

Cell cycle control of PDGF-induced Ca^{2+} signaling through modulation of sphingolipid metabolism

ALESSANDRO FATATIS AND RICHARD J. MILLER¹

Department of Pharmacological and Physiological Sciences, The University of Chicago, Chicago, Illinois 60637, USA

ABSTRACT The effects of growth factors have been shown to depend on the position of a cell in the cell cycle. However, the physiological basis for this phenomenon remains unclear. Here we show that the majority of both CEINGE clone3 (cl3) and human embryonic kidney 293 cells, when arrested in a quiescent phase (G_0), responded to platelet-derived growth factor BB (PDGF-BB) with non-oscillatory Ca^{2+} signals. Furthermore, the same type of Ca^{2+} response was also observed in CEINGE cl3 cells (and to a lesser extent in HEK 293 cells) blocked at the G_1/S boundary. In contrast, CEINGE cl3 cells synchronized in early G_1 or released from G_1/S arrest responded in an oscillatory fashion. This cell cycle-dependent modulation of Ca^{2+} signaling was not observed on epidermal growth factor and G-protein-coupled receptor stimulation and was not due to differences in the expression of PDGF receptors (PDGFRs) during the cell cycle. We demonstrate that inhibition of sphingosine-kinase, which converts sphingosine to sphingosine-1-phosphate, caused G_0 as well as G_1/S synchronized cells to restore the oscillatory Ca^{2+} response to PDGF-BB. In addition, we show that the synthesis of sphingosine and sphingosine-1-phosphate is regulated by the cell cycle and may underlie the differences in Ca^{2+} signaling after PDGFR stimulation.—Fatatis, A., Miller, R. J. Cell cycle control of PDGF-induced Ca^{2+} signaling through modulation of sphingolipid metabolism. *FASEB J.* 13, 1291–1301 (1999)

Key Words: DMS · DHS · epidermal growth factor · PDGF

THE PROGRESSION OF a cell through the cell cycle is regulated by several growth factors. After mitosis (G_1 phase), the absence of these factors causes the exit of cells from the cell cycle and their reversible arrest in a state of quiescence, the G_0 phase (1, 2). Cells then require growth factors to re-enter the cell cycle (3, 4). A crucial step during the course of the cell cycle is the restriction point, located at the end of the G_1 phase. This represents the last opportunity for a cell to decide whether to enter the S phase or not, a decision that depends on growth factor availability (5, 6). After cells traverse the restriction point, they

advance through the G_1/S boundary and enter S phase independently of proliferative signals (7, 8).

There has been great interest in trying to characterize the physiological basis for the different susceptibility to growth factors observed during the course of the cell cycle. Furthermore, an increasing amount of evidence demonstrates that growth factors may exert opposite effects on cellular fate, such as proliferation and programmed cell death (9–11). Stimulation of platelet-derived growth factor receptors (PDGFRs)² increases intracellular free Ca^{2+} ($[\text{Ca}^{2+}]_i$) and activates at least one sphingomyelinase, leading to the production of ceramide, sphingosine (SPH), and sphingosine-1-phosphate (SPP) (12–15). Sphingolipids and their metabolites represent an emerging class of bioactive compounds that are involved in cellular growth and death (16). Moreover, SPH and SPP can release Ca^{2+} from intracellular stores independently from *myo*-inositol-1,4,5-trisphosphate (IP_3) production (17, 18). Ca^{2+} is a second messenger of critical importance during cell cycle progression. Indeed, blocking Ca^{2+} influx through plasma membrane channels or mobilization from intracellular stores stops cells from entering G_1 and proceeding toward the G_1/S transition (19–21). It has been recently shown that different patterns of Ca^{2+} signals may be responsible for transmitting distinct messages to the nucleus (22). This prompted us to investigate whether the characteristics of Ca^{2+} signaling after PDGFR stimulation were dependent on the cell cycle.

Here we show that the kinetics of Ca^{2+} responses to platelet-derived growth factor BB (PDGF-BB) in

¹ Correspondence: Department of Pharmacological and Physiological Sciences, The University of Chicago, 947 East 58th St., MC-0926, Chicago, Illinois 60637, USA. E-mail: rjmx@midway.uchicago.edu

² Abbreviations: ATP, adenosine 5'-triphosphate; BK, bradykinin; BrdU, 5-bromo-2'-deoxyuridine; DHS, DL-*threo*-dihydrosphingosine; DMS, *N,N*-dimethylsphingosine; EGF, epidermal growth factor; ET-1, endothelin-1; FBS, fetal bovine serum; Fura-2/AM, Fura-2/acetoxymethyl ester; GPCR, G-protein-coupled receptor; IP_3 , *myo*-inositol-1,4,5-trisphosphate; PDGF-BB, platelet-derived growth factor BB; PDGFR, platelet-derived growth factor receptor; PKC, protein kinase C; SPH, sphingosine; SPP, sphingosine-1-phosphate; TLC, thin-layer chromatography.

CEINGE c13 and HEK 293 cells are related to the cell cycle. We also obtained evidence for a role for sphingolipid metabolism in this phenomenon.

MATERIALS AND METHODS

Materials

The oligodendroglial cell line CEINGE c13 was obtained as described previously (23). The HEK 293 cell line was from ATCC (Rockville, Md.) (CRL1573).

PDGF-BB, PDGF-AA, epidermal growth factor (EGF), and bradykinin (BK) were from Calbiochem-Novabiochem (San Diego, Calif.). Hoechst 33342 and Fura-2/acetoxymethyl-ester (Fura-2/AM) were from Molecular Probes (Eugene, Oreg.). DL-*threo*-dihydrosphingosine (DHS), *N,N*-dimethylsphingosine (DMS), endothelin-1 (ET-1), adenosine 5'-triphosphate (ATP), DL-sphingosine, bisindolylmaleimide I-HCl, and poly-L-lysine were from Sigma (St. Louis, Mo.). Aphidicolin and mimosine were from Biomol Research Laboratories (Plymouth Meeting, Pa.) and lovastatin was kindly provided by Alfred W. Alberts (Merck, Sharp & Dohme Research Laboratories, Rahway, N.J.).

Cell culture and synchronization

Cells were grown in Dulbecco's modified Eagle's medium (CEINGE c13) or minimum essential medium (HEK 293) (Life Sciences, Grand Island, N.Y.) containing 10% fetal bovine serum (FBS, Hyclone, Logan Utah) and 50 μ g/ml gentamicin. G_0 synchronized CEINGE c13 and HEK 293 cells were obtained by removing serum from the culture medium for 48 and 72 h, respectively (24).

Cells synchronized in early G_1 were obtained by supplying cells in G_0 with culture medium containing 5% FBS and 10 μ M lovastatin for 20 h. This drug reversibly inhibits the synthesis of mevalonic acid, which is essential for isoprenylation of p21^{ras}, a small GTP binding protein crucial for G_0/G_1 transition (25).

Cells were reversibly synchronized in late G_1 by supplying cells in G_0 for 20 h with culture medium containing 5% FBS and 200 μ M mimosine, a drug that can block cells at a point that precedes the G_1/S boundary of ~ 2 h (24). Synchronization at the G_1/S boundary was obtained using the reversible inhibitor of the α -DNA polymerase aphidicolin, added at a 5 μ M concentration to asynchronously growing cultures for 20 h (24).

A population enriched in S phase cells was obtained by removing aphidicolin from cells blocked at the G_1/S boundary and supplying them with fresh culture medium containing 5% FBS (24). The percentage of cells entering the S phase after G_1/S release was monitored by immunocytochemistry using an antibody against 5-bromo-2'-deoxyuridine (BrdU), a thymidine analog that is incorporated into the nucleus of cells actively synthesizing DNA. Using this approach, the entry into S phase was observed as early as 30 min after aphidicolin removal, with $36 \pm 3\%$ and $48 \pm 6\%$ of BrdU-positive cells stained at the first and second hour, respectively. The last time point has been used for all experiments relative to the Ca^{2+} response to PDGF-BB during the S phase.

A population enriched in cells at the G_2/M transition was obtained by monitoring the expression of cyclin B1 after the release from late G_1 block and during the progression through the S phase.

Serum withdrawal as well as pharmacological cell cycle

synchronization might cause variable degrees of apoptotic death in different cell types. Therefore, the percentage of apoptotic cells induced by different synchronizing treatments was assessed by performing a cell viability test using Hoechst 33342 on a representative coverslip before each series of experiments and proved to be negligible for both cell lines used in this study.

Western blot analysis

Nuclear and cytoplasmic extracts for cyclin expression and total cell lysates for PDGFR expression were obtained as described (26, 27). The protein concentration in the clarified lysate was determined by BCA protein assay (Pierce, Rockford, Ill.). Cell lysates containing equal amount of proteins were resolved on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (28) using 10% and 7.5% polyacrylamide gel for cyclins and PDGFRs, respectively. The separated proteins were electroblotted to Hybond-ECL nitrocellulose (Amersham, Arlington Heights, Ill.) or immobilon-P PVDF membrane (Millipore Corporation, Bedford, Mass.). Membranes were then incubated for 1 h in phosphate-buffered saline containing 4% dry milk powder, 1% bovine serum albumin and 0.1% Tween-20. The antibodies for the immunoblotting were used at the following dilutions: anti-cyclin A (Upstate, Lake Placid, N.Y.) at 1:1000; anti-cyclin E, anti-cyclin B1, and anti-cyclin D1 (Santa-Cruz Biotechnology, Santa Cruz, Calif.) at 1:2000, 1:1000, and 1:100, respectively. Anti- β -PDGFR (Santa Cruz Biotechnology) and anti- α -PDGFR (Upstate) rabbit polyclonal antibodies were used at 1:1000 and 1:4000 dilution, respectively. Anti-rabbit and anti-mouse HRP-conjugated secondary antibodies (Promega, Madison, Wis.) were used at 1:20,000 dilution. The binding of the antibodies to the membrane was detected using the SuperSignal Ultra (Pierce) or ECL (Amersham) chemiluminescent substrates. Densitometric analysis was performed using the UN-SCAN-IT software (Silk Scientific, Inc., Utah).

Immunocytochemistry and immunofluorescence

For the immunocytochemistry of BrdU incorporation, cells were incubated for 30 min with 10 μ M BrdU added to the culture medium and fixed with 70% ethanol/50 mM glycine buffer for 30 min at -20°C . Primary antibody directed against BrdU, secondary alkaline-phosphatase-conjugated antibody, and color substrate solutions were from BrdU labeling and detection Kit II (Boehringer Mannheim, Indianapolis, Ind.), and used according to the manufacturer's instructions.

For the immunofluorescence of cyclin B₁, cells were fixed and permeabilized in cold methanol for 20 min, blocked in 5% normal donkey serum, and incubated with anti-cyclin B₁ (Santa Cruz) 1:1000 in blocker for 1 h. The biotin-conjugated secondary antibody (1:500 for 1 h) was followed by streptavidin-Cy3 at 1:3000 for 15 min. Coverslips were mounted with glycerol/phosphate-buffered saline (9:1) containing 1,4 diazabicyclo[2.2.2]octane (2.5% W/V) as anti-fading agent.

Intracellular Ca^{2+} measurement and videomaging analysis

Cells were grown on 25 mm N^o 1 glass coverslips coated with poly-L-lysine (100 μ g/ml). Cell loading with 2 μ M Fura-2/AM, single-cell videomaging of as many as 20–30 cells/field, and calibration of fluorescent signals were performed as described previously (27).

Thin-layer chromatography (TLC) analysis of lipids

Labeling with [³H]serine (20 μ Ci/ml, Amersham), a precursor of cellular sphingolipids, and harvesting of cells exposed

to 10 ng/ml PDGF-BB for various time were performed as reported (29). Lipid extraction was obtained using chloroform/methanol/concentrated HCl (100:200:1 v/v) and the phases were separated as described (30, 31). The lipids, dried under nitrogen, were analyzed by 2-dimensional thin-layer chromatography performed on silica gel 60 G plates (Alltech, Deerfield, Ill.). The plates were developed using chloroform/methanol/ammonium hydroxide (13:7:1) as solvent for the first dimension and chloroform/methanol/water/acetic acid (30:30:2:5) as solvent for the second dimension (31). Lipids were located using iodine vapors and phospholipids were visualized using molybdenum blue spray (Sigma). SPH (Avanti Polar Lipids, Alabaster, Ala.) and SPP (Biomol) were used as standards to identify the silica gel areas to scrape off and count by liquid scintillation spectrometry.

Statistical analysis

Data values are expressed as mean value \pm SE. Student's *t* test or the Mann-Whitney nonparametric test were used and statistical significance was defined as a *P* value of 0.01 or less.

RESULTS AND DISCUSSION

We have previously shown that PDGF-BB induces both non-oscillatory and oscillatory Ca^{2+} responses in CEINGE c13 cells ($46 \pm 4\%$ and $54 \pm 4\%$, respectively, in 188 cells analyzed; see also 32) (Fig. 1). These two types of Ca^{2+} responses were evenly distributed in asynchronously proliferating cell cultures, but never observed in the same cell. We therefore decided to investigate whether the type of

Ca^{2+} signal observed in response to PDGF-BB was related to the position of the cells in the cell cycle.

A first series of experiments was performed using CEINGE c13 cells synchronized in G_0 and early G_1 phase. Cell synchronization was assessed by monitoring the nuclear levels of cyclin D1, whose expression is down-regulated in resting cells. Furthermore, the percentage of cells incorporating BrdU was evaluated (Fig. 2A). When G_0 arrested CEINGE c13 cells were exposed to PDGF-BB, the majority of cells showed non-oscillatory Ca^{2+} responses ($92 \pm 3\%$, 107 cells analyzed in eight experiments). In contrast, $74 \pm 7\%$ of cells blocked in early G_1 responded to PDGF-BB in an oscillatory fashion (84 cells analyzed in six experiments) (Fig. 2B).

Activation of G-protein-coupled receptors (GPCRs) for BK, ET-1, and ATP in asynchronous CEINGE c13 cells exclusively induced a non-oscillatory Ca^{2+} response, as we previously reported (32). Cell synchronization in early G_1 did not modify the pattern of Ca^{2+} signals induced by these agonists (data not shown).

Drugs commonly used as synchronizing agents may induce a certain degree of metabolic perturbation in cells (33, 34). Consequently, it seemed important to verify that the changes in the types of Ca^{2+} signaling we observed were really connected to the cell cycle and not attributable to the methods used to synchronize the cells *per se*. We therefore analyzed the changes in Ca^{2+} signaling in response

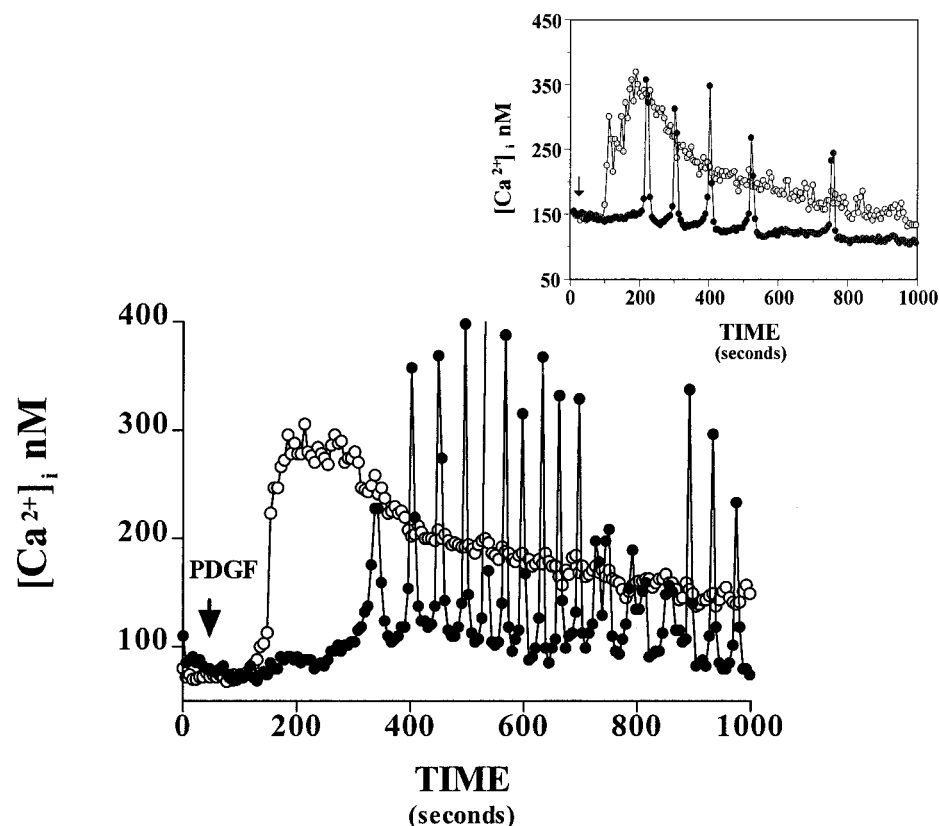


Figure 1. Typical non-oscillatory (open circle) and oscillatory (filled circle) Ca^{2+} responses to 10 ng/ml PDGF-BB (added at the arrow and maintained for 5 min) in asynchronously growing CEINGE c13 cells. The insert shows a cell oscillating with a slower frequency.

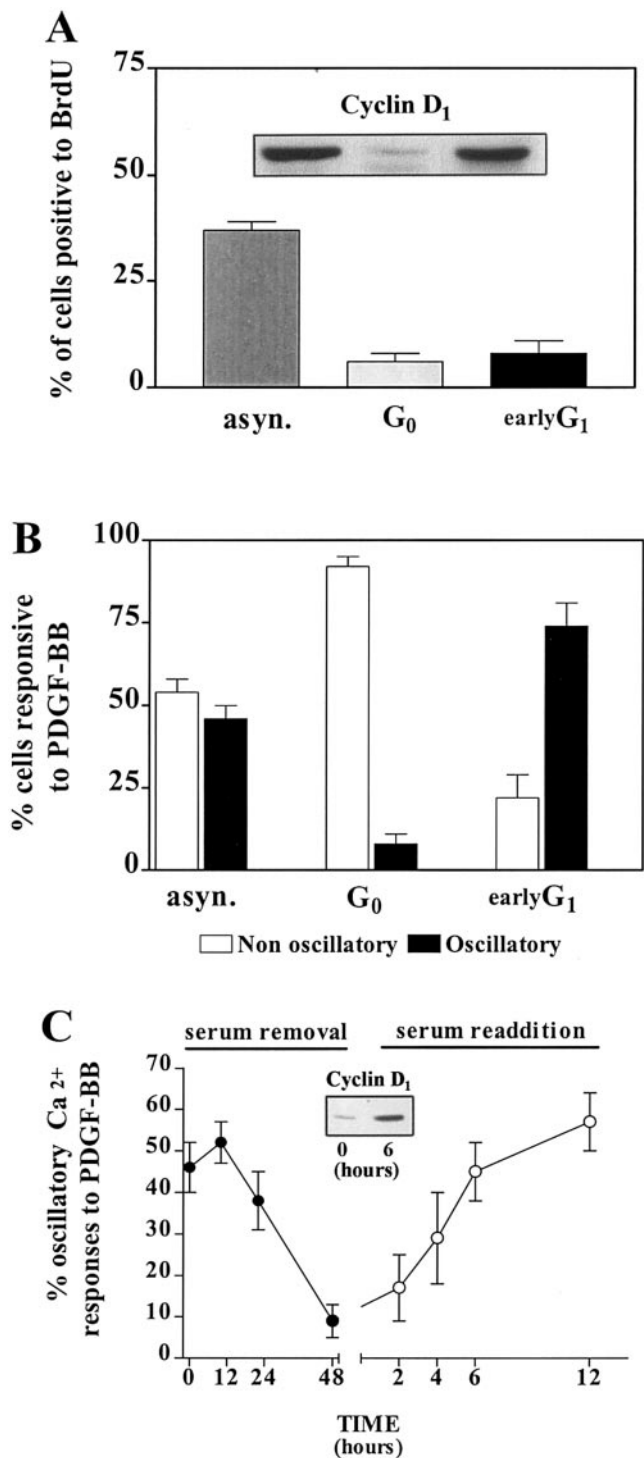


Figure 2. Correlation between cell cycle position and Ca²⁺ response to PDGF-BB in G₀ and early G₁ synchronized CEINGE c13 cells. **A)** Percentage of proliferating cells in asynchronously growing cultures and in cells synchronized in G₀, and early G₁ phase were 37 ± 2%, 6 ± 2%, 8 ± 3%, respectively. Insert: cyclin D₁ was highly expressed in both asynchronous and early G₁ synchronized cells, whereas G₀ resting cells showed negligible expression of this cyclin. **B)** Differences in the percentage of oscillatory and non-oscillatory Ca²⁺ responses to PDGF-BB observed in G₀ and early G₁ synchronized cells compared to asynchronous cultures. **C)** Time-dependent effect of serum removal from asynchronous cells and serum readdition to G₀ quiescent cells on Ca²⁺

to PDGF-BB at fixed time intervals during exit from the cycle as well as during re-entry and progression through G₁ phase in the absence of synchronizing drugs. As shown in Fig. 2C, serum withdrawal produced a progressive disappearance of the oscillatory Ca²⁺ signaling in response to PDGF-BB. Although after 12 h of serum removal the percentage of oscillatory cells was still comparable to that observed in asynchronous cultures, after 24 and 48 h Ca²⁺ oscillations were observed in only 31 ± 6% and 8 ± 3% of cells responsive to PDGF-BB, respectively. The oscillatory Ca²⁺ signaling in response to PDGF-BB gradually reappeared when G₀ arrested cells were induced to re-enter the cycle by serum addition, as shown by the re-expression of cyclin D₁ (see insert in Fig. 2C). For instance, 2, 4, and 6 h after serum addition, 17 ± 8%, 29 ± 11%, and 45 ± 7% of responding cells exhibited an oscillatory Ca²⁺ response (20, 43, and 58 cells analyzed in three experiments for each set of data). Finally, after 12 h, the percentage of oscillatory Ca²⁺ responses to PDGF-BB reached 57 ± 7% (23 cells analyzed in three experiments). The reappearance of oscillatory Ca²⁺ signaling in response to PDGF-BB during the transition from G₀ to G₁ agreed with the results obtained in cells pharmacologically synchronized in early G₁ (Fig. 2B).

A second series of experiments was performed with CEINGE c13 cells pharmacologically synchronized at the G₁/S border as well as on cultures enriched in cells in S phase. Cell synchronization was evaluated by measuring the nuclear expression of cyclin A and cyclin E, whose protein levels peak at the G₁/S border and are thought to be involved in controlling DNA replication (35) (Fig. 3A). Cultures maximally enriched in cells in S phase (48 ± 6% of cells incorporating BrdU) were obtained at the second hour after the release from the pharmacological block at the G₁/S border. Cells blocked at the G₁/S border responded to PDGF-BB mostly in a non-oscillatory fashion, with a percentage of oscillatory Ca²⁺ responses of only 12 ± 6% (53 cells analyzed in five experiments). However, their entry into the S phase was followed by a significant increase in the percentage of oscillatory Ca²⁺ responses (48 ± 10%; 43 cells analyzed in three experiments) (Fig. 3B).

As previously done for G₀/G₁ transition, we decided to analyze the Ca²⁺ responses to PDGF-BB in CEINGE c13 cells at fixed time intervals after the release from late G₁ block and during the progression through the G₁/S border and S phase. When cells blocked in late G₁ phase were exposed to PDGF-BB, the Ca²⁺ signaling observed was mostly

signaling induced by PDGF-BB. The re-entry in the cell cycle of G₀ synchronized cells was confirmed by the re-expression of cyclin D₁ (see insert).

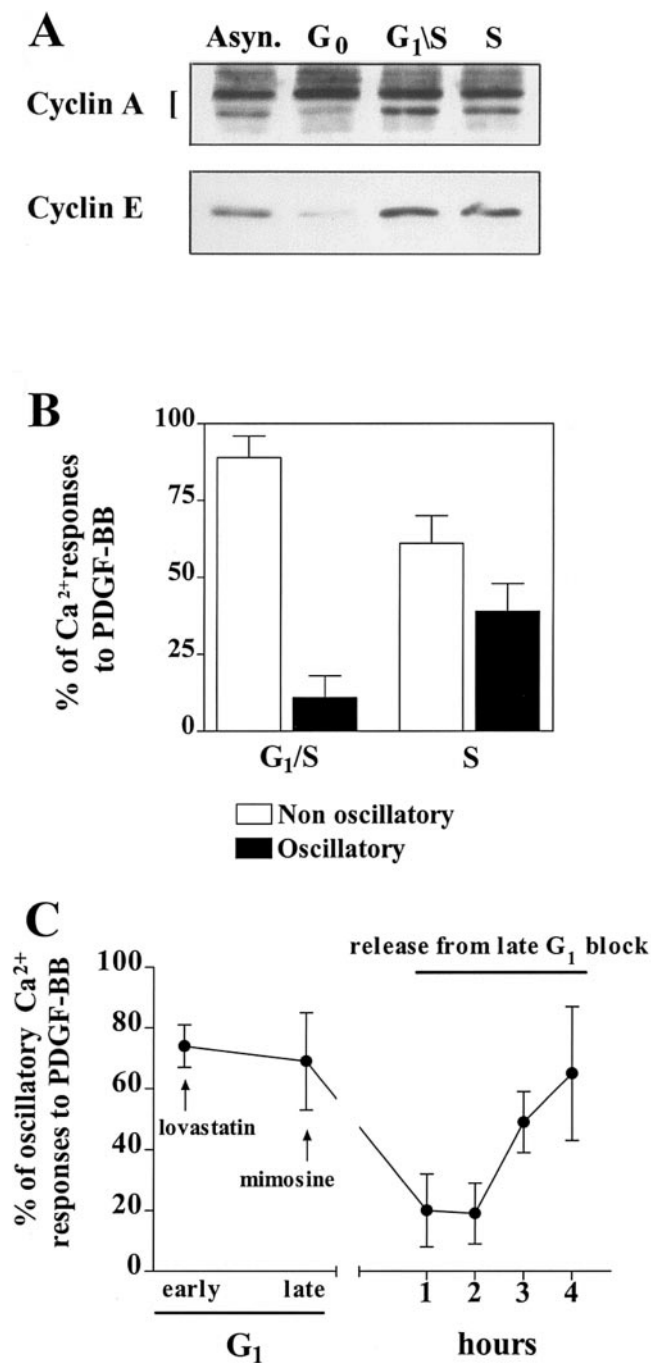


Figure 3. Correlation between cell cycle position and Ca²⁺ response to PDGF in G₁/S and S phase-synchronized CEINGE c13 cells. *A*) Cells synchronized at the G₁/S border showed high levels of cyclin A and cyclin E that were slightly reduced by the passage into the S phase. *B*) Percentage of oscillatory and non-oscillatory Ca²⁺ responses to PDGF-BB observed in G₁/S and S synchronized cells. *C*) Time-dependent changes in the percentage of Ca²⁺ responses to PDGF-BB after release from the arrest in late G₁ phase induced by mimosine.

oscillatory and comparable to that seen in early G₁ cells (69±18%, 19 cells analyzed in three experiments) (Fig. 3C). Cells were released from late G₁ block by the addition of fresh serum-containing medium and entered the S phase at the third hour,

as assessed by BrdU staining (data not shown). At the first and second hour postrelease, probably corresponding to the G₁/S transition, a significant reduction in the percentage of oscillatory Ca²⁺ responses was observed (20±16% and 19±10%, respectively; 24 and 21 cells analyzed in three experiments for each set of data) (Fig. 3C). This was in agreement with the results obtained in cells pharmacologically synchronized at the G₁/S boundary (Fig. 3B). At the third and fourth hour postrelease (a time interval corresponding to the S phase), the percentage of oscillatory Ca²⁺ responses increased (49±10% and 65±22%, respectively; 22 and 15 cells analyzed in three experiments for each set of data). This was in accord with the results obtained in cells released from the pharmacological arrest in G₁/S (Fig. 3B).

A third group of experiments was performed using cultures enriched in CEINGE c13 cells arrested at the G₂/M transition. This was achieved by monitoring the cytoplasmic and nuclear expression of cyclin B1 in cells released from the block in late G₁ phase induced by mimosine. Cyclin B1 is first synthesized during S phase, maximally expressed at the G₂/M transition, and then degraded during anaphase (36). Western blot and densitometric analysis revealed that cyclin B1 levels gradually increased starting from the second hour after the release from late G₁ block. The highest cytoplasmic levels were reached at the fourth hour whereas the nuclear levels reached a peak at the sixth and then declined at the tenth hour (Fig. 4A, B). The nuclear translocation of cyclin B1 was also assessed by immunofluorescence performed using an anti-cyclin B1 specific antibody on cells arrested at late G₁ phase and at the sixth hour after the release from the block (Fig. 4C, D). In the latter cell population the percentage of oscillatory Ca²⁺ responses to PDGF-BB was similar to that observed in cells traversing the S phase (54±6%; 21 responsive cells out of 84 total cells analyzed in three separate experiments). These data therefore indicate that the cell cycle does not exert any modulatory activity on Ca²⁺ signaling induced by PDGF-BB in cells at the G₂/M transition.

It seemed important to ascertain whether such a cell cycle-dependent modulation of Ca²⁺ responses to PDGF-BB was exclusive to CEINGE c13 cells or whether it could also be observed in other cell types. We therefore decided to apply the same experimental paradigm used for CEINGE c13 cells to HEK 293 cells. In these cells, PDGF-BB induces membrane ruffling and activation of the PTP2C protein phosphatase (37), but Ca²⁺ signaling after PDGFR activation has never previously been characterized. When asynchronously growing HEK 293 cells were exposed to PDGF-BB, exclusively oscillatory Ca²⁺ signals were observed (79 cells analyzed in three experiments; Fig. 5A). However, synchronization in

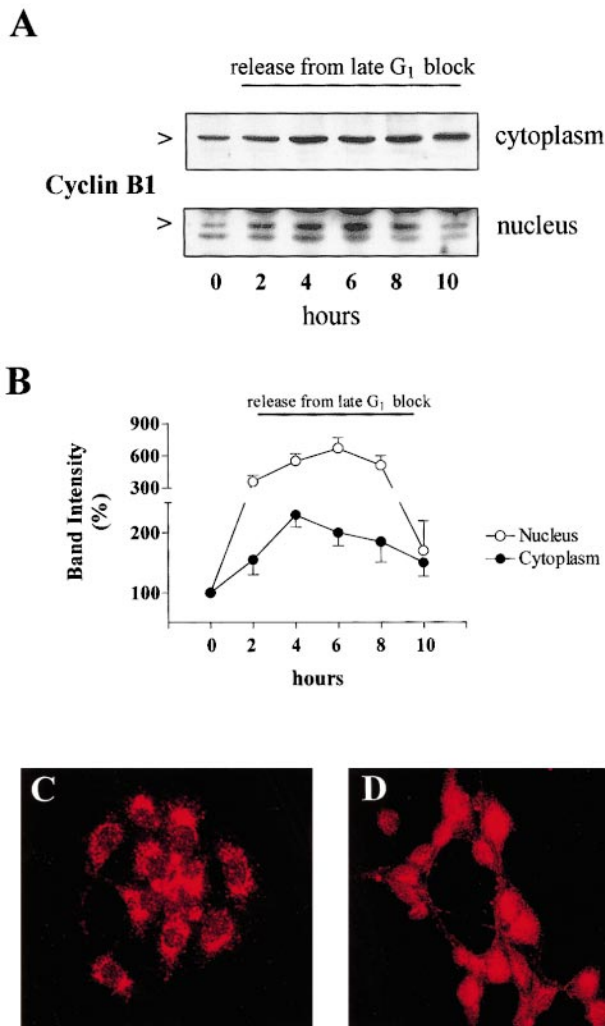


Figure 4. Analysis of cyclin B1 expression as an indication of cell synchronization at the G₂/M transition. *A*) Western blot of the time-dependent expression of cyclin B₁ in the cytoplasm and nucleus of CEINGE c13 cells after release from arrest in late G₁ phase. *B*) Densitometric analysis, performed on two separate experiments, shows that the highest levels of cyclin B1 were observed earlier in the cytoplasmic extracts compared to the nuclear extracts, indicating cytoplasmic synthesis followed by nuclear translocation. *C*) Immunofluorescence performed with an antibody against cyclin B1 shows lack of nuclear staining in cells arrested in late G₁ phase, whereas 6 h after the release from the block an evident nuclear staining was observed in ~40–60% of the cell population (*D*).

G₀ phase of HEK 293 caused the appearance of non-oscillatory Ca²⁺ responses to PDGF-BB (Fig. 5*B*). As observed in CEINGE c13 cells, the decrease in oscillatory Ca²⁺ responses was time dependent but required a prolonged period of serum withdrawal. For instance, although 48 h after serum removal no changes in the percentage of oscillatory Ca²⁺ responses were observed, after 60 h oscillatory Ca²⁺ responses to the growth factor were 51 ± 2%. Finally, after 4 days of starvation, only 26 ± 7% of HEK 293 cells responded in an oscillatory fashion (Fig. 5*C*; 60, 43, and 53 cells analyzed in three separate experi-

ments for each data point). Synchronization in G₀ of HEK 293 cells was confirmed by the progressive decrease in the percentage of cells stained for BrdU and by the reduction of cyclin D1 expression (data not shown). As shown for CEINGE c13 cells, quiescent HEK 293 cells could be induced to re-enter the cell cycle by serum readdition. Indeed, this caused a rapid restoration of the oscillatory Ca²⁺ responses (Fig. 5*C*). Synchronization of HEK 293 cells at the G₁/S border was also able to induce the appearance of non-oscillatory Ca²⁺ responses in 20 ± 8% of cells (56 cells analyzed in three experiments).

To understand whether the control of Ca²⁺ signaling operated by the cell cycle was limited to PDGFRs, we decided to test a different growth factor such as EGF (50 ng/ml). CEINGE c13 cells are unresponsive to EGF, whereas in HEK 293 cells this growth factor induces stimulation of adenylyl cyclase (38), mitogen-activated tyrosine kinases, and *trans*activation of Elk-1 (39).

When asynchronously growing HEK 293 cells were exposed to EGF, both oscillatory and non-oscillatory Ca²⁺ responses were observed (61 ± 19% of oscillatory responses; 75 cells analyzed in three experiments) (Fig. 5*D*). However, cell synchronization in G₀ was unable to modify the relative percentage of occurrence of these two types of Ca²⁺ response (68 ± 10% of oscillatory responses observed in 31 cells analyzed in two experiments).

These results demonstrate that in the two cell lines we tested, there is a correlation between the position of a cell in the cell cycle and the type of Ca²⁺ signaling produced by PDGF-BB. They also indicate that Ca²⁺ responses induced by GPCRs and by the EGF receptor are not influenced by the same mechanism.

Recent studies have shown that in BALB/c-3T3 fibroblasts, expression of the β isoform of PDGFR is up-regulated when cells undergo growth arrest on serum deprivation (40), whereas terminal differentiation of 3T3-L1 fibroblasts induces a down-regulation of both α- and β-PDGFRs (41). In the light of these results, it has been proposed that changes in PDGFR levels may provide a mechanism for regulating cell responsiveness to this growth factor during the cell cycle (41). Since CEINGE c13 cells express both α- and β-PDGFRs (27), we investigated whether cell cycle-dependent changes in the expression of these two receptors could account for the differences in the Ca²⁺ response to PDGF-BB we observed. Western blot analysis performed using cells synchronized in G₀, G₁, and at G₁/S boundary showed that the protein levels of both α- and β-PDGFR did not change significantly in all phases of the cell cycle examined (Fig. 6*A*). This idea was also confirmed by the fact that similar patterns of α and β receptor expression were observed in cells responding with

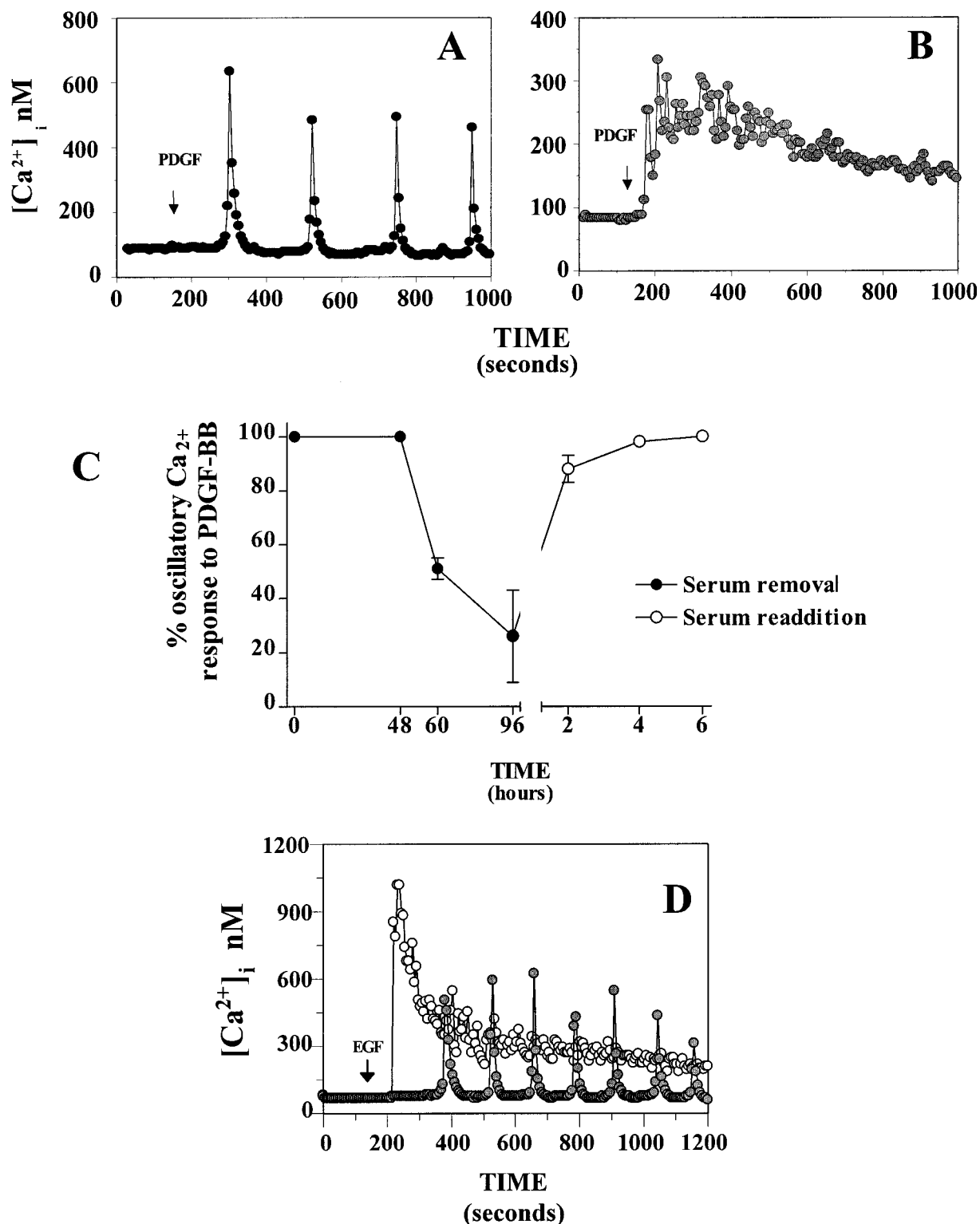


Figure 5. Ca^{2+} signaling induced by PDGF-BB in HEK 293 cells. *A*) Typical oscillatory Ca^{2+} response to PDGF-BB observed in all the asynchronous HEK 293 cells studied and *B*) non-oscillatory Ca^{2+} response observed in quiescent HEK 293 cells exposed to PDGF-BB. *C*) Time-dependent changes in the occurrence of oscillatory Ca^{2+} responses to PDGF-BB induced by serum removal and readdition. *D*) Ca^{2+} responses induced in HEK 293 cells by EGF exposure. Perfusion with the agonists started at the arrow and lasted for 5 min.

opposite kinetics to PDGF-BB, such as G_1 and G_1/S synchronized cells (Fig. 6*B*).

HEK 293 cells lack β -PDGFR (39); therefore, the Ca^{2+} signaling induced by PDGF-BB in these cells

must be due to stimulation of α -PDGFR, which binds the growth factor with an affinity equivalent to that of the β subunit (12). The expression levels of α -PDGFR in asynchronously growing and G_0 syn-

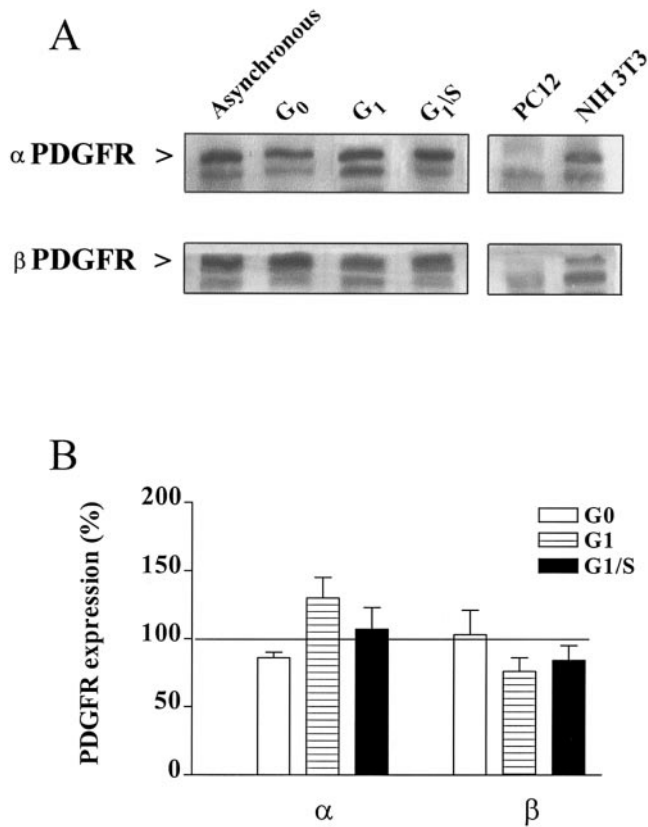


Figure 6. Western blot (A) and densitometric (B) analysis of α - and β -PDGFR expression in CEINGE c13 cells synchronized in different positions of the cell cycle. Proteins obtained from PC12 cells and NIH 3T3 fibroblasts were also included in the gel as a negative and a positive control for PDGFR expression, respectively. Data are from three separate experiments.

chronized HEK 293 cells did not show significant differences (data not shown), suggesting that α -PDGFR is subjected to the same modulation by the cell cycle as the β isoform.

These results therefore indicated that the target(s) involved in the cell cycle control of PDGF Ca^{2+} signaling was located downstream of the plasma membrane receptor.

It is known that PDGF induces an increase in cellular levels of SPH and activation of sphingosine kinase, the enzyme responsible for the conversion of SPH in SPP (29). These two sphingolipids, among other cellular effects, mobilize Ca^{2+} from thapsigargin-sensitive intracellular pools without interacting with the IP_3 binding site of the IP_3 receptor (42, 43).

We have previously shown that in asynchronously growing CEINGE c13 cells, exposure to PDGF-BB in the presence of DHS, a potent inhibitor of sphingosine kinase (44), produced an increase in the percentage of oscillatory Ca^{2+} responses. Furthermore, the addition of 10 μM exogenous SPH, which can be taken up by intact cells (31), induced a Ca^{2+} response with similar oscillatory kinetics and delay to that induced by PDGF-BB itself (32). This led to the hypothesis that SPH and SPP were responsible for

the oscillatory and non-oscillatory Ca^{2+} response, respectively.

We therefore considered the possibility that sphingosine kinase activity, and thus the intracellular levels of SPH and SPP, were regulated in a cell cycle specific fashion, thereby determining the type of Ca^{2+} signaling induced by PDGF in a particular phase of the cell cycle.

The ability of DHS to block the sphingosine kinase was investigated in CEINGE c13 cells by TLC analysis. As shown in Fig. 7A, though DHS was unable to affect the production of SPH or SPP *per se* under basal conditions, it significantly increased the levels of SPH and reduced the levels of SPP when these cells were stimulated with PDGF-BB.

Preincubation with 10 μM DHS induced a significant increase in oscillatory Ca^{2+} responses to PDGF-BB both in G₀ ($50 \pm 7\%$; 75 cells analyzed in four experiments) and G_{1/S} synchronized CEINGE c13 cells ($47 \pm 11\%$; 64 cells analyzed in five experiments) (Fig. 7B).

In parallel experiments, we replaced PDGF-BB with exogenous SPH in the presence of DHS. Under these conditions, oscillatory Ca^{2+} responses were observed in $59 \pm 5\%$ of G₀ and $89 \pm 7\%$ of G_{1/S} synchronized CEINGE c13 cells, respectively (17 and 31 cells analyzed in two separate experiments for each set of data) (Fig. 7B). DHS alone was without effect on intracellular Ca^{2+} , as described previously (32).

Similarly, $78 \pm 7\%$ of G₀ arrested HEK 293 cells responded to PDGF-BB in an oscillatory manner when the production of SPP was inhibited compared with $26 \pm 10\%$ of cells not treated with DHS (41 cells analyzed in two experiments, data not shown).

Thus, both in G₀ and G_{1/S} synchronized cells, the ability to respond with Ca^{2+} oscillations to PDGF-BB could be restored when SPH levels were increased.

A putative inhibitory activity of sphingosine kinase antagonists on protein kinase C (PKC) has been reported (45). However, incubation of G₀ synchronized CEINGE c13 cells for 30 min with the specific inhibitor of PKC bisindolylmaleimide I-HCl (200 nM) (46) was unable to reproduce the increase in the percentage of oscillatory Ca^{2+} responses to PDGF-BB observed when cells were treated with DHS (18 cells studied in 3 separate experiments). An effect similar to that caused by DHS was also produced by 10 μM DMS, another inhibitor of sphingosine kinase, which at this concentration is ineffective on PKC (47). These results suggest that the modulatory role of SPH and SPP on PDGF-BB-induced Ca^{2+} signaling is independent to PKC inhibition.

A clear correlation between the cell cycle and production of SPH and SPP induced by PDGF-BB was established by measuring the levels of these two sphingolipids in G₀ and early G₁ synchronized

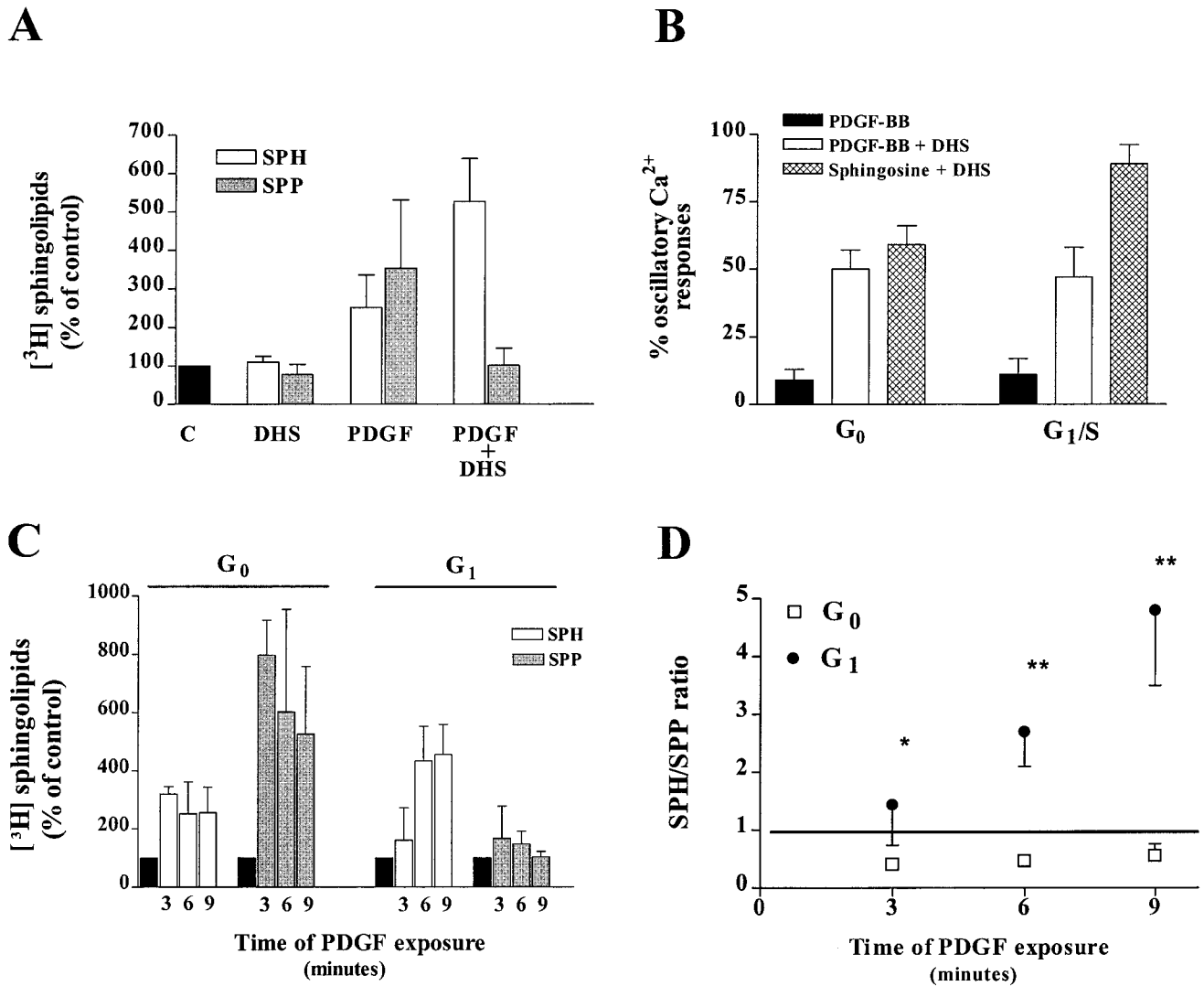


Figure 7. Analysis of sphingolipid production in CEINGE c13 cells. *A*) Production of SPH and SPP measured by TLC in G₀ quiescent cells exposed for 6 min to PDGF-BB or incubated for 15 min with 10 μ M DHS alone or before and during PDGF-BB exposure. *B*) Percentage of oscillatory Ca²⁺ responses induced by PDGF-BB or by exogenous SPH (10 μ M) on inhibition of sphingosine kinase by DHS in G₀ and G_{1/S} synchronized cells. *C*) Time-dependent production of SPH and SPP induced by PDGF-BB in G₀ and early G₁ synchronized cells. Data are means \pm SE of three independent experiments and are expressed as percentage of SPH and SPP production relative to the untreated cells. *D*) SPH/SPP ratios calculated for each time interval considered in G₀ and early G₁ synchronized cells exposed to PDGF-BB. Data are the average of SPH/SPP ratios (means \pm SE) calculated for each single experiment performed.

CEINGE c13 cells, which (as shown above) responded with opposite Ca²⁺ signals to the growth factor stimulation (Fig. 2C).

Indeed, in G₀ synchronized cells, exposure to PDGF-BB produced higher levels of SPP and lower levels of SPH compared with cells synchronized in early G₁ (Fig. 7C). Thus, opposite SPH/SPP ratios were observed in G₀ and in early G₁ synchronized cells (Fig. 7D), suggesting that the relative rather than absolute levels of the two sphingolipids appear to be critical in the modulation of Ca²⁺ signaling induced by PDGF-BB.

The onset of changes in SPH/SPP ratios was observed at 3 min in G₀ synchronized and at 6 min in early G₁ synchronized cells after PDGF-BB exposure.

This correlates with the time delays preceding the appearance of non-oscillatory and oscillatory Ca²⁺ responses (170 \pm 7 and 285 \pm 20 s, respectively in 188 cells examined; see also ref 32).

In conclusion, this study provides the first evidence for a cell cycle-dependent control of the Ca²⁺ signaling and the production of SPH and SPP induced by PDGF-BB. It also strongly suggests that these two sphingolipids determine the type of Ca²⁺ signals transduced in response to this growth factor. The intracellular target(s) for the Ca²⁺ mobilizing action of SPH and SPP has not been identified yet. Modulation or binding to distinct subtypes of IP₃ receptors that have different Ca²⁺ releasing properties (48) or interaction with additional signaling

pathways could explain the differences in the kinetics of the Ca²⁺ responses induced by these two sphingolipids.

It has been shown that PDGF-BB activates sphingomyelinase, ceramidase, and sphingosine kinase, whereas EGF has no effect on sphingolipid metabolism (15, 49). GPCRs have been shown to activate sphingosine kinase. However, this appears to be a receptor-specific phenomenon, also dependent on the cell type (50, 51). Indeed, the lack of activation of the sphingolipid pathway in CEINCE c13 and HEK 293 cells by EGF receptors and the GPCRs tested could explain why Ca²⁺ responses induced by these receptors were not modulated by the cell cycle.

It is interesting that in the cells we tested, it seems that distinct Ca²⁺ signals are required in specific phases of the cell cycle. It is widely recognized that the activity of several intracellular targets can be modulated by both the frequency and amplitude of Ca²⁺ signals (52–54) and that sphingolipids are involved in cell proliferation (29) and programmed cell death (55). Therefore, differences in PDGF-induced signaling during cell cycle progression could participate in regulating the occurrence and/or progression of these events. **[F]**

The authors wish to thank Dr. Olimpia Meucci for helpful discussion and invaluable suggestions and Umar Shakur for excellent technical assistance with cell cultures. This research was supported by U.S. Public Health Service grants DA02121, DA02575, MH40165, NS33502, DK42086, DK44840, NS21442, and NS33826.

REFERENCES

- Zetterberg, A., and Larsson, O. (1991) Coordination between cell growth and cell cycle transit in animal cells. *Cold Spring Harbor Symp. Quant. Biol.* **56**, 137–147
- Pardee, A. B. (1989) G₁ events and regulation of cell proliferation. *Science* **246**, 603–608
- Zetterberg, A., and Larsson, O. (1985) Kinetic analysis of regulatory events in G₁ leading to proliferation or quiescence of Swiss 3T3 cells. *Proc. Natl. Acad. Sci. USA* **82**, 5365–5369
- Sherr, C. (1994) G₁ phase progression: cycling on cue. *Cell* **79**, 551–555
- Pardee, A. B. (1974) A restriction point for control of normal animal cell proliferation. *Proc. Natl. Acad. Sci. USA* **71**, 1286–1290
- Zetterberg, A., Larsson, O., and Wiman, K. G. (1995) What is the restriction point? *Curr. Opin. Cell Biol.* **7**, 835–842
- Temin, H. (1971) Stimulation by serum of multiplication of stationary chicken cells. *J. Cell. Physiol.* **78**, 161–170
- Larsson, O., Lathman, C., Zickert, P., and Zetterberg, A. (1989) Cell cycle regulation of human diploid fibroblasts: possible mechanisms of platelet-derived growth factor. *J. Cell. Physiol.* **139**, 477–483
- Choi Kim, H.-R., Upadhyay, S., Li, G., Palmer, K. C., and Deuel, T. F. (1995) Platelet-derived growth factor induces apoptosis in growth-arrested murine fibroblasts. *Proc. Natl. Acad. Sci. USA* **92**, 9500–9504
- Cassacia-Bonnefil, P., Carter, B., Dobrowsky, R., and Chao, M. (1996) Death of oligodendrocytes mediated by the interaction of nerve growth factor with its receptor p75. *Science* **272**, 542–545
- Frade, J. M., Rodriguez-Tebar, A., and Barde, Y. A. (1996) Induction of cell death by endogenous nerve growth factor through its p75 receptor. *Nature (London)* **383**, 166–168
- Claesson-Welsh, L. (1994) Platelet-derived growth factor receptor signals. *J. Biol. Chem.* **269**, 32023–32026
- Williams, L. T. (1989) Signal transduction by the platelet-derived growth factor receptor. *Science* **243**, 1564–1570
- Hannun, Y. A., and Bell, R. M. (1989) Functions of sphingolipids and sphingolipid breakdown products in cellular regulation. *Science* **243**, 500–507
- Coroneos, E., Martinez, M., McKenna, S., and Kester, M. (1995) Differential regulation of sphingomyelinase and ceramidase activities by growth factors and cytokines. *J. Biol. Chem.* **270**, 23305–23309
- Spiegel, S., and Merrill A. H., Jr. (1996) Sphingolipid metabolism and cell growth regulation. *FASEB J.* **10**, 1388–1397
- Mattie, M., Brooker, G., and Spiegel, S. (1994) Sphingosine-1-phosphate, a putative second messenger, mobilizes calcium from internal stores via an inositol trisphosphate-independent pathway. *J. Biol. Chem.* **269**, 3181–3188
- Sakano, S., Takemura, H., Yamada, K., Imoto, K., Kaneko, M., and Ohshika, H. (1996) Ca²⁺ mobilizing action of sphingosine in Jurkat human leukemia T cells. *J. Biol. Chem.* **271**, 11148–11155
- Whitfield, J. F., MacManus, J. P., Boynton, A. L., Durkin, J., and Jones, A. (1982) In *Functional Regulation at the Cellular and Molecular Levels* (Corradino, R. A., ed) pp. 61–87, Elsevier/North Holland, Inc., New York
- Takuwa, N., Zhou, W., and Takuwa, Y. (1995) Calcium, calmodulin and cell cycle progression. *Cell. Signal.* **7**, 93–103
- Prem Veer Reddy, G. (1994) Cell cycle: regulatory events in G₁ → S transition of mammalian cells. *J. Cell. Biochem.* **54**, 379–386
- Deisseroth, K., Bito, H., and Tsien, R. W. (1996) Signaling from synapse to nucleus: postsynaptic CREB phosphorylation during multiple forms of hippocampal synaptic plasticity. *Neuron* **16**, 89–101
- Russo, T., Mogavero, A. R., Ammendola, R., Mesuraca, M., Fiore, F., Fatatis, A., Salvatore, G., and Cimino, F. (1993) Immortalization of a cell line showing some characteristics of the oligodendrocyte phenotype. *Neurosci. Lett.* **159**, 159–162
- Krek, W., and DeCaprio, J. A. (1995) Cell synchronization. *Methods Enzymol.* **254**, 114–124
- Jakóbisziak M., Bruno, S., Skierski, J. S., and Darzynkiewicz, Z. (1991) Cell cycle-specific effects of lovastatin. *Proc. Natl. Acad. Sci. USA* **88**, 3628–3632
- Masamune, A., Igarashi, Y., and Hakomori, S. (1996) Regulatory role of ceramide in interleukin (IL)-1β-induced E-selectin expression in human umbilical vein endothelial cells. *J. Biol. Chem.* **271**, 9368–9375
- Fatatis, A., and Miller, R. J. (1997) PDGF-induced Ca²⁺ signaling in the CG4 oligodendroglial cell line and in transformed oligodendrocytes expressing the β-PDGF receptor. *J. Biol. Chem.* **272**, 4351–4358
- Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**, 680–685
- Olivera, A., and Spiegel, S. (1993) Sphingosine-1-phosphate as second messenger in cell proliferation induced by PDGF and FCS. *Nature (London)* **365**, 557–560
- Hasegawa-Sasaki, H. (1985) Early changes in inositol lipids and their metabolites induced by platelet-derived growth factor in quiescent Swiss mouse 3T3 cells. *Biochem. J.* **232**, 99–109
- Zhang, H., Desai, N. N., Olivera, A., Seki, T., Brooker, G., and Spiegel, S. (1991) Sphingosine-1-phosphate, a novel lipid involved in cellular proliferation. *J. Cell. Biol.* **114**, 155–167
- Fatatis, A., and Miller, R. J. (1996) Sphingosine and sphingosine-1-phosphate differentially modulate PDGF-BB induced Ca²⁺ signaling in transformed oligodendrocytes. *J. Biol. Chem.* **271**, 295–301
- Pardee, A. B., and Keyomarsi, K. (1992) Modification of cell proliferation with inhibitors. *Curr. Opin. Cell Biol.* **4**, 186–191
- Urbani, L., Sherwood, S. W., and Schimke, R. T. (1995) Dissociation of nuclear and cytoplasmic cell cycle progression by drugs employed in cell synchronization. *Exp. Cell. Res.* **219**, 159–168
- Sheaff, R. J., and Roberts, J. M. (1998) Regulation of G₁ phase. *Res. Prob. Cell Differ.* **22**, 1–34

36. Pines, J. (1998) Regulation of the G₂ to M transition. *Res. Prob. Cell Differ.* **22**, 57–78
37. Cossette, L. J., Hoglinger, O., Mou, L., and Shen, S.-H. (1996) Localization and down-regulating role of the protein tyrosine phosphatase PTP2C in membrane ruffles of PDGF-stimulated cells. *Exp. Cell Res.* **223**, 459–466
38. Chen, Z., Niels, H. S., Sun, H., Barbier, A., and Patel, T. B. (1995) Expression on type V adenylyl cyclase is required for epidermal growth factor-mediated stimulation of cAMP accumulation. *J. Biol. Chem.* **270**, 27525–27530
39. Bennet, A. M., Hausdorff, S. F., O'Reilly, A. M., Freeman, R. M., Jr., and Neel, B. G. (1996) Multiple requirements for SHPTP2 in epidermal growth factor-mediated cell cycle progression. *Mol. Cell. Biol.* **16**, 1189–1202
40. Vaziri, C., and Faller, D. V. (1995) Repression of platelet-derived growth factor β -receptor expression by mitogenic growth factors and transforming oncogenes in murine 3T3 fibroblasts. *Mol. Cell. Biol.* **15**, 1244–1253
41. Vaziri, C., and Faller, D. V. (1996) Down-regulation of platelet-derived growth factor receptor expression during terminal differentiation of 3T3-L1 pre-adipocyte fibroblasts. *J. Biol. Chem.* **271**, 13642–13648
42. Ghosh, T. K., Bian, J., and Gill, D. L. (1990) Intracellular calcium release mediated by sphingosine derivatives generated in cells. *Science* **248**, 1653–1656
43. Mattie, M., Brooker, G., and Spiegel, S. (1994) Sphingosine-1-phosphate, a putative second messenger, mobilizes calcium from internal stores via an inositol trisphosphate-independent pathway. *J. Biol. Chem.* **269**, 3181–3188
44. Olivera, A., Kohama, T., Tu, Z., Milstien, S., and Spiegel, S. (1998) Purification and characterization of rat kidney sphingosine kinase. *J. Biol. Chem.* **273**, 12576–12583
45. Igarashi, Y., Hakomori, S., Toyokuni, T., Dean, B., Fujita, S., Sugimoto, M., Ogawa, T., El-Ghendy, K., and Racker, E. (1989) Effect of chemically well-defined sphingosine and its N-methyl derivatives on protein kinase C and src kinase activities. *Biochemistry* **28**, 6796–6800
46. Kiss, Z., Phillips, H., and Anderson, W. H. (1995) The bisindolylmaleimide GF 109203X, a selective inhibitor of protein kinase C, does not inhibit the potentiating effect of phorbol ester on ethanol-induced phospholipase C-mediated hydrolysis of phosphatidylethanolamine *Biochim. Biophys. Acta* **1265**, 93–95
47. Edsall, L. C., Van Brocklyn, J. R., Cuvillier, O., and Spiegel, S. (1998) N,N-dimethylsphingosine is a potent competitive inhibitor of sphingosine kinase but not protein kinase C: modulation of cellular levels of sphingosine 1-phosphate and ceramide. *Biochemistry* **37**, 12892–12898
48. Miyakawa, T., Maeda, A., Yamazawa, T., Hirose, K., Kurosaki, T., and Lino, M. (1999) Encoding of Ca²⁺ signals by differential expression of IP₃ receptor subtypes. *EMBO J.* **18**, 1303–1308
49. Sheela Rani, C. S., Wang, F., Fuior, E., Berger, A., Wu, J., Sturgill, T. W., Beitner-Johnson, D., LeRoith, D., Varticovski, L., and Spiegel, S. (1997) Divergence in signal transduction pathways of platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) receptors. *J. Biol. Chem.* **272**, 10777–10783
50. Meyer zu Heringdorf, D., Lass, H., Alemany, R., Laser, K. T., Neumann, E., Zhang, C., Schmidt, M., Rauen, U., Jakobs, K. H., and van Koppen, C. J. (1998) Sphingosine kinase-mediated Ca²⁺ signalling by G-protein coupled receptors. *EMBO J.* **17**, 2830–2837
51. Alemany, R., Meyer zu Heringdorf, D., van Koppen C. J., and Jakobs, K. H. (1999) Formyl peptide receptor signaling in HL-60 cells through sphingosine kinase. *J. Biol. Chem.* **272**, 3994–3999
52. Thomas, A. P., Bird, G. J., Hajnóczky, G., Robb-Gasper, L. D., and Putney, J. W., Jr. (1996) Spatial and temporal aspects of cellular calcium signaling. *FASEB J.* **10**, 1505–1517
53. Berridge, M. J. (1997) The AM and FM of calcium signaling. *Nature (London)* **386**, 759–760
54. Li, W., Liopis, J., Whitney, M., Zlokarnik, G., and Tsien, R. Y. (1998) Cell-permeant caged InsP₃ ester shows that Ca²⁺ spike frequency can optimize gene expression. *Nature (London)* **392**, 936–941
55. Cuvillier, O., Pirianov, G., Kleuser, B., Vanek, P. G., Coso, O. A., Gutkind, J. S., and Spiegel, S. (1996) Suppression of ceramide-mediated programmed cell death by sphingosine-1-phosphate. *Nature (London)* **381**, 800–803

Received for publication June 23, 1998.
Revised for publication March 15, 1999.