of PDGF are shown in fig.1. The basal level of $[Ca^{2+}]_i$ was 112–148 nM (mean ± SE, 130 ± 5 nM; n = 12). On addition of 50 ng/ml of PDGF, $[Ca^{2+}]_i$ increased to more than 300 nM within 2 min. After this rapid rise in $[Ca^{2+}]_i$, the c-*myc* mRNA levels had increased markedly (fig.2). The increase of c-*myc* mRNA levels by PDGF was dose-dependent (fig.3), and the amounts of this growth factor needed for this reaction were nearly the same as those needed for the increase of $[Ca^{2+}]_i$. Furthermore, the Ca^{2+} ionophores such as A23187 and ionomycin were able to substitute for PDGF and increased the c-*myc* mRNA levels (figs

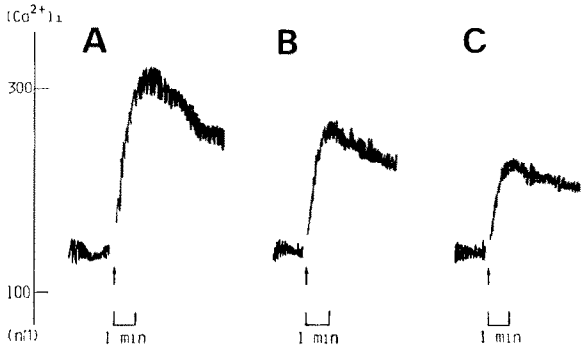

Fig.1. PDGF-induced increase of $[Ca^{2+}]_i$. Swiss 3T3 cells attached onto Cytodex 1 microcarrier beads were loaded with quin2 and stimulated by various amounts of PDGF in Hepes-buffered saline containing 1 mM $CaCl_2$. With: (A) 50 ng/ml of PDGF, (B) 25 ng/ml of PDGF, (C) 12.5 ng/ml of PDGF. Arrows indicate the points of PDGF addition.

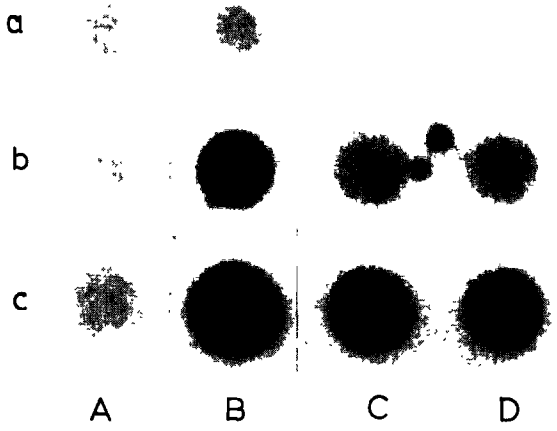

Fig.2. Dot blot analysis of c-*myc* mRNA levels after incubation with PDGF, A23187 and ionomycin. Quiescent cultures of Swiss 3T3 cells were incubated for 3 h with PDGF (50 ng/ml), A23187 (1.2 μM) or ionomycin (1.2 μM). (A) No addition, (B) with PDGF, (C) with A23187, (D) with ionomycin. (a) 2 μg total RNA, (b) 4 μg total RNA, (c) 8 μg of total RNA.

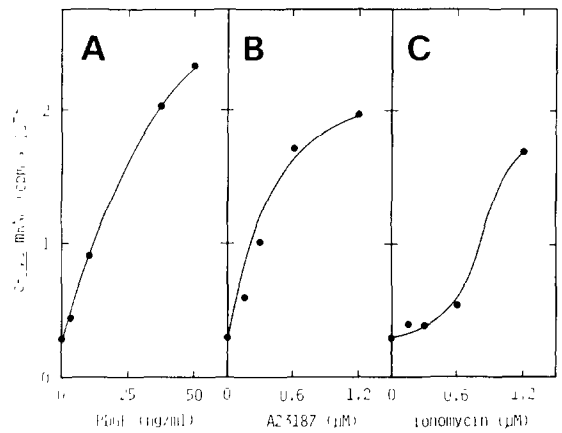

Fig.3. Dose-dependent increase of c-*myc* mRNA levels induced by PDGF, A23187 and ionomycin. Quiescent cultures of Swiss 3T3 cells were incubated for 3 h with various amounts of PDGF, A23187 or ionomycin as indicated. The levels of c-*myc* mRNA are expressed as cpm/4 μg total RNA. Each value is the mean of triplicate determinations. With: (A) PDGF, (B) A23187, (C) ionomycin.

2 and 3). The maximum levels of this mRNA induced by these ionophores were roughly equal to those induced by PDGF. It has been reported that PDGF-induced elevation of c-*myc* mRNA levels is

Table 1

Effect of cycloheximide on PDGF- and A23187-induced increase of c-*myc* mRNA levels

Additions	c-*myc* mRNA (cpm)	
	Cycloheximide (−)	Cycloheximide (+)
None	30	170
PDGF	250	400
A23187	190	320

Quiescent cultures of Swiss 3T3 cells were incubated for 3 h with various additions as indicated. PDGF (62.5 ng/ml), A23187 (1.2 μM) and cycloheximide (10 μg/ml) were employed. The levels of c-*myc* mRNA are expressed as cpm/4 μg total RNA. Each value is the mean of triplicate determinations

further enhanced by the addition of cycloheximide, which by itself increases the levels of c-*myc* mRNA [4]. The A23187-induced increase of c-*myc* mRNA levels was also further enhanced by the addition of this compound as shown in table 1, suggesting that expression of the c-*myc* gene by A23187 as well as by PDGF does not require the synthesis of new proteins.

The results presented above clearly indicate that PDGF induces both Ca^{2+} mobilization and c-*myc* gene expression in Swiss 3T3 cells. Moreover, evidence is presented that expression of the c-*myc* gene is stimulated by the Ca^{2+} ionophores. These results strongly suggest that Ca^{2+} may serve as a messenger for PDGF-induced expression of the c-*myc* gene. PDGF has been shown to stimulate phosphoinositide turnover to produce inositol trisphosphate, which then mobilizes Ca^{2+} from intracellular stores to the cytoplasm [6]. Therefore, it is likely that the cytoplasmic free Ca^{2+} mobilized in this way may be responsible for the activation of this gene. It is generally accepted that the diverse effects of Ca^{2+} may be mediated through the activation of calmodulin, a ubiquitous Ca^{2+}-receptor protein [15]. The involvement of calmodulin in the c-*myc* gene activation is now under investigation.

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