Evidence for involvement of phospholipase C-γ2 in signal transduction of platelet-derived growth factor in vascular smooth-muscle cells

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In order to examine the mechanisms underlying smooth-muscle cell proliferation, we investigated effect of platelet-derived growth factor (PDGF) dimers on proliferation of rabbit vascular smooth-muscle cells (VSMCs) and also involvement of phospholipase C (PLC) isoforms in the signal transduction. PDGF-BB and -AB, but not -AA, stimulated cell proliferation and intracellular production of inositol trisphosphate. Northern and Western analyses demonstrated that VSMCs mainly expressed PLC-γ2 and PLC-δ1 among four PLC isoforms tested. A number of cellular proteins, including PLC-γ2, but not PLC-δ1, were phosphorylated on a tyrosine residue by the stimulation of either PDGF-BB or -AB. These results suggest a functional association of PDGF receptor and PLC-γ2 that might be responsible for PDGF-dependent VSMC growth. In addition, the expression of PLC-γ2 was extremely low in the primary VSMC cultures and was induced during further cultivation of the primary cultures, indicating that an acquisition of PDGF-signal-transducing components, including PLC-γ2, may be an important step for proliferation of smooth-muscle cells.

INTRODUCTION

The production of the second messengers diacylglycerol and Ins(1,4,5)P_3 by the action of inositol-phospholipid-specific phospholipase C (PLC) is one of the most common types of transmembrane signalling in a variety of cell-stimulation systems (for reviews, see [1–4]). Since growth factors such as platelet-derived growth factor (PDGF) and epidermal growth factor initiate the rapid formation of InsP_3, suggesting that PLC is involved in their signalling pathways. Most growth factors bind to receptors and activate intrinsic tyrosine kinases. Mammalian cells contain multiple forms of PLC, among which a γ-type (PLC-γ1) is preferentially phosphorylated [5–13] and activated [14–17] by these receptor tyrosine kinases.

The proliferation of vascular smooth-muscle cells (VSMCs) is essential for the formation of atherosclerotic plaques (for reviews, see [18–21]). VSMCs in the media contract co-operatively in response to vasoactive stimuli, but do not proliferate in response to growth factors. We refer to these VSMCs as the contractile phenotype. On the other hand, VSMCs which have migrated to the intima are phenotypically and functionally different from the contractile phenotype. They acquire proliferative activity in response to growth factors and show no contractile activity. These cells represent the synthetic phenotype rather than contractile one [18,22]. Since such phenotypic modulation of VSMCs can be observed *in vitro* by cultivating primary VSMCs [23,24], there is little knowledge of how VSMCs alter their phenotype so as to acquire the ability to grow. In this study, we present evidence that PLC-γ2, instead of PLC-γ1, is involved in the signalling pathway of PDGF, using synthetic phenotype VSMC cultures and also demonstrate induction of PLC-γ2 expression with phenotypic modulation of VSMCs.

MATERIALS AND METHODS

Cells

Primary cultures of rabbit VSMCs were prepared from fresh aortae of adult Japanese white rabbits by incubating segments of the tissue in a solution of 0.2% (w/v) collagenase in Hanks' balanced salt solution (HBSS) at 37 °C for 30 min under humidified 5%-CO_2 in an incubator [25]. The cells isolated from the tissue were collected, washed, and suspended in Dulbecco's modified Eagle medium containing 10% (v/v) fetal-calf serum. The primary cells were maintained in the same medium with a change of medium every 3 days and used for the experiments of phenotypic modulation. On the other hand, the cultures subcultured with 20 and 30 cell population doubling numbers were used as synthetic phenotype VSMCs. For the examination of the effect of PDGF homo- and hetero-dimers on cell growth, InsP_3 formation and protein phosphorylation, cells were cultivated for 2 days in a starvation medium: Dulbecco's modified Eagle medium containing 1% (w/v) BSA, 30 nM Na_2SeO_4, 5 μg/ml transferin and 5 μg/ml insulin.

Northern and Western analyses

Poly(A)$^+$ RNA was isolated by using a FAST TRACK mRNA isolation kit (Invitrogen). Electrophoresis of 10 μg portions was carried out in 1% (w/v) agarose gels containing 3% (w/v) formaldehyde, and the separated RNAs were transferred to nylon filters by the capillary blotting method. Hybridization was performed at 65 °C for 12 h by using cDNA probes for the four distinct PLC isoforms [26] and a Rapid Hybridization kit (Amersham), and the filters were washed in 2 × SSC (1 × SSC = 150 mM NaCl/15 mM sodium citrate) containing 0.1% SDS

Abbreviations used: VSMC, vascular smooth-muscle cell; PLC, phosphoinositide-specific phospholipase C; PDGF, platelet-derived growth factor; HBSS, Hanks' balanced salt solution.

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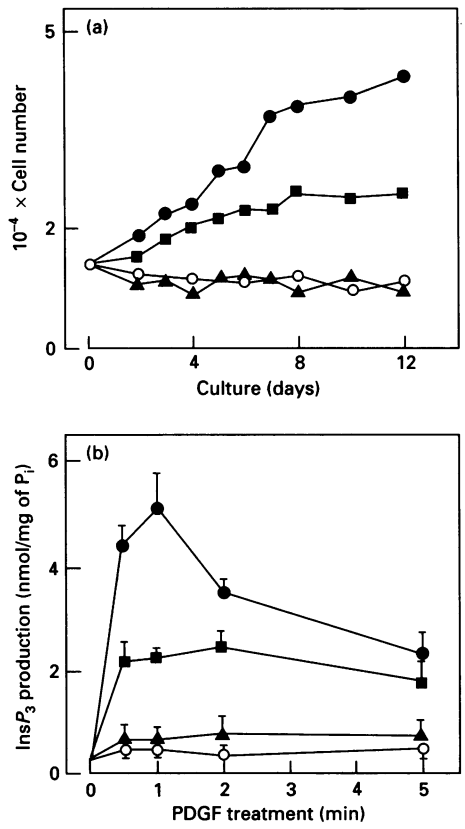

Figure 1 Effect of PDGF dimers on VSMC growth and $InsP_3$ formation

(**a**) VSMCs were seeded at a density of 1×10^4/6 cm dish and cultivated in the serum-containing medium for 24 h and in the starvation medium for 2 days. Then cells were maintained in the starvation medium (○) or stimulated with 5 ng/ml PDGF-AA (▲), -AB (■) or -BB (●) dimer. Cell number was determined by counting every 2 days. Results are means ± S.D. ($n = 3$). (**b**) VSMCs were stimulated with vehicle (○) or 50 ng/ml PDGF-AA (▲), -AB (■) or -BB (●) for the indicated periods, and the reaction was terminated by adding ice-cold trichloroacetic acid. $InsP_3$ was extracted and determined in accordance with the notes accompanying the assay kit (Amersham). Results are means ± S.D. ($n = 3$).

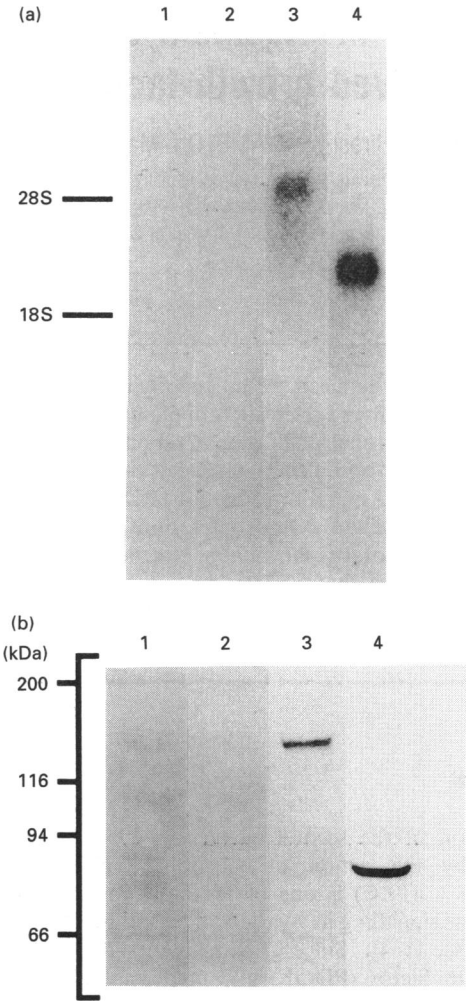

Figure 2 Expression level of PLC isoforms in the synthetic phenotype VSMCs

(**a**) Poly(A)$^+$ RNA (10 μg each) was analysed by Northern blotting using PLC-β1 (lane 1), PLC-γ1 (lane 2), PLC-γ2 (lane 3) and PLC-δ1 (lane 4) cDNAs as probes. The positions of the two major RNA species are indicated. (**b**) Cellular proteins (40 μg each) were separated by SDS/PAGE and immunoblotted with anti-PLC-β1 (lane 1), anti-PLC-γ1 (lane 2), anti-PLC-γ2 (lane 3) and anti-PLC-δ1 (lane 4) antibodies. Positions of molecular size standards (kDa) are indicated.

twice at room temperature and twice at 65 °C [26]. Cellular proteins were prepared as follows. Cells (~ 10^6) were homogenized in 10 mM Tris/HCl (pH 7.4)/1 mM EDTA/1 mM leupeptin and the debris was removed by centrifugation (400 *g*, 5 min). Part of the resultant supernatant (~ 20 μg of protein) was subjected to SDS/PAGE (7.5%-acrylamide gel). After electroblotting of the samples, the filters were immunostained with antibodies against the PLC isoforms [27,28].

Phosphorylation and immunoprecipitation

Cells were labelled with carrier-free $H_3{}^{32}PO_4$ (1 mCi/ml, ICN Biochemicals) for 2 h and washed with HBSS. A cell lysate was prepared from 5×10^6 cells by solubilization with 0.5% (w/v) Nonidet P-40 in a buffer consisting of 20 mM Tris/HCl (pH 7.4), 2 mM sodium vanadate, 2 mM EDTA, 10 mM sodium pyrophosphate, 100 mM NaF, 0.1 mM phenylmethanesulphonyl fluoride, 0.1 mM di-isopropyl fluorophosphate and 1 mM leupeptin. After centrifugation (10000 *g*, 15 min) the lysates were co-incubated at 4 °C for 4 h with anti-phosphotyrosine or anti-PLC antibodies. Immunoprecipitates were obtained by incubating the reaction mixture with Protein-G–Sepharose (Zymed Laboratories) and washed thoroughly. The resultant pellets were analysed by SDS/PAGE (7.5% acrylamide) and autoradiography. In the double-immunoprecipitation assay, immunoprecipitates obtained from experiments with the anti-PLC-γ2 antibody were solubilized by heating (90 °C, 2 min) in a solubilization buffer consisting of 50 mM Tris/HCl (pH 7.4), 2% SDS and 1% (v/v) 2-mercaptoethanol. The resultant solution was diluted with 100 vol. of distilled water and then incubated with anti-phosphotyrosine antibody. The final immunoprecipitates were obtained in the same manner as described above.

Others

Protein was determined by dye binding with a Protein Assay reagent (Bio-Rad Laboratories). $InsP_3$ was determined with an $InsP_3$ assay system (Amersham).

RESULTS

Effect of PDGF on growth and $InsP_3$ formation

In order to examine the biological effect of PDGF dimers, the

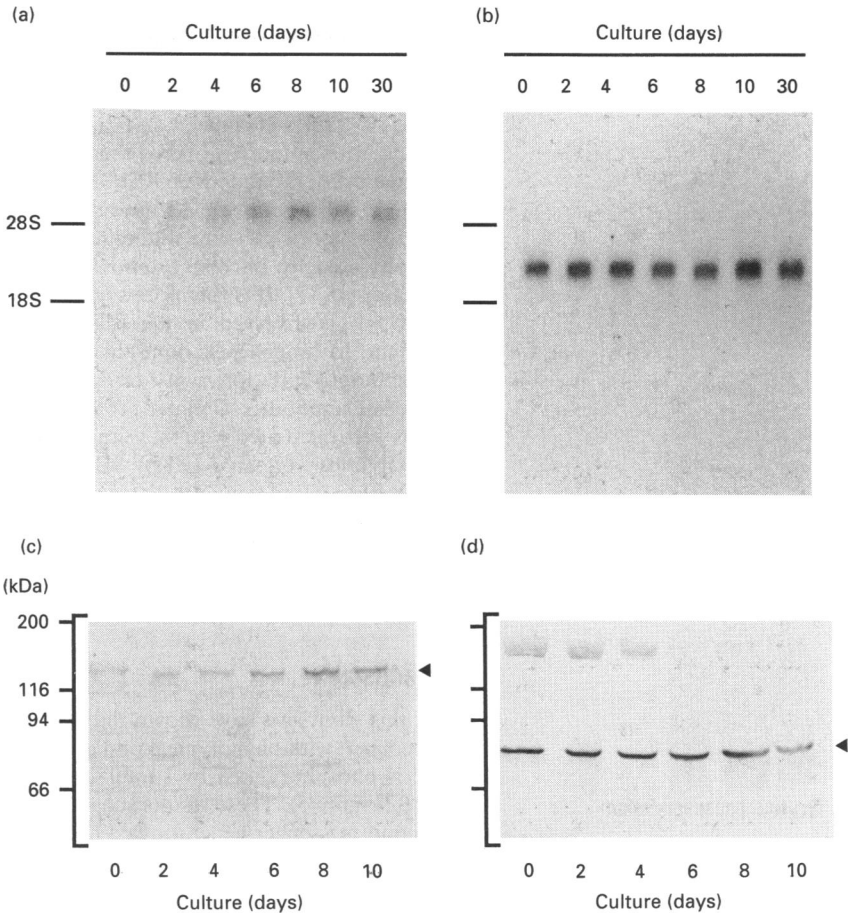

Figure 3 Induction of PLC-γ2 and PLC-δ1 during phenotypic modulation

Poly(A)$^+$ RNA (5 μg each) was analysed by Northern blotting with PLC-γ2 (**a**) and PLC-δ1 (**b**) cDNAs as probes. The positions of the two major RNA species are indicated. Cellular proteins (40 μg each) were separated by SDS/PAGE and immunoblotted with anti-PLC-γ2 (**c**) and PLC-δ (**d**) antibodies. The positions for PLC-γ2 and -δ are indicated by arrowheads. Positions of molecular size standards (kDa) are indicated.

cultures were independently treated with PDGF-AA, -AB and -BB, and the cell numbers were determined every day. The cultures were maintained for 2 days in a starvation medium, and then stimulated with one containing PDGF dimers at a final concentration of 5 ng/ml. The PDGF-containing medium was replaced every other day. As shown in Figure 1(a), PDGF-BB markedly stimulated proliferation of VSMCs. PDGF-AB also induced cell growth, while PDGF-AA did not show any activity on VSMC growth. Since PDGF is thought to transduce its signals via stimulation of the phosphatidylinositol systems, we determined the InsP_3 formation induced by these stimulations. When cells were treated with PDGF-BB, a rapid production of InsP_3 was observed (Figure 1b). The InsP_3 level was highest about 1 min after stimulation and subsequently decreased to the control level. PDGF-AB also induced InsP_3 formation, but its effect was smaller than with PDGF-BB. The activity of PDGF-AA was quite low. These results are consistent with cell growth shown in Figure 1(a).

Expression of PLC isoforms in VSMCs

The expression levels of four PLC isoforms were examined by using cDNA probes and specific antibodies [26–28], which are also effective for recognition of four equivalent PLC isoforms in the rabbit. Northern and Western analyses demonstrated that, among the PLC isoforms tested, PLC-γ2 and PLC-δ1 were the major PLC species in rabbit VSMC cultures (Figure 2). We could not detect any significant signals for PLC-β1 and -γ1, indicating their expression levels are, if any, extremely low. Therefore it is likely that PLC-γ2 and/or PLC-δ1 are involved in the signal transduction of PDGF-BB and -AB.

On the other hand, expression of PLC-γ2 was undetectable in normal rabbit aorta. Thus it may happen that expression of PLC-γ2 is induced during cultivation of primary VSMC cultures. When the expression level of PLC isoforms was analysed, only PLC-δ1 was detected in the primary cultures and its expression level remained constant throughout the cultivation periods (Figures 3b and 3d). The expression of PLC-γ2 was not detected in the primary cultures, but its level increased during cultivation and reached a plateau by day 8 (Figures 3a and 3c). This is consistent with the time when the primary cultures begin to respond to PDGF (results not shown). At no period of cultivation was the expression of PLC-β1 and PLC-γ1 detected.

Phosphorylation induced by PDGF stimulation

We analysed cellular proteins whose phosphorylation at a tyrosine residue was enhanced by PDGF treatment. Phosphotyrosine-

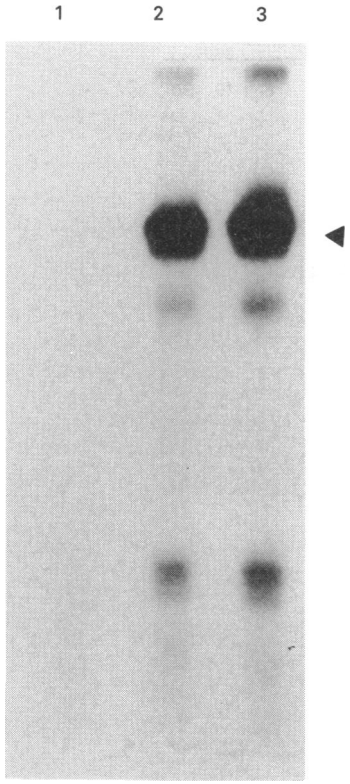

Figure 4 PDGF-dependent protein tyrosine phosphorylation

The cultures were prelabelled with $H_3{}^{32}PO_4$ and stimulated with PDGF-AA (lane 1), -AB (lane 2), or -BB (lane 3). Cell lysates prepared from these cells were incubated with anti-phosphotyrosine antibody. The resultant immunoprecipitates were analysed by SDS/PAGE (7.5% acrylamide). Position of PDGF receptor is indicated by an arrowhead.

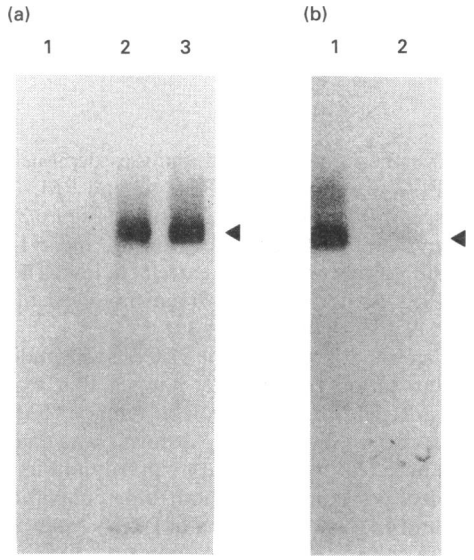

Figure 5 Detection of PLC-γ2 phosphorylation

(**a**) The cultures were prelabelled with $H_3{}^{32}PO_4$ and stimulated with PDGF-AA (lane 1), -AB (lane 2) or -BB (lane 3). Cell lysates prepared from these cells were incubated with anti-PLC-γ2 antibody. The resultant immunoprecipitates were analysed by SDS/PAGE (7.5% acrylamide). (**b**) The cultures were prelabelled with $H_3{}^{32}PO_4$ and stimulated with PDGF-BB. Cell lysates prepared from the stimulated cells were incubated with anti-PLC-γ2 (lane 1) or anti-PLC-δ1 antibody (lane 2). The resultant immunoprecipitates were solubilized and further incubated with anti-phosphotyrosine antibody. The final immunoprecipitates were analysed by SDS/PAGE (7.5% acrylamide). PLC-γ2 was indicated by an arrowhead.

containing proteins were obtained as immunoprecipitates after incubating cellular proteins with anti-phosphotyrosine antibody and then analysed by SDS/PAGE. A number of proteins with different molecular masses, including PDGF receptors (~ 180 kDa), were phosphorylated at tyrosine residues by PDGF stimulation, though none of these were detected in non-stimulating cells. Of the three PDGF dimers tested, the effect of PDGF-BB was most evident and that of PDGF-AA was quite small (Figure 4). Recent studies have demonstrated that PLC-γ1 is activated by phosphorylation induced by receptor tyrosine kinases [15,17]. It is therefore suggested that PLC-γ2, instead of PLC-γ1, is involved in the PDGF signalling in this VSMC system. In order to examine this possibility, we carried out an immunoprecipitation assay using anti-PLC and anti-phosphotyrosine antibodies. Cellular proteins obtained from ^{32}P-labelled cells were incubated with these antibodies and resultant immunoprecipitates were analysed by SDS/PAGE. As shown in Figure 5(a), PLC-γ2 was actually phosphorylated at tyrosine residues by PDGF-AB or -BB treatment. Phosphorylation of PLC-δ1 was not detected by stimulation with any PDGF dimers (Figure 5b). These results suggest that PLC-γ2 may be responsible for $InsP_3$ formation induced by PDGF-AB or -BB.

DISCUSSION

In this study, we have shown that expression of PLC-γ2 occurs in concert with phenotypic modulation (Figure 3), and that PLC-γ2 is phosphorylated by stimulation with either PDGF-AB or -BB (Figure 5). These results suggest that PLC-γ2 functions as a second-messenger-generating component in PDGF-BB and -AB signalling in rabbit VSMCs. It was previously demonstrated that PLC-γ2 was phosphorylated and activated by PDGF-BB stimulation, using rat fibroblasts over-expressing PLC-γ2 [29]. Our results are consistent with that finding. PLC-γ2 is structurally similar to PLC-γ1, which is known to play a critical role in the signal transduction of PDGF and EGF. Phosphorylation of PLC-γ1 at a tyrosine residue by intrinsic receptor kinases has been detected in a variety of cells [5–13]. PDGF-dependent tyrosine phosphorylation of PLC-γ2 was observed in the immunoprecipitates in this VSMC system (Figure 5). Thus PLC-γ2 would be activated by PDGF receptors in the same manner as PLC-γ1 [15,17]. On the other hand, VSMCs mainly express another type of PLC, PLC-δ1, whose expression level remained constant throughout phenotypic modulation (Figure 3a). PLC-δ1 was neither phosphorylated by the receptor kinase nor included in the PDGF-receptor–PLC complex. Since both contractile and synthetic-phenotype VSMCs respond to angiotensin II and generate $InsP_3$ (results not shown), PLC-δ1 may be involved in the signal transduction for angiotensin II rather than that for PDGF.

Results shown in this study indicate the existence of PDGF receptors on the cell surface of synthetic-phenotype VSMCs. In fact, when the binding activity of PDGF-BB was measured in the primary cultures maintained for different periods, PDGF receptors seemed to be expressed with phenotypic modulation of VSMCs (results not shown). Therefore, primary cultures (within ~ 2 days after preparation of VSMCs) which do not respond to PDGF undergo a change to the synthetic phenotype after further cultivation in serum-containing medium. During this modulation, VSMCs express in their plasma membrane a functional signal-transducing complex for PDGF-BB and -AB that includes PDGF receptor and PLC. In fact, we demonstrate the expression of PLC-γ2 that occurs during the acquisition of proliferative activity in VSMCs.

Extracellular factors that regulate the phenotype of VSMCs

are still unknown. When primary VSMC cultures are maintained in the serum-free synthetic medium, expression of PLC-γ2 and PDGF receptor is induced in the same manner as was observed in this study. Thus it is certain that PDGF itself is not involved in the phenotypic modulation of VSMCs. Rather, the mechanical and structural environment, such as extracellular matrix, may be important for maintaining the contractile phenotype. On the other hand, various lines of evidence have suggested that PDGF-dependent proliferation of VSMCs is an early event in the pathogenesis of atherosclerosis [30–35]. Ross and co-workers [36] have recently shown the existence of PDGF B-chain protein in all stages of lesion development in both clinical and experimental atherosclerosis. This observation suggests that the PDGF-dependent system is also required for the chronic conversion of lesions; that is, repeated stimulation of VSMCs with PDGF results in pathological progression. In this study we have demonstrated the expression of PLC-γ2 and its functional association with PDGF receptor. Hence it is conceivable that the expression of the PDGF-signalling complex is enhanced during progression of atherosclerotic lesions. Further analyses are required to identify the components at all stages of atherogenesis.

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