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CYCLIC AMP REGULATES PDGF-STIMULATED SIGNAL TRANSDUCTION AND DIFFERENTIATION OF AN IMMORTALIZED OPTIC-NERVE-DERIVED CELL LINE

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Summary

To facilitate the study of the molecular events underlying the development of optic-nerve-derived oligodendrocytes and their growth-factor-related signal transduction events, we immortalized perinatal rat optic nerve cells with a temperature-sensitive simian virus 40 large T-antigen, carrying the tsA58 and U19 mutations, *via* a retrovirus vector. The line, tsU19-9, was selected on the basis of the expression of the neural precursor marker nestin. At the permissive temperature, 33 °C, tsU19-9 cells had a flat epithelial morphology. In contrast, following exposure to platelet-derived growth factor (PDGF), a factor important in the lineage progression of oligodendrocytes, or in the presence of dibutyryl cyclic AMP at 39 °C (the non-permissive temperature), the cells underwent morphological and antigenic differentiation to cells characteristic of the oligodendrocyte lineage. We used this cell line to investigate the binding characteristics of PDGF and related signalling cascades. Competition binding, phosphoinositide hydrolysis and intracellular Ca^{2+} mobilization assays all demonstrated that the three different isoforms of PDGF (AA, AB and BB) bound to and acted on the cell line. Overnight exposure to forskolin, a treatment that initiated morphological and phenotypic progression into an oligodendrocyte lineage, decreased PDGF-BB-induced intracellular Ca^{2+} mobilization and inhibited basal and PDGF-stimulated [^{3}H]thymidine incorporation. Our results demonstrate that tsU19-9 may serve as a resource to study early optic-nerve oligodendrocyte development.

Key words: oligodendrocyte precursor, cell line, SV40 large T-antigen, immortalization, proliferation, differentiation, PDGF receptor, growth factor.

Introduction

There is growing interest in the mechanisms regulating the proliferation and differentiation of multipotential cells that can give rise to the diverse neuronal and glial cells of the adult brain (Frederiksen et al., 1998; Renfranz et al., 1991; Ohtani et al., 1992; Louis et al., 1992; Sawamura et al., 1995; Barnett and Crouch, 1995; Newman et al., 1995). Cell lines representing different stages of neural cell development can be useful tools in the study of the molecular and physiological mechanisms regulating the differentiation of stem cells. Previously, we have shown that a temperature-sensitive oncogene, simian virus 40 (SV40) large T-antigen carrying both the tsA58 and U19 mutations, was able successfully to immortalize vimentin-positive/A_2B_5-positive cells from the optic nerve of neonatal rat (Almazan, 1990; Almazan and McKay, 1992). The selected clones had the potential to express some properties characteristic of oligodendrocytes. At the non-permissive temperature, 39 °C, a number of these immortalized cells differentiated and expressed oligodendrocyte-specific molecules, such as 2′3′-cyclic nucleotide phosphohydrolase (CNP, Kurihara and Tsukada, 1987) and myelin basic protein (MBP, Sternberger et al., 1978). They also reacted positively with R-mAb, a monoclonal antibody that recognizes differentiating oligodendrocytes (Ranscht et al., 1982). Similarly, the tsA58 SV40 large T-antigen mutant can immortalize neural precursor cells expressing nestin (Frederiksen et al., 1988; Lendahl et al., 1990; Renfranz et al., 1991) as well as Schwann cells derived from newborn rat sciatic nerve (Do Thi et al., 1998). The immortalized neural cells were capable of differentiating into either neuronal or astrocytic-like phenotypes (Frederiksen et al., 1988; Lendahl et al., 1990), while the Schwann cell line had the capability of forming myelin structures *in vitro* and *in vivo* (Do Thi et al., 1998). Additionally, Campagnoni and coworkers have used a similar approach to immortalize oligodendrocytes from normal, shiverer (Verity et al., 1993) and Jimpy mouse brains (Bongarazone et al., 1997). Most significantly, when an immortalized hippocampal stem cell line was transplanted into a host brain, it integrated into the

implant site and acquired the morphology of neurons and glial cells (Renfranz et al., 1991).

Since the rat optic nerve does not contain neuronal cell bodies, it was of interest to determine whether SV40 large T-antigen immortalized nestin-positive cells could also differentiate into oligodendrocytes. Optic nerve oligodendrocytes arise from O2A precursor cells, which in culture can give rise to both oligodendrocytes and type 2 astrocytes (Raff et al., 1983). *In vitro* culture conditions influence the choice of developmental pathways taken by the bipotential O2A cell (Raff et al., 1985; Lillien et al., 1988; Noble et al., 1988; Richardson et al., 1988): in 10 % fetal calf serum (FCS), most differentiate into type 2 astrocytes, whereas on a monolayer of type 1 astrocytes or in serum-free medium (SFM), they differentiate and develop into oligodendrocytes (Raff et al., 1983). Furthermore, the addition of platelet-derived growth factor (PDGF) stimulates division and motility and inhibits the premature differentiation of O2A cells in culture (Raff et al., 1985; Noble et al., 1988; Richardson et al., 1988; Hart et al., 1989a; Bögler et al., 1990; McKinnon et al., 1990; Bhat et al., 1992; Barres et al., 1993; Grinspan et al., 1993). *In vivo*, the level of mRNA coding for the PDGF A peptide increases in rat embryonic brain prior to oligodendroglia ontogenesis, and several cells, including astrocytes (Richardson et al., 1988; Mudhar et al., 1993) and neurons (Vignais et al., 1995; Mudhar et al., 1993), have the potential to synthesize PDGF. The O2A precursor cells express (Hart et al., 1989b; Pringle et al., 1992, 1993) high-affinity membrane receptors for PDGF, and it is clear that this growth factor regulates the lineage progression of the progenitors *in vivo* as well as *in vitro* (Barres et al., 1993). Active PDGF consists of homodimers of A and B chains (AA, AB, BB) that differentially activate PDGF-Rα and PDGF-Rβ. All the different PDGF forms bind and activate PDGF-Rα, whereas PDGF-Rβ is only activated by PDGF-BB.

In this study, we describe the morphological and immunocytochemical properties of an immortalized optic-nerve-derived cell line (tsU19-9) and the physiological responses elicited by PDGF. The cell line has the potential to differentiate into R-mAb-positive cells, suggesting that they can acquire an oligodendrocyte phenotype. In addition, PDGF stimulates the proliferation and morphological alterations of the tsU19-9 cells. Furthermore, as in primary oligodendrocyte culture (Raible and McMorris, 1989, 1990, 1993), forskolin, an agent that raises intracellular cyclic AMP levels, favors morphological and antigenic differentiation of tsU19-9 cells. Therefore, this cell line may serve as a resource to study the underlying molecular and genetic events responsible for early optic-nerve-derived oligodendrocyte differentiation using techniques that may require large pools of starting material otherwise virtually unobtainable from primary cultures of optic-nerve-derived oligodendrocytes. We have previously used this cell line to study the adhesion properties of myelin-associated glycoprotein (Almazan et al., 1992).

Materials and methods

Materials

The anti-glial fibrillary acidic protein (GFAP) antibodies were obtained from ICN. Secondary antibodies were purchased from Southern Biotechnology. The A_2B_5 hybridoma cells were obtained from ATCC, and R-mAb supernatant was donated by Dr B. Ranscht (Ranscht et al., 1982). Construction of retrovirus recombinant plasmid (pZipNeoSV40U19tsA58) and immortalization of cell lines have been described in detail previously (see Fig. 1 for a summary of methods; Almazan and McKay, 1992).

Cell culture and immunofluorescence

Cells were grown on poly-D-ornithine-coated coverslips in Dulbecco's modified Eagle medium (DMEM) + 5 % calf serum/5 % fetal calf serum (complete medium) or in DMEM:F12, containing 15 mmol l^{-1} Hepes, 50 μg ml^{-1} transferrin, 5 μg ml^{-1} insulin, 300 nmol l^{-1} triiodothyronine, 30 nmol l^{-1} sodium selenite, 20 nmol l^{-1} progesterone, 0.1 nmol l^{-1} putrescine and 20 nmol l^{-1} hydrocortisone (serum-free medium, or SFM). All cells were grown at 33 °C unless otherwise indicated. Immunostaining for surface components was carried out on living cells or cells fixed for 5 min in 4 % paraformaldehyde in phosphate-buffered saline (PBS). Staining for intracellular antigens was carried out on cell cultures fixed for 10 min at room temperature (21 °C) in 30 % methanol/70 % acetone for the T-antigen or 4 % paraformaldehyde in PBS for nestin. After fixation, the cells were washed with PBS and blocked for 15 min with PBS containing 0.2 % bovine serum albumin (BSA), 5 % FCS and 0.2 % Triton X-100. The monoclonal antibodies A_2B_5 (Eisenbarth et al., 1979), 401 (Hockfield and McKay, 1985) and anti-R-mAb (Ranscht et al., 1982) were used as hybridoma supernatants. The secondary antibodies were conjugated to fluorescein or Texas Red and diluted 1:100 in the same buffer. Coverslips were mounted with Immuno-mount containing 2 % 1,4-diazobicyclo(2,2,2)-octane to prevent fading and were examined under a Leitz Diaplan microscope. The photographs were taken using Kodak Panatomic-X or TMY-400 ASA.

Binding assays

The tsU19-9 cells were plated in 96-well plates and grown at 33 °C in complete medium until 70–75 % confluent. The cells were grown for an additional 2 days in SFM to deplete serum-derived growth factors. For competition binding experiments, cells were incubated with increasing amounts of unlabeled PDGF-AA, PDGF-AB or PDGF-BB (UBI) with 4 ng ml^{-1} (0.13 nmol l^{-1}) of [^{125}I]PDGF-BB (NEN, 3.8×10^4 GBq $mmol^{-1}$) for 1 h at 33 °C. Labeled PDGF without or with 1 mmol l^{-1} suramin (Miles Scientific) was used to determine total or non-specific binding, respectively. The cells were washed three times with ice-cold PBS, once with ice-cold 5 % trichloroacetic acid (TCA) and air-dried. The radioactivity was extracted by solubilizing the cells in 250 μl of 0.2 mol l^{-1} NaOH + 1 % SDS, and then measured in

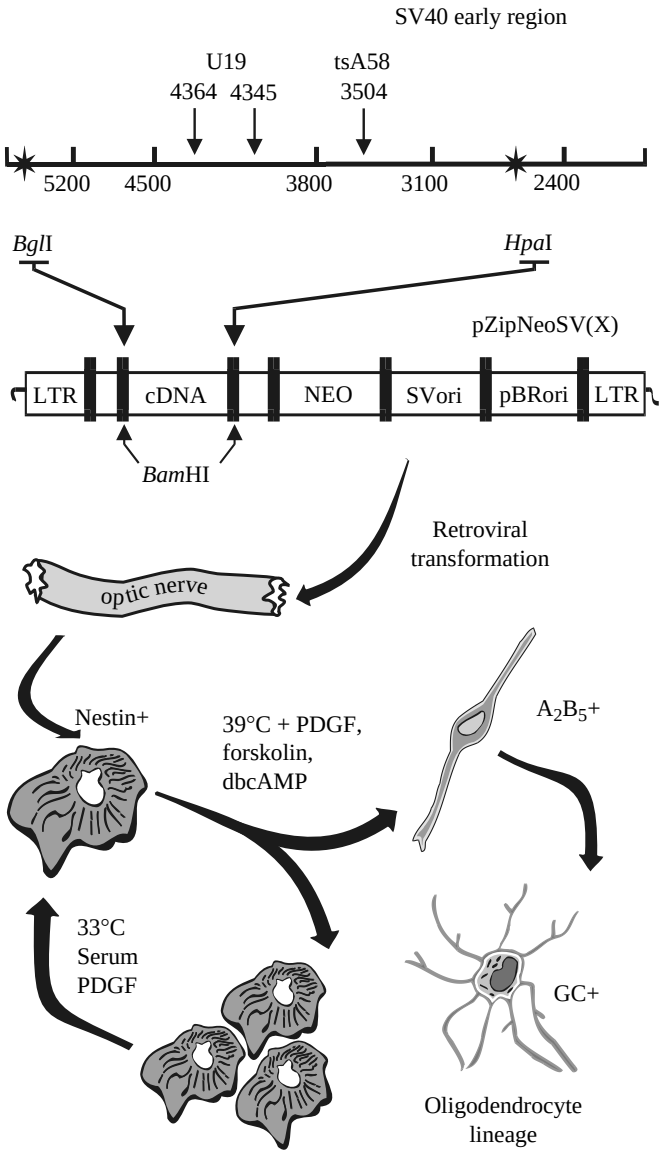

Fig. 1. Immortalization of an optic nerve cell *via* retroviral transformation with SV40U19tsA58 plasmid. The *Bgl*1-*Hpa*1 fragment of the early region of SV40 large T-antigen was inserted into the *Bam*H1 cloning site of pZipNeoSV(X) vector. The temperature-sensitive mutations are on nucleotides 4364, 4345 (U19) and 3504 (tsA58). The plasmid was then propagated in JS4 *Escherichia coli*. Psi 2 viral producer cells were transfected with the plasmid using the calcium phosphate method, and then selected with G418. Optic nerve cells were obtained from postnatal day 3 rat pups and infected with Psi 2 supernatant containing replication-defective retrovirus with the pZipNeoSV40U19tsA58 plasmid. G418-resistant colonies were expanded and selected on the basis of their expression of nestin. At 39 °C or in the presence of platelet-derived growth factor (PDGF), forskolin or dibutyryl cyclic AMP (dbcAMP), the flattened cells undergo morphological and antigenic transformation and resemble oligodendrocyte precursors. Cells become $A_2B_5^+$ or R-mAb$^+$(GC$^+$). The cells are propagated at the permissive temperature, 33 °C, in the presence of serum-containing medium. Full details of this procedure appear in Almazan and McKay (1992).

a γ counter. Values are presented as the means of three samples ± S.E.M.

Inositol phosphate measurements

The tsU19-9 cells were plated at approximately 80×10^3 cells per well on six-well (35 mm) dishes and grown for 24–48 h in complete medium. The cells were radiolabeled with 74 kBq ml^{-1} of myo-[^{3}H]inositol (NEN, 4.5×10^2 GBq mmol^{-1}, 37 MBq ml^{-1}) for 5 days, with a change of medium after 48 h. The medium was changed to SFM + 2 μCi ml^{-1} of myo-[^{3}H]inositol during the last 24 h period to deplete serum and growth factors. Prior to the assay, the cells were incubated for 2 h with DMEM containing 0.1 % BSA and 10 mmol l^{-1} LiCl. PDGF (AA or BB; 50 ng ml^{-1}) was added to the cells, and the reaction was stopped after 30 min by placing the dishes on ice, aspirating the medium and adding 0.5 ml of ice-cold methanol. The dishes were scraped, and the lysates were transferred to 15 ml conical tubes. The plates were washed with 0.5 ml methanol, and 0.9 ml of distilled deionized water and the wash liquid were transferred to the 15 ml tube. To partition the aqueous and organic phases in the samples, 1 ml of chloroform was added, and the tubes were vortexed for 30 s. The samples were centrifuged for 5 min at 1000 revs min^{-1}, and the aqueous layer was transferred to a second 15 ml tube containing 1 ml of Dowex-1 (X8 formate form, 200–400 mesh Bio-Rad). The slurry was incubated at room temperature for 30 min, during which the beads were gently mixed with the sample to maximize adsorption. Following the incubation, the tubes were centrifuged for 30–60 s at 250 ***g*** and the supernatant was discarded. Total inositol phosphate levels were determined using a method similar to that described by Berridge et al. (1983). Briefly, the beads were washed twice with 5 ml of 60 mmol l^{-1} ammonium formate/5 mmol l^{-1} sodium tetraborate to remove free inositol and glycerophosphate contaminants. Total inositol phosphate was eluted in five washes of 1 ml, one wash with 2.4 mol l^{-1} ammonium formate/0.1 mol l^{-1} formic acid and four washes with 1.2 mol l^{-1} ammonium formate/0.1 mol l^{-1} formic acid. The 1 ml eluates were transferred to minivials containing 5 ml of Optiphase Highsafe III (Fisher) and counted in a β counter. Values are presented as the mean of triplicate samples ± S.E.M.

Determination of intracellular Ca^{2+} levels ($[Ca^{2+}]_i$)

The tsU19-9 cells were grown to confluency on 10 cm dishes (approximately 2×10^6 cells per dish) in complete medium. The cells were washed in PBS/citrate, to remove the remaining serum, and lightly trypsinized (0.025 % trypsin + 1 mmol l^{-1} EDTA) for 1 min. The cells were removed from the culture dish by triturating with 2×3 ml of complete medium, centrifuged at 150 ***g*** for 5 min, and resuspended in Hanks balanced salt solution (HBSS) containing 1.3 mmol l^{-1} Ca^{2+} (approximately 2×10^6 cells per 0.5 ml). The cells were loaded with 5 μmol l^{-1} Fura-2 (acetoxymethyl ester, Molecular Probes, Eugene, OR, USA) by incubation at 37 °C for 30 min on a rotary shaker. The cells were washed three times with HBSS containing 1.3 mmol l^{-1} Ca^{2+}, resuspended in the same

buffer to a final concentration of approximately 4×10^6 cells per 2 ml, and transferred to cuvettes containing stir bars. Changes in Fura-2 fluorescence were measured at room temperature using a Perkin-Elmer LS-50 fluorometer. Excitation and emission wavelengths were 340 nm and 500 nm with 10 nm slits, respectively. The maximum level of $[Ca^{2+}]$ was determined by permeabilization of the cell suspension with 0.01 % Triton X-100 in 20 mmol l^{-1} Tris buffer, while the minimum level was estimated by the further addition of 10 mmol l^{-1} EGTA. The calibration curve and all calculations were carried out using FLDM software (Perkin-Elmer). In a series of experiments, tsU19-9 cells were pretreated with 50 ng ml^{-1} of PDGF-AA, -AB or -BB for 30 min, with 200 μmol l^{-1} genistein for 5 min, with 4 mmol l^{-1} EGTA for 1 min or with 10 μmol l^{-1} forskolin (Sigma) and/or 0.5 mmol l^{-1} isobutylmethylxanthine (IBMX) (Sigma) for 24 h.

Measurement of DNA synthesis

The effect of PDGF and forskolin/IBMX on the proliferation of tsU19-9 cells was measured by [^{3}H]thymidine incorporation. Cells were plated on 24-well dishes at a density of approximately 10×10^3 to 15×10^3 cells per well and grown in complete medium until 70–75 % confluent. The cells were deprived of serum for 48 h and preincubated with or without 10 μmol l^{-1} forskolin/0.5 mmol l^{-1} IBMX for 24 h prior to stimulation with PDGF-BB. The cells were incubated overnight (18–20 h) with 37 kBq ml^{-1} of [^{3}H]thymidine (Amersham, 3.1×10^{-3} GBq $mmol^{-1}$, 37 MBq ml^{-1}) prior to determination of levels of incorporation. Labeling medium was aspirated, and the cells were washed three times with ice-cold 5 % TCA prior to solubilization in 250 μl of 0.2 mol l^{-1} NaOH + 0.1 % SDS. The lysate was transferred to minivials containing 5 ml of Ecolite (ICN), vortexed and counted in a β counter. Values are presented as the means of 3–4 determinations ± S.E.M.

Data analysis

One-way analysis of variance (ANOVA) using Students–Newman–Keuls multiple-comparison test or two-tailed paired Student's *t*-test were used to determine statistical significance as indicated in the figure legends. $P<0.05$ was considered significant. When indicated, the control data were normalized to 0 % for comparison with treated cultures. All experiments were reproduced 2–4 times.

Results

PDGF, forskolin and dibutyryl cyclic AMP regulate the morphological and antigenic phenotype of tsU19-9 cells

TsU19-9 cells have been grown in culture for several months and do not form foci *in vitro*. Their growth requirements were explored at 33 °C and 39 °C. Cells proliferate on polyornithine-coated dishes in complete medium and in SFM in the presence or absence of PDGF-BB (Fig. 2) at 33 °C. At this temperature, tsU19-9 cells appear flat and epitheloid in nature and often grow in clusters (Fig. 2A). Under these culture conditions, the cells express nestin, a neural precursor intermediate filament protein (Fig. 2B), and T-antigen in the nucleus (not shown). At 39 °C, in the presence of PDGF-BB (or PDGF-AA), the cells undergo morphological alterations and appear bipolar or multipolar in nature (Fig. 2C). The change in morphology is accompanied by positive staining of cells with multiple processes for R-mAb (Fig. 2D). At 39 °C, treatment with forskolin (not shown) or dibutyryl cyclic AMP (dbcAMP) (Fig. 3A) for 3–4 days enhances process formation, and the majority

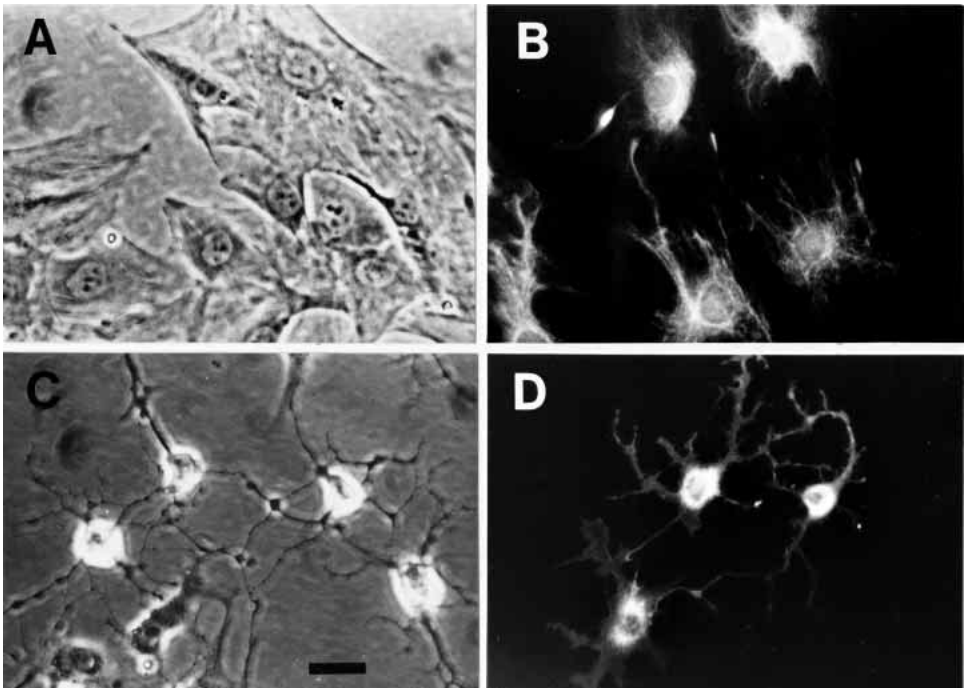

Fig. 2. Effects of a temperature shift on the morphological and immunological phenotype of tsU19-9 cells. Phase-contrast micrographs of tsU19-9 cells growing on polyornithine-coated coverslips at 33 °C show a flat epithelial morphology (A) and nestin expression (B). Shifting the temperature from 33 °C to 39 °C in the presence of platelet-derived growth factor (PDGF) in SFM (see Materials and methods) causes gradual and dramatic changes in morphology of tsU19-9 cells (C), and multipolar cells become positive for R-mAb (D). Scale bar, 15 μm.

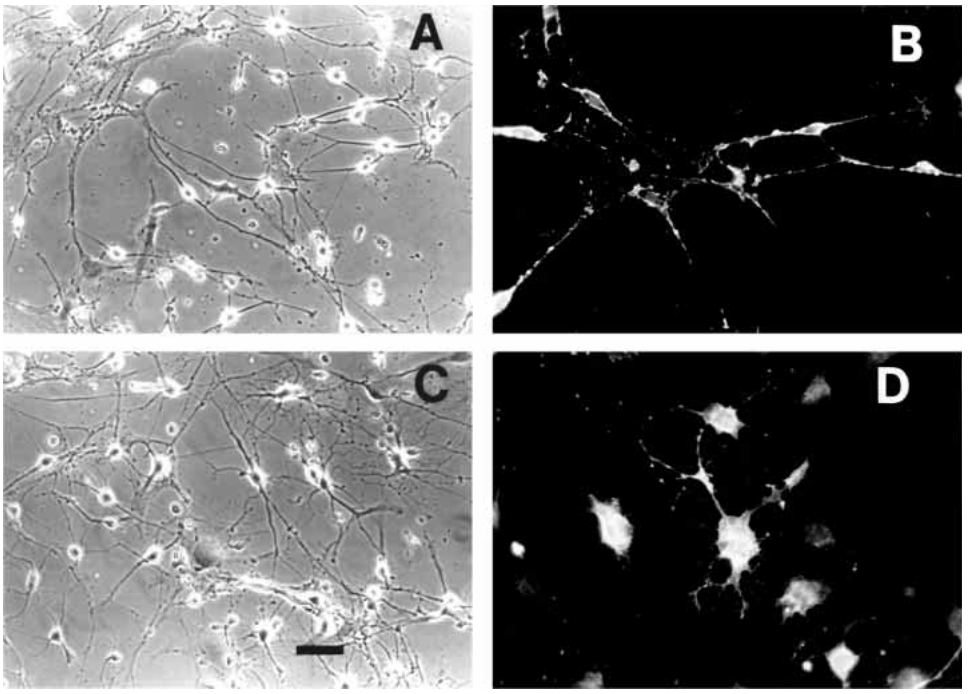

Fig. 3. Effects of platelet-derived growth factor (PDGF) on the phenotype of tsU19-9 at the non-permissive temperature. (A) Phase-contrast micrograph of tsU19-9 cells growing on polyornithine-coated coverslips at 39 °C in the presence of 1 mmol l^{-1} dbcAMP for 3–4 days. Many cells become bipolar and positive for A_2B_5 (B). The addition of both dbcAMP and PDGF-BB (10 ng ml^{-1}) causes some of the cells to extend multiple processes (C) and to become positive for R-mAb (D). Double-labeling experiments have not been carried out. Scale bar, 50 μm.

of the bipolar cells become positive for A_2B_5 (Fig. 3B). Further morphological and antigenic differentiation is produced by the combination dbcAMP and 10 ng ml^{-1} of PDGF-BB (Fig. 3C). The cells elaborate numerous processes and many become R-mAb-positive (Fig. 3D).

All forms of PDGF bind to tsU19-9 cells: competition binding of PDGF-AA, -AB and -BB to [^{125}-I]PDGF-BB

Since PDGF-BB enhanced the differentiation of tsU19-9 cells, we examined the binding characteristics of all forms of PDGF to quiescent cells at 33 °C (Fig. 4). Competition curves with increasing concentrations of PDGF reveal that all isoforms displace [^{125}-I]PDGF-BB binding in the following rank order of potency; BB≈AB>AA. PDGF-AB (Fig. 4, middle panel) and PDGF-BB (Fig. 4, bottom panel) at the maximal concentration used, 400 ng ml^{-1} (13 nmol l^{-1}), were able to displace binding to 34±2 % and 37±5 %, respectively, of the control level. However, PDGF-AA (Fig. 4, top panel), at the same concentration, could only displace binding to 66±9 % of the control level.

PDGF-AA and -BB stimulate inositol phosphate formation and intracellular Ca^{2+} mobilization in tsU19-9 cells

Competition binding studies indicated the possibility of different binding sites for PDGF-AA and PDGF-BB and, therefore, we wanted to determine whether these factors also stimulated PDGF-associated signal transduction cascades, such as inositol phosphate formation, and intracellular Ca^{2+} mobilization in a similar manner.

The tsU19-9 cells were incubated with 74 kBq ml^{-1} [^{3}H]myo-inositol for 5 days and then stimulated with PDGF-AA or -BB in the presence of 10 mmol l^{-1} LiCl for 30 min (Fig. 5A). PDGF-BB (50 ng ml^{-1}) increased the formation of inositol phosphate 6.3-fold (P<0.001) (Fig. 5A), while PDGF-AA (50 ng ml^{-1}) was much less efficacious and stimulated formation only 1.3-fold (P<0.05) (Fig. 5A).

The addition of 50 ng ml^{-1} of either PDGF-AA or -BB (Fig. 5B) stimulated a delayed increase in $[Ca^{2+}]_i$. Approximately 30–40 s after the addition of either factor, the maximal intracellular Ca^{2+} levels were similar with both forms of PDGF. However, the effect of PDGF-BB was sustained for more than 4 min, while the levels in the PDGF-AA-stimulated cultures returned to near baseline levels within this period. Since PDGF-AA and -AB displaced [^{125}I]PDGF-BB binding, we wanted to determine whether these ligands could also regulate the binding sites for PDGF-BB used to release intracellular Ca^{2+} (Fig. 6). Cells were pretreated with 50 ng ml^{-1} PDGF-AA, -AB or -BB for 30 min to downregulate the receptors and were then challenged with PDGF-BB to measure Ca^{2+} responses. Pretreatment with PDGF-AA had no effect on the PDGF-BB-stimulated $[Ca^{2+}]_i$ transients, while AB nearly abolished and BB completely abolished the response (Fig. 6). These results suggested that PDGF-AA binds to and regulates signal transduction mechanisms through a different PDGF receptor isoform from that for PDGF-AB and -BB.

PDGF-BB stimulates intracellular Ca^{2+} mobilization in a biphasic dose-dependent fashion via *a tyrosine-kinase-dependent mechanism*

The addition of PDGF-BB resulted in a dose-dependent increase in $[Ca^{2+}]_i$ in tsU19-9 cells (Fig. 7A). The minimum discernible response was stimulated by 1 ng ml^{-1} (33 pmol l^{-1}), and maximum values were obtained at 50 ng ml^{-1} (1.67 nmol l^{-1}). Concentrations above 50 ng ml^{-1} did not further significantly increase the release of intracellular Ca^{2+}

(data not shown). Pretreatment with 4 mmol l^{-1} EGTA (Fig. 7B) 1 min prior to the addition of PDGF-BB chelated the Ca^{2+} present in the medium (HBSS containing 1.3 mmol l^{-1} Ca^{2+}) and revealed two phases of the intracellular Ca^{2+} mobilization: an initial release from intracellular stores, followed by influx of Ca^{2+} from the extracellular medium (Estacion and Mordan, 1993; Kozawa et al., 1995).

Genistein, a tyrosine kinase inhibitor, blocks autophosphorylation of the PDGF receptor and subsequent downstream second-messenger events, which include stimulation of phospholipase C (PLC)-γ, formation of inositol 1,4,5-trisphosphate ($InsP_3$) and intracellular Ca^{2+} release (Lane et al., 1991). Pretreatment of tsU19-9 cells with a low dose of genistein (between 1 and 20 μmol l^{-1}) 5 min prior to PDGF-BB challenge altered the time course of intracellular Ca^{2+} release (data not shown); at higher concentrations (200 μmol l^{-1}), the release of Ca^{2+} was completely abolished (Fig. 8). To determine whether genistein affected cellular viability or dye uptake, we challenged the cells with thrombin and ionomycin. Thrombin, a serine protease, binds to its cognate receptor and initiates a signal transduction cascade resulting in Ca^{2+} mobilization *via* G-proteins. In contrast, ionomycin, a Ca^{2+} ionophore, directly permits the entry of extracellular Ca^{2+} into the cell and efflux of intracellular Ca^{2+} stores. Although genistein pretreatment slightly reduced the increases in

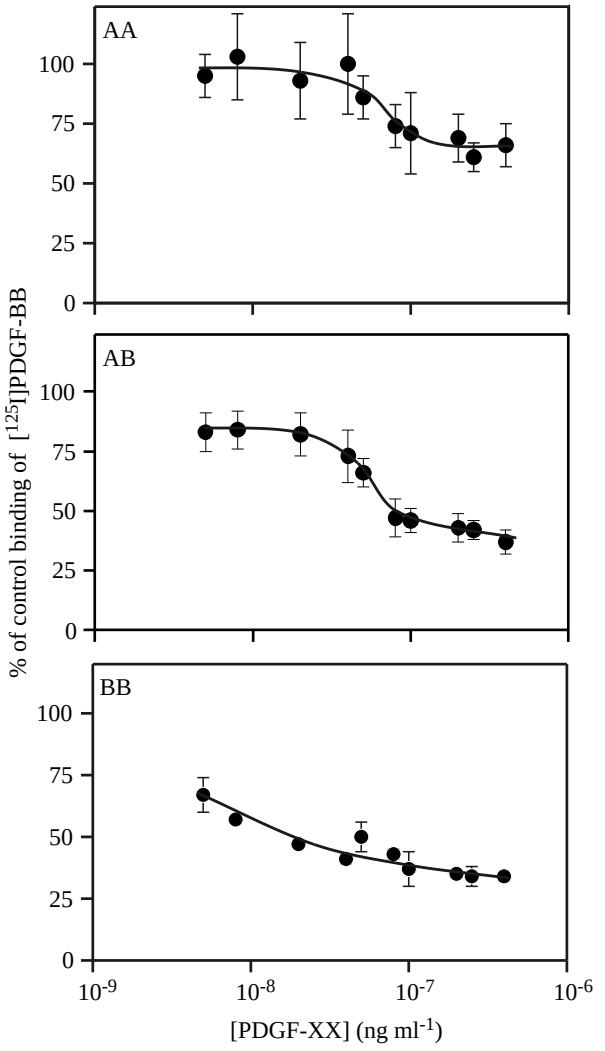

Fig. 4. Competition binding of [^{125}I]PDGF-BB with PDGF-AA, -AB and -BB. tsU19-9 cells were incubated with 4 ng ml^{-1} (130 pmol l^{-1}) [^{125}I]PDGF-BB with increasing concentration of PDGF-AA, -AB or -BB. Binding was determined as described in Materials and methods. Values are means of three determinations ± S.E.M.

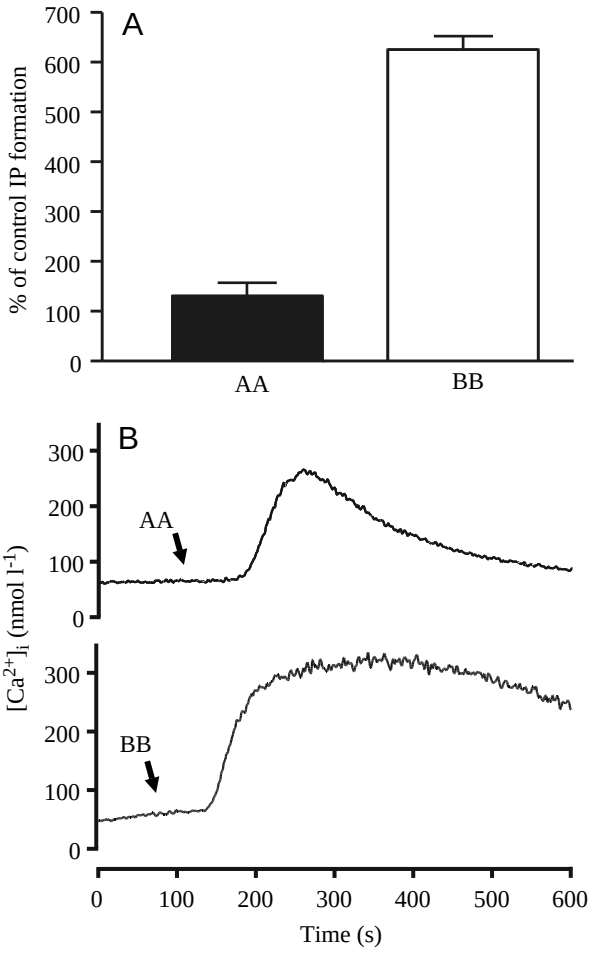

Fig. 5. Platelet-derived growth factor (PDGF)-induced inositol phosphate formation and mobilization of intracellular Ca^{2+}. (A) Cells were prelabeled with 74 kBq ml^{-1} of myo-[^{3}H]inositol for 5 days in complete medium and for 1 day in SFM (see Materials and methods). The cultures were washed three times with DMEM + 10 mmol l^{-1} LiCl and incubated with PDGF-AA or -BB (50 ng ml^{-1}) for 30 min. The reaction was arrested with ice-cold methanol, and total inositol phosphates (IP) were extracted and separated as described in Materials and methods. Results are expressed as a percentage increase over control (unstimulated) values and represent mean values + S.E.M. of three separate determinations. Difference from control value: PDGF-BB, $P<0.001$; PDGF-AA, $P<0.05$. (B) Ca^{2+} mobilization. $[Ca^{2+}]_i$ levels were determined using the Fura-2 method. At 60 s, the cells suspensions were challenged with 50 ng ml^{-1} PDGF-AA (top trace) or 50 ng ml^{-1} PDGF-BB (bottom trace).

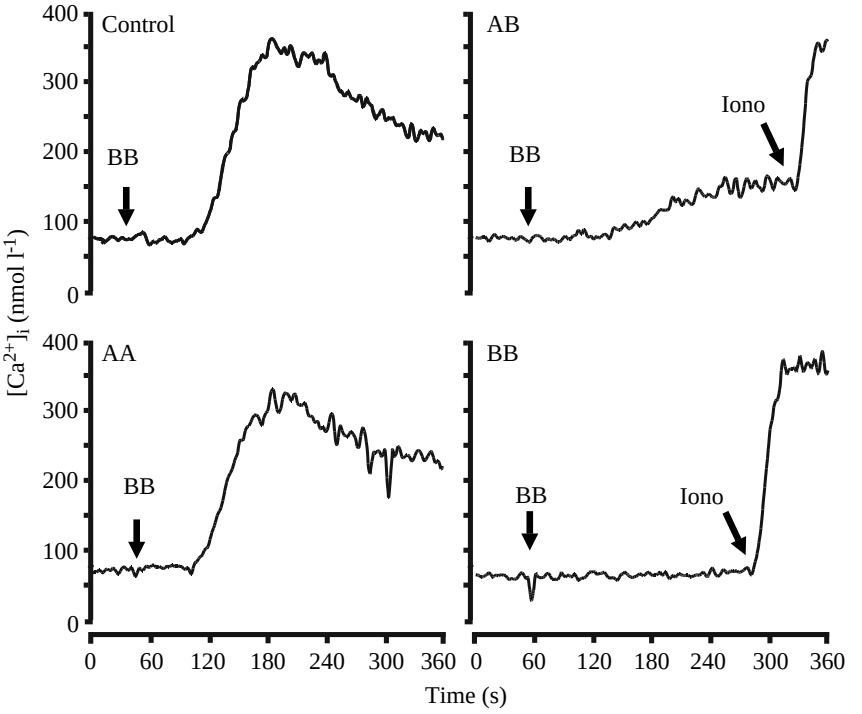

Fig. 6. Regulation of PDGF-BB-stimulated intracellular Ca^{2+} mobilization by different isoforms of platelet-derived growth factor (PDGF). Cell suspensions were challenged with 50 ng ml^{-1} PDGF-BB without (top left) or with a 30 min pretreatment with 50 ng ml^{-1} PDGF-AA (bottom left), 50 ng ml^{-1} PDGF-AB (top right) or 50 ng ml^{-1} PDGF-BB (bottom right). When indicated, the cells were challenged with 100 nmol l^{-1} ionomycin (Iono) to ensure cell viability and responsiveness.

intracellular Ca^{2+} evoked by thrombin or ionomycin, the cells were always responsive, demonstrating that their viability was unaffected.

Forskolin modulates the PDGF-BB-stimulated intracellular Ca^{2+} mobilization

Forskolin, an adenylyl cyclase activator, and IBMX, a phosphodiesterase inhibitor, are two factors known to increase intracellular cyclic AMP levels, a treatment that favored antigenic progression of tsU19-9 cells. The accumulation of cyclic AMP may result in activation of cyclic-AMP-dependent protein kinase (PKA) which phosphorylates and inactivates the $InsP_3$ receptor (Ferris et al., 1991). We therefore wanted to determine whether this pathway could modulate PDGF-BB-stimulated release of intracellular Ca^{2+}. Pretreatment of tsU19-9 cells with 10 μmol l^{-1} forskolin, 0.5 mmol l^{-1} IBMX or both for 24 h partially inhibited PDGF-stimulated intracellular Ca^{2+} release compared with control cultures (Fig. 9).

Forskolin inhibits PDGF-stimulated DNA synthesis in tsU19-9 cells

Forskolin stimulates PDGF-induced proliferation in Schwann cells (Davis and Stroobant, 1990), while it induces growth arrest and differentiation in oligodendrocyte precursors (Raible and McMorris, 1989, 1990, 1993). We therefore examined the effect of co-incubation with 10 μmol l^{-1} forskolin/0.5 mmol l^{-1} IBMX on PDGF-stimulated DNA synthesis in tsU19-9 cells (Fig. 10). Although PDGF-AA increased inositol phosphate hydrolysis and intracellular Ca^{2+}, it had no observable effect on DNA synthesis compared with the control level. In contrast, both PDGF-AB and -BB increased incorporation, to 333 %, and 271 % respectively, above control values (Fig. 10). Forskolin alone or in combination with PDGF-AA decreased DNA synthesis by 42 % below control values. The mitogenic properties of PDGF-AB and PDGF-BB were effectively neutralized by co-incubation with forskolin. These results suggest that forskolin regulates PDGF-mediated signal transduction and may inhibit the proliferation of tsU19-9 cells by favoring their growth arrest and differentiation.

Discussion

In the present study, we used a temperature-sensitive SV40 large T-antigen to immortalize cells from postnatal day 2 rat optic nerve to establish an early optic-nerve progenitor cell model. One of the clones, tsU19-9, was selected on the basis of the expression of nestin, a neural precursor intermediate filament protein. Since the optic nerve contains no neuronal cell bodies, which also express nestin, this cell line may reflect an early glial progenitor or an O2A progenitor with the potential to differentiate into cells of the oligodendrocyte or astrocyte lineage. The expression of nestin in cells of the oligodendrocyte lineage has recently been confirmed in our laboratory (Almazan et al., 1993) and by Gallo and Armstrong (1995).

There has been a growing interest in isolating early neural progenitor cells to study the factors that regulate the genetic changes during differentiation into oligodendrocytes, astrocytes and neurons (Frederiksen et al., 1998; Renfranz et al., 1991; Ohtani et al., 1992; Louis et al., 1992; Sawamura et al., 1992; Barnett and Crouch, 1995; Newman et al., 1995). Growth factors, such as PDGF (Raff et al., 1985; Noble et al., 1988; Richardson et al., 1988; Hart et al., 1989a; Bögler et al.,

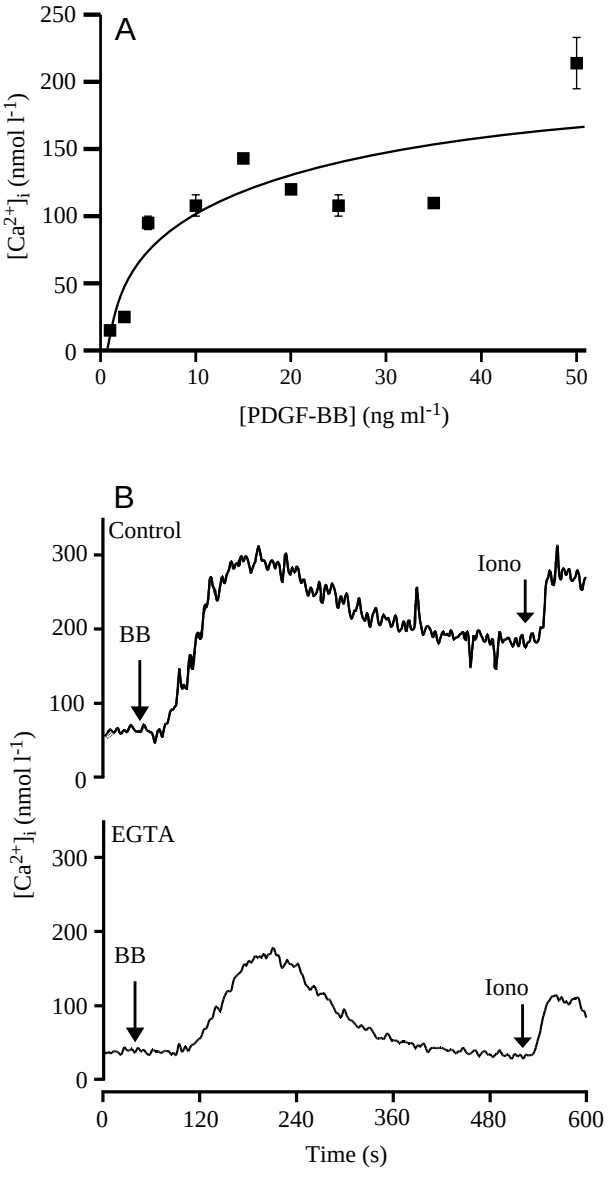

Fig. 7. Concentration-dependent stimulation of $[Ca^{2+}]_i$ by PDGF-BB. (A) Cells suspensions were challenged with increasing doses of PDGF-BB (1–50 ng ml^{-1} or 3.33×10^{-11} to 1.67×10^{-9} mol l^{-1}) and increases in $[Ca^{2+}]_i$ were measured. The data represents the peak $[Ca^{2+}]_i$ obtained in each separate determination. The maximal increase (N>25) for 50 ng ml^{-1} PDGF-BB was between 150 and 500 nmol l^{-1}. Values are means ± S.E.M. of three determinations. (B) Effect of EGTA on PDGF-BB-stimulated intracellular Ca^{2+}. Cell suspensions were given no pretreatment (top panel) or pretreated with 4 mmol l^{-1} EGTA (B, bottom panel) for 1 min prior to the addition of 50 ng ml^{-1} of PDGF-BB (first arrow). The cells were challenged with 100 nmol l^{-1} ionomycin (Iono, second arrow) to ensure cell viability.

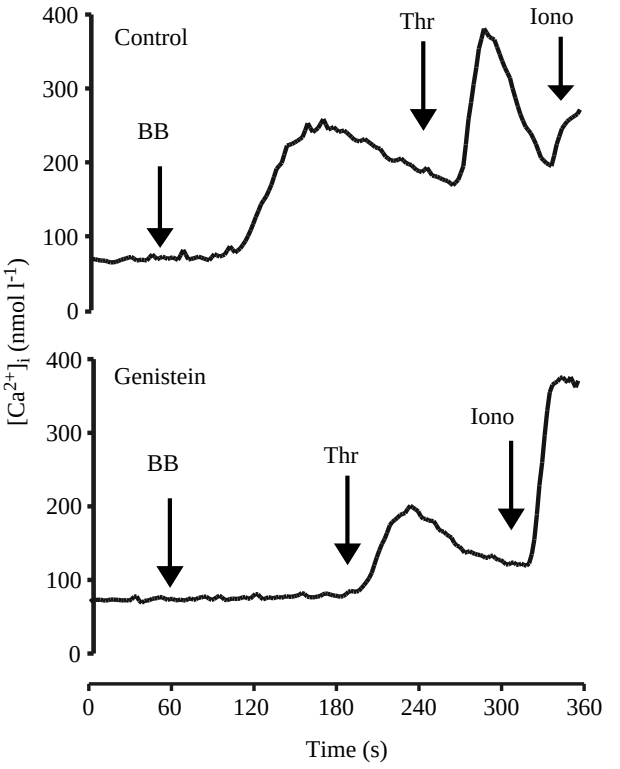

Fig. 8. Effect of genistein on PDGF-BB-stimulated intracellular Ca^{2+} mobilization. Cell suspensions were challenged with 50 ng ml^{-1} PDGF-BB without (top) or with a 5 min pretreatment with 200 μmol l^{-1} genistein (bottom). The cells were challenged with 0.2 units ml^{-1} thrombin (Thr) and 100 nmol l^{-1} ionomycin (Iono) to ensure cell viability and the specificity of the effect of genistein.

1990; McKinnon et al., 1990, 1993; Bhat et al., 1992; Barres et al., 1993; Grinspan et al., 1993), and agents that elevate intracellular cyclic AMP levels (Raible and McMorris, 1989, 1990, 1993) regulate the growth and differentiation of oligodendrocyte precursors *in vitro*. Furthermore, others have shown that these factors induce glial cell lines, immortalized by several different methods, to express oligodendrocyte-specific markers, such as 2′,3′-cyclic-nucleotide 3′-phosphodiesterase (CNP), myelin basic protein (MBP) and proteolipid protein (Jung et al., 1994; Jensen et al., 1993; Russo et al., 1993; Satoh et al., 1994), and regulate their growth (Louis et al., 1992; Satoh et al., 1994). To compare our cell line with both brain-derived primary culture and reported oligodendrocyte-like cell lines, we therefore examined the effect of PDGF and forskolin on the antigenic phenotype of tsU19-9.

At the non-permissive temperature, 39 °C, PDGF-BB, forskolin or a combination of both factors favored the phenotypic and antigenic differentiation of tsU19-9 cells into the oligodendrocyte lineage. Previously, PDGF-AA has been shown to be a mitogen and a survival factor for early oligodendrocyte precursors, but was not reported to enhance their morphogenic or antigenic differentiation (McKinnon et al., 1990; Barres et al., 1993; Grinspan et al., 1993; Grinspan and Franceschini, 1995; Gallo and Armstrong, 1995). In this case, therefore, PDGF-BB may act as a permissive factor allowing the survival of tsU19-9 cells that no longer divide under the control of the denatured SV40 large T-antigen. Thus, the tsU19-9 cells that were once immortalized resume their

interrupted course of differentiation, and their survival is enhanced in the presence of PDGF-BB. In contrast, Raible and McMorris (1989, 1990, 1993) clearly demonstrated that factors that raise intracellular cyclic AMP levels accelerate the differentiation of oligodendrocyte precursors. Therefore, forskolin may alter either the rate or the potential for tsU19-9 cells to become cells of the oligodendrocyte lineage; however, further work is needed to test the role of both PDGF-BB and forskolin in the differentiation of tsU19-9 cells.

Since there are two types of PDGF receptors, α and β (Claesson-Welsh et al., 1989), we first determined which of the PDGF growth factor isoforms shows greatest binding activity by measuring their ability to displace radiolabeled PDGF-BB. Although all forms of PDGF (AA, AB and BB) bind to the PDGF-αα receptor, it has the highest affinity for PDGF-AA. In contrast, the PDGF-ββ receptor binds to PDGF-BB, but not to the AA form (Claesson-Welsh et al., 1989; Heidaran et al., 1990a,b; Olashaw et al., 1991; Pringle et al., 1991). We found that PDGF-BB and -AB displaced approximately 70 % of labeled PDGF-BB binding, while PDGF-AA, at 100 times the concentration of radioligand, was only able to inhibit approximately 30 % of the binding. Therefore, PDGF-AA may displace the binding from the available PDGF-αα receptors, while the other forms of PDGF displace the binding from the PDGF-ββ or -αβ receptors. The ability of PDGF-AB to displace the binding of [^{125}I]PDGF-BB almost as well as PDGF-BB is surprising. It is generally thought that PDGF-AB binds to PDGF-αα and PDGF-αβ receptors and has no effect on PDGF-ββ homodimers (Kanakaraj et al., 1991; Heidaran et al., 1993; Pringle et al., 1991). However, PDGF-AB may accomplish this inhibition by two mechanisms; by displacing [^{125}I]PDGF-BB from the PDGF-αα homodimers, as does PDGF-AA, or by preventing the dimerization of PDGF-β receptors into functional homodimers to bind the radioligand (Heidaran et al., 1993; Pringle et al., 1991).

Others have shown previously that the majority of the PDGF binding sites expressed in centrally derived oligodendrocyte precursors are PDGF-αα receptors (Hart et al., 1989b; McKinnon et al., 1990; Pringle et al., 1992). Therefore, tsU19-9 cells, which are derived from the optic nerve, differ from the previously described primary cultures, since only 30 % of the PDGF binding sites prefer PDGF-AA. However, further analysis of tsU19-9 cells must be performed to confirm the types and quantity of PDGF receptors present. Since we determined that there were more binding sites for PDGF-BB, we used PDGF-BB for further analysis of PDGF-stimulated signal transduction, and other forms of PDGF were used for comparative purposes.

Activation of the PDGF receptor results in stimulation of a diverse spectrum of early events, including an increase in inositol phosphate formation and in $[Ca^{2+}]_i$ (Diliberto et al., 1992; Chow and Powis, 1993; Estacion and Mordan, 1993). We therefore determined the effect of PDGF binding on these signal transduction intermediates in tsU19-9 cells. PDGF-AA stimulated a 1.3-fold increase (significant at $P<0.05$), while PDGF-BB stimulated a 6.3-fold increase in inositol phosphate levels. This difference may reflect a lower number of binding sites for PDGF-AA or a lower coupling efficiency of the receptors (Olashaw et al., 1991; Simm et al., 1992; Heidaran et al., 1993). However, the activation of both PDGF-αα and -ββ receptors is coupled to PLC-γ (Heidaran et al., 1993), the enzyme that hydrolyses phosphatidylinositol to $InsP_3$ and

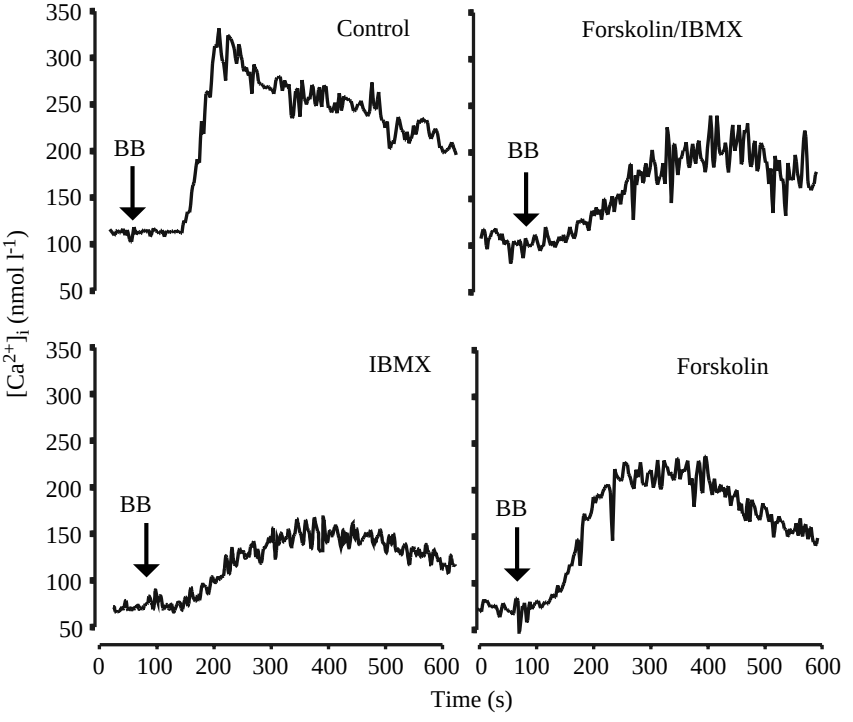

Fig. 9. Effect of forskolin and/or isobutylmethylxanthine (IBMX) pretreatment on PDGF-BB-stimulated intracellular Ca^{2+} levels. Cultures were given no pretreatment (top left) or pretreated with 0.5 mmol l^{-1} IBMX (bottom left), 10 μmol l^{-1} forskolin (bottom right) or 0.5 mmol l^{-1} IBMX + 10 μmol l^{-1} forskolin (top right) for 24 h prior to assay. The cell suspensions were challenged with 50 ng ml^{-1} PDGF-BB (all panels, arrow).

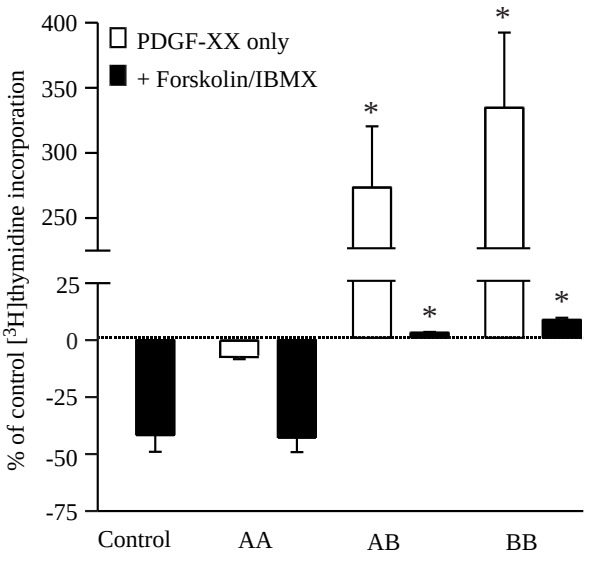

Fig. 10. Effect of forskolin/IBMX on PDGF-stimulated DNA synthesis. Quiescent cultures of tsU19-9 cells were incubated with 10 ng ml^{-1} platelet-derived growth factor (PDGF) (AA, AB, BB) without (open columns) or with 10 μmol l^{-1} forskolin + 0.5 mmol l^{-1} isobutylmethylxanthine (IBMX) (filled columns) for 48 h. [^{3}H]thymidine incorporation was determined as described in Materials and methods. Controls values were determined in cultures treated with DMEM + 0.1 % BSA alone and are normalized to 0 %. Values are means for four determinations ± S.E.M. Differences were determined by ANOVA compared with control or forskolin-treated values: *P<0.001. The values obtained with each growth factor in combination with forskolin are not significantly different from the values for control or forskolin-treated cultures (i.e. AA + forskolin *versus* control or forskolin) as determined by paired Student's *t*-test.

diacylglycerol. Although PDGF-AA was much weaker compared with PDGF-BB in stimulating inositol phosphate hydrolysis, they were nearly equipotent in stimulating intracellular Ca^{2+} mobilization. The discrepancy between inositol phosphate hydrolysis and intracellular Ca^{2+} mobilization may reflect the result of signal thresholds in transduction amplification. Although there may be fewer binding sites for PDGF-AA, or fewer PDGF-αα receptors, there were sufficient numbers to elicit the propagation of early short-term signal transduction (Olashaw et al., 1991; Diliberto et al., 1992). Therefore, PDGF-AA was able to elicit the release of Ca^{2+} from intracellular pools in a manner similar to PDGF-BB because the amount of $InsP_3$ formed was sufficient to stimulate release from intracellular stores. Further evidence for two different PDGF receptors was revealed by the absence of PDGF-AA-mediated downregulation of PDGF-BB-stimulated signal transduction (Olashaw et al., 1991). Pretreatment of tsU19-9 cells with PDGF-AA did not affect PDGF-BB-stimulated intracellular Ca^{2+} mobilization; however, PDGF-AB, which can bind to PDGF-β receptors (Pringle et al., 1991), almost abolished the response.

It is interesting to note that, although PDGF-AA stimulated inositol phosphate hydrolysis and intracellular Ca^{2+} mobilization, it had no effect on the proliferation of tsU19-9 cells. This finding is surprising since PDGF-AA is a potent mitogen for oligodendrocytes (Noble et al., 1988; Richardson et al., 1988; Hart et al., 1989b; Bögler et al., 1990; McKinnon et al., 1990; Pringle et al., 1991; Grinspan et al., 1993; Grinspan and Franceschini, 1995). However our findings are similar to those of Simm et al. (1992), who used a fibroblast cell line expressing equal amounts of both PDGF-α and -β receptor subunits. They found that PDGF-AA stimulated 'early events', such as receptor autophosphorylation; however, unlike PDGF-BB, it weakly stimulated inositol phosphate hydrolysis and had no effect on 'late events' such as proliferation. Another explanation for the difference in response to PDGF isoforms may be due to the limiting amounts of the PDGF-αα receptor. Furthermore, PDGF-AA-stimulated intracellular Ca^{2+} mobilization was transient compared with the sustained response stimulated by PDGF-BB and, as the findings of Simm et al. (1992) suggest, the transient increase in $[Ca^{2+}]_i$ may not be sufficient to activate late events such as proliferation.

Since activation of PKA (Graves et al., 1993) is implicated in the regulation of PDGF-mediated signal transduction, we determined the role of these pathways in PDGF-BB-stimulated Ca^{2+} mobilization and proliferation of tsU19-9 cells. Overnight exposure of tsU19-9 cells to forskolin partially inhibited PDGF-BB-stimulated intracellular Ca^{2+} mobilization and arrested basal and PDGF-stimulated rates of proliferation. The mechanisms controlling the antagonistic effects of PKA and other intermediates governing forskolin-mediated growth arrest in oligodendrocyte precursors and other cell types are not clear. Recently, Graves et al. (1993) have narrowed the scope of targets for PKA in PDGF-induced signaling using smooth muscle cells. Their findings suggested that activated PKA blocked specific signaling downstream from the receptor complex (i.e. autophosphorylation of the PDGF-ββ receptor and activation of PLC-γ or Raf-1), but upstream from activation of mitogen-activated protein kinase (MAPK). Furthermore, Burgering et al. (1993) clearly demonstrated, in NIH-3T3 and rat-1 fibroblasts, that protein kinase A, in contrast to PKC, negatively modulated the activity of the nucleotide exchange factor mSos, which can normally permit the GAP/p21Ras pathway to activate the MAPK pathway, potentially resulting in cell division. The cyclic-AMP-mediated phosphorylation and inhibition of mSos may help explain why newly differentiated oligodendrocytes *in vitro* become mitotically unresponsive to PDGF, although they still show PDGF-stimulated Ca^{2+} transients (Hart et al., 1989a). It is possible that the substratum-adhesion-mediated increase in intracellular cyclic AMP levels *in vitro*, as reported by Vartanian et al. (1988), stimulates PKA activity, which in turn desensitizes mSos and the PDGF-stimulated signal transduction pathway. Therefore, the absence of the PDGF-stimulated signal transduction could allow other factors, such as the autocrine-derived TGF-β, an inhibitor of oligodendrocyte progenitor proliferation (McKinnon et al., 1993), to promote the growth arrest and differentiation of the precursor cells. In addition, activation of PKA could result in

more long-term changes in oligodendrocyte progenitors. Thus, PKA phosphorylates and activates cyclic-AMP-responsive element binding proteins (Sheng and Greenberg, 1990; Kerr et al., 1992; Meinkoth et al., 1993; Lee and Masson, 1993; Papavassiliou, 1994), which in turn regulate gene expression and, in the case of oligodendrocyte precursors, differentiation.

In conclusion, our results demonstrate that forskolin, an agent that elevates intracellular cyclic AMP levels, favors the morphological and antigenic differentiation of tsU19 cells and antagonizes PDGF-stimulated signal transduction and cell division. These findings demonstrate that tsU19 cells may serve as an optic-nerve-derived glial progenitor model to study the role of PDGF and signals that regulate intracellular cyclic AMP levels with respect to glial cell differentiation. Furthermore, these cells may be useful for transplantation purposes for studies of nerve remyelination (Crang et al., 1992).

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