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The induction of early response genes in rat smooth muscle cells by PDGF-AA is not sufficient to stimulate DNA-synthesis

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The effect of the three platelet-derived growth factor (PDGF) isoforms AA, AB and BB on the induction of the early growth response genes c-*fos*, *egr*-1 and c-*myc* mRNA in vascular smooth muscle cells from rat was compared with their respective mitogenic potency. The three PDGF isoforms strongly stimulated the induction to a similar extent. In contrast, PDGF-AB and -BB provoked a marked DNA synthesis whereas PDGF-AA exerted only a poor mitogenic effect in smooth muscle cells. PDGF-AA-stimulated receptor autophosphorylation was not detectable in comparison with the strong effect elicited by PDGF-AB or -BB and correlated with its low mitogenicity but not with the almost equal induction of the early response genes. It is discussed that no or only very low receptor phosphorylation is required to link receptor activation to the induction of c-*fos*, *egr*-1 or c-*myc*. Furthermore the induction of the investigated gene does not seem to be sufficient for an optimal mitogenic response.

Platelet-derived growth factor (PDGF) isoform; c-*fos*; *egr*-1; c-*myc*; Receptor autophosphorylation

1. INTRODUCTION

Platelet-derived growth factor (PDGF) is a major mitogen in serum which promotes the proliferation of fibroblasts, glia cells, and smooth muscle cells in vitro [1–3]. The two homologous chains, termed A and B, which have been identified and sequenced, can combine to three different dimeric isoforms: AA, AB, and BB. The dimeric structure is stabilized by disulfide bridges, and after destruction of these bridges the biological activity is lost [1–3]. The fact that only dimers are biologically active, along with the discovery of two different PDGF receptors (α and β) led to the hypothesis that one dimeric PDGF may bind two receptor molecules (receptor subunit model) [4–7]. The model predicts that PDGF-AA binds only α-type PDGF receptors, PDGF-AB mainly binds one α-type and one β-type receptor, and PDGF-BB predominantly binds β-type PDGF receptors.

The two receptors with a molecular weight of 180 kDa share a common structure, including an extracellular domain that is composed of five immunoglobulin-like repeats, a single transmembrane and a cytoplasmic segment that includes split tyrosine kinase sequences [4–6]. But despite extensive amino acid sequence homology, the two receptors display considerable heterogeneity in their extracellular parts and their cytoplasmic non-catalytic domains, such as the kinase insertion stretches and the C-termini. These cytoplasmic regions participate in the interaction between the activated receptor and cytoplasmic proteins (for review see [3]) and thus may confer distinct substrate specificities upon the two homologous receptors.

In order to identify different signalling pathways of the two types of PDGF receptors we compared the effects of the three PDGF isoforms on the induction of the protooncogenes c-*fos*, c-*myc* and *egr*-1 [8], which belong to the class of early response genes.

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Abbreviations: VSMC, vascular smooth muscle cells; DMEM, Dulbecco's modified Eagle's medium; PDGF, platelet-derived growth factor; *egr*-1, early growth response gene-1; SH_2, src-kinase homologous region 2; PLC-γ, phospholipase Cγ; PKC, protein kinase C; $InsP_3$, inositoltrisphosphate; PC, phosphatidylcholine.

2. MATERIALS AND METHODS

2.1. *Materials*

PDGF-AA, PDGF-AB and PDGF-BB from *E. coli* were prepared as previously described [9,10]. Dulbecco's modified Eagle's medium (DMEM), Ham's F-10 and Dulbecco's phosphate-buffered saline were obtained from Gibco BRL (Eggenstein, Germany).

2.2. *Culture of vascular smooth muscle cells (VSMC)*

VSMC were isolated from rat aorta (strain, female Wistar–Kyoto, 6–10 weeks old, Charles River Wiga GmbH, Sulzfeld, Germany) by the media explantate method and cultured over several passages according to Ross [11]. The culture medium was DMEM supplemented with 20% fetal calf serum.

2.3. *Measurement of DNA synthesis in VSMC*

The effect of the PDGF isoforms on DNA synthesis was measured

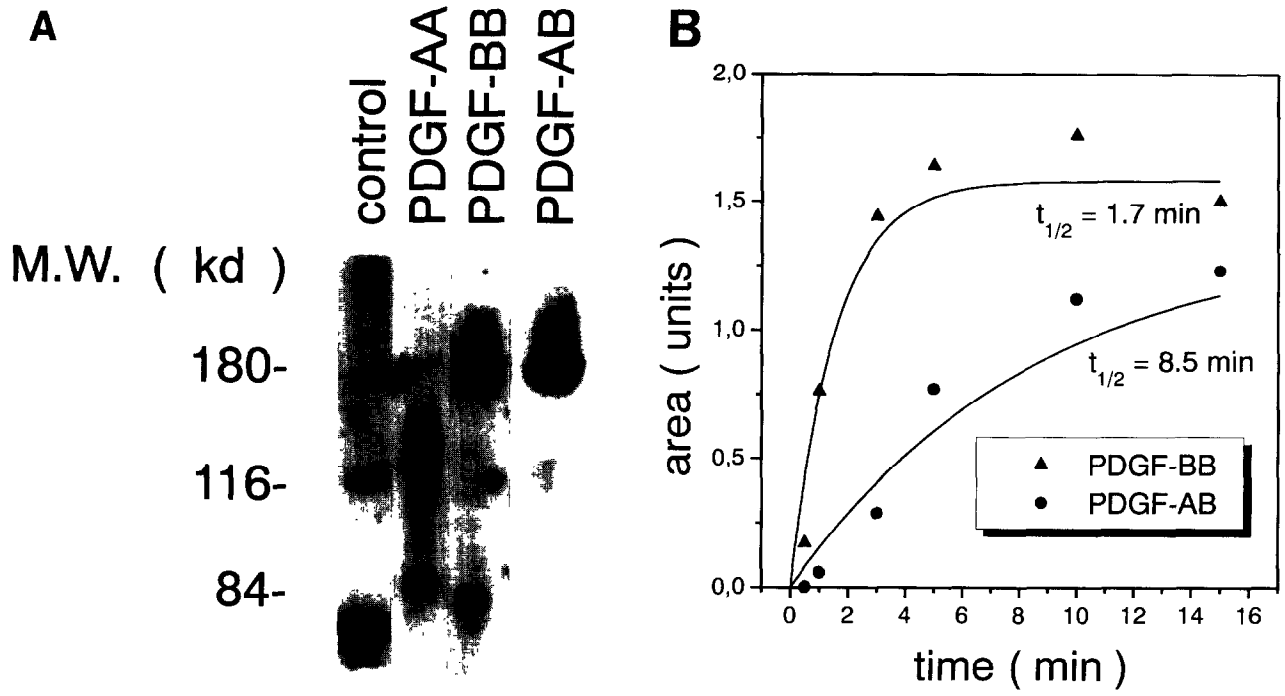

Fig. 1. (A) Receptor autophosphorylation stimulated by PDGF isoforms. Cells grown in 12-well plates were lysed in SDS-containing buffer. 50% of the lysate was used for Western blot analysis [13]. Stimulation was done for 15 min at 50 ng/ml of each PDGF isoform The star indicates the location of the PDGF receptors. (B) Densitometric analysis of the kinetic of receptor autophosphorylation stimulated by PDGF-AB or PDGF-BB (50 ng/ml). Areas were scanned by an LKB Laser densitometer. Kinetic constants were evaluated assuming first order kinetics (using the program Origin (Microcal)).

by a slight modification of the method of Nemecek et al. [12]. VSMC were seeded in 24-well culture plates and cultivated in culture medium until confluent. Then the medium was replaced by serum-free (quiescent) medium consisting of a mixture of DMEM and Ham's F-10 medium (1:1). Following another 24 h cultivation in quiescent medium cells were stimulated with 50 ng/ml of the PDGF isoforms. After 20 h 3 μCi/ml [^{3}H]thymidine was added to the quiescent medium. 4 h after [^{3}H]thymidine addition, experiments were terminated as previously described [12].

2.4. *Analysis of tyrosine-phosphorylated proteins*

This was done as described [13].

2.5. *RNA extraction and analysis*

The expression of c-*fos* and *egr*-1 mRNA was studied after preincubation of the cells for 24 h in serum-free quiescent medium in 75 cm^2 culture flasks. Then the quiescent cells were stimulated with the PDGF isoforms. Total RNA was extracted from VSMC by the guanidinium isothiocyanate/CsCl procedure [14]. Typically, between 50–70 μg total RNA was obtained from the cells of a 75 cm^2 flask. 10 μg of total RNA was separated by electrophoresis in a 6% formaldehyde/1.2% agarose gel, blotted on Hybond N$^+$ membranes (Amersham, Little Chalfont, UK), washed at room temperature in 5 × SSC (1 × = 0.15 M NaCl, 0.015 M sodium citrate) for 5 min, and fixed with UV irradiation. After fixing, the blots were washed at 60°C in 0.1 × SSC, 0.1% sodium dodecylsulfate (SDS) for 5 min. This wash greatly reduced background. Prehybridization and hybridization were performed overnight at 60°C in 5 × SSC, 0.2% SDS, 50 mM sodium phosphate, 10 × Denhardt's solution, 200 μg/ml salmon sperm (ss) DNA. The DNA probes were labelled with [^{32}P]deoxycytidine triphosphate ([α-^{32}P]dCTP) (Amersham Buchler, Braunschweig, Germany) by random oligonucleotide priming (Amersham) to a specific activity of 2–4 × 10^9 dpm/μg DNA. The stringency of the final wash was 0.2 × SSC containing 0.1% SDS at 65°C for 2 × 45 min. A 2.1 kb fragment (OC 68 insert) of *egr*-1 including three zinc-fingerdomains, a 1.0 kb fragment of v-*fos* and a 1.56 kb fragment of c-*myc* (Dianova/Oncor Science, Hamburg, Germany) were used as probes. Blots were exposed to Kodak films (Kodak X-Omat, 8 × 10 inch, Rochester, USA) for 3–7 days at −70°C. Blots were standardized using a 0.77 kb cDNA probe for β-actin (Dianova/Oncor, Hamburg, Germany). Densitometric analysis was performed with Hoefer GS-300 Scanning Densitometer (Hoefer Scientific Instruments, San Francisco, CA). The size in kilobases (kb) of the detected mRNA was calculated by the molecular weight standard RNA (7.4, 5.3, 2.8, 1.6 kb, Boehringer-Mannheim, Germany) as well as by the 18 S (1.8 kb) and 28 S (4.6 kb) ribosomal RNA migration from the gel wells.

3. RESULTS

PDGF-AB and PDGF-BB (50 ng/ml) induced a similar marked increase in [^{3}H]thymidine incorporation from 234 ± 16 (basal value) to 1,982 ± 124 and 2,020 ± 109 cpm/μg protein (mean ± S.D., $n = 3$, $P < 0.05$), respectively. PDGF-AA induced only a minor increase from 234 ± 16 to 432 ± 38 cpm/μg protein (mean ± S.D., $n = 3$, $P < 0.05$), demonstrating the poor mitogenic effect of PDGF-AA in VSMC compared with two other PDGF isoforms. The extent of tyrosine autophosphorylation stimulated by the PDGF isoforms correlated with their respective mitogenic po-

tency, i.e. PDGF-AB and -BB caused a strong stimulation whereas the effect of PDGF-AA was below detection (Fig. 1A). The half time for total tyrosine phosphorylation was 1.7 min for PDGF-BB and 8.5 min for PDGF-AB. Maximum phosphorylation was observed after 8 min for PDGF-BB and 15 min for PDGF-AB, respectively (Fig. 1B). These times are considerably longer than those observed for $[Ca^{2+}]_i$ increase which is at maximum after 1 or 2 min for PDGF-BB or -AB, respectively [15]. Maximum $[Ca^{2+}]_i$ signalling apparently occurs already at low total receptor phosphorylation.

Fig. 2 shows the time–course of the induction of the 2.2 kb c-*fos* mRNA at a concentration of 50 ng/ml of the three PDGF-isoforms. The time–course of the c-*fos* mRNA induction by all three isoforms was very similar showing a maximum at 30 min. After 60 min the values returned to control levels. Fig. 3 shows a quantitative evaluation of the c-*fos* mRNA induction by densitometric analysis. In addition to the identical time–course, the dose–response for the three isoforms was quite similar, PDGF-AA being somewhat less potent than the other two isoforms. Furthermore, Fig. 3 shows the effects of PDGF isoforms on the induction of *egr*-1 and c-*myc* mRNAs. Evidently the kinetics are identical and typical for the respective mRNA species, i.e. *egr*-1 mRNA content was maximum between 30 and 60 min and then declined gradually, and c-*myc* mRNA content increased up to 120 min. Remarkably the PDGF-AA-induced synthesis of c-*myc* mRNA was as strong as with the two other isoforms. PDGF-AA at lower concentrations was somewhat less effective at stimulating *egr*-1 mRNA production. At higher concentrations (50 ng/ml) there was no difference. Clearly all three PDGF isoforms strongly stimulated the induction of the three protooncogenes.

4. DISCUSSION

We recently described that both the human recombinant PDGF-BB and PDGF-AB stimulated the phosphoinositide (PI) signalling system leading to an increase of intracellular inositol 1,4,5-triphosphate ($InsP_3$), diacylglycerol formation, intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) and DNA synthesis in VSMC [15]. Although PDGF-AA lacked any effects on the PI signalling system ($InsP_3$ and $[Ca^{2+}]_i$) it induced diacylglycerol formation in VSMC but with different kinetics compared to that observed with the other PDGF isoforms. PDGF-AB and PDGF-BB exerted a biphasic formation of diacylglycerol peaking at 1 min and 5 min whereas the PDGF-AA-induced diacylglycerol formation was monophasic with a maximum at 5 min. In this context, it has been suggested that the first phase of the PDGF-BB- and -AB-induced diacylglycerol formation originates from the phosphatidylinositol 4,5-bisphosphate whereas the second phase of diacylglycerol formation originates from phosphatidylcholine (PC) [15,16].

There are 5,000 binding sites per cell for PDGF-AA, 45,000 binding sites per cell for PDGF-AB, and 31,000 binding sites per cell for PDGF-BB [15]. The relatively low number of PDGF α-receptors, together with the lower intrinsic autophosphorylation capacity [13], could explain the failure to detect phosphotyrosine after stimulation by PDGF-AA.

The propagation of the mitogenic signal from the receptor very often involves proteins such as phospholipase Cγ (PLC-γ), GTPase activating protein, phosphatidylinositol-3 kinase, carrying an SH_2-domain that is capable of binding to specific regions of the receptor containing autophosphorylated tyrosines

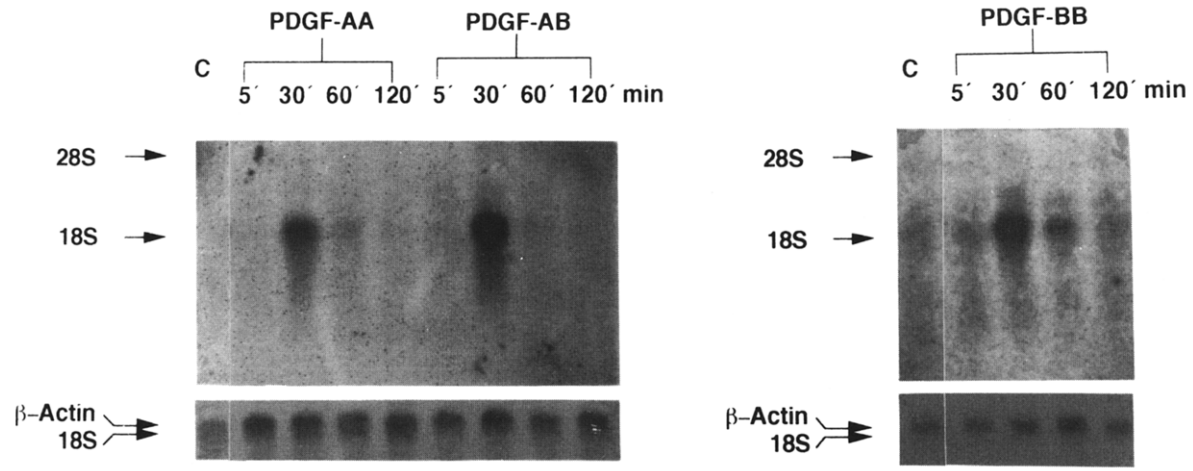

Fig. 2. Time–course of the c-*fos* induction in VSMC after cell stimulation with the PDGF isoforms. Quiescent VSMC were stimulated with 50 ng/ml of each PDGF isoform for the indicated times. 10 μg of total RNA were separated on a formaldehyde–agarose gel, blotted onto Hybond N^+ membranes and probed with a ^{32}P-labelled 1.0 kb fragment of v-*fos* which hybridized to the 2.2 kb of c-*fos* mRNA. The same blot previously hybridized with v-*fos* cDNA probe was rehybridized with a 0.77 kb cDNA probe for β-actin. Arrows indicate the RNA markers, the 28 S (4.6 kb), the 18 S ribosomal RNA (1.8 kb), the 2.2 kb c-*fos* mRNA and the 2.9 kb β-actin mRNA.

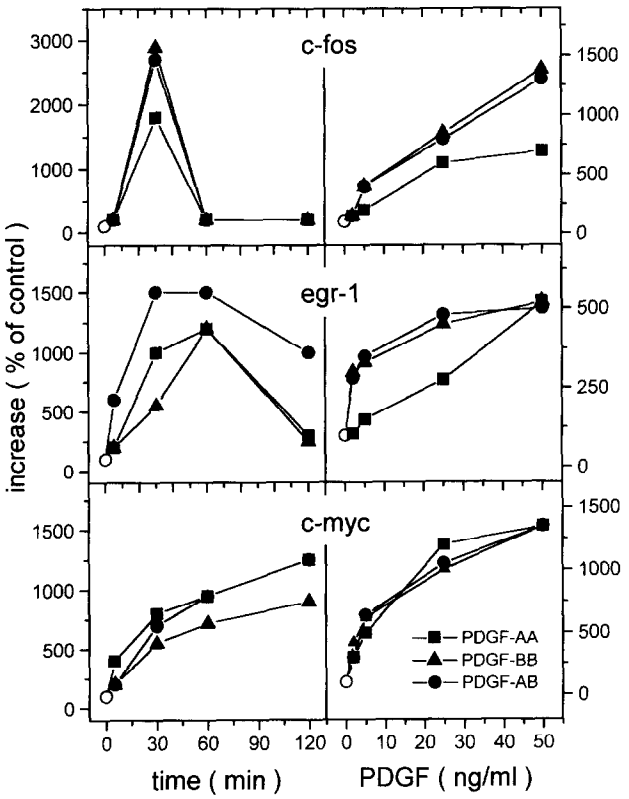

Fig. 3. Densitometric analysis of time- and concentration-dependent induction of c-*fos*, c-*myc* and *egr*-1 mRNA. For time dependency the concentrations of the PDGF isoforms were 50 ng/ml. The concentration dependency was determined after 30 min for c-*fos*, 60 min for *egr*-1 and 120 min for c-*myc*, respectively. (■) PDGF-AA; (●) PDGF-AB; (▲) PDGF-BB; (○) control.

[17,18]. For example, for the activation of PLC-γ its association via its SH_2-domains with the autophosphorylated PDGF receptor and subsequent phosphorylation at tyrosine residues is required [19]. A detailed analysis showed that tyrosine residues 1,009 and 1,021 in the β-type receptor are the targets of PLC-γ binding. Interestingly, only one of these tyrosine residues (1,021) is present in the sequence of the α-type receptor [20]. This structural difference adds a further explanation to the failure of PDGF-AA to raise the concentration of $InsP_3$ or $[Ca^{2+}]_i$. But remarkably, the second phase of diacylglycerol accumulation, which most likely originates from hydrolysis of PC [15,16], was the same for all three PDGF isoforms. The latter observation corroborates the results presented here that all three isoforms induce the expression of the three chosen early response genes to a similar extent. Since also protein kinase C (PKC) activity is stimulated to a similar extent by all three PDGF isoforms [21], it is possible that the induction of the early response genes proceeds via diacylglycerol release with subsequent PKC activation. This hypothesis is in line with the results from Hall et al. [22] showing that c-*fos* mRNA induction in mammalian cells is channeled predominantly through PKC activation. But activation of this pathway alone apparently does not result in a strong mitogenic response [22], explaining the low effect of PDGF-AA on DNA synthesis. On the other hand, PDGF-AB and -BB are able to activate additional signalling pathways that, together with the PKC pathway, might lead to cell division.

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