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Interrelationships of Platelet-Derived Growth Factor Isoform-Induced Changes in c-*fos* Expression, Intracellular Free Calcium, and Mitogenesis

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Both increases in c-*fos* proto-oncogene expression and intracellular free calcium ($[Ca^{2+}]_i$) have been implicated as necessary components of the signal transduction pathway by which platelet-derived growth factor (PDGF) stimulates DNA synthesis in cultured BALB/c3T3 fibroblasts. To determine the interrelationship between PDGF-induced increases in c-*fos* proto-oncogene expression and $[Ca^{2+}]_i$, purified, recombinant BB and AA homodimeric isoforms of PDGF were used to evaluate the dose-response relationships and mechanisms of growth factor-induced changes in these two parameters as well as DNA synthesis. Concentration-dependent increases in $[Ca^{2+}]_i$, c-*fos* expression, and [^{3}H]thymidine incorporation were observed with both BB and AA PDGF isoforms. BB PDGF was consistently more potent and efficacious than the AA isoform in eliciting a given response. The $[Ca^{2+}]_i$ dependency of PDGF-induced increases in c-*fos* expression and DNA synthesis was determined by pretreatment of cells with agents that inhibit increases in $[Ca^{2+}]_i$: BAPTA, Quin-2, and TMB-8. Under these conditions, PDGF-induced DNA synthesis was blocked, whereas c-*fos* expression was enhanced. Conversely, in cells made deficient in protein kinase C (PKC) activity by prolonged treatment with phorbol ester, BB and AA PDGF-induced c-*fos* expression was inhibited by 75–80%, while PDGF-induced increases in $[Ca^{2+}]_i$ and DNA synthesis were unaffected or enhanced. Additionally, the PKC-independent component of PDGF-stimulated c-*fos* expression was found to be independent of increases in $[Ca^{2+}]_i$. These data suggest that 1) both BB and AA PDGF isoforms elicit alterations in $[Ca^{2+}]_i$ and c-*fos* proto-oncogene expression through the same or similar mechanisms in BALB/c3T3 fibroblasts, 2) PDGF-stimulated increases in $[Ca^{2+}]_i$ are not required for c-*fos* expression, and 3) distinct pathways regulate PDGF-induced c-*fos* expression and mitogenesis, with c-*fos* expression being substantially PKC-dependent yet $[Ca^{2+}]_i$-independent, while mitogenesis is $[Ca^{2+}]_i$-dependent yet PKC-independent.

Abbreviations used: $[Ca^{2+}]_i$, intracellular free calcium concentration; DMEM-H, Dulbecco's modified Eagle's medium with high glucose; DVM, digitized video microscopy; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; PKC, protein kinase C; PPP, platelet-poor plasma; TMB-8, 8-(diethylamine)octyl-3,4,5-trimethoxybenzoate hydrochloride; TPA, 12-tetradecanoylphorbol-13-acetate.

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The process of cell proliferation is positively regulated by a variety of extracellular signals including peptide growth factors such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (FGF), and insulin [1]. PDGF is a potent peptide mitogen for cells of mesenchymal origin [2]. Much of the current information available on the mechanism of PDGF action has been obtained using the heterodimeric form of PDGF (AB), the predominant form found in human platelets, consisting of disulfide-linked A and B peptide chains [3,4]. Naturally occurring, mitogenically active homodimeric forms of PDGF (AA and BB) have also been identified in osteosarcoma cells [5] and porcine platelets [6], respectively. Differences in the biological activities of the PDGF isoforms with respect to mitogenic activity, chemotactic response, and transforming potential have been recently reported [7,8].

Early cellular events occurring upon PDGF stimulation have been extensively examined and include receptor autophosphorylation of tyrosine residues, hydrolysis of plasma membrane phospholipids, increases in intracellular free calcium concentration ($[Ca^{2+}]_i$), activation of PKC, increases in pH_i by activation of Na^+/H^+ exchange, and induction of cytoskeletal alterations [9–13]. Increased $[Ca^{2+}]_i$, but not PKC activation or cytosolic alkalinization, has been shown to be essential for PDGF-induced DNA synthesis in BALB/c-3T3 cells [13]. A number of growth factors, including PDGF, have also been shown to stimulate the expression of several cellular homologues (i.e., c-*fos* and c-*myc*) of viral oncogenes within minutes to hours after receptor binding [14,15]. One of these proto-oncogenes, c-*fos,* encodes a nuclear protein whose normal cellular function is thought to involve the transcriptional control of other unidentified growth related genes [16]. The precise role and regulation of growth factor-induced c-*fos* expression in mitogenesis is currently unclear, but the necessity and/or permissivity of this gene product in cell proliferation is supported by the transforming ability of its retroviral homologue [17,18].

A number of second messenger pathways can apparently mediate growth factor-stimulated proto-oncogene expression. Activation of protein kinase C (PKC), either indirectly through the action of growth factors such as PDGF and FGF or directly by administration of phorbol ester tumor promotors, induces c-*fos* and c-*myc* mRNA at the transcriptional level in a variety of cell types [14,19,20]. Induction of these genes, however, can also occur through PKC-independent pathways as demonstrated by EGF-induced gene expression (EGF does not stimulate phosphatidylinositol hydrolysis or activate PKC), and by PKC down-regulation experiments in which cells are made deficient in the enzyme by pretreatment with high concentrations of phorbol esters [21]. Cyclic AMP has also been identified as one of the second messengers involved in PKC-independent proto-oncogene induction by growth factors [22,23]. The regulatory role of cAMP in growth factor-stimulated gene expression (as well as mitogenesis) is apparently cell type specific, as both positive and negative effects have been reported [23–25]. Lastly, cellular Ca^{2+} fluxes have been found to stimulate c-*fos* in some systems [26] and it has recently been demonstrated that Ca^{2+}-induced c-*fos* expression in rat pituitary cells is independent of PKC activity [27].

Recently, the existence of multiple PDGF receptors possessing different binding affinities and specificities for the different isoforms has been demonstrated [28–30]. Whether the manifestation of different biological activities of the PDGF isoforms is a

result of the activation of unique signaling pathways through distinct receptors or a consequence of cell-specific differences in receptor number and populations (that may possess similar signal-transduction mechanisms) and, therefore, binding capacities for a given isoform, remains to be clarified. The present study was designed to 1) examine the mitogenic signal-transduction pathway(s) of purified, recombinant PDGF isoforms in BALB/c-3T3 fibroblasts to determine whether different PDGF isoforms activate distinct pathways for stimulation of DNA synthesis, 2) determine the interrelationships between PDGF-induced changes in two mitogenesis-associated parameters, $[Ca^{2+}]_i$ and c-*fos* expression, and 3) correlate isoform-specific alterations in $[Ca^{2+}]_i$ and c-*fos* expression with isoform-specific induction of DNA synthesis.

MATERIALS AND METHODS

Materials

Recombinant PDGF homodimers, $AA_{endothelial}$ and $BB_{c\text{-}sis}$, were a generous gift from Dr. W. Kusmik of Creative BioMolecules, Inc. (Hopkinton, MA). Fura-2-AM and BAPTA-AM were obtained from Molecular Probes (Eugene, OR). [^{3}H]thymidine and [α-^{32}P]dCTP were purchased from Amersham (Chicago, IL) and ICN (Irvine, CA), respectively. Formamide, phenol, and agarose were from Bethesda Research Laboratories (Gaithersburg, MD). All other reagents were purchased from either Calbiochem (San Diego, CA) or Sigma Chemical Co. (St. Louis, MO).

Cell Culture

BALB/c murine 3T3 fibroblasts (clone A31) were grown as described [11]. Cells were maintained in Dulbecco's modified Eagle's medium with high glucose (DMEM-H) supplemented with 10% heat-inactivated Colorado calf serum, 4 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin, in humidified 5% CO_2/95% air at 37°C. Cells were deprived of fresh serum addition for 5 days prior to growth factor exposure and were between passage number 6 and 16. In PKC down-regulation experiments, 4-day serum deprived cultures were pretreated for 24 h with 12-tetradecanoyl-phorbol-13-acetate (TPA), 600 nM, in 10% spent medium from the same cells/90% DMEM-H.

RNA Isolation and Northern Blot Analysis

Incubations of confluent, serum-deprived cells with growth factor and pharmacological agents were terminated by two rapid washes with ice-cold phosphate-buffered saline. Total RNA was then immediately isolated according to the method of Chomczynski and Sacchi [31]. Quantitation and purity were assessed spectrophotometrically by absorbance at 260 and 280 nM. RNA precipitates were redissolved in RNA sample buffer containing 1× gel running buffer (40 mM morpholinopropanesulfonic acid, pH 7.0, 10 mM sodium acetate, 1 mM EDTA), 50% formamide, and 2.2 M formaldehyde, and heat denatured at 65°C for 10 min. For Northern blot analyses, RNA (20 μg/lane) was size-fractionated by electrophoresis on agarose/formaldehyde gels and transferred by blotting onto Nytran nylon membranes (Schleicher & Schuell) as described previously [32]. The location of 18S and 28S rRNA on the gels was determined by ethidium bromide staining prior to transfer.

Membranes were prehybridized at 42°C for 2–3 h in a solution containing 50% formamide, 5× SSPE (1× SSPE: 0.18 M NaCl, 10 mM sodium phosphate, pH 7.7, 1

mM EDTA), 5× Denhardt's solution, 0.1% SDS, and 150 μg/ml salmon testis DNA. Hybridizations were performed with ^{32}P-labeled DNA probes (2–5 × 10^6 cpm/ml) for 18 h at 42°C in essentially the same buffer (2.5× Denhardt's replacing 5×). Following hybridization, membranes were washed once in 6× SSPE/0.1% SDS at ambient temperature, once in 1× SSPE/0.1% SDS at 42°C, and twice in 0.1× SSPE/0.1% SDS at 55°C. Blots were autoradiographed using Kodak XAR film at −70°C with intensifying screen (DuPont). The intensities of the major bands were determined by scanning densitometry.

Probes

The *fos* probe employed was generated by excising and purifying a 2.3 kb rat c-*fos* insert from the pSP65c-*fos*1A plasmid originating from T. Curran (Roche Institute, Nutley, NJ) and kindly supplied by S. Hall (University of North Carolina, Chapel Hill). The actin probe was prepared using a 770 bp chicken actin sequence purchased from Oncor Probes (Gaithersburg, MD). Probes were labeled by random priming (Boehringer Mannheim kit; Promega Klenow enzyme) to a specific activity greater than 10^8 cpm/μg.

Determination of Intracellular Calcium

$[Ca^{2+}]_i$ was measured using digitized video microscopy (DVM) in individual or small collections of individual, living cells (grown on glass coverslips) loaded with the Ca^{2+}-sensitive fluorophore, Fura-2. Details of the Fura-2 loading protocol, as well as the $[Ca^{2+}]_i$ measurements, have been described previously [13,33–35]. In brief, 5 day serum-deprived cells on coverslips were prechilled for 10 min at 4°C to prevent subsequent uptake of fluorophore into intracellular organelles. Cells were then loaded with Fura-2-AM (5 μM) for 20 min at 37°C. Cells on coverslips were then washed, transferred to incubation chambers, and placed on the stage of an inverted fluorescence microscope. Incubations were performed in DMEM supplemented with 10% spent medium and 10 mM HEPES and maintained at approximately 37°C using an air curtain incubator. $[Ca^{2+}]_i$ was measured by the ratio technique [35,36]. Pairs of images were obtained every 10 s over a period of 5 min at two excitation wavelengths (340 nm, Ca^{2+}-bound; 380 nm, Ca^{2+}-free Fura-2) and the ratio was determined. This method provides a measure of $[Ca^{2+}]_i$ over the 25 nM to 1 μM range and corrects for differences in accessible volume or cytoplasmic pathlength. Changes in the ratioed Fura-2 fluorescence (340/380 nM) were calibrated against EGTA-buffered solutions containing defined free Ca^{2+} concentrations [35].

DNA Synthesis

DNA synthesis was determined by [^{3}H]thymidine incorporation [11]. BALB/c-3T3 cells were grown in 96-well tissue culture plates for 5 days without medium change. Confluent cultures were treated with fresh DMEM-H containing 0.5% platelet-poor plasma (PPP) and the indicated mitogens in the absence or presence of specified antagonists for 1 h at 37°C. Where indicated, cells were preincubated with antagonists prior to PDGF addition. The incubation medium was removed and cells were washed and then labeled with [^{3}H]thymidine (1 μCi/ml, 10 Ci/mmol) in DMEM-H/0.5% PPP for 24 h at 37°C. The amount of [^{3}H]thymidine incorporation was determined by liquid scintillation counting as previously described [35].

RESULTS

PDGF Isoform Dose-Response Relationships

Quiescent, serum-deprived BALB/c-3T3 cells were treated with varying concentrations of AA and BB PDGF for 30 min and total RNA was isolated and analyzed for the abundance of c-*fos* and actin mRNA. The expression of c-*fos* message is rapid and transient in response to PDGF stimulation [14] and was found to be maximal at 30 min under the present conditions (data not shown). Constitutive actin mRNA levels detected in untreated, quiescent cells remained unchanged in cultures treated with growth factor or other agents under the conditions presented. Actin expression, therefore, was used to correct for quantitative differences in RNA gel loading and all c-*fos* data presented has been so normalized. Figure 1 shows an autoradiogram depicting the concentration dependence of c-*fos* expression induced by AA and BB PDGF isoforms. Untreated control cultures contained barely detectable levels of c-*fos* mRNA. Stimulation with PDGF isoforms (1–40 ng/ml) resulted in large increases in c-*fos* levels (Fig. 2A). The BB isoform was significantly more potent and efficacious in eliciting this response than AA PDGF (BB: ED_{50} = 5 ng/ml, maximum response = 115-

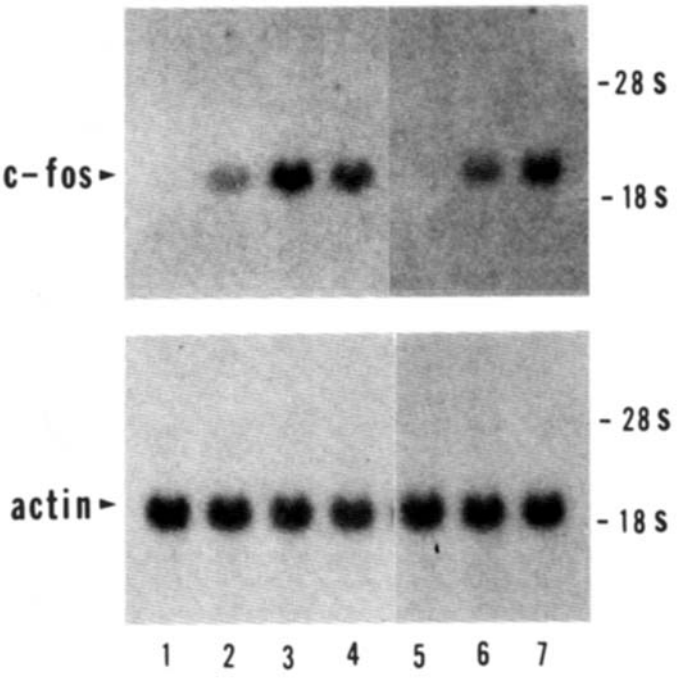

Fig. 1. Induction of c-*fos* expression by BB and AA PDGF isoforms. Quiescent BALB/c3T3 fibroblasts were exposed to various concentrations of BB or AA PDGF for 30 min. Total RNA was extracted and analyzed (20 μg/lane) for c-*fos* expression using a ^{32}P-labeled cDNA probe. The blot was then stripped and reprobed for actin mRNA. The markers 28S and 18S refer to the positions of the major species of ribosomal RNA on the stained gel. The c-*fos* signal corresponds to a 2.2 kb message, the actin signal to 1.9 kb β-actin mRNA. **Lane 1:** Unstimulated control cells. **Lanes 2–4:** BB PDGF—2.5, 10, 40 ng/ml. **Lanes 5–7:** AA PDGF—2.5, 10, 40 ng/ml. Induction of c-*fos* expression from very low basal levels was maximal at 10 ng/ml with BB, whereas AA PDGF stimulation requires higher doses.

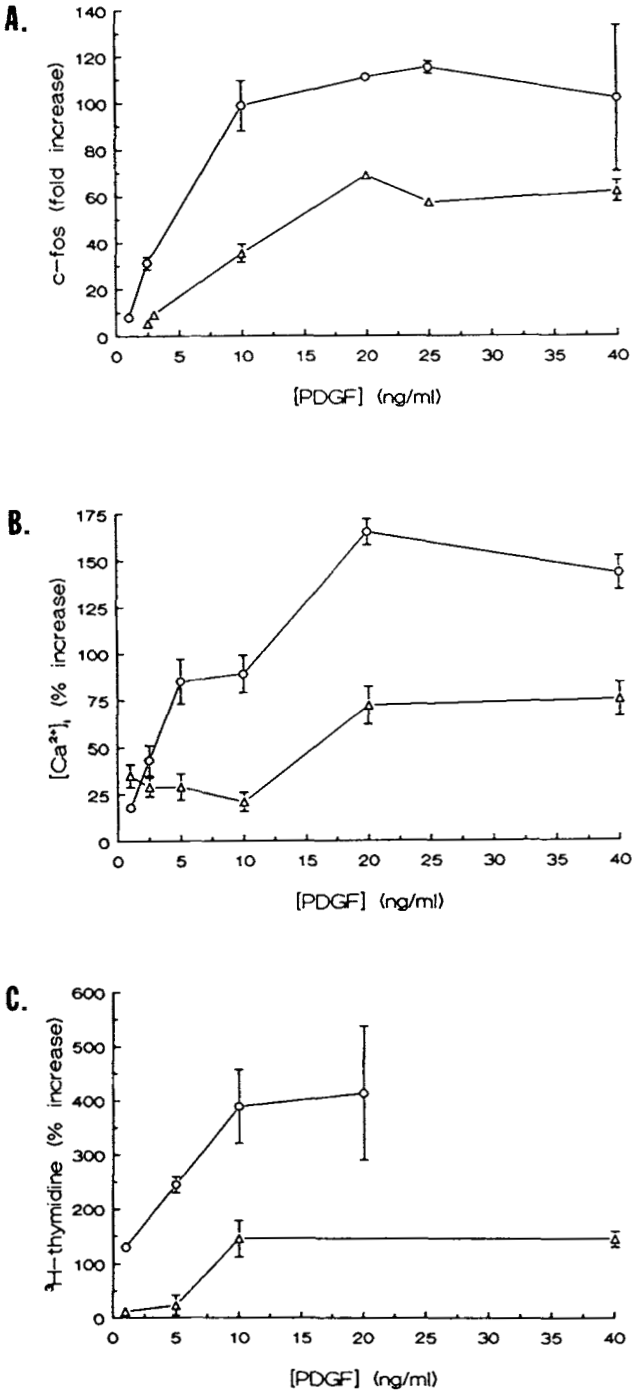

Fig. 2. PDGF isoform dose-response relationships for induction of increased c-*fos* expression, $[Ca^{2+}]_i$, and DNA synthesis. Quiescent BALB/c3T3 cells were exposed to BB (circles) and AA (triangles) PDGF isoforms, 1–40 ng/ml, and the three parameters measured as described in "Materials and Methods." **A:** c-*fos* mRNA. **B:** $[Ca^{2+}]_i$ increase. **C:** DNA synthesis. DNA synthesis was assessed by [^{3}H]thymidine incorporation. Data are expressed as changes from the unstimulated control values (0). Each point represents the mean ± SE obtained from at least 3 independent experiments.

fold increase; AA: ED_{50} = 10 ng/ml, maximum response = 69-fold increase). Concentrations of AA PDGF as high as 80 ng/ml failed to increase c-*fos* levels any further (data not shown).

PDGF isoform-induced increases in $[Ca^{2+}]_i$ over the same concentration range are shown in Figure 2B. Control values for basal, unstimulated levels of $[Ca^{2+}]_i$ were 156 ± 24 nM. Again, BB PDGF elicited Ca^{2+} responses at lower doses than AA, and the maximal response obtained was approximately twice as high with BB compared to AA. Maximal $[Ca^{2+}]_i$ responses occurred at concentrations similar to those which induced maximal c-*fos* expression.

DNA synthesis as assessed by [^{3}H]thymidine incorporation was also stimulated by both PDGF isoforms (Fig. 2C). The BB isoform was more potent than AA with maximum ^{3}H incorporation levels 5-fold above untreated controls as opposed to a 2-fold increase induced by AA. Thus, similar dose-response relationships exist for a given PDGF isoform in effecting increases in c-*fos* expression, $[Ca^{2+}]_i$ and DNA synthesis in BALB/c-3T3 cells. In addition, a consistent hierarchy of BB over AA is observed for all three parameters in terms of the maximum response elicited.

Effect of Ca^{2+} Antagonists on PDGF Isoform-Induced Changes in c-*fos* Expression and DNA Synthesis

In order to assess the role of Ca^{2+} in PDGF isoform induced c-*fos* expression, cells were preincubated prior to PDGF stimulation with agents that inhibit $[Ca^{2+}]_i$ increases. Cells preloaded with BAPTA or Quin-2, chelators of intracellular Ca^{2+} [33], were stimulated with BB or AA PDGF and processed for c-*fos* mRNA abundance and [^{3}H]thymidine incorporation. As shown in Figure 3, under conditions that block [^{3}H]thymidine incorporation, c-*fos* expression induced by maximal concentrations of either PDGF isoform was enhanced by the presence of Ca^{2+} chelator. The ability of BAPTA (10 μM) to block BB and AA PDGF-stimulated increases in $[Ca^{2+}]_i$ was confirmed by DVM with Fura-2 (Table I). The effects of BAPTA on PDGF-induced changes in c-*fos*, $[Ca^{2+}]_i$ and DNA synthesis (stimulatory for c-*fos*, inhibitory for $[Ca^{2+}]_i$ and [^{3}H]thymidine increases) were determined to be concentration dependent with maxima at 10 μM and partial effects at 1 μM for all three parameters (data not shown). Quin-2 has previously been reported to block $[Ca^{2+}]_i$ increases [37] and its fluorescence properties preclude $[Ca^{2+}]_i$ determinations by the Fura-2 method employed in the present study. Similar results were obtained in cells pretreated with 8-(diethylamine)octyl-3,4,5-trimethoxybenzoate hydrochloride (TMB-8) (Fig. 3), which has been shown to inhibit IP_3-induced release of Ca^{2+} from nonmitochondrial intracellular storage sites [38]. The inhibitory effect of Ca^{2+}-antagonist pretreatment on PDGF-induced [^{3}H]thymidine incorporation is not likely a consequence of toxicity of the treatment, as we have recently demonstrated that exposure of cells to the antagonists, at the same concentrations and for the same time period, 4 h following PDGF treatment does not inhibit PDGF-induced DNA synthesis [39]. The effect of increased $[Ca^{2+}]_i$ in the absence of PDGF stimulation was also investigated by treating cells with the Ca^{2+} ionophore ionomycin. Increased $[Ca^{2+}]_i$ stimulated by ionomycin was not associated with increases in c-*fos* levels or [^{3}H]thymidine incorporation (Table I). Thus, modulation of $[Ca^{2+}]_i$ by Ca^{2+} antagonists or a Ca^{2+} ionophore did not demonstrate a Ca^{2+}-dependency for c-*fos* expression.

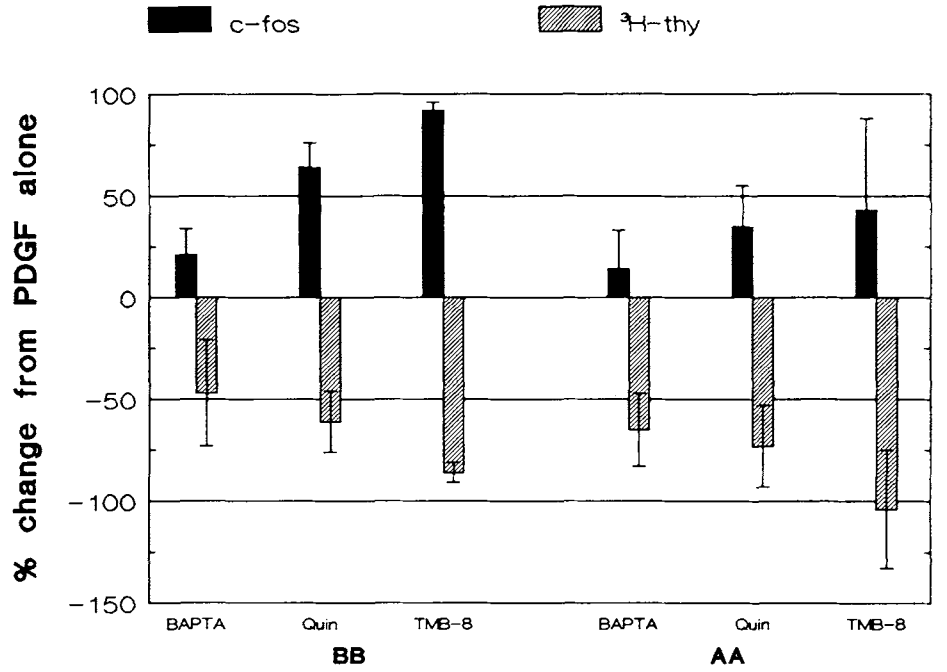

Fig. 3. Opposite effects of calcium antagonists on PDGF isoform-induced c-*fos* expression and DNA synthesis. BB (10 ng/ml) and AA (40 ng/ml) PDGF-stimulated changes in c-*fos* expression (solid bars) and [^{3}H]thymidine incorporation (hatched bars) were assessed in the absence and presence of agents that inhibit $[Ca^{2+}]_i$ increases. Cells were preincubated with 10 μM antagonist for either 30 min (BAPTA/AM or Quin-2/AM) or 15 min (TMB-8) prior to PDGF addition. RNA was extracted for analysis of c-*fos* expression immediately following 30 min exposure to PDGF. DNA synthesis was measured 24 h after a 1 h PDGF incubation period (PDGF and Ca^{2+}-antagonist removed at end of 1 h incubation). Results are expressed as the percent change from PDGF-stimulated values obtained in the absence of Ca^{2+}-antagonist. Positive values (c-*fos*) indicate further stimulation in the presence of the indicated agents, whereas negative values ([^{3}H]thymidine) indicate an inhibition of PDGF stimulation of this parameter (with −100% equaling unstimulated control values). Values represent the mean ± SE from three or more independent experiments.

Role of PKC in PDGF-Induced c-*fos* Expression: Dissociation From $[Ca^{2+}]_i$ and Mitogenesis

Activation of protein kinase C (PKC) is thought to be an important component in the signaling pathway(s) of many growth factor-induced biological responses. In BALB/c3T3 fibroblasts, PDGF promotes the production of the endogenous PKC activator, diacylglycerol, by both phosphatidylinositol hydrolysis and metabolism of phosphatidylcholine. Cells can be made deficient in PKC activity through down-regulation of the enzyme by prolonged treatment with phorbol esters, exogenous PKC activating agents [40]. Such treatment has been shown to prevent PKC-dependent influx of Ca^{2+}, cytosolic alkalinization, and protein phosphorylation in BALB/c3T3 cells [13]. The effect of PKC down-regulation (24 h pretreatment of cells with 600 nM TPA) on PDGF isoform-induced c-*fos* expression is shown in Figure 4. Under these conditions, both BB and AA PDGF isoform-induced gene expression were inhibited by greater than 75%. Down-regulation of enzyme activity was confirmed by the ability of the TPA pretreatment protocol to block (> 98%) the induction of c-*fos* mRNA by

TABLE I. Antagonism of PDGF Isoform-Induced Increases in $[Ca^{2+}]_i$ is Associated With Inhibition of DNA Synthesis but Not c-*fos* Expression*

Condition	c-*fos*	$[Ca^{2+}]_i$	[^{3}H]thymidine
	(fold increase)	*(% increase)*	*(% increase)*
Serum (10%)	139 ± 17	164 ± 13	314 ± 31
BB (10 ng/ml)	116 ± 13	80 ± 13	207 ± 28
BB + BAPTA (10 μM)	148 ± 23	4 ± 1	44 ± 19
AA (40 ng/ml)	80 ± 16	84 ± 7	159 ± 8
AA + BAPTA (10 μM)	109 ± 21	3 ± 1	25 ± 18
Ionomycin (0.1 μM)	1.1 ± 0.4	128 ± 10	−3 ± 10

*Quiescent BALB/c3T3 cells were stimulated with PDGF (BB or AA isoform), serum, or ionomycin at the indicated concentrations. The effect of the $Ca^{2+}{}_i$-antagonist, BAPTA, was assessed by preincubation with BAPTA/AM 30 min prior to PDGF addition. c-*fos* expression, $[Ca^{2+}]_i$, and [^{3}H]thymidine incorporation were measured as described in "Materials and Methods." Results are expressed as changes from unstimulated controls (0) and are mean ± SE values.

acute TPA stimulation (15 min, 150 nM). The inhibitory effect of phorbol ester treatment was specific for the active compound, TPA, as no such inhibition was observed when the inactive analog, 3-O-methyl-TPA, was substituted (data not shown). Additionally, the inhibitory effect of TPA pretreatment on c-*fos* expression is shown not to be due to a non-specific effect on mRNA levels as illustrated by Northern analysis of actin abundance in Figure 4A.

The PKC-independent component of c-*fos* stimulation by the PDGF isoforms was also found to be independent of $[Ca^{2+}]_i$ increases as illustrated by the inability of BAPTA to alter c-*fos* levels in down-regulated cells (Fig. 4; Table II). Table II summarizes the effects of PDGF isoform-induced alterations in c-*fos* expression, $[Ca^{2+}]_i$, and DNA synthesis in PKC down-regulated cells and demonstrates a dissociation between c-*fos* expression and alterations in $[Ca^{2+}]_i$ or DNA synthesis: PKC-deficient cells exhibit unchanged or enhanced PDGF-induced Ca^{2+} and mitogenic responses while at the same time gene expression is substantially inhibited.

DISCUSSION

The cellular signaling pathway of the human AB heterodimeric form of PDGF in BALB/c3T3 fibroblasts has been demonstrated to involve receptor activation of phospholipase C with consequent hydrolysis of phosphatidylinositol 4,5-bisphosphate to inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) [13,41,42]. IP_3 causes a rapid, transient increase in $[Ca^{2+}]_i$ by releasing nonmitochondrial intracellular calcium stores [43], while DAG activates protein kinase C (PKC). Both increases in $[Ca^{2+}]_i$ and PKC activity have been postulated to be important and/or necessary processes in cellular proliferation and the induction of growth-associated gene expression in a number of systems [14,20,26]. In the present study, we have compared the effects of recombinant PDGF homodimers AA and BB on growth factor-induced increases in $[Ca^{2+}]_i$, c-*fos* expression, and DNA synthesis in BALB/c3T3 cells in an attempt to determine whether differences exist in the signaling pathways used by these PDGF molecules that are important in the growth factor's mitogenic activity. Specifically addressed in the present study were the roles of $[Ca^{2+}]_i$ and PKC in the induction of c-*fos* proto-oncogene expression and mitogenesis.

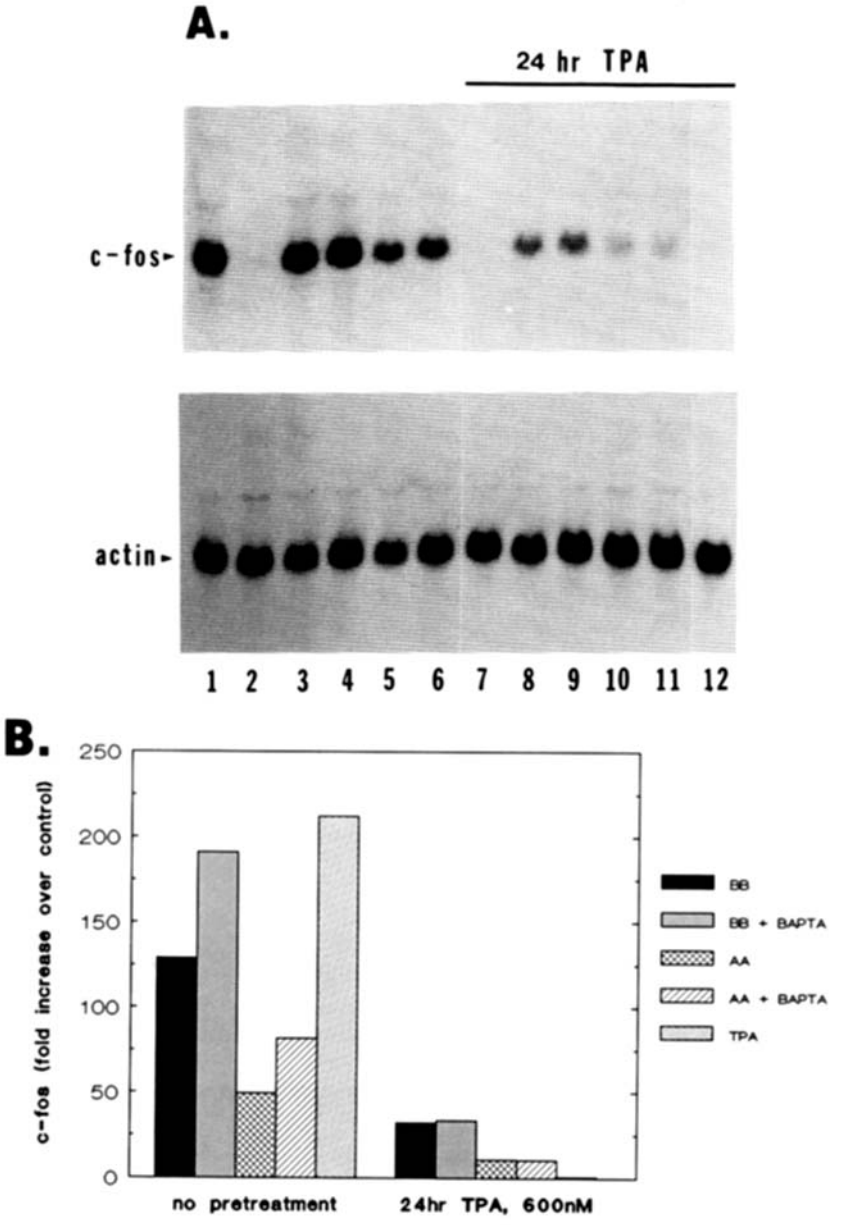

Fig. 4. PDGF isoform-induced c-*fos* expression exhibits partial dependence on PKC activity but is independent of increases in $[Ca^{2+}]_i$. Serum-starved cells were pretreated for 24 h with TPA, 600 nM, or vehicle alone (10% spent medium). PDGF or acute TPA stimulation was for 30 min; BAPTA treatments (where indicated) were 30 min preincubations with 10 μM BAPTA/AM which remained in the subsequent PDGF incubation. Total RNA was analyzed for c-*fos* and actin abundance as in Figure 1. **A:** Northern blot analysis: **Lanes 1–6** represent non-TPA pretreated control cultures; **lanes 7–12** are from cells made PKC-deficient by 24 h TPA pretreatment. Lanes 1,12: Acute TPA stimulation (150 nM). Lanes 2,7: Unstimulated controls. Lanes 3,8: BB PDGF (10 ng/ml). Lanes 4,9: BB PDGF + BAPTA. Lanes 5,10: AA PDGF (40 ng/ml). Lanes 6,11: AA PDGF + BAPTA. **B:** Shows the densitometric quantitation of c-*fos* mRNA normalized to the amount of actin message detected in the same sample. PKC down-regulated cells show virtually no c-*fos* stimulation by acute TPA treatment, confirming inhibition of enzyme activity. PKC deficiency results in a substantial inhibition of c-*fos* induction by both BB and AA PDGF isoforms. Chelation of Ca^{2+}_i (BAPTA) results in enhanced expression in control cultures and has little or no effect in PKC-deficient cells. Similar results were obtained with two additional, different cell preparations.

TABLE II. Protein Kinase C Down-Regulation Inhibits PDGF Isoform-Induced c-*fos* Expression but Not Increases in $[Ca^{2+}]_i$ or DNA Synthesis*

Stim.	TPA pretreat	BAPTA	c-*fos*	$[Ca^{2+}]_i$	[^{3}H]thy
	(24 h)	*(10 μM)*	*(fold increase)*	*(% increase)*	*(% increase)*
TPA	–	–	167 ± 44	31 ± 5	28 ± 7
—	+	–	1.2 ± 0.3	5 ± 8	27 ± 33
TPA	+	–	2.3 ± 1.2	5 ± 9	28 ± 3
BB	–	–	156 ± 28	76 ± 6	290 ± 65
BB	+	–	38 ± 6	74 ± 19	309 ± 145
BB	+	+	26 ± 8	1 ± 1	33 ± 57
AA	–	–	106 ± 57	74 ± 9	159 ± 8
AA	+	–	25 ± 15	98 ± 4	259 ± 22
AA	+	+	16 ± 6	2 ± 1	0 ± 22

*Untreated controls and cells made deficient in PKC by 24 h pretreatment with 600 nM TPA were stimulated acutely with TPA (150 nM), BB PDGF (10 ng/ml), or AA PDGF (40 ng/ml). Additionally, some PKC-deficient cells were preincubated with the Ca^{2+} chelator, BAPTA, for 30 min prior to PDGF stimulation. c-*fos* mRNA abundance, $[Ca^{2+}]_i$, and [^{3}H]thymidine incorporation were measured as described in "Materials and Methods" and are expressed as changes from their respective unstimulated controls (0). Values are the mean ± SE of at least two separate determinations. PDGF-induced c-*fos* expression exhibited a substantial PKC dependency, whereas $[Ca^{2+}]_i$ increases and DNA synthesis were actually enhanced in PKC-deficient cells. The PKC-independent component of induced c-*fos* expression was not dependent on increased $[Ca^{2+}]_i$.

No discernible *mechanistic* differences in PDGF isoform activities could be elucidated as our results demonstrate that both AA and BB stimulation result in concentration-dependent increases in $[Ca^{2+}]_i$, c-*fos* expression, and DNA synthesis, as has been previously documented for human AB PDGF in this system [14,44,45]. However, differences in efficacy were noted in terms of stimulation of alterations in $[Ca^{2+}]_i$, c-*fos* expression, and DNA synthesis with BB eliciting larger maximal responses than AA (Fig. 2). Dose-response relationships for a given isoform were similar for the three parameters measured. Similar dose-response relationships have been reported for receptor autophosphorylation [46] and IP_3 production [47] by AA and BB homodimers in BALB/c3T3 cells. Comparing receptor autophosphorylation and mitogenic responses of BALB/c3T3 cells to CHO cells that had been transfected with cloned human PDGF receptor cDNA, Escobedo and colleagues [46] proposed that the responses of BALB/c3T3 cells to AA and BB stimulation could be attributed to a single type of PDGF receptor. Differences in potencies of the isoforms to elicit a particular response were thought to arise from a lower apparent affinity of AA for the receptor. The ability of BB to consistently produce larger maximum responses than AA at presumed saturating concentrations (Fig. 2), however, favors a model that includes the presence of multiple PDGF receptor types, one capable of binding both isoforms, the other preferentially binding BB [28–30]. The ratio of the two classes of receptors or receptor subunits (α:β or αα:αβ:ββ [if dimerization of the receptor is required for activity]) as determined by [^{125}I]PDGF binding studies varies with cell type and has been reported to range from 1:1 for Swiss 3T3 fibroblasts and MG-63 osteosarcoma cells to 20:1 in human dermal and foreskin fibroblasts [30]. Making the (over-)simplifying assumptions that 1) the different receptor subtypes possess equal

mitogenic potencies or transducing abilities ($[Ca^{2+}]_i$ and c-*fos* responses), and 2) ligand binding is translated into biological response with 100% efficiency (no spare receptors), our data would suggest that BALB/c3T3 fibroblasts possess an apparent ratio of BB:AA binding (effector) sites of 2–2.5:1 (see Fig. 2). Preliminary ^{125}I-labeled BB and AA PDGF binding data from this laboratory indicates a ratio of approximately 3:1 (BB:AA) in BALB/c3T3 cells (S.H. Bernacki and B. Herman, unpublished observation). Such findings are consistent with differences in the PDGF isoform maximal responses reflecting proportional differences in receptor numbers.

Another possible explanation for the apparent efficacy differences between the isoforms is that distinct subpopulations of cells with different receptor compositions exist within the bulk cultures. One subpopulation, representing approximately one-third of the total number of cells, may be responsive only to the AA isoform. Clarification of the number and types of PDGF receptors and their abilities to effect a given response in BALB/c3T3 cells will entail further examination of isoform binding characteristics and manipulation of specific receptor types by down-regulation and antibody blocking approaches. Pilot studies designed to address these questions, using single cell $[Ca^{2+}]_i$ measurements, indicate that a single BALB/c3T3 cell can possess both α and β type receptors, as cells that responded to saturating concentrations of the AA isoform were subsequently able to exhibit a $[Ca^{2+}]_i$ response upon addition of BB PDGF (P. Diliberto and B. Herman, unpublished observations). It therefore does not appear that the difference in maximum responses elicited by the two isoforms merely reflects the presence of a subpopulation of cells that is responsive only to the AA isoform.

Alternatively, or in addition to differences solely in receptor subtype number, the different PDGF isoforms and their corresponding receptors could be eliciting common responses through distinct signaling mechanisms. Increases in $[Ca^{2+}]_i$ (presumably arising from IP_3-sensitive storage sites) were found to be obligatory for the induction of DNA synthesis by both BB and AA isoforms (Table I), a finding previously reported for human AB PDGF [13]. PKC activation, in contrast, was not found to be an essential component of the mitogenic signaling pathway of either isoform as demonstrated in cells in which PKC down-regulation was achieved by prolonged phorbol ester pretreatment (Table II). These data are consistent with results obtained with human AB PDGF, as well as with the observations that all three isoforms are capable of inducing increases in $[Ca^{2+}]_i$ with subsequent DNA synthesis by a PKC-independent mechanism [Table II; ref. 13].

As proto-oncogene expression induced by PDGF is thought to be an important and/or necessary event in mitogenic signaling, the present findings concerning the regulation of c-*fos* expression are intriguing. Although a fairly close correlation between PDGF-induced changes in $[Ca^{2+}]_i$ and c-*fos* abundance were demonstrated for both isoforms (Fig. 2), prevention of increases in $[Ca^{2+}]_i$ (BAPTA and Quin-2) had no inhibitory effect on c-*fos* expression. Additional support for the dissociation of c-*fos* expression and $[Ca^{2+}]_i$ includes the inability of TMB-8 (blockade of intracellular Ca^{2+} release) to alter PDGF-induced c-*fos* levels and the inability of ionomycin (a Ca^{2+} ionophore) to induce c-*fos* expression in the absence of growth factor. Prior studies linking increased $[Ca^{2+}]_i$ to c-*fos* expression have been performed in excitable cell systems such as PC12 pheochromocytoma and GH_3 pituitary cell lines that utilize extracellular calcium via voltage-dependent Ca^{2+} channels as the major source of

$[Ca^{2+}]_i$ increases [26,27]. Interestingly, in PC12 cells, c-*fos* protein and mRNA can be induced either by depolarizing agents or by the polypeptide growth factor, NGF. The induction of c-*fos* by NGF is independent of extracellular calcium and is unaffected by dihydropyridine Ca^{2+} channel blockers; in contrast, c-*fos* induction by depolarizing agents such as high extracellular K^+ and veratridine or the dihydropyridine Ca^{2+} channel agonist, BAY K 8644, is dependent on extracellular Ca^{2+} and is totally blocked by dihydropyridine Ca^{2+} channel antagonists [26]. Similarly, BAY K 8644 induction of c-*fos* in GH_3 cells was found to occur through a mechanism distinct from that of PDGF-induced c-*fos* expression in the same cells (Ca^{2+}-dependent, PKC-independent vs. PKC-dependent, vide infra) [27]. Increased $[Ca^{2+}]_i$ has also been implicated in the mechanism(s) of proto-oncogene induction in thymocytes and Swiss 3T3 fibroblasts by virtue of the ability of calcium ionophores (A23187 and ionomycin) to induce gene expression [24,48]. In both cell types, these effects were dependent on influx of Ca^{2+} from extracellular stores. Thus, a pattern emerges in which the involvement of Ca^{2+} in the positive regulation of c-*fos* expression is determined not by cell type or the presence (or magnitude) of an increased $[Ca^{2+}]_i$ response per se, but is associated with a specific Ca^{2+}-signaling pathway (influx of Ca^{2+} from extracellular sources). Our data demonstrating Ca^{2+}-independent induction of c-*fos* by PDGF isoforms in BALB/c3T3 cells are consistent with such a pattern, as the major source of PDGF stimulated increases in $[Ca^{2+}]_i$ has been identified as an IP_3-mediated release of intracellular calcium stores and not extracellular Ca^{2+} entry mediated by voltage-sensitive Ca^{2+} channels [13].

Addition of the intracellular Ca^{2+} chelator, BAPTA, not only failed to inhibit but actually increased c-*fos* mRNA levels stimulated by PDGF (Figs. 3, 4). This superinduction was also observed with other intracellular Ca^{2+} antagonists, Quin-2 and TMB-8 (Fig. 3). Interestingly, similar findings have been reported for the effect of EGTA in combination with A23187 on PDGF-induced c-*myc* expression in MG-63 osteogenic sarcoma cells [49]. PDGF-stimulated c-*myc* expression in this system was found to be independent of both increased cytoplasmic $[Ca^{2+}]$ and PKC. Superinduction of growth factor-stimulated proto-oncogene expression by removal or chelation of intracellular Ca^{2+} may point to a negative regulatory role for basal $[Ca^{2+}]_i$ in induced gene expression.

Protein kinase C, in contrast to $[Ca^{2+}]_i$, appears to play a major regulatory role in PDGF isoform-induced c-*fos* expression in BALB/c3T3 cells (Fig. 4; Table II). This again is interesting in light of the role, or lack thereof, that PKC plays in PDGF-stimulated mitogenesis in these same cells. An inverse relationship exists between c-*fos* expression and [^{3}H]thymidine incorporation under conditions in which PKC is directly activated or in PDGF-stimulated cells that have been made deficient in PKC. Similar to the results obtained with the AA and BB isoforms of PDGF, reduction but not elimination of c-*fos* induction by human AB PDGF has been reported in a variety of cell types made deficient in PKC [21,27,50]. These data indicate that PKC-dependent second messengers play a significant but not absolute role in PDGF-induced c-*fos* expression but at the same time are not required for PDGF-induced stimulation of cell proliferation. The data point to the existence of multiple pathways for the induction of c-*fos* expression. It is not possible to rule out a role for c-*fos* in the PDGF mitogenic pathway due to the small but significant PKC-independent component of gene expression observed. The mechanism of the PKC-independent pathway has not been

identified although it does appear to be independent of $[Ca^{2+}]_i$ (Fig. 4). Cyclic AMP has been shown to act as a positive regulator of PDGF-induced c-*fos* expression in Swiss 3T3 fibroblasts [23,24]. PDGF stimulation in BALB/c3T3 cells has not been found to increase cAMP levels under normal conditions, although similar information in PKC down-regulated cells is presently unavailable. A cAMP dependent pathway has, however, been identified in BALB/c3T3 cells for EGF-stimulated c-*fos* and c-*myc* expression, although this effect apparently requires the simultaneous influx of extracellular Ca^{2+} [22]. More recently, a negative regulatory role for cAMP has been reported for PDGF stimulated c-*myc* expression and DNA synthesis in human fibroblasts [25]. Inducible c-*fos* expression was found to be unaffected by cAMP levels in this system. Thus, it remains that, even though an apparent dissociation exists between c-*fos* expression and increases in either DNA synthesis or $[Ca^{2+}]_i$ stimulated by the AA and BB PDGF isoforms in BALB/c3T3 fibroblasts, the necessity of c-*fos* in the mitogenic signaling pathway cannot be dismissed due to the persistence of a minor PKC-independent component of PDGF-inducible c-*fos* expression, the mechanism of which requires further clarification.

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