

Platelet-derived Growth Factor (PDGF)-induced Ca^{2+} Signaling in the CG4 Oligodendroglial Cell Line and in Transformed Oligodendrocytes Expressing the β -PDGF Receptor*

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Ca^{2+} signaling induced by platelet-derived growth factor (PDGF) was investigated in the oligodendroglial cell lines CG4 and CEINGE clone 3, using fura-2 microfluorimetry and video imaging. CEINGE cl3 cells, immortalized with polyoma middle T antigen, were found to uniformly express the polyoma middle T antigen protein as well as 2',3'-cyclic nucleotide 3'-phosphodiesterase, a specific marker for oligodendroglia. PDGF-BB induced both oscillatory and non-oscillatory Ca^{2+} responses in CEINGE cl3 cells as well as in CG4 cells, grown either as O-2A progenitors or differentiated oligodendrocytes. However, in CG4 cells the percentage of oscillatory Ca^{2+} responses was higher than that observed in CEINGE cl3 cells. In contrast, oscillatory Ca^{2+} responses were not observed in PC-12 cells transfected with β -PDGF receptor (PDGFR) or in NIH 3T3 fibroblasts. CG4 cells expressed only the α -PDGFR, whereas CEINGE cl3 cells expressed both α and β isoforms. When CEINGE cl3 cells were exposed to PDGF-AA, which binds only to the α -PDGFR, the percentage of oscillatory Ca^{2+} responses was higher than that observed after PDGF-BB stimulation. We previously reported that block of the enzyme sphingosine kinase, and a consequent increase in intracellular sphingosine levels in CEINGE cl3 cells caused an increase in the percentage of oscillatory Ca^{2+} responses induced by PDGF-BB. However, in CG4 cells block of sphingosine kinase did not increase the oscillatory Ca^{2+} response elicited by PDGF-BB, although the addition of exogenous sphingosine induced an oscillatory Ca^{2+} response in 77% of cells studied. We hypothesize that the α -PDGFR is less effective than the β -PDGFR in stimulating the activity of sphingosine kinase. The results also suggest that α - and β -PDGFRs may differently regulate sphingolipid metabolism.

PDGF¹ plays a fundamental role in the development of oligodendroglia. Oligodendrocyte progenitors, which originate from the subventricular zones, undergo several PDGF-sensi-

tive steps while acquiring the ability to produce myelin during migration and proliferation (1–4). PDGF is mitogenic and chemotactic for several cell types, including fibroblasts, smooth muscle, and glial cells (5–7). Three PDGF isoforms have been identified, being homo- or heterodimers of related A and B polypeptide chains and named AA, -AB, and -BB (8). Two separate isoforms of the PDGFR, named α and β , have also been identified (for review see Ref. 8). These receptors consist of transmembrane tyrosine kinases that dimerize upon binding to the growth factor (9). This is a prerequisite for the reciprocal phosphorylation of the receptors on different tyrosine residues localized in the intracellular portion of the molecule (10, 11). This process leads to the formation of docking sites for molecules with catalytic activity such as PLC- γ , phosphatidylinositol 3-kinase, and Ras-GAP or molecules that function as adaptors for other substrates like Grb2, Nck, and Shc (for review see Ref. 12). It has been shown that the α -PDGFR binds both PDGF-AA and PDGF-BB with comparable affinity, whereas the β -PDGFR binds PDGF-BB exclusively (13, 14); PDGFRs also differ in their functional interaction with other molecules, like PLC- γ_1 (15) and in the induction of several phenomena like chemotaxis, membrane edge ruffling, and mitogenesis, depending on the cell type studied (for review see Ref. 8).

It has been previously shown that oligodendroglial cells only express the α isoform of PDGFR, which is down-regulated when O-2A glial progenitors differentiate into oligodendrocytes (16–18).

An increase in $[\text{Ca}^{2+}]_i$ is one of the first events that occurs following the stimulation of PDGFRs (19). The role of Ca^{2+} as second messenger in chemotaxis, cell proliferation, and immediate early gene expression has been well documented (20–23). For example, a clear correlation between changes in mRNA levels of a set of immediate early genes and the modulation of $[\text{Ca}^{2+}]_i$ induced by glutamate receptor stimulation in oligodendrocyte precursors has been observed (24). Activation of different sets of genes following the stimulation of distinct intracellular Ca^{2+} signaling pathways has also been described in neurons (25). It is possible that not only the magnitude of the $[\text{Ca}^{2+}]_i$ increase but also its kinetics could play a crucial role in this process. In fact, different Ca^{2+} signaling kinetics could activate separate biochemical pathways leading to diverse messages at the nuclear level. Considering the multiple events regulated by PDGF during oligodendroglial development, this possibility appears to be very interesting. However, few studies have investigated PDGF-induced Ca^{2+} signaling during oligodendroglial differentiation (26). We have previously shown that mT-transformed oligodendroglial CEINGE cl3 cells responded to PDGF-BB exposure with two kinetically distinct types of Ca^{2+} signals. The relative intracellular levels of the two sphingolipids, sphingosine and sphingosine 1-phosphate (SPP), which are modulated by the activity of the enzyme sphingosine kinase, appeared to be responsible for the oscillatory and non-

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¹ The abbreviations used are: PDGF, platelet-derived growth factor; R, receptor; $[\text{Ca}^{2+}]_i$, intracellular free Ca^{2+} concentration; PLC, phospholipase C; CNP, 2',3'-cyclic nucleotide 3'-phosphodiesterase; mT, polyoma middle T antigen; GalC, galactocerebroside; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium; SPP, sphingosine 1-phosphate.

oscillatory Ca^{2+} responses, respectively (27).

In this paper, we show that CEINGE cl3 cells express both α - and β -PDGFR. The Ca^{2+} response induced by PDGF and by exogenous sphingosine and SPP in these cells was compared with that observed in the oligodendroglial cell line CG4 (28), in PC-12 cells transfected with the β -PDGFR (29), and in NIH 3T3 fibroblasts. This was done in order to ascertain whether the patterns of Ca^{2+} signaling observed in CEINGE cl3 cells are a peculiarity of this clone or a particular feature of the oligodendroglial lineage. Furthermore, the consequences of blocking sphingosine kinase on Ca^{2+} signaling caused by α - or β -PDGFR activation were investigated.

EXPERIMENTAL PROCEDURES

Materials—PDGF-BB, PDGF-AA, and Tween 20 were from Calbiochem-Novabiochem (San Diego, CA). ATP, sphingosine, DL-threo-dihydrosphingosine, and neomycin sulfate were from Sigma. Neomycin chloride was used to avoid possible nonspecific effect of high sulfate concentrations and was prepared, as described (30), by dissolving neomycin sulfate at 1 M and precipitating the sulfate with 3 volumes of 1 M $BaCl_2$. The supernatant was neutralized to pH 7.4 with 1 M HCl. SPP was from Biomol (Plymouth Meeting, PA). Fura-2/acetoxymethyl ester (fura-2/AM) was from Molecular Probes (Eugene, OR). The sources of primary antibodies were as follows: anti-mT mouse monoclonal antibody (PAb 750) was from Dr. Steve Dilworth; anti-CNP and anti-GalC mouse monoclonal antibodies were from Boehringer Mannheim; anti- α -PDGFR rabbit polyclonal antibody was from Upstate Biotechnology (Lake Placid, NY); anti- β -PDGFR mouse monoclonal antibody was from Transduction Laboratories (Lexington, KY).

Cell Culture—CEINGE clone 3 and NIH 3T3 fibroblasts were grown in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Inc.) containing 10% fetal calf serum (Life Technologies, Inc.) and 50 μ g/ml gentamycin on uncoated plastic Petri dishes (Falcon).

PC-12 pheochromocytoma cells transfected with the β -PDGFR (wild type) and parental PC-12 cells were grown in DMEM containing 5% newborn calf serum and 5% horse serum (Life Technologies, Inc.) on poly-L-lysine-coated (100 μ g/ml) plastic Petri dishes (29).

CG4 cells as O-2A progenitors were grown in DMEM containing 30% of a medium conditioned by the neuroblastoma cell line B-104, biotin, insulin, and a mixture of hormones and growth factors (N1), as described previously (31). To differentiate CG4 to oligodendrocytes, cells were grown in DMEM containing biotin, N1, and a concentration of insulin three times higher than that used for O-2A progenitors (32). Cells were grown on poly-D-ornithine-coated (100 μ g/ml) plastic Petri dishes. Fetal calf serum (1%) was eventually added to the growth medium after the 3rd day of differentiation. CG4/oligodendrocytes were maintained in culture from 8 to 12 days, and cell differentiation was evaluated by microscopic observation and by immunofluorescence staining against galactocerebroside C (GalC).

All cells were maintained in a humidified incubator at 36.5 °C in a 10% CO_2 atmosphere and were fed twice weekly.

Immunofluorescence—Cells were grown on 15-mm round glass coverslips coated with polylysine and fixed with 4% paraformaldehyde added to the growth medium for 10 min (2% final concentration). Cells were then treated with fresh paraformaldehyde (4%) for 20 min and after being washed were incubated for 1 h in phosphate-buffered saline (PBS) containing 10% normal goat serum (Jackson Immunoresearch, West Grove PA) and 0.05% Tween 20. Cells were then incubated for 1 h with the primary antibodies at the following dilutions: anti-mT 1:100; anti-CNP 10 μ g/ml. A biotinylated secondary antibody was incubated for 1 h, followed by a 30-min incubation with streptavidin conjugated with indocarbocyanine (CY3) (Jackson Immunoresearch, West Grove PA). Nuclear counterstaining was obtained using Hoechst 33342 (Molecular Probes, Eugene, CA). Coverslips were mounted using an aqueous mounting solution (10% glycerol in PBS) containing 2.5% (w/v) of 1,4-diazabicyclo[2.2.2]octane (Sigma) as antifading agent. Cell fluorescence was detected using an Axioskop microscope equipped for epifluorescence (Zeiss, Germany).

Western Blot Analysis—Cells growing in 100-mm tissue culture dishes were washed twice with cold PBS and then scraped from the dish. After a brief centrifugation, the pellet was resuspended in a lysis buffer of the following composition: 25 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM NaF, 1 mM dithiothreitol, 1% Nonidet P-40, 2 μ g/ml aprotinin, 20 μ M leupeptin, 1 mM [4-(2-aminoethyl)benzenesulfonyl]fluoride, HCl (Calbiochem). The protein concentration in the clarified lysate was determined by Bio-Rad protein assay. Cell lysates

containing equal amounts of protein were resolved on a SDS-polyacrylamide gel electrophoresis according to the method of Laemmli (33) using 5, 10, and 12.5% polyacrylamide gel for PDGFRs, mT, and CNP, respectively. The separated proteins were electroblotted to Hybond-enhanced chemiluminescence nitrocellulose membrane (Amersham Corp.) or Hybond-PVDF (Amersham Corp.) for PDGFRs. Membranes were then incubated for 1 h in PBS containing 5% milk powder, 2% bovine serum albumin, and 0.1% Tween 20. This buffer was also used for the incubation with primary antibodies at the following dilutions: anti-mT 1:1000, anti-CNP 10 μ g/ml, anti- β -PDGFR (Transduction Lab) 1 μ g/ml, anti- α -PDGFR (Upstate) 1:2000. Results with anti-PDGFRs were also compared with anti- α -PDGFR (1890) and with anti- β -PDGFR (7649) rabbit polyclonal antibodies from Dr. Daniel F. Bowen-Pope, both used at 1:2000 dilution. After a wash with PBS containing 0.1% Tween 20, a secondary antibody conjugated with horseradish peroxidase (Promega) was used at 1:10,000 dilution. The binding of the antibodies to the membrane was detected using the enhanced chemiluminescence system (Amersham Corp.).

Intracellular Ca^{2+} Measurement and Video Imaging—Cells were grown on 25-mm no. 1 glass coverslips coated with poly-L-lysine (100 μ g/ml) and were loaded with 2 μ M fura-2 acetoxymethyl ester (fura-2/AM) using a balanced salt solution (standard buffer) of the following composition (in mM): NaCl 157, KCl 5, $MgSO_4$ 0.4, $MgCl_2$ 0.5, KH_2PO_4 0.6, $NaHCO_3$ 3, HEPES 20, glucose 10, $CaCl_2$ 2, and bovine serum albumin 0.2% (osmolality 320–340 mosm/kg), pH adjusted to 7.4 with Tris 1 M. Cells were incubated with fura-2/AM for 25–45 min at room temperature to avoid probe compartmentalization and then incubated for an additional 10–15 min with standard buffer to allow the complete de-esterification of the fluorescent probe. The single cell video imaging of as many as 10–30 cells/field and the calibration of fluorescent signals were performed as described previously (27).

Statistical Analysis—Data values expressed as mean \pm S.E. Student's *t* test or Mann-Whitney non-parametric test were used, and statistical significance was defined as a *p* value of 0.01 or less.

RESULTS

Characterization of CEINGE cl3 Cells, Immunofluorescence and Western Blot Analysis of Middle T and CNP Protein Expression—The CEINGE cl3 cell line was obtained by infecting E14 rat brain cells with a murine leukemia virus carrying the gene encoding the polyoma mT antigen (34). Although CEINGE cl3 cells have been previously found to express a retroviral mRNA hybridizing to the polyoma mT gene (34), we decided to verify that the protein was effectively expressed.

An equal amount of protein (60 μ g) from a total cell lysate of CEINGE cl3 cells and NIH 3T3 fibroblasts (as a negative control) was loaded onto a 10% polyacrylamide gel. As shown in Fig. 1, CEINGE cl3 cells expressed a protein with a molecular weight of approximately 55,000, whereas in NIH 3T3 fibroblasts the protein was not detected. Immunofluorescent staining obtained using CEINGE cl3 cells shows that a significant amount of the protein was detectable in every cell in the field (Fig. 1). No staining was obtained using NIH 3T3 fibroblasts (not shown).

The expression of the oligodendroglial marker GalC by CEINGE cl3 cells has been previously reported (34). To further characterize the clone, we investigated the expression of CNP as a specific marker, since this enzyme is expressed early and at high levels during the development and differentiation of oligodendrocytes and appears to be involved in the biogenesis of myelin (35, 36). For this purpose, an equal amount of protein (40 μ g) from the total cell lysate of CEINGE cl3 cells, NIH 3T3 fibroblasts (as a negative control), and CG4 cells differentiated to oligodendrocytes (as a positive control) was loaded onto a 12.5% polyacrylamide gel. Fig. 2 shows that both CEINGE cl3 cells and CG4/oligodendrocytes expressed a protein with a similar molecular weight of approximately 45,000–50,000. The amount of protein expressed appeared to be significantly higher in CG4/oligodendrocytes when compared with CEINGE cl3 cells, whereas NIH 3T3 fibroblasts did not express this protein at all. These results were confirmed by immunofluorescence staining, showing that CEINGE cl3 cells stained posi-

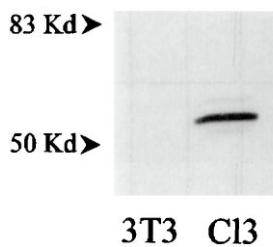
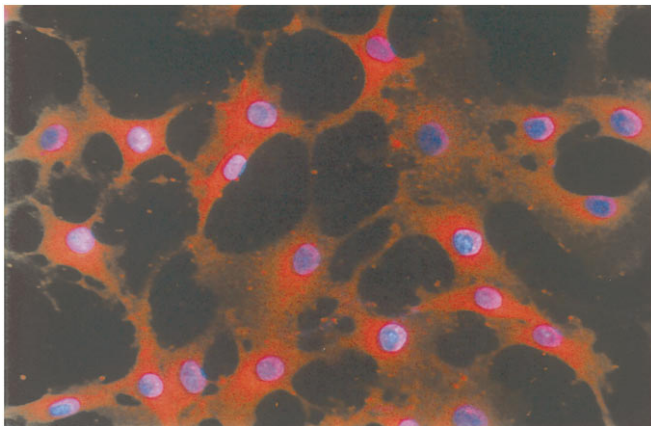


FIG. 1. Immunofluorescence and Western blot analysis of mT protein expression. The upper panel shows a representative microscopic field in which all CEINGE cl3 cells stained uniformly positively using the pAB750 antibody directed against the mT protein. The lower panel shows an immunoblot of the total cell lysate (60 μ g per lane) of NIH 3T3 fibroblasts and CEINGE clone 3 cells probed with the same antibody.

tively for CNP (Fig. 2). Similar positive staining was obtained using CG4/oligodendrocytes, whereas NIH 3T3 fibroblasts appeared completely negative (not shown).

Single Cell Video Imaging of the Ca^{2+} Response Induced by PDGF-BB in CEINGE Clone 3 and CG4 Cells—As described previously (27), exposure of CEINGE cl3 cells to 10 ng/ml PDGF-BB for 5 min was able to induce Ca^{2+} responses with two different types of kinetics. The first response was characterized by an early single increase in $[Ca^{2+}]_i$, which was frequently followed by a plateau. In contrast, the pattern of the second Ca^{2+} response was oscillatory, with several spikes of different frequencies and magnitudes beginning significantly later than the non-oscillatory response (Fig. 3, A and B). Similarly, the growth factor caused $[Ca^{2+}]_i$ increases, both in CG4/O-2A progenitors and CG4/oligodendrocytes, with a similar dual pattern (Fig. 3, C and D). The number of cells responsive to the agonist was significantly lower in CG4 cells in comparison to CEINGE cl3 cells (see Table I).

Finally, the percentage of oscillatory cells in CG4/O-2A progenitors and CG4/oligodendrocytes was higher than in CEINGE cl3 cells (Table I), and the delays preceding the onset of the two distinct Ca^{2+} responses were significantly different, as observed in CEINGE cl3 cells.

Single Cell Video Imaging of the Ca^{2+} Response Induced by PDGF-BB in NIH 3T3 Fibroblasts and β -PDGFR-transfected PC-12 Cells—We investigated whether Ca^{2+} responses with different kinetics were also induced by PDGF-BB in other cell types such as NIH 3T3 fibroblasts and PC-12 cells transfected with the β isoform of PDGFR (29). 3T3 fibroblasts responded with a single Ca^{2+} rise frequently followed by a plateau (Fig. 4B), as previously reported by others (37). Comparable responses were observed in PC-12 cells, although the magnitude

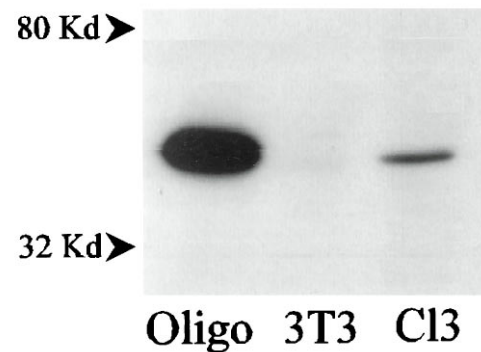
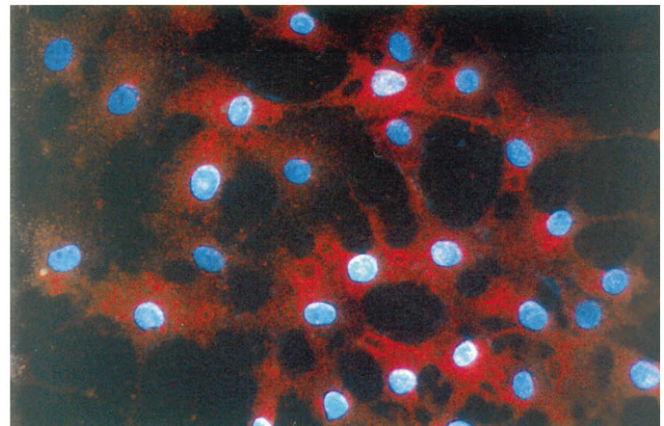


FIG. 2. Immunofluorescence and Western blot analysis of CNP protein expression. The upper panel shows a representative microscopic field in which CEINGE cl3 cells stained positively using an antibody directed against the CNP protein. The lower panel shows an immunoblot of the total cell lysate (40 μ g per lane) of CG4 cells differentiated to oligodendrocytes, NIH 3T3 fibroblasts, and CEINGE clone 3 cells probed with the same antibody.

of the $[Ca^{2+}]_i$ increase was smaller than in 3T3 fibroblasts (Fig. 4A). However, the oscillatory pattern observed in CEINGE cl3 and CG4 cells was never detected in NIH 3T3 or PC-12 cells.

Expression of Both α and β Receptors for PDGF in CEINGE Clone 3 Cells, Western Blot Analysis—We investigated the expression of α and β receptors for PDGF in CG4 and CEINGE cl3 oligodendroglial cells. Positive control NIH 3T3 fibroblasts that express both PDGFR isoforms (38) and negative control parental PC-12 cells which have no detectable expression of PDGFRs (39) were also included in the immunoblots. Fig. 5 shows that the α isoform of PDGFR was detected in both CG4/O-2A progenitors and CG4/oligodendrocytes as well as in CEINGE cl3 cells. The latter cells showed an amount of protein that was comparable to that found in 3T3 fibroblasts, whereas CG4 cells expressed an even higher level of the protein regardless of their state of differentiation. In all cell types two separate proteins with an apparent molecular weight of about 180,000 and 160,000 were detected. It is likely that a mature glycosylated form and a partially modified form of the receptor are expressed as described previously in primary oligodendroglia (16). However, neither protein was detected in PC-12 cells. Although the β isoform of PDGFR was not detected in CG4 cells, in agreement with previous studies in primary oligodendroglia (16), CEINGE cl3 cells clearly expressed the β isoform of the PDGFR. The protein which has an apparent molecular weight of 190,000 was also present in 3T3 fibroblasts, whereas it was undetectable in PC-12 cells (Fig. 5).

Effect of Neomycin on PDGF-BB-induced Ca^{2+} Response in

CG4 and CEINGE cl3 Cells—A pharmacological approach was adopted for studying the consequences of the difference in PDGFR expression in CG4 and CEINGE cl3 cells. The aminoglycoside neomycin is able to inhibit the binding of PDGF-BB to the α receptor, whereas binding to the β receptor is not affected by this drug (30). When CG4/oligodendrocytes were preincubated with 5 mM neomycin for 15 min before starting perfusion with PDGF-BB (10 ng/ml), the Ca^{2+} response was totally abolished in all the 46 cells analyzed (Fig. 6A). However, subsequent exposure of the same cells to 100 μ M ATP elicited a $[Ca^{2+}]_i$ increase, usually a rapid peak followed by a plateau, in more than 90% of cells studied. This demonstrates that neomycin neither inhibited PLC β nor exerted any toxic effect. However, preincubation of CEINGE cl3 cells with neomycin did not inhibit the Ca^{2+} signaling induced by PDGF-BB stimulation (Fig. 6B), and the usual pattern of both oscillatory and non-oscillatory Ca^{2+} response was observed.

Exposure of CEINGE cl3 Cells to PDGF-AA—When CEINGE cl3 cells were perfused for 5 min with 20 ng/ml PDGF-AA, which is able to bind only the α isoform of PDGFR, 149 out of 247 cells studied (60%) showed a $[Ca^{2+}]_i$ increase (data not shown). In 69% of the responsive cells, the kinetics were oscillatory and comparable with those induced by PDGF-BB exposure but preceded by a significantly shorter delay (146 ± 7 versus 278 ± 9 s). Similarly, a shorter delay was also detected in the remaining 31% of the cells responding in a non-oscillatory fashion, when compared with the non-oscillatory response induced by PDGF-BB in CEINGE cl3 cells (93 ± 7 versus 200 ± 7 s).

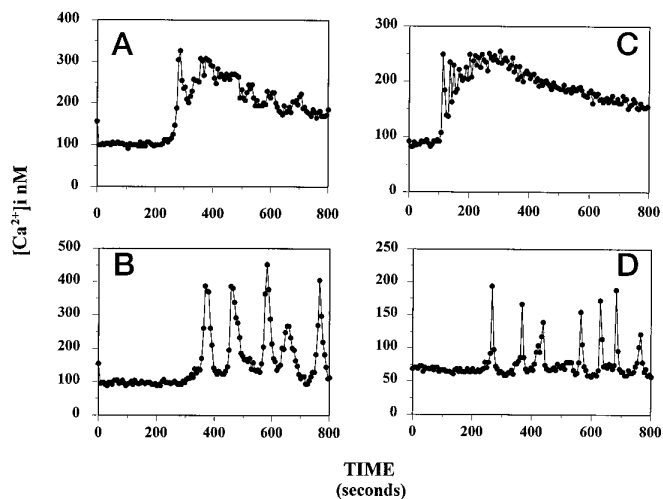


FIG. 3. Effect of PDGF-BB on $[Ca^{2+}]_i$ in CEINGE cl3 cells and CG4/oligodendrocytes. Single cell fura-2 analysis of cells exposed to 10 ng/ml PDGF-BB is shown. Perfusion with the agonist started at 30 s and was maintained for 5 min. An acquisition time of one data point every 5 s was used. Ca^{2+} responses with non-oscillatory and oscillatory kinetics were observed both in CEINGE cl3 cells (A and B) and in CG4/oligodendrocytes (C and D). Each trace shown is from a single cell and represents a typical response for a given microscopic field of cells. Data are from 168 CEINGE cl3 cells analyzed in 11 separate experiments and from 56 CG4/oligodendrocytes analyzed in 8 separate experiments.

Sequential Exposure of CEINGE cl3 Cells to PDGF-AA and BB—CEINGE cl3 cells were perfused with either 20 ng/ml PDGF-AA or 10 ng/ml PDGF-BB for 5 min during a 15-min experiment. After a 20-min interval, they were restimulated with the same or the other PDGF isoform. A homologous second stimulation with PDGF-AA was unable to induce a Ca^{2+} response in all the analyzed cells which were responsive to the first pulse (Fig. 7). These results are similar to those we previously observed after a homologous second stimulation with PDGF-BB (27). PDGF-AA also failed to induce a Ca^{2+} response when perfused after PDGF-BB (Fig. 7).

In contrast, 61% of the cells previously exposed to PDGF-AA were able to respond with a $[Ca^{2+}]_i$ increase to a subsequent stimulation with PDGF-BB.

Effect of DL-threo-Dihydrosphingosine on PDGF-BB-induced Ca^{2+} Signaling—Incubation for 15 min with the inhibitor of the sphingosine kinase, DL-threo-dihydrosphingosine (10 μ M) (40), did not increase the percentage of oscillatory Ca^{2+} responses induced by 10 ng/ml PDGF-BB in CG4/O-2A progenitors and CG4/oligodendrocytes, in contrast to results previously observed in CEINGE cl3 (27). In CEINGE cl3 cells incubated with DL-threo-dihydrosphingosine, the percentage of oscillatory responses evoked by PDGF-BB was 87 ± 4 (70% of responsive cells in 62 cells analyzed), and in CG4/O-2A progenitors and CG4/oligodendrocytes oscillatory kinetics were observed in 49 ± 9 and $33 \pm 8\%$ of cells, respectively (Fig. 8; see also Table I). The percentage of responsive CG4/O-2A progenitors was 51% in 39 cells analyzed with a delay of 268 ± 35 s, while the percentage of responsive CG4/oligodendrocytes was 23% in 31 cells analyzed with a delay of 255 ± 48 s. DL-threo-Dihydrosphingosine was also ineffective in inducing an oscillatory Ca^{2+} response to PDGF-BB in NIH 3T3 and β -PDGFR-transfected PC-12 cells. Despite a 15-min incubation with the sphingosine kinase inhibitor, all the cells analyzed responded uniformly in a non-oscillatory fashion to PDGF-BB exposure (data not shown).

Effect of Sphingosine and Sphingosine 1-Phosphate on $[Ca^{2+}]_i$ in CG4/Oligodendrocytes, NIH 3T3 Fibroblasts, and β -PDGFR-transfected PC-12 Cells—Exogenous sphingosine was perfused onto the cells after a 15-min incubation with 10 μ M DL-threo-dihydrosphingosine to block its conversion to SPP. A 5-min perfusion with 10 μ M sphingosine elicited an oscillatory Ca^{2+} response in the majority of the CG4/oligodendrocytes studied (Table II). NIH 3T3 fibroblasts and β -PDGFR-transfected PC-12 cells responded with a single $[Ca^{2+}]_i$ increase, never showing any oscillatory Ca^{2+} signaling (data not shown).

Exposure of cells for 5 min to 1 μ M SPP produced a non-oscillatory Ca^{2+} response in 82% of the CG4/oligodendrocytes (Table II) and in all the responsive NIH 3T3 fibroblasts and β -PDGFR-transfected PC-12 cells analyzed (data not shown).

DISCUSSION

In these experiments we studied the Ca^{2+} responses elicited by stimulation of PDGFRs in the CG4 cell line and compared them with analogous effects induced in CEINGE cl3 cells and in cells of non-oligodendroglial origin such as NIH 3T3 fibroblasts and β -PDGFR-transfected PC-12 cells. Our results

TABLE I
 Ca^{2+} responses elicited by PDGF-BB

Cell type	Responsive cells	Oscillatory cells and delay (s)	Non-oscillatory cells and delay (s)
CG4/O-2A ($n = 87$)	22 (25%)	64% 198 ± 11	36% 129 ± 22
CG4/oligodendrocyte ($n = 227$)	56 (25%)	52% 266 ± 15	48% 183 ± 12
CEINGE clone 3 ($n = 269$)	188 (70%)	46% 278 ± 9	54% 200 ± 7

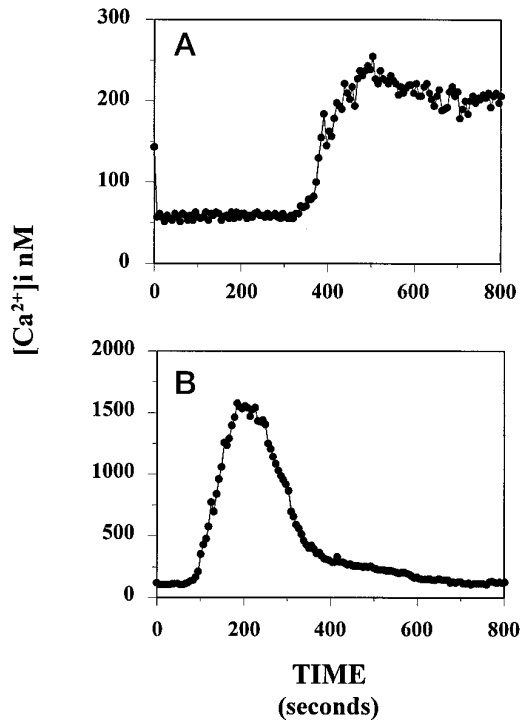


FIG. 4. Effect of exposure to PDGF-BB of PC-12 cells transfected with the β -PDGFR and NIH 3T3 fibroblasts. After perfusion with the agonist both PC-12 cells transfected with the β -PDGFR (A) and NIH 3T3 fibroblasts (B) showed a uniform non-oscillatory Ca²⁺ response. PDGF-BB was added at 30 s and removed after 5 min. Traces are from typical single cells. Data are from 42 PC-12 cells transfected with the β -PDGFR analyzed in three separate experiments and from 38 NIH 3T3 fibroblasts analyzed in three separate experiments.

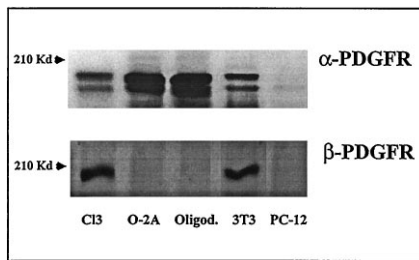


FIG. 5. Western blot analysis of PDGFR expression. Immunoblot analysis of total cell lysate (40 μ g per lane) from CEINGE cl3 cells (Cl3), CG4/O-2A progenitors (O-2A), CG4/oligodendrocytes (Oligod.), NIH 3T3 fibroblasts (3T3), and parental PC-12 cells (PC-12) probed with anti-PDGFR α (upper panel) and anti-PDGFR β (lower panel). Bands are all from the same immunoblot. Data are representative of other two experiments showing similar results.

clearly demonstrate the oligodendroglial origin of CEINGE cl3 cells and also suggest that the oscillatory Ca²⁺ response induced by PDGF is a peculiarity of oligodendroglia. The results also suggest that α -PDGFR may be less effective than β -PDGFR in stimulating the enzyme sphingosine kinase.

First we characterized the CEINGE clone 3 cells. Western blot analysis for the mT antigen clearly showed expression of the protein in the CEINGE cl3 cell line. Uniform detection of the protein in all cells was evident using immunofluorescence (Fig. 1). Thus the differences in PDGF-BB-induced Ca²⁺ responses previously described (27) are not a consequence of unequal protein levels in the cell population. It has been previously shown that CEINGE cl3 cells stain positively for GalC antigen, a specific immunological marker for oligodendrocytes (34). As we now also show the CEINGE cl3 cell line also expresses the specific oligodendroglial marker CNP. Two isoforms of this enzyme have been identified, with molecular

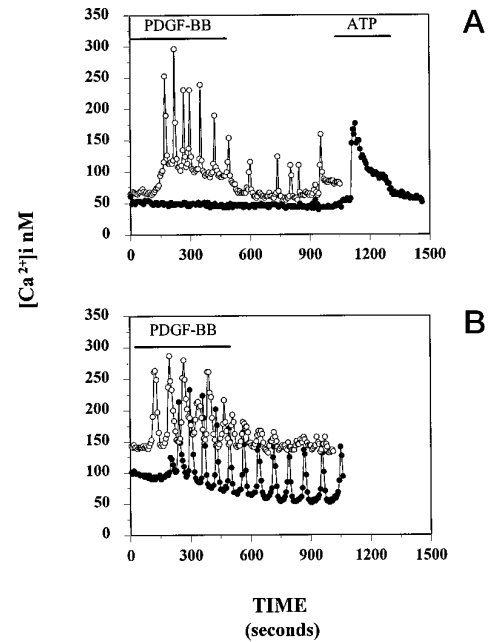


FIG. 6. Effect of neomycin on PDGF-BB induced Ca²⁺ signaling. In CG4/oligodendrocytes (A) the Ca²⁺ response induced by 10 ng/ml PDGF-BB (open circles) was totally abolished by 5 mM neomycin which was preincubated with the cells for 15 min before starting the experiment and also perfused during the agonist exposure (filled circles). When the same cells were successively stimulated with 100 μ M ATP a [Ca²⁺]_i increase was observed. In CEINGE cl3 cells (B), the Ca²⁺ response induced by PDGF-BB (open circles) was unaffected by neomycin (filled circles). Traces are from typical single cells representative of 46 CG4/oligodendrocytes and 53 CEINGE cl3 cells analyzed in three separate experiments for each cell type.

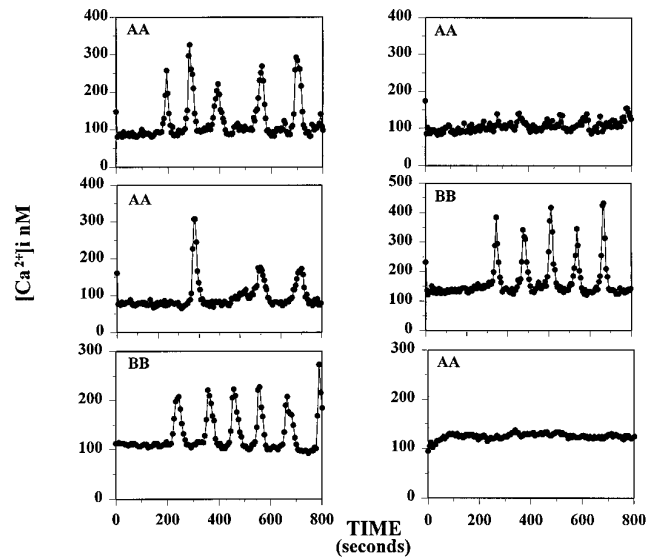


FIG. 7. Effects on [Ca²⁺]_i of sequential exposure of CEINGE cl3 cells to PDGF-AA and -BB. When 20 ng/ml PDGF-AA was perfused for a second time after a 20-min interval to the same CEINGE cl3 cells, it was unable to induce a second Ca²⁺ response (upper panel). When CEINGE cl3 cells were exposed for 5 min to PDGF-AA and, subsequently, after a 20-min interval to 10 ng/ml PDGF-BB, a Ca²⁺ response was observed (middle panel). When PDGF-AA was perfused to CEINGE cl3 cells previously exposed to PDGF-BB, it was unable to induce a Ca²⁺ response (lower panel). Data are from at least three separate experiments for each PDGF-AA and -BB combination.

weights of 46,000 and 48,000, both being derived from a single gene (41). In O-2A precursors only the mRNA encoding the larger isoform has been found, whereas in differentiated oligodendrocytes both CNP mRNAs have been described (42). West-

ern blot analysis showed that a single protein band was identified in CEINGE cl3 cells, showing a molecular weight of approximately 45,000–50,000 and corresponding to a similar band identified in CG4/oligodendrocytes (Fig. 2).

CEINGE cl3 cells responded to PDGF-BB exposure with Ca^{2+} signals characterized by oscillatory or non-oscillatory kinetics. We have shown that this may be due to different intracellular levels of the two sphingolipids, sphingosine and SPP (27). It was interesting to ascertain whether this phenomenon was a general feature of cells of the oligodendroglial lineage or was just a peculiarity of CEINGE cl3 cells, related for example to mT expression. This possibility was suggested by the fact that mT antigen is able to bind intracellular substrates like phosphatidylinositol 3-kinase and PLC- γ 1 (43, 44) and that its expression in NIH 3T3 fibroblasts confers an enhanced responsiveness to growth factors (45). However, the glial cell line CG4 also responded to PDGF-BB exposure with dual oscillatory and non-oscillatory kinetics, as observed in CEINGE cl3 cells (Fig. 3). In contrast an oscillatory Ca^{2+} response was never observed when NIH 3T3 fibroblasts, as well as PC-12 cells transfected with β -PDGFR, were exposed to PDGF-BB or to exogenous sphingosine. These data indicate that the dual Ca^{2+} signaling induced by PDGFR stimulation and the modulatory action of sphingosine and SPP are indeed properties of cells of the oligodendroglial lineage. The most likely explanation for this is that in oligodendroglial cells, Ca^{2+} mobilization elicited by PDGFR stimulation is much more sensitive to intracellular

levels of sphingosine and SPP than in other cell types. On the other hand, in the light of our results, the possibility of a modulatory action operated by sphingolipids on other pathways, such as PLC- γ -inositol trisphosphate, cannot be ruled out.

It has been reported that cells of the oligodendroglial lineage only express the α isoform of PDGFR (17, 46) and that oligodendrocytes show a lower level of mRNA for this receptor and protein expression compared with O-2A progenitors (16). In agreement with these observations, we found that CG4 cells grown both as O-2A progenitors and oligodendrocytes expressed α -PDGFR. However, no decrease in α -PDGFR protein level was observed in CG4/oligodendrocytes, as also suggested by the similar percentages of CG4/O-2A progenitors and CG4/oligodendrocytes that responded with a $[Ca^{2+}]_i$ increase to PDGF-BB (Table I). This could be explained by a peculiarity of this cell line in which α -PDGFR expression has never been previously investigated or by the fact that after the 3rd day of differentiation to oligodendrocytes 1% of fetal calf serum was added to the culture medium to increase the cell survival (28, 47). The presence of basic fibroblast growth factor in the serum may have contributed to an up-regulation of α -PDGFR expression, as previously reported in primary oligodendroglia (16). Interestingly, CEINGE cl3 cells also expressed the β isoform of PDGFR. This finding was confirmed by experiments performed using neomycin as an inhibitor of PDGF-BB binding to the α -PDGFR (30). Thus, neomycin completely blocked PDGF-BB-induced Ca^{2+} signaling in CG4 cells, whereas it was ineffective in CEINGE cl3 cells (Fig. 6), suggesting that the Ca^{2+} response observed in these latter conditions was due solely to β -PDGFR activation. In light of these results, we exposed CEINGE cl3 cells to PDGF-AA, in order to selectively stimulate the α -PDGFR and verify whether this could evoke a Ca^{2+} response different from that observed when both α - and β -PDGFRs were stimulated by PDGF-BB. In these experiments we also observed both oscillatory and non-oscillatory Ca^{2+} responses, although the percentage of oscillatory cells was higher and the delays preceding both Ca^{2+} responses were shorter than those observed using PDGF-BB. Interestingly, a second homologous PDGF stimulation of the same CEINGE cl3 cells was ineffective in increasing the $[Ca^{2+}]_i$, regardless of the PDGF isoform used. This suggests desensitization of the transductional mechanisms linking PDGFRs with the Ca^{2+} signaling machinery. On the other hand, PDGF-BB exposure following a previous PDGF-AA pulse induced a Ca^{2+} response in the majority of the cells (Fig. 7). These data can be explained by invoking a selective desensitization of α -PDGFR by PDGF-AA and further supports the presence of β -PDGFRs in CEINGE cl3 cells.

When PDGF-BB exposure of CG4 cells was preceded by incubation with DL-threo-dihydrosphingosine, no increase in the percentage of oscillatory cells was observed in contrast to CEINGE cl3 cells (Fig. 8). Nevertheless, when exogenous sphingosine was perfused alone an oscillatory Ca^{2+} response

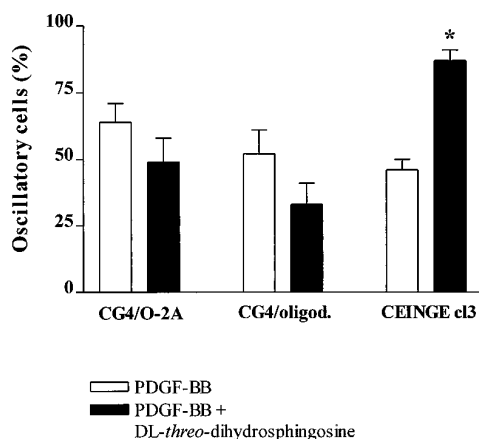


FIG. 8. Effect of sphingosine kinase blockade on Ca^{2+} responses induced by PDGF-BB in CG4 and CEINGE cl3 cells. Cells were incubated with 10 μ M DL-threo-dihydrosphingosine for 15 min before exposure to 10 ng/ml PDGF-BB. The percentage of CEINGE cl3 cells showing an oscillatory Ca^{2+} response in the presence of sphingosine kinase blockade was higher than in untreated cells ($p < 0.001$), whereas both CG4/O-2A progenitors and CG4/oligodendrocytes showed no significant changes in the percentage of oscillatory Ca^{2+} responses when incubated with the sphingosine kinase inhibitor before PDGF-BB exposure.

TABLE II
 Ca^{2+} responses induced by exogenous sphingosine and SPP

Cell type	Responsive cells	Oscillatory cells and delay (s)	Non-oscillatory cells and delay (s)
Ca^{2+} responses induced by exogenous sphingosine			
CG4/oligodendrocyte ($n = 201$)	92 (46%)	77% 188 \pm 15	23% 118 \pm 21
CEINGE clone 3 ^a ($n = 88$)	53 (60%)	100% 129 \pm 9	
Ca^{2+} responses elicited by exogenous SPP			
CG4/oligodendrocyte ($n = 101$)	44 (43%)	18% 56 \pm 8	82% 43 \pm 4
CEINGE clone 3 ^a ($n = 65$)	38 (58%)		100% 30 \pm 6

^a Data from Fatatis and Miller, J. Biol. Chem. 271, 295–301, 1996.

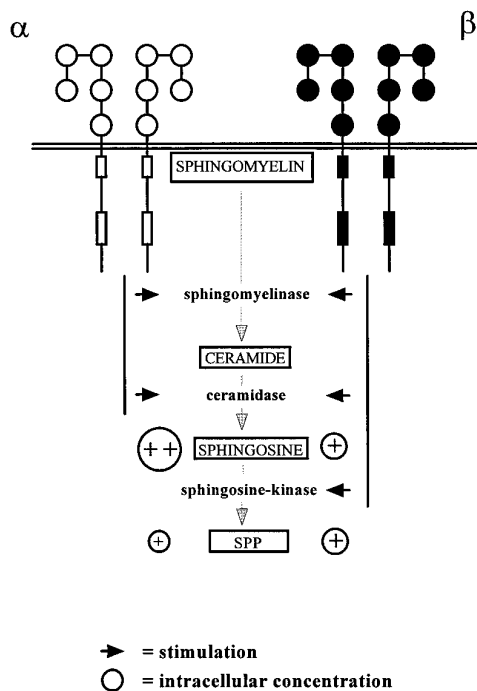


FIG. 9. A possible model for different modulation of the sphingolipid metabolic pathway by α - and β -PDGFRs. Both PDGFR isoforms may stimulate sphingomyelinase and ceramidase, thus inducing an increase in ceramide and sphingosine. However, lack of stimulation of sphingosine kinase, following α -PDGFR activation, may lead to a higher intracellular level of sphingosine compared with that obtained as a consequence of β -PDGFR stimulation. This phenomenon could explain the higher percentage of oscillatory Ca²⁺ responses induced by PDGF-BB in CG4 cells, which express only the α -PDGFR, and in CEINGE cl3 cells when the α -PDGFR is the only isoform stimulated by PDGF-AA.

was detected in most of the cells, whereas exposure to SPP mostly produced non-oscillatory responses (Table II). These data suggest that although sphingosine and SPP are able to elicit two distinct types of Ca²⁺ response, as observed in CEINGE cl3 cells (27), block of sphingosine kinase did not modulate the Ca²⁺ signaling following the stimulation of α -PDGFR. This result is difficult to explain in the light of information available on the mechanism of coupling of PDGFRs to the sphingolipid metabolic pathway. Although a clear correlation between PDGFR stimulation and increases in the intracellular levels of ceramide, sphingosine, and SPP have been reported (48–50), the biochemical mechanisms underlying these phenomena are still unclear. Furthermore, although stimulation of sphingosine kinase activity has been observed during β -PDGFR stimulation (51), no data are available regarding the α isoform of this receptor. A possible explanation of our observations could involve lack of stimulation of sphingosine kinase activity by α -PDGFR. This could result in higher intracellular levels of sphingosine and account for a higher percentage of oscillatory cells (see Fig. 9). It is interesting to note that after exposure to PDGF-BB of both CG4/O-2A progenitors and CG4/oligodendrocytes, which express only the α -PDGFR, the percentage of oscillatory cells was higher than that observed when CEINGE cl3 cells were stimulated with the same PDGF isoform. This could be because the β -PDGFR in CEINGE cl3 stimulates sphingosine kinase to produce more SPP, thus accounting for the higher number of non-oscillatory responses. This hypothesis is supported by the observation that when CEINGE cl3 cells were stimulated with PDGF-AA instead of PDGF-BB, a higher percentage of oscillatory cells were detected. However, it seems reasonable to hypothesize that

despite the absence of stimulation of sphingosine kinase activity, some SPP is still produced, accounting for the non-oscillatory Ca²⁺ responses observed during α -PDGFR activation.

The possibility that different PDGF-induced Ca²⁺ responses in oligodendroglia are coupled to distinct cellular phenomena such as mitogenesis, chemotaxis, and differentiation seems very attractive. Future studies will be essential in order to correlate the kinetics of Ca²⁺ signaling induced by PDGFRs to particular cytoplasmic and nuclear events.

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