

Sphingosine and Sphingosine 1-Phosphate Differentially Modulate Platelet-derived Growth Factor-BB-induced Ca^{2+} Signaling in Transformed Oligodendrocytes*

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The roles of sphingosine and sphingosine 1-phosphate in Ca^{2+} signaling following platelet-derived growth factor (PDGF) receptor stimulation were investigated in the oligodendrocyte cell line CEINGE c13, using single-cell fura-2 microfluorimetry and videoimaging. Two different Ca^{2+} responses were observed, which differed in their delays and kinetics. The first response, which occurred after a shorter delay, exhibited a single Ca^{2+} peak often followed by a plateau, while the second type of response was characterized by a longer delay and by Ca^{2+} spikes with different frequencies and amplitudes. The latter phenomenon was never observed after stimulation of G protein-coupled receptors for ATP, ET-1, and BK. The incubation with the inhibitor of sphingosine kinase, DL-threo-dihydrosphingosine, significantly increased the percentage of cells responding to PDGF-BB exposure with Ca^{2+} spikes (87 versus 47%), while it did not modify the Ca^{2+} response elicited by exposure to ATP, ET-1, or BK. Exposure to exogenous 10 μM sphingosine or 1 μM sphingosine 1-phosphate produced oscillatory and non-oscillatory Ca^{2+} responses, respectively, similar to those elicited by PDGF-BB. A second application of PDGF-BB, 30 min after the first, was normally ineffective in producing a Ca^{2+} response. However, if the second exposure was preceded by the inhibition of sphingosine 1-phosphate formation, an oscillatory Ca^{2+} response occurred in all cells. We conclude that intracellular levels of sphingosine and sphingosine 1-phosphate may differentially modulate Ca^{2+} signaling triggered by PDGF receptor stimulation in CEINGE c13-transformed oligodendrocytes.

Polypeptide growth factors regulate cellular functions by binding to transmembrane receptors, which possess tyrosine kinase activity (RTKs) (1, 2). PDGF¹ is able to promote growth in fibroblasts (3), smooth muscle cells (4), and glial cells (5). One of the first cellular events that occurs following the stimulation of PDGF receptors is an increase in $[\text{Ca}^{2+}]_i$ (6, 7). This increase has been shown to be correlated with PDGF-induced cell proliferation (8–10). The $[\text{Ca}^{2+}]_i$ increase caused by PDGF receptor stimulation is mainly the result of Ca^{2+} mobilization

from intracellular stores, a mechanism recognized as being a consequence of an IP_3 formation, at least in smooth muscle cells (10, 11). In the case of PDGF receptors, this involves the γ_1 isoform of phospholipase C (1, 12), which is directly phosphorylated on both serine and tyrosine residues by the activated receptor (13). Overexpression of this enzyme potentiates IP_3 production following PDGF exposure in fibroblasts (14).

It has recently become clear, however, that a second and possibly equally important biochemical pathway may be involved in PDGF-evoked Ca^{2+} signaling. It was initially shown that the sphingomyelin metabolite sphingosine was able to mobilize intracellular Ca^{2+} when exogenously added to smooth muscle cells. It has also been hypothesized that a further modification of the molecule is necessary for its Ca^{2+} mobilizing action (15). A subsequent study demonstrated that SPP produced from sphingosine is a potent mitogen for 3T3 fibroblasts. Like sphingosine, SPP mobilizes Ca^{2+} by an intracellular mechanism (16). Several further studies have confirmed that sphingosine and SPP are able to mobilize Ca^{2+} from intracellular stores in different cell types (17, 18). The recent observation that intracellular levels of sphingosine and SPP increase after PDGF and fetal calf serum stimulation of Swiss 3T3 fibroblasts is consistent with these sphingolipids mediating the mitogenic action of PDGF (19). Furthermore, sphingosine kinase, the enzyme responsible for converting sphingosine to SPP, shows a transient increase in activity after PDGF stimulation. Blocking this enzyme with the competitive inhibitor DL-threo-dihydrosphingosine markedly reduces the DNA synthesis induced by PDGF (19). This suggests that SPP is the main sphingolipid in the signal transduction pathway, which follows PDGF receptor stimulation in these cells. It is possible that both sphingolipids might mobilize Ca^{2+} through a modulatory action on the IP_3 pathway (18), or they could act in a completely independent fashion, as recently suggested for SPP in fibroblasts (20). However, the exact mechanism by which these sphingolipids act still needs to be elucidated. It is also important to understand whether each lipid has an active role in intracellular signaling or whether SPP is the effective mediator and sphingosine is only an intermediate metabolite.

Although several studies have examined the intracellular Ca^{2+} signaling triggered by PDGF in vascular smooth muscle cells (10) and 3T3 fibroblasts (6), data regarding this transductional mechanism in oligodendroglial cells are not available. The development and proliferation of oligodendrocytes are critically regulated by PDGF (21–23), which, besides its mitogenic and chemotactic activity, stimulates c-Jun and c-Fos expression in oligodendrocyte progenitor cells (24).

In this study, single-cell microfluorimetry and videoimaging were used to investigate Ca^{2+} signaling as a result of PDGF receptor stimulation in the cell line CEINGE c13. These cells, immortalized with polyoma middle T antigen, have been identified as oligodendrocytes by using antibodies directed against

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¹ The abbreviations used are: PDGF, platelet-derived growth factor; SPP, sphingosine 1-phosphate; IP_3 , inositol 1,4,5-trisphosphate; ET-1, endothelin-1; BK, bradykinin.

galactocerebroside C (25), a specific marker for oligodendroglia.

EXPERIMENTAL PROCEDURES

Materials—PDGF-BB and ryanodine were from Calbiochem; ATP, BK, ET-1, caffeine, sphingosine, and DL-threo-dihydrosphingosine were from Sigma. SPP was from Biomol. Fura-2/acetoxymethyl ester (fura-2/AM) was from Molecular Probes, Inc.

Cell Culture—Immunofluorescence studies performed on CEINGE c13 cells using antibodies directed against galactocerebroside C, myelin basic protein, and 2'-3'-cyclic nucleotide 3'-phosphohydrolase showed a positive staining for all three specific oligodendroglia markers (25).²

These cells were cultured as a monolayer in polystyrene dishes and maintained in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) containing 10% heat-inactivated fetal calf serum (Life Technologies, Inc.) and 50 μ g/ml gentamycin. Cells were grown in a humidified incubator at 36.5 °C in a 5% CO₂ atmosphere and were fed twice weekly. All experiments were performed with cells from passages 60–80.

Intracellular Ca^{2+} Measurements and Videoimaging—Cells were grown on 25-mm clean no. 1 glass coverslips, previously coated with poly-L-lysine (50 μ g/ml), and transferred in a 35-mm Petri plastic dish. Cells were loaded with 2 μ M fura-2/AM (Calbiochem) using a balanced salt solution (standard buffer) of the following composition (mM): 157 NaCl, 5 KCl, 0.4 MgSO₄, 0.5 MgCl₂, 0.6 KH₂PO₄, 3 NaHCO₃, 20 HEPES, 10 glucose, 2 CaCl₂, and 0.2% bovine serum albumin (osmolality 330–340 mOsm/kg), pH adjusted to 7.4 with 1 M Tris. Cells were incubated with fura-2/AM for 45 min at room temperature to avoid probe compartmentalization and then incubated for further 15 min with standard buffer to allow the complete deesterification of the fluorescent probe. The cytoplasmic fluorescence appeared uniform throughout the cells.

The single-cell videoimaging was performed as described previously (26) with some modifications. Coverslips were mounted on a coverslip chamber (Medical Systems Co., Greenvale, NY), and the fluorescence measurements were performed at 23–26 °C. Cells were continuously superfused using a peristaltic pump (Gilson) with a flow rate of 650 μ l/min, and the perfusion medium was directed on cells under observation by a microtube positioned by a micromanipulator (Narishige, Japan). The removal of experimental solutions from the coverslip chamber (500 μ l, volume) was achieved using a microaspirator (Medical System Co., Greenvale, NY) connected to a vacuum pump, and the entire volume of the chamber turned over in less than 60 s. A two-way valve (Thomson, Springfield VA) controlled the flow from a separate injection loop, which was used to perfuse cells with different experimental solutions.

Ca^{2+} -free experiments were performed using a nominally Ca^{2+} -free standard buffer containing 0.2 mM EGTA. Before starting the experiment, cells were perfused with this buffer for 2–3 min to completely remove Ca^{2+} from the extracellular environment. Fura-2 fluorescence was imaged with an inverted Nikon Diaphot microscope using a Nikon 20 \times /1.3 NA Fluor DL objective lens. The cells were illuminated with a 100-watt Xenon lamp (Oriental Corp., Stratford, CT) with quartz collector lenses. A shutter and a filter wheel containing the two different interference filters (340 and 380 nm) were controlled by a computer. Emitted light was passed through a 400-nm dichroic mirror, filtered at 480 nm, and collected by a video camera (MTI, Michigan City, IN) connected with a KS 1381 light intensifier (Videoscope Intl.). Images of as many as 10–30 cells/field were averaged using an Avio Image S (Nippon Avionic) real time image processor set at 16 frames for each data point and digitized in an image analyzer (Applied-Imaging Ltd., Dukesway Gateshead, United Kingdom) connected to a computer equipped with Tardis software. Each cell in the image was independently analyzed for each time point in the captured sequence. All individual $[Ca^{2+}]_i$ traces shown are from a single cell and are representative responses for a given field of cells. It should be mentioned that, as previously reported, some CEINGE c13 cells show spontaneous changes in the $[Ca^{2+}]_i$, represented by Ca^{2+} spikes with different amplitudes and frequencies (27). Although this phenomenon has not been frequently observed, a previous data acquisition of 5 min was performed before each experiment, and those cells showing such spontaneous Ca^{2+} spiking activity were not considered for the analysis.

For the calibration of fluorescent signals we used cells loaded with fura-2; R_{max} and R_{min} are, respectively, the ratios at saturating and zero $[Ca^{2+}]_i$, and were obtained perfusing cells with a salt solution containing 10 mM CaCl₂ and 5 μ M ionomycin for 3 min and subsequently with a

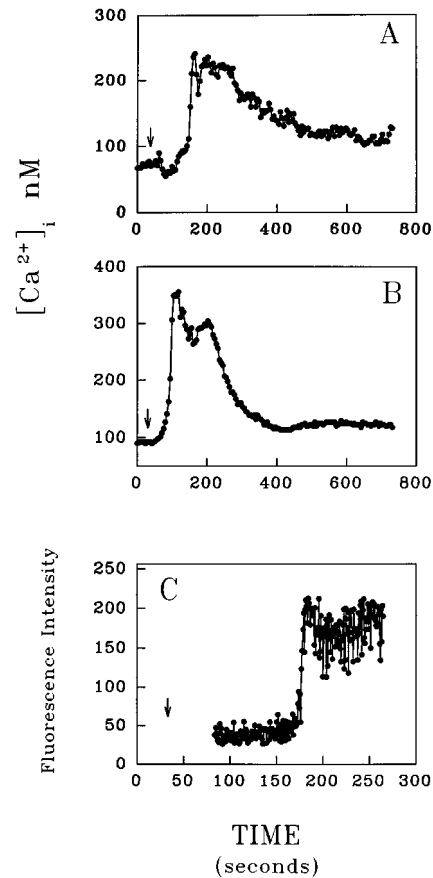


FIG. 1. Non-oscillatory Ca^{2+} responses induced by PDGF-BB. Single-cell analysis is shown of fura-2 fluorescence in cells exposed for 5 min to 10 ng/ml PDGF-BB (added at the arrow). An acquisition time of one data point every 5 s was used. *A*, a single increase in $[Ca^{2+}]_i$ was followed by a small plateau, which outlasted PDGF-BB removal; *B*, a single increase in $[Ca^{2+}]_i$ was followed by a return to basal levels while PDGF-BB was still present; *C*, a $[Ca^{2+}]_i$ trace obtained with an acquisition time of one data point every 800 ms during a non-oscillatory response.

Ca^{2+} free salt solution containing 10 mM EGTA for 20 min. The values of the obtained R_{max} and R_{min} , expressed as gray level mean, were used to calculate the calibration curve using the Tardis software. Intracellular calcium concentration was determined as previously reported (28).

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

RESULTS

PDGF-BB Exposure Causes Two Different Types of Ca^{2+} Responses in CEINGE c13 Cells—When CEINGE c13 cells were exposed to PDGF-BB (10 ng/ml) for 5 min, two different Ca^{2+} responses were observed. The first type of response was characterized by a progressive increase in $[Ca^{2+}]_i$, which was frequently followed by a sustained plateau. The delay between the start of PDGF-BB exposure and the $[Ca^{2+}]_i$ increase was 166 ± 4 s ($n = 196$) (Fig. 1, *A* and *B*). The second type of Ca^{2+} response was characterized by a series of Ca^{2+} spikes exhibiting variable frequency and amplitude and a delay of 251 ± 7 s ($n = 148$) (Fig. 2). Among responding cells (which represented 80% of the whole population), non-oscillatory and oscillatory responses were observed in 57 and 43% of the cells, respectively (344 responding cells analyzed).

To exclude the possibility that the non-oscillatory response consisted of a Ca^{2+} spiking response at very high frequency, an acquisition time of 800 ms was adopted. Using this faster analysis, non-oscillatory Ca^{2+} responses with a shorter delay

² A. Fatatis and R. J. Miller, manuscript in preparation.

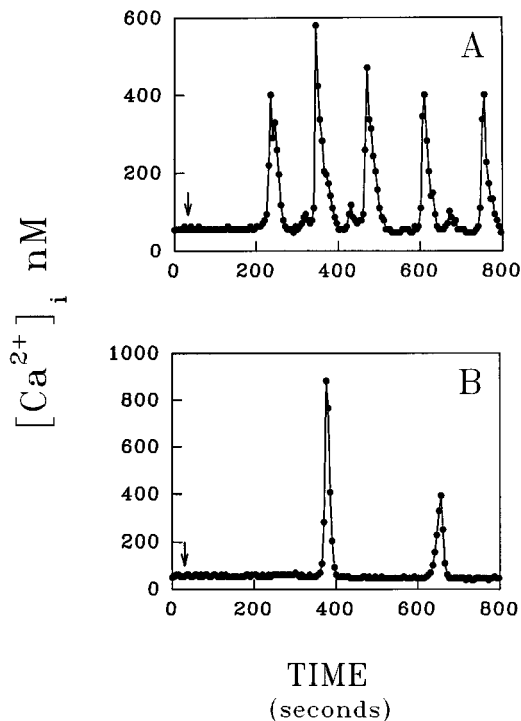


FIG. 2. **Oscillatory Ca^{2+} responses induced by PDGF-BB.** In 43% of responding cells, a 5-min exposure to 10 ng/ml PDGF-BB (added at the arrow) evoked a Ca^{2+} spiking activity with different frequencies and amplitudes. The response shown in A was typical. Less than 5% of oscillating cells showed only two spikes during the 14 min following the PDGF-BB addition (B). The delay preceding the Ca^{2+} responses was 251 ± 7 s, significantly longer than the delay of 166 ± 4 s preceding non-oscillatory responses (Fig. 1).

could still be demonstrated (Fig. 1C). Furthermore, following the initial non-oscillatory response, no further increases in $[Ca^{2+}]_i$ were observed during the remaining data acquisition period.

A very small percentage of oscillatory cells (<5%) displayed only two Ca^{2+} spikes in the 14 min following the PDGF-BB addition (Fig. 2B). However, even in this case, the average delay of the Ca^{2+} response was similar to that observed in cells responding with multiple Ca^{2+} spikes.

Perfusion of the growth factor for a shorter period of time (1 min) did not significantly modify the percentage of cells responding with non-oscillatory and oscillatory patterns and the relative time course of these responses (63%, delay = 133 ± 7 s and 37%, delay = 266 ± 22 s, respectively; $n = 55$).

Effect of Extracellular Ca^{2+} Removal on Ca^{2+} Responses—When cells were exposed to PDGF-BB in the absence of extracellular Ca^{2+} , a reduction in the magnitude of the Ca^{2+} response was observed. In non-oscillatory cells (Fig. 3A), the plateau phase following the peak disappeared. In oscillatory cells, the magnitude of the spikes was reduced (Fig. 3B). In cells showing the oscillatory response, the spiking activity was also significantly abbreviated (Fig. 3C). In experiments in which cells were studied for 21 min, the last Ca^{2+} spike was observed 700 ± 52 s after the exposure to PDGF-BB in normal medium, while after extracellular Ca^{2+} removal the last spike was observed after 462 ± 29 s (42 cells analyzed). There was no change in the delay preceding both types of response.

Absence of Ca^{2+} Oscillations upon Stimulation of G Protein-coupled Receptors or Exposure to Ryanodine and Caffeine—As previously reported, CEINGE c13 cells also possess different G protein-coupled receptors (27). Exposure of these cells to ATP (100 μ M), ET-1 (100 nM), and BK (1 μ M) for 5 min elicited a $[Ca^{2+}]_i$ increase after a very short delay (10–15 s) (Fig. 4). After

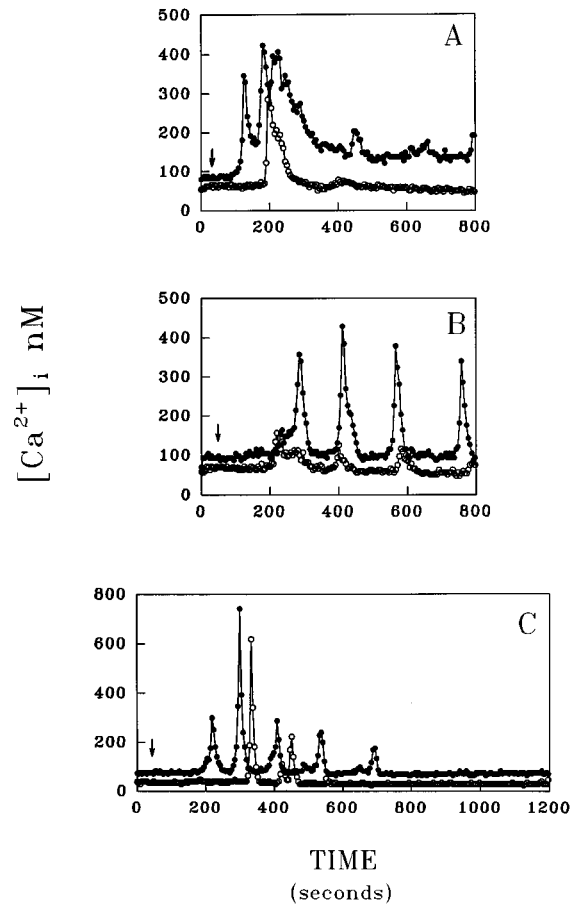


FIG. 3. **Effect of extracellular Ca^{2+} removal on PDGF-BB-induced Ca^{2+} signaling.** Prior to exposure to 10 ng/ml PDGF-BB (added at the arrow), cells were perfused for 3 min with a Ca^{2+} free solution containing 200 μ M EGTA. Each panel represents a typical response of cells exposed to PDGF-BB in absence of extracellular Ca^{2+} (empty circles) compared to an analogous response obtained in different cells when extracellular Ca^{2+} was present (filled circles). A, a non-oscillatory response represented by a monophasic increase in $[Ca^{2+}]_i$ not followed by the plateau phase when the extracellular Ca^{2+} was removed; B, an oscillatory Ca^{2+} response with spikes of reduced amplitude when cells were exposed to PDGF-BB after extracellular Ca^{2+} removal. Panel C compares the response of two typical oscillatory cells and shows that in the presence of extracellular Ca^{2+} , the spiking activity lasts longer than following extracellular Ca^{2+} removal. In these series of experiments, the images were acquired for longer time periods (21 min instead of 14 min). Data are from 110 cells analyzed in six separate experiments.

ATP exposure, return to basal level was clearly slower than after the stimulation with ET-1 or BK. However, in all three circumstances, the $[Ca^{2+}]_i$ increase was monophasic, and no repetitive Ca^{2+} spikes were observed. Other studies have indicated that ryanodine/caffeine-sensitive intracellular Ca^{2+} stores participate in the production of $[Ca^{2+}]_i$ oscillations. To ascertain whether they played a role in CEINGE c13 cells, the effect of 20 μ M ryanodine was studied. Ryanodine did not induce increases in $[Ca^{2+}]_i$ when perfused for 5 min (data not shown). Similar results were obtained when perfusing cells for 5 min with 20 mM caffeine, a drug that releases Ca^{2+} from the same stores as ryanodine (data not shown).

Inhibition of Sphingosine Kinase Increases the Percentage of Cells Showing an Oscillatory Response after Exposure to PDGF-BB—Incubation of CEINGE c13 cells for 10 min with 10 μ M DL-threo-dihydrosphingosine, an inhibitor of the sphingosine kinase, substantially increased the percentage of cells responding with oscillations when subsequently exposed to 10 ng/ml of PDGF-BB (87%) (Fig. 5). The delay of the Ca^{2+} response was 242 ± 13 s. The remaining 13% of cells still re-

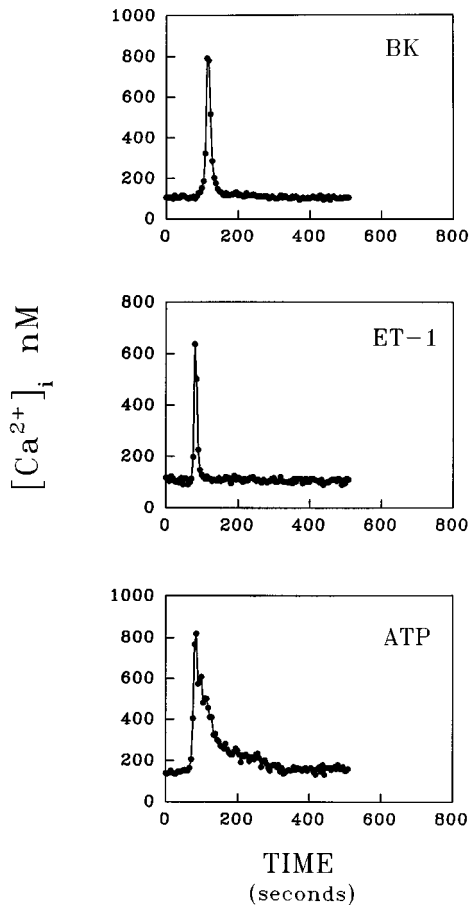


FIG. 4. Typical Ca^{2+} responses evoked by stimulation of G protein-coupled receptors for BK, ET-1, and ATP. Exposure of cells to 1 μM BK, 100 nM ET-1, and 100 μM ATP is shown. Perfusion of each agonist started at 30 s and was maintained for 5 min.

spending in the non-oscillatory mode showed a delay of 142 ± 21 s (62 responding cells analyzed).

In contrast, a 10-min incubation with 10 μM DL-*threo*-dihydrosphingosine did not modify the Ca^{2+} responses evoked by following exposure to 100 μM ATP, 100 nM ET-1, or 1 μM BK (data not shown).

Finally, when cells were exposed to 10 μM DL-*threo*-dihydrosphingosine alone, no changes in the $[Ca^{2+}]_i$ were observed (data not shown).

Effect of Exposure of Cells to Sphingosine and Sphingosine 1-Phosphate—We examined whether exogenous sphingosine and SPP were able to modify $[Ca^{2+}]_i$ in CEINCE c13 cells. Prior to perfusion with sphingosine, cells were incubated for 10 min with 10 μM DL-*threo*-dihydrosphingosine to block its conversion to SPP. As illustrated in Fig. 6, sphingosine exposure caused Ca^{2+} spiking activity in 100% of responding cells, with a delay of 129 ± 9 s (53 cells analyzed, representing 60% of the whole population). In contrast, all cells responding to SPP exposure showed a monophasic $[Ca^{2+}]_i$ increase (38 cells analyzed, representing 58% of the whole population). In the latter case, the initial Ca^{2+} peak was not followed by a further Ca^{2+} response and was characterized by a much shorter delay (30 ± 6 s; Fig. 6).

Multiple Exposures to PDGF-BB and Effect of Sphingosine Kinase Inhibition—Exposure of cells to two consecutive 5-min additions of 10 ng/ml PDGF-BB, separated by an interval of 30 min, was usually characterized by the absence of a second Ca^{2+} response (Fig. 7). In fact, in 42 cells studied using this type of paradigm, only 1 responded with a small $[Ca^{2+}]_i$ increase to a second growth factor exposure. Furthermore, all cells that were

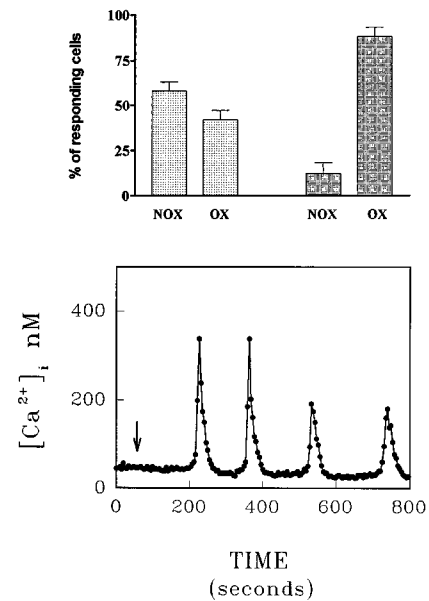


FIG. 5. Effect of sphingosine kinase inhibition on PDGF-BB-induced Ca^{2+} responses. Upper panel, percentage of non-oscillatory (NOX) and oscillatory (OX) cells in response to 10 ng/ml PDGF-BB with (■) or without (□) a previous 5-min incubation with the sphingosine kinase inhibitor DL-*threo*-dihydrosphingosine (10 μM); lower panel, a typical oscillatory Ca^{2+} response in a cell incubated with 10 μM DL-*threo*-dihydrosphingosine prior to being exposed for 5 min to 10 ng/ml PDGF-BB. The inhibitor was perfused during the entire experiment. Data are from 62 cells analyzed in five separate experiments.

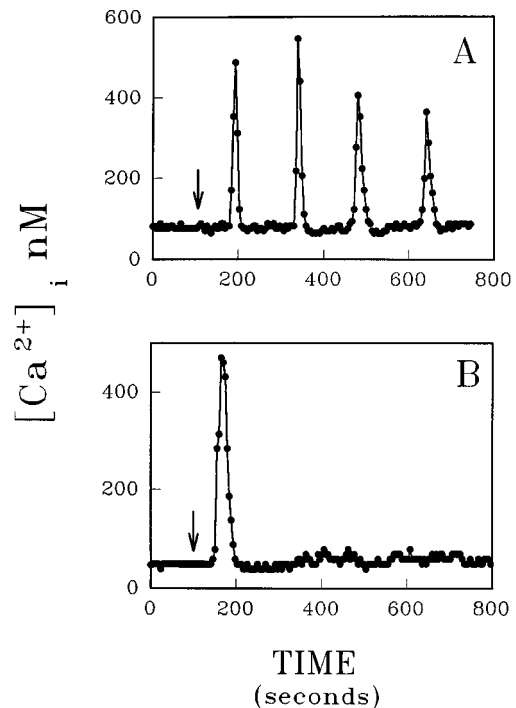
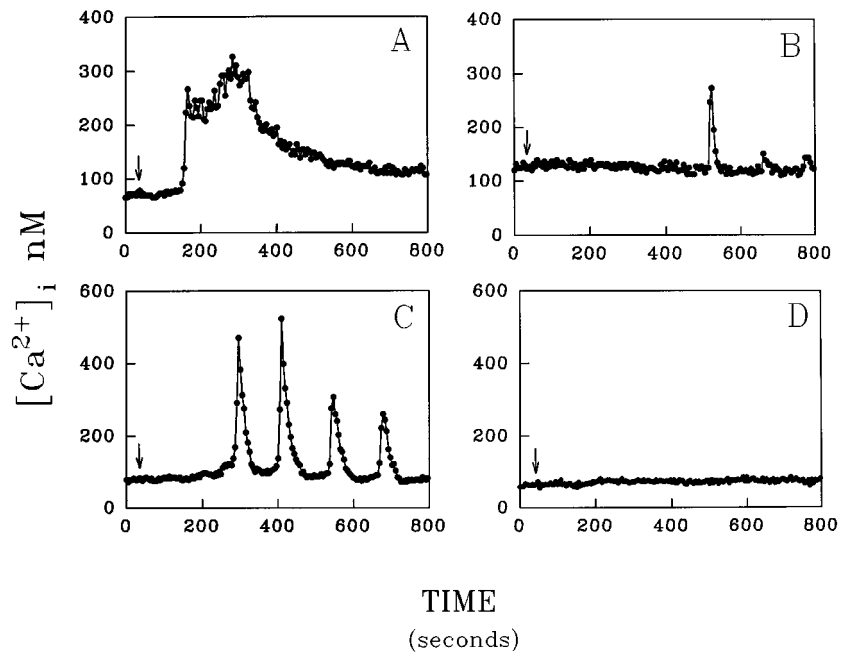


FIG. 6. Effects of perfusion with exogenous sphingosine and SPP. A, oscillatory Ca^{2+} responses caused by exposing cells for 5 min to 10 μM sphingosine, following a previous 10-min incubation with 10 μM DL-*threo*-dihydrosphingosine. Data are from 53 cells analyzed in four different experiments. B, a monophasic Ca^{2+} increase elicited perfusing cells for 5 min to 1 μM SPP. Each substance was added to the cells as indicated by the arrow. Data are from 38 cells analyzed in three separate experiments.

unresponsive to the first addition were also unresponsive to the second one. Incubation with 10 μM DL-*threo*-dihydrosphingosine for 10 min prior to the second PDGF-BB addition significantly

FIG. 7. Effect of two consecutive additions of PDGF-BB on Ca^{2+} signaling. After an initial Ca^{2+} response elicited by a 5-min exposure to PDGF-BB in non-oscillatory (A) and oscillatory cells (C), a second addition to the same cells, after a 30-min interval, was unable to cause a further $[Ca^{2+}]_i$ increase (B and D). Panels A and B show the only cells out of 42 cells analyzed in three separate experiments that responded with a single Ca^{2+} peak to the second PDGF-BB addition.



increased the percentage of responding cells. Under these circumstances, 19 out of 55 cells, including some cells that had previously responded with a non-oscillatory pattern to the first PDGF addition, showed an oscillatory Ca^{2+} response to the second addition (Fig. 8). Interestingly, after the incubation with DL-threo-dihydrospingosine, six cells that were not responsive to the first PDGF-BB exposure showed an oscillatory Ca^{2+} response to the second one.

DISCUSSION

This study shows that in cells belonging to the oligodendrocyte lineage, PDGF-BB induces two distinct patterns of Ca^{2+} signaling, possibly as a consequence of different modulatory roles played by sphingosine and SPP.

Both Ca^{2+} response patterns were partially dependent on the presence of extracellular Ca^{2+} . Removal of extracellular Ca^{2+} reduced both their magnitude and duration (Fig. 3), suggesting that some Ca^{2+} influx following PDGF-BB exposure must take place. This influx may occur via the "capacitative" pathway for refilling intracellular Ca^{2+} pools, which can be induced by their depletion (29, 30).

The non-oscillatory pattern observed in CEINGE cl3 cells exposed to PDGF-BB is very similar to analogous Ca^{2+} responses induced by the same growth factor in fibroblasts (6) and vascular smooth muscle cells (7). However, the oscillatory Ca^{2+} responses induced by PDGF-BB in CEINGE cl3 cells have never been described in any other cell type. Ca^{2+} spikes have frequently been observed upon stimulation of G protein-coupled receptors and are generally considered to be a consequence of some interplay between Ca^{2+} influx and Ca^{2+} mobilization from intracellular stores (30). However, exposure of CEINGE cl3 cells to three different agonists at G protein-coupled receptors (Fig. 4), or to ryanodine or caffeine, was unable to elicit oscillatory Ca^{2+} responses. Thus, this phenomenon seems to be peculiar to PDGF-BB stimulation of this cell type. Furthermore, it also suggests that the mechanisms involved in the genesis of Ca^{2+} oscillations are unrelated to Ca^{2+} mobilization from ryanodine/caffeine-sensitive intracellular pools.

We might hypothesize that the oscillatory Ca^{2+} response elicited by PDGF-BB in CEINGE cl3 cells involves a signaling pathway different from the activation of phospholipase C by G proteins. Indeed, the evidence suggests that the two different

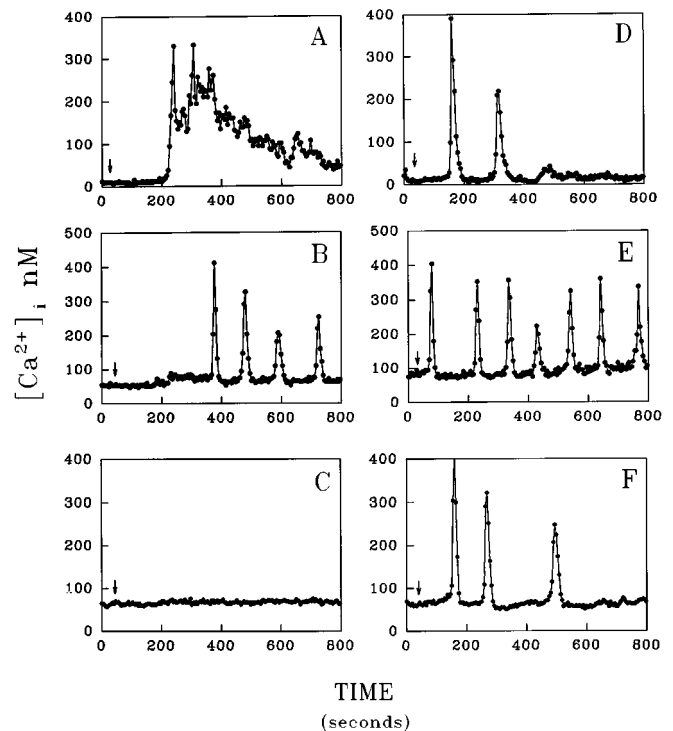


FIG. 8. Effect of sphingosine kinase inhibition on Ca^{2+} responses induced by a second PDGF-BB exposure. After an initial Ca^{2+} response produced by PDGF-BB (A and B), the same cells were incubated for 10 min with $10 \mu M$ DL-threo-dihydrospingosine during the 30-min interval preceding a second PDGF-BB exposure, which is shown in panels D and E. Both non-oscillatory and oscillatory cells responded to a second PDGF-BB addition in an oscillatory fashion. Panel C shows one single cell out of six that was unresponsive to a first PDGF-BB exposure but showed an oscillatory Ca^{2+} response to the second PDGF-BB addition after incubation with DL-threo-dihydrospingosine (F). Data are from 55 cells analyzed in three separate experiments.

Ca^{2+} responses observed after PDGF-BB exposure may be the result of a common biochemical pathway. The different delays associated with the two responses could result from the different time required for the production of two different second

messengers such as sphingosine and SPP. Indeed, an increase in the production of both of these substances has been described in 3T3 fibroblasts in response to PDGF-BB (19).

Sphingosine kinase has been proposed to catalyze the generation of SPP from sphingosine (31). 10 μM DL-*threo*-dihydro-sphingosine inhibited the activity of this enzyme by 50% in isolated platelets. In the same system, generation of SPP in response to exogenous sphingosine was inhibited up to 25% (32). Similar results were obtained in 3T3 fibroblasts, where 10 μM DL-*threo*-dihydro-sphingosine inhibited the production of SPP induced by exogenous sphingosine by 63% and also completely eliminated the generation of SPP elicited by PDGF-BB (19). Incubation of CEINGE c13 cells with DL-*threo*-dihydro-sphingosine did not affect the Ca^{2+} response to ATP, BK, and ET-1. This suggests that sphingolipids are not involved in the Ca^{2+} signaling following G protein-coupled receptor stimulation, as previously observed in fibroblasts (19). However, sphingosine kinase inhibition caused a significant increase in the percentage of cells responding to PDGF-BB with $[Ca^{2+}]_i$ oscillations (Fig. 5). Interestingly, the delay preceding this response was identical to that observed in cells spontaneously responding to PDGF-BB in an oscillatory fashion. These data strongly suggest that an increase in intracellular sphingosine levels or the consequent reduction in SPP levels facilitates the occurrence of oscillatory Ca^{2+} responses to PDGF-BB. To further verify this hypothesis, exogenous sphingosine and SPP were separately added to the cells. Sphingosine and SPP caused Ca^{2+} responses that were oscillatory and non-oscillatory, respectively (Fig. 6). SPP exposure never caused an oscillatory Ca^{2+} response in any of the responsive cells analyzed. However, the $[Ca^{2+}]_i$ increase elicited by exogenous SPP was characterized by a faster Ca^{2+} peak and the absence of a plateau, thereby differing kinetically from the non-oscillatory response observed in the 57% of cells exposed to PDGF-BB. This discrepancy might be due to a further signal transduction mechanism, such as the IP_3 pathway, which also participated in mobilizing Ca^{2+} during the non-oscillatory response induced by PDGF. It has also been shown that SPP can be produced by a sphingosine kinase localized at the level of the endoplasmic reticulum (33). This might allow for more effective Ca^{2+} mobilizing actions of SPP produced by PDGF receptor activation in comparison to that shown by exogenously added SPP.

On the other hand, the oscillatory responses caused by perfusion with exogenous sphingosine exactly mimicked those induced by PDGF-BB. The shorter delays that preceded both responses, in comparison to the analogous responses elicited by PDGF-BB, can be easily explained by the fact that in these experiments all the steps involving binding of PDGF-BB to its receptor, receptor activation, and sphingomyelinase recruitment do not take place.

Previous studies have suggested that SPP is the effective Ca^{2+} releasing metabolite, while sphingosine only represented an intermediate precursor for sphingosine kinase activity (15, 20, 33). However, another study performed on dermal fibroblasts in the presence of DL-*threo*-dihydro-sphingosine, showed that sphingosine could indeed mobilize Ca^{2+} from intracellular stores (18). It should also be noted that no studies on the role of sphingolipids in PDGF-stimulated Ca^{2+} signaling have been previously performed using oligodendroglial cells. It seems reasonable to hypothesize that in these cells the mechanism of Ca^{2+} signaling following PDGF receptor stimulation differs in some respects to that observed in 3T3 fibroblasts and vascular smooth muscle cells.

Sphingosine kinase activity has been shown to be stimulated by PDGF-BB in 3T3 fibroblasts, suggesting that this growth factor may be able to stimulate sphingosine production from

sphingomyelin as well as to modulate the synthesis of SPP (19). Thus, the two separate Ca^{2+} responses produced by PDGF-BB in CEINGE c13 cells could be due to different rates of sphingosine kinase activity. In cells showing oscillatory Ca^{2+} responses, sphingosine kinase might be resistant to the stimulatory action of PDGF-BB, causing a progressive elevation in sphingosine levels. This could explain the longer delay preceding the oscillatory Ca^{2+} response due to the slow and continuous accumulation of sphingosine until it reached an effective Ca^{2+} -releasing threshold. In contrast, in non-oscillatory cells, a sphingosine kinase sensitive to the stimulatory action of PDGF-BB could more rapidly transform sphingosine to SPP, increasing its concentration and lowering that of sphingosine. It should be noted that in fibroblasts, PDGF-BB induces a maximal increase in SPP levels after about 3 min (19) and that this time course is in perfect accordance with the duration of delay preceding the non-oscillatory response observed in the present study.

A point of particular interest is that most of the cells responding with a non-oscillatory pattern could be persuaded to oscillate if the intracellular levels of sphingosine were augmented (or those of SPP were reduced). Experiments such as those in Fig. 8 show that inhibition of SPP formation produced an oscillatory response in cells that had responded with a non-oscillatory pattern to a previous PDGF-BB addition. Furthermore, it seems that a threshold level may exist in the Ca^{2+} -releasing concentration of sphingosine. This is suggested by (i) the unresponsiveness of cells to the second PDGF-BB addition in the absence of sphingosine kinase inhibition (when sphingosine levels were increased by inhibition of its transformation in SPP, a second PDGF-BB pulse elicited a Ca^{2+} response) and (ii) some cells unresponsive to an initial PDGF-BB exposure showed a Ca^{2+} response to a second exposure when sphingosine levels were increased by DL-*threo*-dihydro-sphingosine incubation. This indicates that sphingosine production induced by PDGF-BB in some cells may not be sufficient to trigger a Ca^{2+} response.

In conclusion, the results presented here show the involvement of sphingosine and SPP in the Ca^{2+} responses induced by PDGF-BB in cells of the oligodendrocyte lineage. PDGF stimulation of oligodendroglial cells has a mitogenic effect (21) but is also of fundamental importance in development of oligodendrocyte progenitors (23, 34). Thus, the different Ca^{2+} mobilization patterns evoked by sphingosine and SPP may reflect the complex actions of PDGF in these cells. Several reports have recently shown that increases in intracellular Ca^{2+} can activate separate biochemical pathways, such as those regulating the expression of immediate early genes (35, 36). The data reported here suggest that growth factors may cause distinct cytoplasmic and/or nuclear events depending on the kinetics of Ca^{2+} signaling produced.

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