PDGF and Intracellular Signaling in the Timing of Oligodendrocyte Differentiation

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Abstract. In the rat optic nerve, bipotential O-2A progenitor cells give rise to oligodendrocytes and type 2 astrocytes on a precise schedule. Previous studies suggest that PDGF plays an important part in timing oligodendrocyte development by stimulating O-2A progenitor cells to proliferate until they become mitotically unresponsive to PDGF, stop dividing, and differentiate automatically into oligodendrocytes. Since the loss of mitotic responsiveness to PDGF has been shown not to be due to a loss of PDGF receptors, we have now examined the possibility that the unresponsiveness results from an uncoupling of these receptors from early intracellular signaling pathways.

We show that (*a*) although PDGF does not stimulate newly formed oligodendrocytes to synthesize DNA, it induces an increase in cytosolic Ca^{2+} in these cells; (*b*) a combination of a Ca^{2+} ionophore plus a phorbol ester mimics the effect of PDGF, both in stimulating O-2A progenitor cell division and in reconstituting the normal timing of oligodendrocyte differentiation in culture; and (*c*) the same combination of drugs does not stimulate newly formed oligodendrocytes to proliferate, even in the presence of PDGF or dibutyryl cAMP. The most parsimonious explanation for these results is that O-2A progenitor cells become mitotically unresponsive to PDGF because the intracellular signaling pathways from the PDGF receptor to the nucleus are blocked downstream from the receptor and some of the early events that are triggered by receptor activation.

THE rat optic nerve contains three types of macroglial cell, which develop on a precise schedule. Type 1 astrocytes develop before birth from their own precursor cells (Raff et al., 1984), whereas both oligodendrocytes and type 2 astrocytes develop after birth from bipotential O-2A progenitor cells (Raff et al., 1983*b*; Miller et al., 1985). Oligodendrocytes first appear around the time of birth, while type 2 astrocytes first appear in the second postnatal week (Miller et al., 1985).

The timing and direction of O-2A progenitor cell differentiation depend on both an intrinsic developmental program in the cell and on cell-cell interactions (Raff, 1989). When progenitor cells in vitro are deprived of their normal environmental signals, they stop dividing and differentiate prematurely into oligodendrocytes (Temple and Raff, 1985), suggesting that this is the constitutive pathway of differentiation, which is triggered automatically when progenitor cells stop dividing. Type 2 astrocyte differentiation, by contrast, seems to depend on extracellular inducing signals, which begin to operate in the second postnatal week (Temple and Raff, 1985; Hughes and Raff, 1987; Hughes et al., 1988; Lillien et al., 1988).

PDGF, secreted by type 1 astrocytes, plays a crucial role in amplifying the number of O-2A progenitor cells (Noble et al., 1988; Richardson et al., 1988; Pringle et al., 1989) and in timing oligodendrocyte differentiation in culture (Raff et al., 1988). PDGF is thought to act directly on O-2A progenitor cells (Hart et al., 1989) to stimulate their proliferation until an intrinsic timer in the cells causes them to become mitotically unresponsive to PDGF; as a result, the cells stop dividing and differentiate into oligodendrocytes (Raff et al., 1985, 1988).

What is the nature of the timer that controls the onset of mitotic unresponsiveness to PDGF? One possibility is that a molecule required for the proliferative response to PDGF decreases with time, or with each successive cell division, until it falls below a threshold level (Raff et al., 1985; Temple and Raff, 1986). We have shown previously that many newly formed oligodendrocytes, which no longer divide in response to PDGF, still express large numbers of PDGF receptors, making it unlikely that receptor loss is responsible for the loss of mitotic responsiveness to PDGF (Hart et al., 1989).

In 3T3 cells, PDGF activates multiple intracellular signaling pathways (Rozengurt, 1986; Williams, 1989), including the inositol phospholipid pathway (Habenicht et al., 1981; Matuoka et al., 1988; Meisenhelder et al., 1989), which leads to protein kinase C (C-kinase)[1] activation (Rozengurt et al., 1983*a*) and an elevation of cytosolic Ca^{2+} (Ives and

1. *Abbreviations used in this paper*: A23187, Ca^{2+} ionophore A23187; BrdU, bromodeoxyuridine; C-kinase, protein kinase C; dbcAMP, dibutyryl cAMP; GC, galactocerebroside; GFAP, glial fibrillary acidic protein; PDB, phorbol 12,13 dibutyrate.

Daniel, 1987), and the cAMP pathway (Rozengurt et al., 1983*b*), which leads to the activation of cAMP-dependent protein kinase (A-kinase). In addition, PDGF receptors are tyrosine kinases (Yarden et al., 1986; Claesson-Welsh et al., 1988; Gronwald et al., 1988; Matsui et al., 1989), which phosphorylate both themselves and other target proteins in the cell (Escobedo et al., 1988; Williams, 1989) including the tyrosine kinase *c-src* (Ralston and Bishop, 1985), a phosphatidylinositol kinase (Coughlin et al., 1989), and the serine/threonine kinase Raf-1 (Morrison et al., 1988). It is not known how these various intracellular signaling pathways lead to cell division.

In the present study, we have examined the possibility that O-2A progenitor cells become mitotically unresponsive to PDGF because their PDGF receptors become uncoupled from the inositol phospholipid or cAMP signaling pathways. Because the other signaling pathways activated by PDGF are poorly understood, they were not specifically investigated. We show that, although newly formed oligodendrocytes do not respond mitotically to PDGF, PDGF can stimulate a rapid increase in cytosolic Ca^{2+} in these cells as well as in progenitor cells. We also show that O-2A progenitor cells can be stimulated to divide and differentiate into oligodendrocytes on schedule when their cytosolic Ca^{2+} levels are raised with a Ca^{2+} ionophore and, at the same time, their C-kinases are activated by a phorbol ester; the same combination of drugs, however, does not stimulate newly formed oligodendrocytes to synthesize DNA, even in the presence of dibutyryl cAMP (dbcAMP) or PDGF. Finally, we demonstrate that PDGF, but not the combination of Ca^{2+} ionophore and phorbol ester, is still mitogenic for O-2A progenitor cells in which C-kinase has been downregulated. Taken together, these findings suggest that the reason O-2A progenitor cells become mitotically unresponsive to PDGF cannot be solely that their PDGF receptors are functionally uncoupled from early intracellular signaling events; there must be a block in the intracellular mitotic response pathways downstream from these events.

Materials and Methods

Cell Culture

Optic nerves were dissected from embryonic day-18, newborn, or postnatal day-2, -7, or -15 Sprague–Dawley rats and dissociated into single cells using trypsin, EDTA, and collagenase, as previously described (Miller et al., 1985). About 5,000–20,000 cells were cultured at 37°C in 5% CO_2 on poly-D-lysine–coated glass coverslips in 0.5 ml serum-free DME containing additives previously described by Bottenstein (1986).

Cells for culture in microwells were diluted in L-15 medium (Gibco Laboratories, Grand Island, NY) containing 10% FCS. About five cells in 10 μl medium were transferred to single wells of a Terasaki microculture plate (Falcon Labware, Oxnard, CA) that had previously been coated with poly-D-lysine and then incubated with supplemented DME for 2 h at 37°C. The cells were incubated at 37°C in air for 30 min to allow them to adhere, the medium was then changed to astrocyte-conditioned medium diluted 1:20 with supplemented DME, and the plates were then incubated at 37°C in 5% CO_2. The astrocyte-conditioned medium was prepared as previously described (Raff et al., 1988); it was used to increase the survival of isolated cells but had no mitogenic effect at this dilution.

Downregulation of C-kinase

For C-kinase downregulation experiments (Collins and Rozengurt, 1984), newborn optic nerve cells were cultured on coverslips in supplemented DME with a high concentration of phorbol 12,13 dibutyrate (PDB; 10^{-6} M) for 12 h. The cells were then transferred to supplemented DME containing 0.1% BSA for 30 min to remove the PDB, then rinsed, transferred to supplemented DME, and cultured for an additional 36 h with either PDB (10^{-8} M) plus the Ca^{2+} ionophore A23187 (A23187) (10^{-9} M) or human PDGF (2 ng/ml).

Bromodeoxyuridine (BrdU) Incorporation and Immunofluorescence Staining

BrdU (Boehringer Mannheim GmbH, Mannheim, FRG), which is incorporated into replicating DNA (Gratzner, 1982), was added to cultures of optic nerve cells to a final concentration of 5×10^{-5} M for the last 24 h of the culture period. After fixation with 4% paraformaldehyde for 2 min at room temperature, cells were surface stained either with monoclonal anti-galactocerebroside (GC) antibody (Ranscht et al., 1982; ascites fluid diluted 1:100) followed by fluorescein-coupled, class-specific, goat anti-mouse IgG3 (Nordic Immunological Laboratories, Tilburg, The Netherlands; diluted 1:100) or with the A2B5 monoclonal antibody (Eisenbarth et al., 1979; ascites fluid diluted 1:100) followed either by fluorescein-coupled goat anti-mouse Ig (Cappel Laboratories, Malvern, PA; diluted 1:100) or by biotin-coupled sheep anti-mouse Ig (Amersham Corp., Arlington Heights, IL; diluted 1:50) followed by fluorescein-coupled streptavidin (Amersham Corp., diluted 1:50). The antibodies were diluted in MEM buffered with 0.02 M Hepes and containing 10% FCS and 0.2% (wt/vol) sodium azide. Cells were postfixed in glacial acetic acid/ethanol (5:95) at −20°C for 10 min. They were then treated with 2 N HCl to denature the nuclear DNA (Yong and Kim, 1987) followed by 0.1 M sodium borosilicate, pH 8.5, both for 10 min at room temperature. The cells were then labeled with monoclonal anti-BrdU antibody (Magaud et al., 1988; culture supernatant diluted 1:2 in PBS containing 1% Triton X-100 and 2% FCS) followed by rhodamine-coupled goat anti-mouse Ig (Cappel Laboratories; diluted 1:100 in the same solution). In some experiments, cells were stained with a rabbit antiserum against glial fibrillary acidic protein (GFAP) (Pruss, 1979; diluted 1:1,000). In these cases, the cells were prefixed with 70% ethanol for 30 min at −20°C, the bound anti-GFAP antibodies were detected with fluorescein-coupled sheep anti-rabbit Ig (Wellcome Reagent Ltd., Beckenham, England; diluted 1:1,000), and the cells were then prepared for BrdU labeling as before.

Cells on coverslips were mounted in Citifluor (City University, London, England) on glass slides and sealed with nail varnish, while cells in microwells were viewed after the addition of PBS containing 0.1% *p*-phenylenediamine to prevent fading of fluorescence. The cells were then examined in a Universal fluorescence microscope (Carl Zeiss, Inc., Thornwood, NY) using a 40× or 63× objective and photographed using Tri-X film (ASA 400) (Eastman Kodak Co., Rochester, NY).

Glial cell types were identified by their characteristic morphologies and antigenic phenotypes: astrocytes were labeled by anti-GFAP antiserum (Bignami et al., 1972), O-2A progenitor cells by A2B5 monoclonal antibody (Raff et al., 1983*b*), and oligodendrocytes by anti-GC antibody (Raff et al., 1978).

Measurement of Cytosolic Ca^{2+}

Postnatal day-2 optic nerve cells were cultured for 1 d in supplemented DME (without PDGF) on poly-D-lysine–coated glass coverslips on which a grid had been drawn in indelible ink. The cells were incubated with the tetra-acetoxymethyl ester of the fluorescent Ca^{2+} indicator fura-2 (fura-2AM; 4 μM) in supplemented DME for 30 min at room temperature. The fura-2AM was aspirated, and the cells were incubated in L-15 medium for a further 90 min at 37°C in air before they were examined in a fluorescence microscope (Carl Zeiss, Inc.) adapted to alternate rapidly between the excitation wavelengths of 350 and 380 nm. A field of cells was visualized via a television camera connected to an image processor, and the fluorescence was recorded for at least 10 min before and 10 min after the addition of PDGF (2 ng/ml). Free Ca^{2+} levels were calculated for individual cells within the field as described previously (Silver et al., 1989). In control experiments, the vehicle used to suspend the PDGF (10 mM acetic acid containing 2 mg/ml BSA) was added to the cultures. Photographs of the cells were taken on Instamatic film (Polaroid Corp., Cambridge, MA), and the cells were then immunolabeled as described above to aid identification; the field of interest was relocated using the photographs and the ink grid on the coverslip.

Although 4 μM was the lowest concentration of fura-2AM that gave adequate loading of O-2A lineage cells, these cells did not tolerate the procedure well: some showed a spontaneous gradual rise in cytosolic free Ca^{2+}

concentration and, in some cases, degenerated before the experiment ended. Such cells were not included in the analysis.

Materials

Human PDGF was obtained from R and D Systems Inc. (Minneapolis, MN) and goat anti-human PDGF antibodies (an IgG fraction prepared by ion exchange chromatography) were purchased from Collaborative Research Inc. (Waltham, MA). Forskolin, PDB, phorbol 12-myristate 13 acetate, 4α-phorbol 12,13-didecanoate, dbcAMP, A23187, and arachidonic acid were obtained from Sigma Chemical Co. (St. Louis, MO), and fura-2AM was from Molecular Probes Inc. (Junction City, OR).

Results

PDGF Causes a Rapid Increase in Cytosolic Ca^{2+} in Newly Formed Oligodendrocytes

We found previously that many newly formed oligodendrocytes have large numbers of PDGF receptors even though they are mitotically unresponsive to PDGF (Hart et al., 1989). To determine if the PDGF receptors on these cells are functional, we studied the effect of PDGF on cytosolic Ca^{2+} levels in postnatal day-2 optic nerve cells that were cultured for 1 d and then loaded with fura-2AM. The cytosolic free Ca^{2+} concentration was determined in individual cells before and after PDGF was added to the cultures. The oligodendrocytes were initially identified by their characteristic morphology, and the assignments were later checked by labeling with anti-GC antibodies (Raff et al., 1978). In 12 experiments, 12 out of 22 GC^+ oligodendrocytes showed a rise in cytosolic free Ca^{2+} when PDGF (2 ng/ml) was added. In some of these cases, the increase was small (<100 nM), occurred slowly (reaching a peak in >200 s), or quantitative data were incomplete. In the 17 cells where quantitative data were available, 5 showed an increase in cytosolic free Ca^{2+} (from 82 ± 19 nM to 277 ± 15 nM) within 21–200 s after addition of PDGF (Fig. 1). Although there was a mixture of strongly and weakly GC^+ oligodendrocytes in these cultures, all of the oligodendrocytes that showed a Ca^{2+} response were weakly GC^+, suggesting that they had only recently differentiated. This is consistent with our previous findings that oligodendrocytes tend to lose PDGF receptors as they mature (Hart et al., 1989). In control experiments, no change in cytosolic free Ca^{2+} levels were observed in 20 out of 20 oligodendrocytes after addition of the vehicle used to dissolve the PDGF. Thus, PDGF receptors on at least some newly formed oligodendrocytes could be activated by PDGF to cause an increase in cytosolic Ca^{2+}.

As expected, some of the O-2A progenitor cells in these cultures (identified by immunolabeling with A2B5 antibody; Raff et al., 1983*b*; Temple and Raff, 1986) also showed a Ca^{2+} response to PDGF, although fewer cells responded than anticipated. In 12 experiments, 13 out of 52 progenitor cells showed a response, and, in the 48 cells in which quantitative data were complete, 8 showed a rise in cytosolic free Ca^{2+} (from 64 ± 8 nM to 152 ± 33 nM) within 28–266 s after addition of PDGF. In control experiments, 25 out of 25 progenitor cells showed no response when vehicle was added to the cultures without PDGF. The low proportion of progenitor cells that responded to PDGF, compared with the much higher proportion (~70–80%) that would be expected to have PDGF receptors (Hart et al., 1989), was probably due to the sensitivity of these cells to the damaging effects of loading with fura-2AM (see Materials and Methods). O-2A progenitor cells have been found to be unusually sensitive to suboptimal culture conditions and to various physical and chemical manipulations (Raff, M. C., unpublished observations).

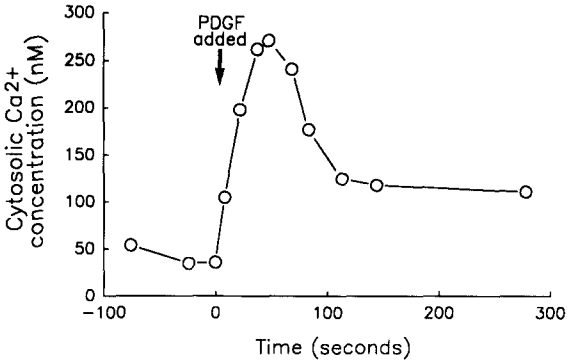

Figure 1. Effect of PDGF on cytosolic Ca^{2+} in a newly formed oligodendrocyte in culture. Postnatal day-2 optic nerve cells were cultured in serum-free medium for 1 d and then loaded with fura-2AM. The cytosolic free Ca^{2+} concentration was determined in this cell before and after PDGF (2 ng/ml) was added to the culture. The oligodendrocyte was initially identified by morphology and was found to be weakly stained after the culture was labeled with anti-GC antibody.

64 out of 64 type 1 astrocytes showed no change in cytosolic free Ca^{2+} levels after addition of PDGF (from an average resting level of 87 ± 6 nM). These results are consistent with our previous finding that type 1 astrocytes do not have PDGF receptors (Hart et al., 1989).

A Combination of PDB and A23187 Stimulates O-2A Progenitor Cells To Synthesize DNA

To study the effects of manipulating intracellular signaling pathways on DNA synthesis in O-2A progenitor cells, we cultured optic nerve cells from newborn rats in serum-free medium for 2 d in the presence of various agents. BrdU was added for the last 24 h of the culture period and the cells were then double labeled by indirect immunofluorescence with the A2B5 monoclonal antibody to identify the progenitor cells and with monoclonal anti-BrdU antibody to identify cells that had incorporated BrdU into DNA (Magaud et al., 1988) (Fig. 2).

When newborn optic nerve cells were cultured in the absence of drugs, <5% of the O-2A progenitor cells incorporated BrdU. When added individually, none of the following agents had a detectable effect on BrdU incorporation in progenitor cells: arachidonic acid (10^{-7}–10^{-4} M), dbcAMP (10^{-6}–10^{-3} M), forskolin (an activator of adenylate cyclase; 10^{-8}–10^{-3} M), PDB (a phorbol ester that activates C-kinase; 10^{-12}–10^{-6} M), or A23187 (a Ca^{2+} ionophore; 10^{-12}–10^{-6} M) (not shown). A combination of PDB and A23187, however, did stimulate progenitor cells to synthesize DNA, with a maximal effect at 10^{-8} and 10^{-9} M, respectively (Fig. 3). This stimulation almost equaled the effect of PDGF, which has been shown previously to be a potent mitogen for O-2A progenitor cells in culture (Noble et al., 1988; Richardson et al., 1988; Pringle et al., 1989). When 4α-phorbol 12,13-didecanoate, an inactive isomer of PDB, was used in place of PDB, no stimulation was seen with A23187 (not shown),

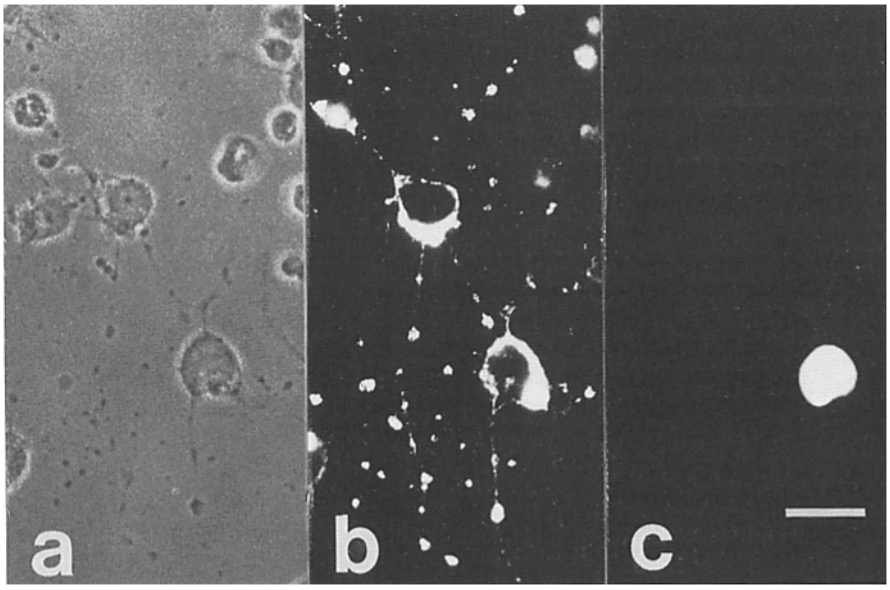

Figure 2. Immunofluorescence micrographs of O-2A progenitor cells stimulated to incorporate BrdU in response to A23187 plus PDB. Newborn day-7 optic nerve cells were cultured in serum-free medium for 2 d with A23187 (10^{-9} M) plus PDB (10^{-8} M). BrdU was added for the last 24 h of the culture period, and the cells were double labeled with A2B5 antibody, followed by biotin-coupled sheep anti-mouse Ig and then fluorescein-coupled streptavidin, and then with anti-BrdU antibody, followed by rhodamine-coupled goat anti-mouse Ig. The cells were photographed using phase-contrast (*a*), fluorescein (*b*), or rhodamine (*c*) optics. One of the two A2B5$^+$, process-bearing progenitor cells shown in *a* and *b* is seen to be BrdU$^+$ in *c*. Bar, 20 μm.

suggesting that the observed effect of PDB (when used with A23187) depended on the specific activation of C-kinase. Another phorbol ester, phorbol 12-myristate 13-acetate, when used together with A23187, also stimulated O-2A progenitor cells to synthesize DNA, but only if 0.5% FCS was added to the medium (not shown). We have no explanation for this difference between PDB and phorbol 12-myristate 13-acetate.

A23187 or PDB were without effect when they were combined with forskolin or dbcAMP (not shown), suggesting that the activation of A-kinase, in combination with either an increase in cytosolic Ca^{2+} or the activation of C-kinase, does not stimulate DNA synthesis in these cells.

PDB and A23187 Act Directly on O-2A Progenitor Cells To Stimulate DNA Synthesis

It was important to determine whether PDB and A23187 other cell type. It was possible, for example, that the drugs were stimulating type 1 astrocytes to secrete PDGF, which in turn stimulated the progenitor cells to synthesize DNA. To study the responses of progenitor cells in the absence of type 1 astrocytes, we studied low density cultures (<5,000 cells per coverslip) of optic nerve cells from postnatal day-15 rats. It has been shown previously that relatively few type 1 astrocytes are released from later than postnatal day-7 optic nerves by the dissociation procedure used (Miller et al., 1985). In three experiments on postnatal day-15 cells, 34 ± 5% of the O-2A progenitor cells were stimulated to incorporate BrdU by the combination of PDB and A23187 compared with <1% in control cultures; in sister cultures, PDGF stimulated 23 ± 6% of the progenitor cells to incorporate BrdU. There were no type 1 astrocytes (as determined by anti-GFAP staining and morphology; Raff et al., 1983*a*) in any of these cultures, although GFAP$^-$ fibroblast-like cells accounted for ~5% of the population. Moreover, in three experiments, the addition of goat anti-PDGF antibodies (90 μg/ml of IgG) to 2-d-old bulk cultures of postnatal day-7 optic nerve cells did not inhibit the ability of PDB plus A23187 to stimulate O-2A progenitor cells to synthesize DNA, even though the same concentration of anti-PDGF antibodies almost completely neutralized the effect of 4 ng/ml PDGF (not shown).

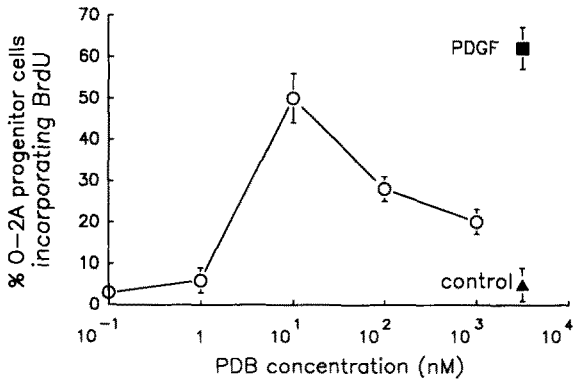

Figure 3. Effect of A23187 plus PDB on BrdU incorporation in O-2A progenitor cells in culture. Newborn optic nerve cells were cultured in serum-free medium for 2 d without additives or with either A23187 (10^{-9} M) plus varying concentrations of PDB or PDGF (2 ng/ml). BrdU was added for the last 24 h of the culture period, and the cells were double labeled with A2B5 and anti-BrdU antibodies. The O-2A progenitor cells were identified as A2B5$^+$, process-bearing cells (see Fig. 2). The results in this figure and in Table II are expressed as mean ± SEM of three experiments.

To further assess whether PDB and A23187 acted directly on O-2A progenitor cells to stimulate them to synthesize DNA, single-cell cultures were prepared from postnatal day-7 optic nerves by limiting dilution in microwells, as previously described (Hughes and Raff, 1987). In microwells that contained only O-2A progenitor cells, as assessed by morphology and immunofluorescence staining with A2B5, PDB plus A23187 stimulated BrdU incorporation almost to the same extent as PDGF (Table I), suggesting that all of these agents act directly on the progenitor cells to induce DNA synthesis.

A Combination of PDB and A23187 Reconstitutes Normal Timing of Oligodendrocyte Differentiation in Culture

To determine whether PDB plus A23187 could also stimulate

Table I. Effect of PDB Plus A23187 on BrdU Incorporation by O-2A Progenitor Cells in Single-cell Microwell Cultures

Agent added	O-2A progenitor cells incorporating BrdU (the number of $BrdU^+$ progenitor cells out of total progenitor cells assessed)
	%
Control	6 (8 out of 127)
PDGF	38 (96 out of 256)
PDB + A23187	30 (54 out of 188)

Postnatal day-7 optic nerve cells were plated in microwells (less than or equal to five cells per well) and cultured for 2 d without additives or with either PBD (10^{-8} M) plus A23187 (10^{-9} M) or PDGF (2 ng/ml). BrdU was added for the last 24 h of the culture period and the cells were quadruple labeled with A2B5, anti-GC, anti-GFAP, and anti-BrdU antibodies. Only microwells that contained a single O-2A progenitor cell ($A2B5^+$, GC^-, $GFAP^-$, process bearing) and no other cells were included in the results shown.

progenitor cell proliferation and reconstitute the normal in vivo timing of oligodendrocyte differentiation in vitro, we cultured embryonic day-18 optic nerve cells for 1, 2, and 3 d with PDB and A23187. A small amount of FCS (0.5%) was added to the medium since it improved cell survival. As described previously (Raff et al., 1985, 1988), in cultures maintained without added mitogens, many O-2A progenitor cells prematurely stopped dividing and differentiated into oligodendrocytes (identified by staining with anti-GC antibody); in cultures maintained in PDGF, O-2A progenitor cells continued to divide, and the first oligodendrocytes appears after 3 d in vitro, which is the equivalent to the day of birth, when the first oligodendrocytes develop in vivo (Miller et al., 1985). As shown in Table II, cultures maintained in PDB plus A23187 behaved similarly to those maintained in PDGF: progenitor cells continued to proliferate, and the first oligodendrocytes were seen after 3 d in vitro.

A Combination of PDGF, PDB, and A23187 Does Not Stimulate Newly Formed Oligodendrocytes To Synthesize DNA

To determine if newly formed oligodendrocytes could be stimulated to synthesize DNA in response to a combination of PDB and A23187 (with or without PDGF), cultures of newborn optic nerve cells were maintained in these reagents and double labeled with anti-GC and anti-BrdU antibodies after 2 d in vitro, having been exposed to BrdU for the last 24 h of the culture period. In three out of three experiments, no oligodendrocytes were induced to incorporate BrdU. Adding dbcAMP (10^{-6}–10^{-3} M) together with these agents did not affect this result. Thus, newly formed oligodendrocytes, which have been shown previously not to synthesize DNA in response to PDGF (Hart et al., 1989), also do not respond to a combination of PDB and A23187, whether or not PDGF and dbcAMP are present.

Downregulation of C-kinase Does Not Inhibit the Ability of PDGF To Stimulate O-2A Progenitor Cells To Synthesize DNA

To determine whether the mitotic effect of PDGF on O-2A progenitor cells requires the activation of C-kinase, we cultured newborn optic nerve cells for 12 h at 37°C in a high concentration of PDB (10^{-6} M) to downregulate C-kinase (Collins and Rozengurt, 1984). The cultures were then washed and maintained for an additional 36 h with either PDGF (2 ng/ml) or PDB (10^{-8} M) plus A23187 (10^{-9} M). BrdU was added for the last 24 h of the culture period. Whereas downregulation of C-kinase decreased the DNA synthesis response of O-2A progenitor cells to PDB plus A23187 from 46 ± 3% to 3 ± 1%, it did not affect their response to PDGF (62 ± 6% compared with 58 ± 5% in control cultures). Thus, the ability of PDGF to stimulate O-2A progenitor cells to synthesize DNA seems not to depend on the activation of C-kinase.

Discussion

In the present study we have continued our efforts to understand the molecular nature of the timing mechanism that controls the onset of oligodendrocyte differentiation. Previous studies have suggested that the mechanism depends on an intrinsic timer in each O-2A progenitor cell that operates by limiting the time, or the number of times, that the cell can divide in response to PDGF (Raff et al., 1985, 1988; Temple and Raff, 1986). The question of why many types of cells stop dividing and differentiate in the continuing presence of growth factors is a fundamental one in vertebrate development.

Two types of PDGF receptor proteins have been defined (Claesson-Welsh et al., 1988; Gronwald et al., 1988; Hart et al., 1988; Heldin et al., 1988)—type A and type B—both of which are single-pass transmembrane tyrosine kinases (Yarden et al., 1986; Claesson-Welsh et al., 1988; Gronwald et al., 1988; Matsui et al., 1989). Cells of the O-2A lineage have type A receptors (Hart et al., 1989; Pringle et al., 1989). Although much more is known about the functional properties of type B receptors (Williams, 1989), given that both types of receptor have very similar structures (Claesson-Welsh et al., 1989), it seems likely that they use similar signal transduction mechanisms. In experiments reported here, we have examined the possibility that O-2A progenitor cells eventually become mitotically unresponsive to PDGF because their PDGF receptors become functionally uncoupled from the inositol phospholipid or cAMP signaling pathways. Our results suggest that this is not the case.

Using fura-2AM to measure cytosolic free Ca^{2+}, we dem-

Table II. Effect of PDB Plus A23187 on O-2A Progenitor Cell Proliferation and the Timing of Oligodendrocyte Development in Cultures of Embryonic Day-18 Optic Nerve Cells

Time in culture	Cell type	Cells in cultures containing: No additives	PDGF (2 ng/ml)	PDB (10^{-8} M) + A23187 (10^{-9} M)
d		*n*	*n*	*n*
1	O-2A progenitors	133	173	148
	Oligodendrocytes	0	0	0
2	O-2A progenitors	184	299	233
	Oligodendrocytes	14	0	0
3	O-2A progenitors	168	522	378
	Oligodendrocytes	58	15	18

The results represent the total number of progenitor cells and oligodendrocytes seen in three experiments. The cells were double labeled with A2B5 and anti-GC antibodies. Oligodendrocytes were identified as GC^+ cells, whereas O-2A progenitors were identified as $A2B5^+$, GC^-, process-bearing cells.

onstrated that PDGF stimulates a rapid increase in cytosolic Ca^{2+} in a proportion of both O-2A progenitor cells, which are mitotically responsive to PDGF, and newly formed oligodendrocytes, which are not. This indicates that, when they are activated, the PDGF receptors on at least some newly formed oligodendrocytes are capable of increasing cytosolic Ca^{2+}. If the increase in Ca^{2+} is mediated by inositol trisphosphate (Berridge, 1988), then this finding would indicate that the PDGF receptors on these newly formed oligodendrocytes can activate the inositol phospholipid signaling pathway. Even if the increase in cytosolic Ca^{2+} is mediated by some other signaling pathway (Nånberg and Rozengurt, 1988), our finding raises the possibility that the block of mitotic responsiveness in these cells lies downstream from the PDGF receptor and some of the early intracellular signaling events that follow its activation.

A second line of experiments strongly supports this possibility. We found that we could mimic the effects of PDGF on O-2A progenitor cells in culture by both increasing cytosolic Ca^{2+} with A23187 and activating C-kinase with PDB. Single-cell experiments showed that both PDGF and the two drugs act directly on progenitor cells to stimulate DNA synthesis. Not only did the drugs mimic PDGF in stimulating progenitor cells to synthesize DNA and to proliferate, they also mimicked PDGF in reconstituting the normal in vivo timing of oligodendrocyte differentiation in cultures of embryonic optic nerve cells. Thus, the timing mechanism that controls when progenitor cells stop dividing and differentiate into oligodendrocytes apparently operates normally when the cells are stimulated to divide by drugs that bypass both the PDGF receptors and the earliest signaling events that they activate. This suggests that these receptors and events might not be involved in the timing mechanism. Consistent with this view was the finding that the same drugs did not stimulate newly formed oligodendrocytes to synthesize DNA, even in the presence of PDGF, indicating that these cells are blocked in their mitotic response somewhere distal to the earliest intracellular signaling events in the mitotic pathway.

All of the results discussed thus far are consistent with the possibility that O-2A progenitor cells eventually become unresponsive to PDGF and to the combination of A23187 plus PDB because C-kinase decreases in concentration or is inactivated. To indirectly test this possibility, we treated optic nerve cultures with a high concentration of PDB for 12 h to downregulate C-kinase in O-2A progenitor cells. Whereas this treatment abolished the ability of the progenitor cells to synthesize DNA in response to A23187 plus PDB, it had no effect on the response of these cells to PDGF. This result suggests that in O-2A progenitor cells, as in other cells, there are multiple, redundant, intracellular signaling pathways from the plasma membrane to the nucleus that lead to cell division (Rozengurt, 1986). It also suggests that the ability of PDGF to stimulate progenitor cells to synthesize DNA does not depend on C-kinase activation, which makes it unlikely that the failure of PDGF to stimulate newly formed oligodendrocytes to synthesize DNA is due solely to a decrease or inactivation of C-kinase.

We were unable to stimulate O-2A progenitor cells to synthesize DNA by increasing the intracellular concentration of cAMP with dbcAMP or forskolin, either alone or in combination with A23187 or PDB. Nor were we able to stimulate newly formed oligodendrocytes to synthesize DNA by these treatments, even when combined with PDGF, making it unlikely that the mitotic unresponsiveness of these cells is due to a block in generating cAMP.

Taken together, our results suggest that O-2A progenitor cells normally become mitotically unresponsive to PDGF because they develop a block downstream from the PDGF receptor and some of the early events that follow its activation, including the increase in cytosolic Ca^{2+} and the activation of C-kinase. Although we cannot exclude the possibility that some early events that normally follow PDGF receptor activation (that we did not examine) fail to occur, this could not be the sole cause of the mitotic unresponsiveness. We need to look next at those parts of the mitotic intracellular signaling pathways that lie closer to the nucleus or within the nucleus itself.

We thank Julia Burne, Angela Lamb, Laura Lillien, Jacqueline Marvel, Ann Mudge, Nigel Pringle, Enrique Rozengurt, and John Wood for their advice.

I. K. Hart is supported by a Medical Research Council Training Fellowship.

Received for publication 6 July 1989 and in revised form 11 September 1989.

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