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*Original Citation:*

The mitogenic effect of platelet-derived growth factor in human hepatic stellate cells requires calcium influx / FAILLI P; RUOCCO C; DE FRANCO R; CALIGIURI A; GENTILINI A; GENTILINI P; GIOTTI A; M. PINZANI. - In: AMERICAN JOURNAL OF PHYSIOLOGY. CELL PHYSIOLOGY. - ISSN 0363-6143. - ELETTRONICO. - 269:(1995), pp. C1133-C1139.

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# The mitogenic effect of platelet-derived growth factor in human hepatic stellate cells requires calcium influx

PAOLA FAILLI, CARLO RUOCCO, RAFFAELLA DE FRANCO, ALESSANDRA CALIGIURI, ALESSANDRA GENTILINI, ALBERTO GIOTTI, PAOLO GENTILINI, AND MASSIMO PINZANI  
*Centro Interuniversitario Ipossie, Dipartimento di Farmacologia, and Istituto di Medicina Interna-Centro Interuniversitario di Fisiopatologia Epatica, Università di Firenze, I-50134 Florence, Italy*

**Failli, Paola, Carlo Ruocco, Raffaella De Franco, Alessandra Caligiuri, Alessandra Gentilini, Alberto Giotti, Paolo Gentilini, and Massimo Pinzani.** The mitogenic effect of platelet-derived growth factor in human hepatic stellate cells requires calcium influx. *Am. J. Physiol.* 269 (*Cell Physiol.* 38): C1133–C1139, 1995.— Platelet-derived growth factor (PDGF) is a key mitogen for hepatic stellate cells (HSC) and has been shown to be implicated in liver tissue repair and fibrogenesis. In this study the relationship between PDGF-induced intracellular  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ) increase and mitogenesis in cultured human HSC was evaluated. In high-density cell cultures (80–90% subconfluence), PDGF induced a significant increase in  $[\text{Ca}^{2+}]_i$ , characterized by a short-lasting peak phase, which was followed by a long-lasting plateau phase. The plateau phase was abolished in the absence of extracellular  $\text{Ca}^{2+}$ . However, in low-density cell cultures (30–40% subconfluence), the plateau phase was absent or markedly less pronounced. In parallel sets of experiments, PDGF was significantly less effective in inducing mitogenesis in low-density cell cultures than in high-density cell cultures and was totally ineffective in the absence of extracellular  $\text{Ca}^{2+}$ . These results suggest that 1) spatial and time dynamics of PDGF-induced  $[\text{Ca}^{2+}]_i$  increase are dependent on cell density and 2) PDGF-induced mitogenesis requires extracellular  $\text{Ca}^{2+}$  influx.

intracellular calcium; image analysis; thymidine incorporation; cell density; mitogenesis; hepatic fibrogenesis

HEPATIC STELLATE CELLS (HSC), also referred to as Ito cells, fat-storing cells, or lipocytes, have been shown to play a key role in the development of liver fibrosis (5, 9–11). Among other growth factors potentially involved in chronic tissue inflammation, platelet-derived growth factor (PDGF) is the most potent mitogen for HSC isolated from rat or mouse liver and maintained in culture (13, 16). The importance of these in vitro observations is confirmed by the recent demonstration of a marked upregulation of both PDGF-B chain and PDGF-receptor  $\beta$ -subunit mRNAs in rat liver tissue, following a  $\text{CCl}_4$ -induced acute injury (19), and in cirrhotic human liver (18).

In rat, as well as in human HSC, all PDGF dimeric forms (AA, AB, and BB) are able to induce mitogenesis, PDGF-BB being the most potent isoform (17). In agreement with the results of studies performed in other cell types responsive to PDGF (20), the mitogenic effect of each PDGF dimeric form appears to be proportionally correlated to the effect of early signal transduction pathways such as phosphoinositide turnover and intracellular  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ) release (17). However, as a consequence of the elucidation of the structure

and function of the PDGF receptor tyrosine kinase domain (1), the possible direct relationship between PDGF-induced increase in  $[\text{Ca}^{2+}]_i$  and mitogenesis began to be questioned. Accordingly, studies performed in aortic smooth muscle cells showed that elevation of  $[\text{Ca}^{2+}]_i$  does not appear to be a prerequisite condition for PDGF to induce *c-fos* and *c-myc* mRNA expression (12).

More recently, several studies have demonstrated that the induction of replicative competence by PDGF is dependent on the maintenance of a sustained increase in  $[\text{Ca}^{2+}]_i$  due to  $\text{Ca}^{2+}$  entry rather than to the release from intracellular stores (3, 4, 8, 21). Extracellular  $\text{Ca}^{2+}$  entry induced by PDGF is related to the opening of low threshold voltage-gated  $\text{Ca}^{2+}$  channels consistent with “T” type designation, whose expression appears to be dependent on cell density in culture (4, 21).

In the present study we have evaluated the temporal and spatial dynamics of PDGF-BB-induced intracellular  $\text{Ca}^{2+}$  transients in cultured human HSC plated at different densities and their relationship with PDGF-induced mitogenesis.

## METHODS

Low osmolality stractan (Larex-LO) was provided by Larex International (Tacoma, WA). Plastic culture dishes were purchased from Falcon, Becton Dickinson (Lincoln Park, NJ). Cell culture media were provided by GIBCO Laboratories (Grand Island, NY), and fetal bovine serum (FBS) from Eurobio (Les Ulis, France). [*Methyl*- $^3\text{H}$ ]thymidine ( $^3\text{H}$ ]TdR, 6.7 Ci/mmol) was purchased from New England Nuclear (Boston, MA).  $\gamma$ -Globulin-free and fatty acid-free bovine serum albumin was obtained from Sigma Chemical (St. Louis, MO). Recombinant human PDGF-BB produced in *Escherichia coli* and purified to homogeneity was purchased from Boehringer Mannheim (Mannheim, Germany). Fura 2-acetoxymethyl ester (AM) and Pluronic F-127 were provided by Molecular Probes (Eugene, OR). All other reagents were of analytic grade.

**Isolation and culture of human HSC.** Human HSC were isolated from wedge sections of normal human liver unsuitable for transplantation, as previously reported (2, 14). Briefly, after a combined digestion with collagenase-pronase, HSC were separated from other liver nonparenchymal cells by ultracentrifugation over gradients of stractan (6). Extensive characterization was performed as described elsewhere (2). Cells were cultured on plastic in Iscove’s modified Dulbecco’s medium supplemented with 0.6 U/ml insulin, 2.0 mmol/l glutamine, 0.1 mmol/l nonessential amino acids, 1.0 mmol/l sodium pyruvate, antibiotic antifungal solution, and 20% FBS. Experiments described in this study were performed on cells between the first and fourth serial passages using three independent cell lines.

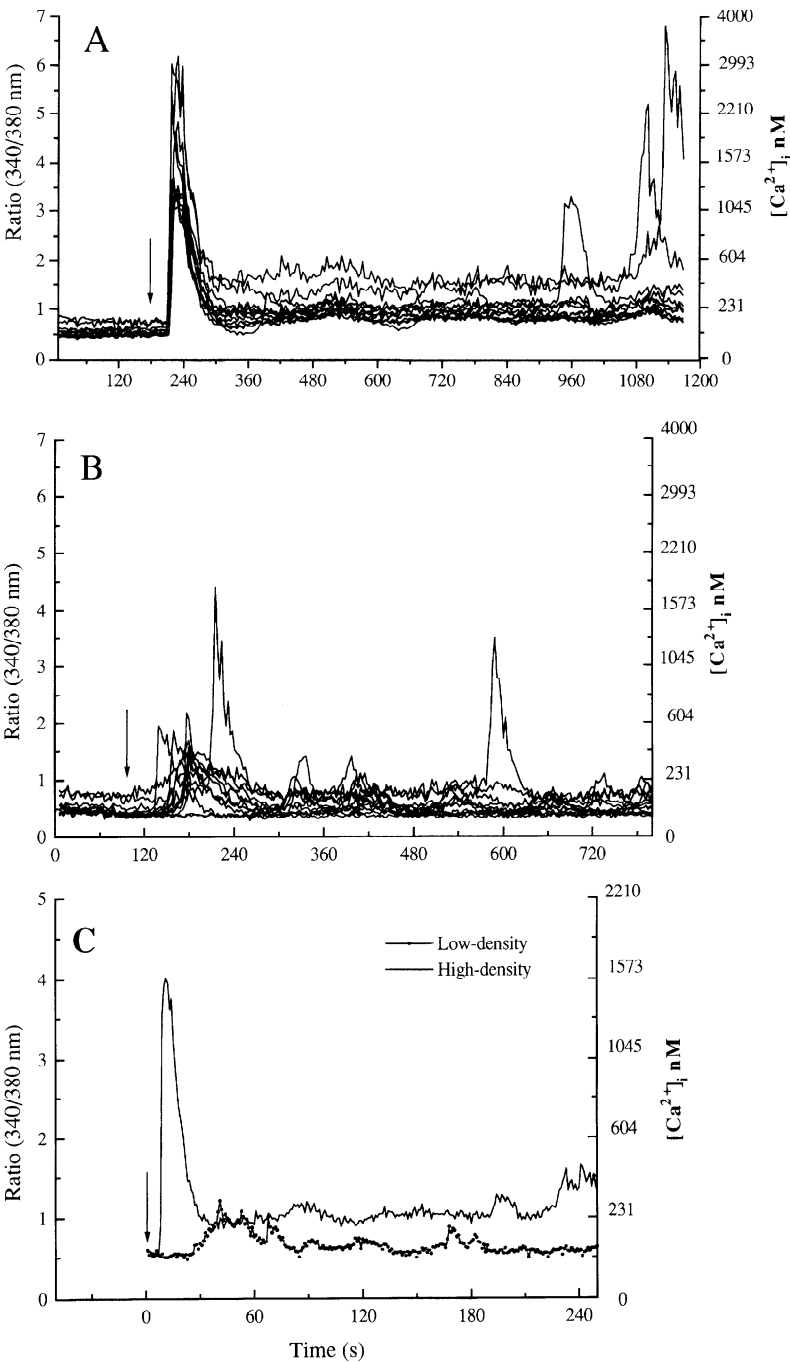


Fig. 1. Effect of platelet-derived growth factor (PDGF)-BB on intracellular  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ) in high- (A) and low-density (B) human hepatic stellate cell (HSC) cultures in the presence of extracellular  $\text{Ca}^{2+}$  (1 mM  $\text{CaCl}_2$ ). PDGF-BB (10 ng/ml) was added at the arrow. Each trace is the single response measured in individual cell loaded with fura 2-acetoxymethyl ester (AM) ( $n = 12$ ). C: mean of the analyzed cells.

Digital video imaging of intracellular free  $\text{Ca}^{2+}$  in individual human HSC. Digital video imaging of  $[\text{Ca}^{2+}]_i$  in individual human HSC was performed as described (7, 14). Cells were grown on round glass coverslips (25 mm diameter, 0.2 mm thick) in complete culture medium and used at two different degrees of confluence: 1) high-density cell cultures (80–90% confluency,  $1.4\text{--}1.6 \times 10^5$  cells) and 2) low-density cell cultures (30–40% confluency,  $3\text{--}5 \times 10^4$  cells). After incubation in serum-free/insulin-free (SFIF) medium for 48 h, cells were incubated with 10  $\mu\text{M}$  fura 2-AM and 15% Pluronic F-127 for 30 min at 22°C.  $[\text{Ca}^{2+}]_i$  was measured in fura 2-loaded cells in *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)- $\text{NaHCO}_3$  buffer containing 140 mM NaCl, 3 mM KCl, 0.5 mM  $\text{NaH}_2\text{PO}_4$ , 12 mM  $\text{NaHCO}_3$ , 1.2 mM  $\text{MgCl}_2$ , 1 mM  $\text{CaCl}_2$ , 10 mM HEPES, and 10 mM glucose, pH 7.4. For experiments performed in the absence of extracellular  $\text{Ca}^{2+}$ , 0.5 mM  $\text{Na}_2\text{EDTA}$  was added to nominally  $\text{Ca}^{2+}$ -free HEPES-

$\text{NaHCO}_3$  buffer. Ratio images (340/380 nm) were collected every 3 s; calibration curves were performed as described (7, 14).

**DNA synthesis.** DNA synthesis was measured as the amount of [*methyl*- $^3\text{H}$ ]thymidine ( $^3\text{H}$ ]TdR) incorporated into trichlo-

Table 1. Effect of PDGF-BB on  $[\text{Ca}^{2+}]_i$  in high-density cell cultures of human hepatic stellate cells

Dose, ng/ml	Basal $[\text{Ca}^{2+}]_i$ , nM	Peak Increase	Plateau Phase	<i>n</i>
5	$82 \pm 46$	$9.6 \pm 2.01$	$2.1 \pm 0.49$	20
10	$85 \pm 42$	$18.3 \pm 3.01$	$2.6 \pm 0.28$	12
20	$80 \pm 47$	$8.8 \pm 3.34$	$2.2 \pm 0.45$	17

Values are means  $\pm$  SD; *n*, number of analyzed cells. Peak increase and plateau phase are fold increase from basal values. PDGF, platelet-derived growth factor;  $[\text{Ca}^{2+}]_i$ , intracellular  $\text{Ca}^{2+}$  concentration.

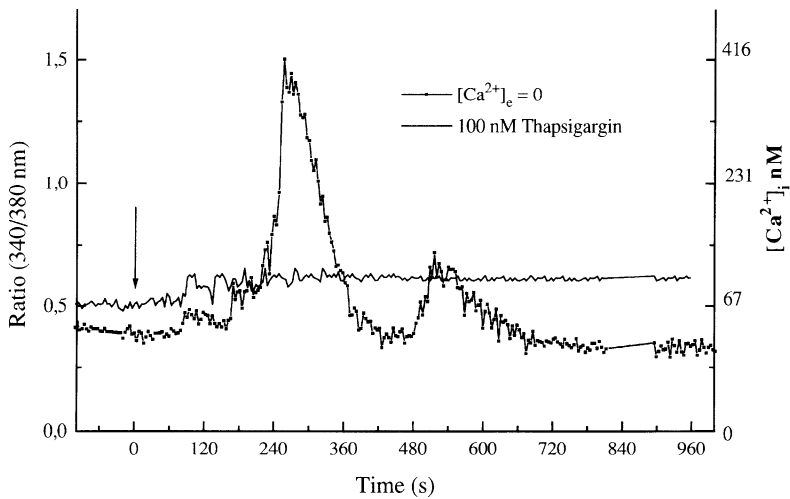


Fig. 2. Effect of PDGF-BB on  $[Ca^{2+}]_i$  in high-density cultures of human HSC in the absence of extracellular  $Ca^{2+}$  [0.5 mM  $Na_2EDTA$ , extracellular  $Ca^{2+}$  concentration ( $[Ca^{2+}]_e = 0$ ) and after a 10-min preincubation with 100 nM thapsigargin. PDGF-BB (10 ng/ml) was added at the arrow (time = 0). Traces are the mean of at least 10 single responses measured in individual fura 2-AM-loaded HSC.

roacetic acid-precipitable material. Cells were plated in 24-well dishes at a density of  $2 \times 10^4$  cells/well in complete culture medium containing 20% FBS. Cells were then allowed to reach 80–90% confluency (high-density cell cultures:  $9 \times 10^4$ – $1 \times 10^5$  cells/well) and were then made quiescent by incubation in SFIF medium for 48 h. In experiments aimed at establishing the mitogenic potential of PDGF-BB on low-density HSC cultures, cells were plated at the same starting density ( $2 \times 10^4$  cells/well), maintained in complete culture medium for 24 h, and then incubated in SFIF medium for an additional 48 h. At this stage of culture, the number of cells per well was on average  $3 \times 10^4$  (30–40% confluency). In experiments investigating the time-course kinetics of PDGF-induced  $[^3H]TdR$  uptake, both low- and high-density cell cultures were pulsed for 12, 24, 36, and 48 h with 1.0  $\mu Ci/ml$   $[^3H]TdR$ , whereas in other experiments cells were incubated with or without PDGF for 20 h and then pulsed for an additional 4 h with  $[^3H]TdR$ . At the end of the pulsing period  $[^3H]TdR$  incorporation into cellular DNA was measured as previously reported (16). Cell number was determined in three separate wells from each dish after trypsinization and counting. Results were always normalized as counts per minute (cpm) per  $10^5$  cells. In experiments requiring 24 h incubation in the absence of extracellular  $Ca^{2+}$  ( $EDTA$ ), the percentage of detached cells at the end of the incubation period, although slightly higher, was not statistically different from that observed in cells maintained with a normal concentration of extracellular  $Ca^{2+}$  in both low- and high-density cell cultures (low-density: no

$EDTA$  0.85%,  $EDTA$  0.90%; high-density: no  $EDTA$  1.0%,  $EDTA$  1.2%).

**Statistical analysis.** All values are means  $\pm$  SD of the indicated number of experiments. Values were compared using Student's *t*-test for paired and unpaired data or analysis of variance.  $P < 0.05$  was considered statistically significant.

## RESULTS

$[Ca^{2+}]_i$  in unstimulated human HSC ranged from 25 to 180 nM (mean  $86 \pm 53$  nM,  $n = 135$ ). Figure 1A shows the typical time course effect of 10 ng/ml of PDGF-BB on 12 different cells grown until high density on coverglass. In this experiment, the basal  $[Ca^{2+}]_i$  was  $81 \pm 34$  nM. As shown, an earlier cell synchronous  $Ca^{2+}$  spike occurred (mean  $[Ca^{2+}]_i$  at the peak:  $1,576 \pm 450$  nM,  $n = 12$ ) followed by a long-lasting plateau (mean  $[Ca^{2+}]_i$ :  $227 \pm 92$  nM,  $n = 12$ ). The  $[Ca^{2+}]_i$  during the plateau phase (calculated for each cell as the mean of deeper points of  $[Ca^{2+}]_i$  oscillations) was significantly higher than the basal value ( $P < 0.05$ , paired data). In several cells, slow  $[Ca^{2+}]_i$  oscillations were superimposed to the plateau phase.

In other similar experiments, the time course characteristics of the increase in  $[Ca^{2+}]_i$  induced by PDGF-BB were always confirmed, although the maximum of the

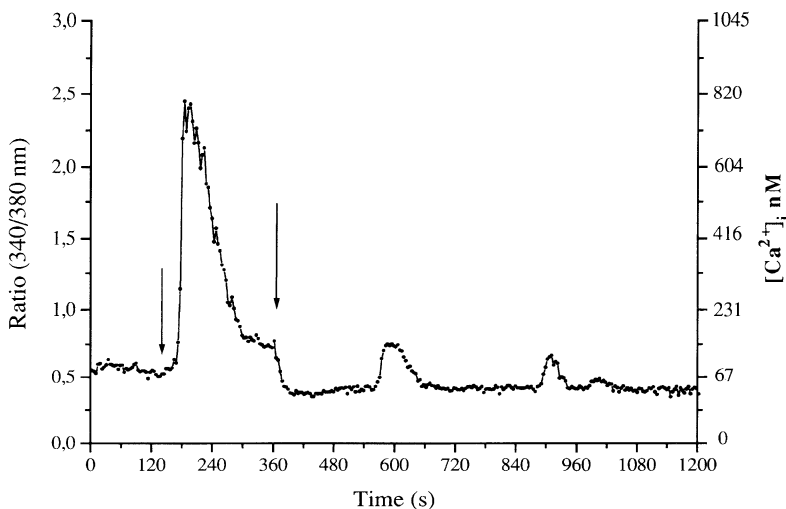


Fig. 3. Effect of  $Na_2EDTA$  on PDGF-BB-induced  $[Ca^{2+}]_i$  increase in high-density cultures of human HSC. PDGF-BB (10 ng/ml) was added at the first arrow in the presence of extracellular  $Ca^{2+}$  (1 mM  $CaCl_2$ ).  $Na_2EDTA$  (1.5 mM) was added at the second arrow. The trace is the mean of 6 single responses measured in individual fura 2-AM-loaded HSC.

peak increase occurred within a wider range (from 80 to 3,150 nM, mean  $808 \pm 614$  nM,  $n = 46$ ); the plateau phase of the increase in  $[Ca^{2+}]_i$  showed a more uniform behavior, reaching a steady-state value of about two- to threefold the unstimulated  $[Ca^{2+}]_i$  value. The first phase of the increase in  $[Ca^{2+}]_i$  was characterized by a lag time of  $\sim 30$  s. In other experiments longer lag phases were also recorded, although cells still showed an earlier synchronized  $[Ca^{2+}]_i$  spike. Interestingly, the plateau increase in  $[Ca^{2+}]_i$  persisted for at least 8 h after the administration of 10 ng/ml PDGF-BB ( $239 \pm 37$  nM,  $n = 84$ , vs. basal levels  $74 \pm 19$  nM,  $n = 84$ ;  $P < 0.001$ , unpaired data).

In high-density cell cultures, PDGF-BB at both a 20 and a 5-ng/ml dose induced an increase in  $[Ca^{2+}]_i$  characterized by a peak phase, which was followed by a plateau phase (Table 1). However, at 5 ng/ml a higher incidence of  $[Ca^{2+}]_i$  oscillations was observed (from 3 to 9 oscillations/10-min period in 50% of analyzed cells).

In low-density cell cultures, 10 ng/ml PDGF-BB induced a different pattern in the time-course of the increase in  $[Ca^{2+}]_i$ : 1) the earlier, rapid phase was not synchronously recorded in all the analyzed cells (time to the maximum  $120 \pm 42$  s,  $n = 12$ , ranging from 67 and 208 s); 2) lag times of the different cells were longer (up to 3 min); 3) the plateau phase was absent or markedly less pronounced, whereas  $Ca^{2+}$  oscillations were still observed (Fig. 1B). In these experiments, the basal and poststimulation  $[Ca^{2+}]_i$  were  $53 \pm 44$  and  $71 \pm 42$  nM, respectively ( $n = 21$ ,  $P = 0.064$ , not significant, paired data). The effects of 10 ng/ml PDGF-BB in low- and high-density cell cultures are compared in Fig. 1C (representative experiments with typical characteristics). Because the peak increase in  $[Ca^{2+}]_i$  in low-density cell cultures was recorded at different times, the time-course profile (mean of the analyzed cells) was markedly different from that observed in high-density cultures.

In high-density cultures, PDGF-BB (10 ng/ml) was still active in increasing  $[Ca^{2+}]_i$  in the absence of external  $Ca^{2+}$  (Fig. 2). Although reduced, the peak phase of  $[Ca^{2+}]_i$  was still present, whereas the plateau phase that followed the peak reached a  $[Ca^{2+}]_i$  that was lower than baseline values ( $20 \pm 23$  and  $31 \pm 26$  nM, respectively,  $P < 0.01$ , paired data). The plateau phase was also abolished when 1.5 mM  $Na_2EDTA$  was added 3 min after 10 ng/ml PDGF-BB (Fig. 3). As clearly shown in Fig. 3, the addition of  $Na_2EDTA$  reduced the  $[Ca^{2+}]_i$  to a value that was lower than the baseline value ( $66 \pm 33$  and  $37 \pm 24$  nM, respectively,  $P < 0.05$ , paired data). The absence of extracellular  $Ca^{2+}$  did not abolish  $Ca^{2+}$  oscillations, suggesting that an internal  $Ca^{2+}$  release accounted for this feature. In addition, the overall contribution of internal  $Ca^{2+}$  pools in the  $Ca^{2+}$  signal induced by PDGF-BB was also analyzed using the sarcoendoplasmic  $Ca^{2+}$  adenosinetriphosphatase inhibitor thapsigargin; preincubation with 100 nM thapsigargin abolished the increase in  $[Ca^{2+}]_i$  induced by PDGF-BB (Fig. 2).

Because of the differences in the pattern of the PDGF-BB-induced  $[Ca^{2+}]_i$  increase between high- and low-density cell cultures, we evaluated whether

PDGF-BB elicited its mitogenic potential to a different extent in the two types of cell culture densities. Figure 4 summarizes the time-course and dose-response characteristics of the mitogenic effect of PDGF-BB, evaluated as  $[^3H]TdR$  incorporation into cellular DNA, in low- and high-density HSC cultures. As shown in Fig. 4A, 10 ng/ml PDGF-BB induced a progressive increase in  $[^3H]TdR$  incorporation, reaching a peak effect after 24 h in both high- and low-density cell cultures. However, PDGF-induced mitogenesis in high-density cell cultures was significantly higher than that observed in low-density cell cultures at any time point studied beyond 12 h ( $P < 0.001$ , unpaired data). The lower responsiveness

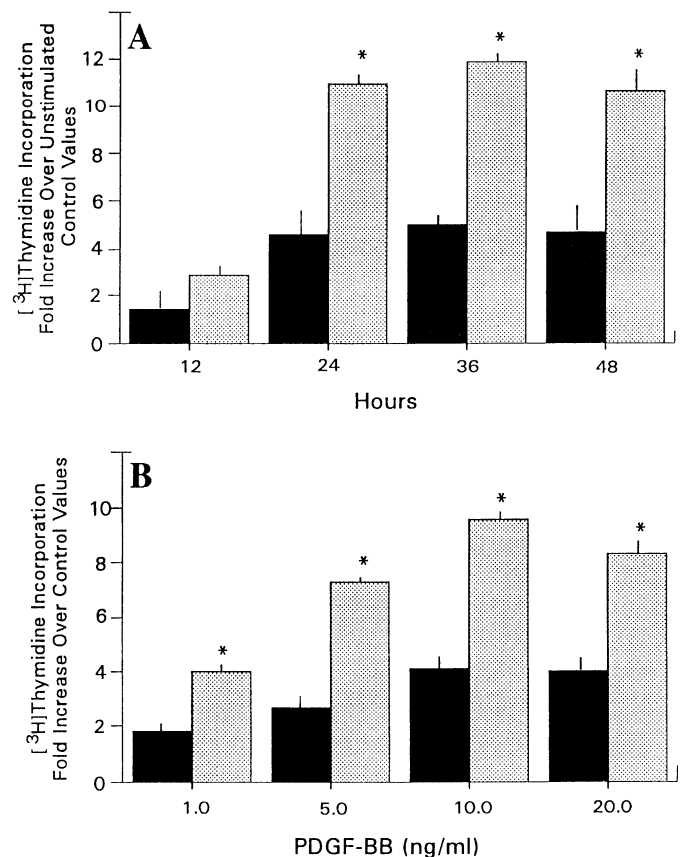


Fig. 4. Mitogenic effect of PDGF-BB in low- (black bars) and high-density (gray bars) HSC cultures. A: time course for the effect of PDGF-BB (10 ng/ml). Cells at the required densities were incubated in serum-free/insulin-free medium for 48 h and then pulsed with 1  $\mu$ Ci/ml [*methyl- $^3$ H*]thymidine ( $[^3H]TdR$ ) in the presence or absence (unstimulated control) of PDGF for the indicated time points. Data (means  $\pm$  SD), relative to 3 experiments performed in triplicate, are expressed as fold increase over unstimulated control values obtained at each time point. For each point considered, fold increase was calculated after normalization of the data as counts per minute (cpm)/ $10^5$  cells. \* $P < 0.001$  vs. fold increase in low-density cells. B: dose-response for the effect of PDGF-BB. After 48 h incubation in serum-free/insulin-free medium, cells were stimulated with increasing doses of PDGF-BB for a total of 24 h and pulsed with  $[^3H]TdR$  during the last 4 h of incubation. Each bar is the mean  $\pm$  SD of 4 experiments performed in triplicate and expressed as fold increase over absolute control values. For each point considered, fold increase was calculated after normalization of the data as cpm/ $10^5$  cells. In this series of experiments, absolute control values were low density,  $179 \pm 37$  cpm/ $10^5$  cells; high-density,  $255 \pm 40$  cpm/ $10^5$  cells. \* $P < 0.001$  vs. fold increase in low-density cells (analysis of variance).

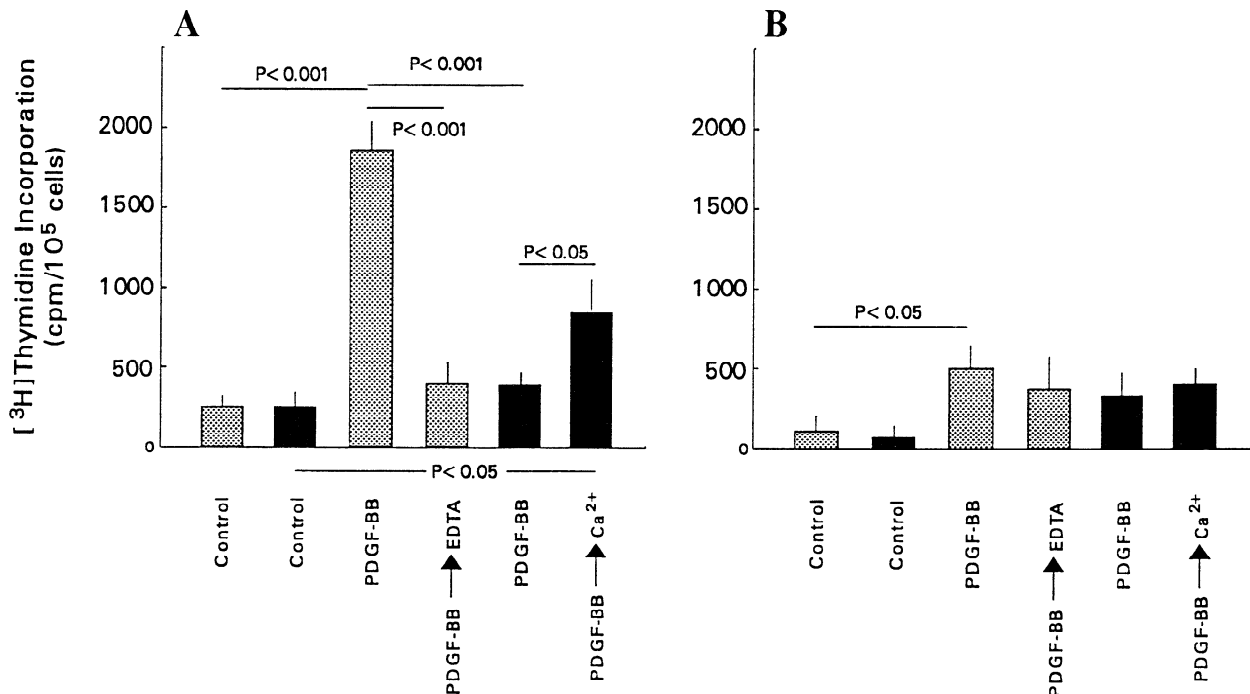


Fig. 5. Mitogenic effect of PDGF-BB in high- and low-density HSC cultures in the presence of extracellular  $\text{Ca}^{2+}$  and in its absence. *A*: high-density cell cultures. *B*: low-density cell cultures. Cells at the required densities were incubated for 48 h in serum-free/insulin-free medium and then stimulated with PDGF-BB (10 ng/ml) for 24 h. Cells were pulsed with [ $^3\text{H}$ ]TdR during the last 4 h of incubation. Experiments were performed in the presence of 1 mM extracellular  $\text{Ca}^{2+}$  (gray bars) or in its absence (EDTA, black bars).  $\text{Na}_2\text{EDTA}$ , 2 mM (bar 4 from left), and  $\text{CaCl}_2$ , 2 mM (bar 6 from left), were added 30 min after PDGF. Each bar is the mean  $\pm$  SD of experiments performed in triplicate (high density, 5 experiments; low density, 3 experiments).

of low-density cell cultures was confirmed in dose-response experiments (Fig. 4*B*), in which, at each dose tested, PDGF-BB induced a significantly higher response in high-density cell cultures ( $P < 0.001$ , analysis of variance), with 10 ng/ml being the most effective dose for both culture conditions. In these experiments, absolute control levels of [ $^3\text{H}$ ]TdR uptake in low- and high-density cell cultures were not statistically different, although a trend to a lower uptake was observed in low-density cultures (means  $\pm$  SD, low-density  $179 \pm 37$  cpm/ $10^5$  cells, high-density  $255 \pm 45$  cpm/ $10^5$  cells).

The main difference in the pattern of the PDGF-induced  $[\text{Ca}^{2+}]_i$  increase between the two types of cell culture densities was the occurrence of the plateau phase. Accordingly, further experiments were specifically designed to assess the role of  $\text{Ca}^{2+}$  influx from the extracellular medium in PDGF-induced mitogenesis. As shown in Fig. 5*A*, the addition of  $\text{Na}_2\text{EDTA}$ , both before and 30 min after PDGF-BB, abolished the increase in [ $^3\text{H}$ ]TdR incorporation, whereas the restitution of  $\text{Ca}^{2+}$  in the external medium after the administration of PDGF-BB (administered in the presence of 0.5 mM  $\text{Na}_2\text{EDTA}$ ) partially restored the proliferative effect of PDGF-BB. As illustrated in Fig. 5*B*, PDGF-BB-induced [ $^3\text{H}$ ]TdR uptake was not significantly affected by the addition of  $\text{Na}_2\text{EDTA}$  in low-density cell cultures, either before or after PDGF-BB. In addition, restitution of  $\text{Ca}^{2+}$  to the external medium did not even partially restore the mitogenic response to PDGF.

## DISCUSSION

The results of the present study provide evidence that induction of replicative competence by PDGF-BB in human HSC is dependent on the maintenance of elevated and sustained  $[\text{Ca}^{2+}]_i$  levels due to extracellular  $\text{Ca}^{2+}$  entry. In addition, PDGF-BB-induced mitogenesis appears to be strongly influenced by cell density.

As already demonstrated in rat HSC (17) and in other potentially related cell types (15), the increase in  $[\text{Ca}^{2+}]_i$  induced by PDGF-BB in human HSC is characterized by two main components: 1) a consistent and transient increase (peak increase), likely related to  $\text{Ca}^{2+}$  release from internal stores (probably the endoplasmic reticulum), and 2) a lower but longer lasting increase (plateau phase) due to an influx from the external medium.

The temporal and spatial dynamics of  $[\text{Ca}^{2+}]_i$  increase induced by PDGF-BB were significantly affected by the degree of cell density. Indeed, in contrast to what was observed in low-density cell cultures, the PDGF-induced increase in  $[\text{Ca}^{2+}]_i$  in high-density cultures was characterized by a shorter lag time and a synchronous  $\text{Ca}^{2+}$  spike constantly followed by a long-lasting plateau phase, this latter being the major distinctive element. In recent studies, Estacion and Mordan (4, 21) have clearly shown that the extent and duration of the plateau phase induced by PDGF are related to the opening of "T" type  $\text{Ca}^{2+}$  channels and that, importantly, the expression of these channels appears to be dependent on cell density.

The results of our experiments investigating PDGF-induced mitogenesis in high-density cell cultures strongly suggest that this effect is largely dependent on the maintenance of a sustained influx of extracellular  $\text{Ca}^{2+}$ . This possibility is further suggested by the good correlation existing between the dose response of PDGF as  $\text{Ca}^{2+}$ -mobilizing agonist and mitogen (Table 1, and Fig. 4B) and by the observation that PDGF-induced mitogenesis was abrogated not only when cells were stimulated in the virtual absence of extracellular  $\text{Ca}^{2+}$  but also when  $\text{Ca}^{2+}$  was removed from the external medium 30 min after stimulation. Interestingly, in these latter conditions, the sustained  $[\text{Ca}^{2+}]_i$  increase was abolished, reaching values of  $[\text{Ca}^{2+}]_i$  lower than those preceding PDGF stimulation. This observation suggests the existence of an outward  $\text{Ca}^{2+}$  driving force possibly generated by the gradient in  $\text{Ca}^{2+}$  concentration existing between the cytosol and the external medium, communicating through the opening of membrane channels. The occurrence of this phenomenon may explain, at least in part, the complete abrogation of the PDGF-BB-induced mitogenic effect in the absence of extracellular  $\text{Ca}^{2+}$  and its partial recovery when a physiological concentration of  $\text{Ca}^{2+}$  in the extracellular medium is restored.

The role of the plateau phase of the  $\text{Ca}^{2+}$  signal (lasting at least 8 h after stimulation) in PDGF-induced mitogenesis is also supported by the results of experiments performed in low-density cell cultures of human HSC, in which PDGF is ineffective in inducing this sustained increase and is significantly less effective in inducing DNA synthesis. In addition, in these culture conditions, the mitogenic effect of PDGF is unaffected by changes in the concentration of extracellular  $\text{Ca}^{2+}$ . Therefore, it is possible that, after stimulation with PDGF-BB, high- and low-density cell cultures behave differently because of different possibilities to exchange  $\text{Ca}^{2+}$  with the external medium through membrane channels, probably linked to different "phenotypical" expression of  $\text{Ca}^{2+}$  channels. An alternative explanation could be that, in low-density cell cultures, PDGF receptors are "downregulated," i.e., lower in number and/or with reduced affinity for the ligand. However, this possibility is not in agreement with results obtained in our laboratory and published in part (15), indicating that at cell densities between 40 and 70% ( $4-8 \times 10^4$  cells), there are no significant differences in the number of PDGF-BB binding sites (on average 75,000/cell) and their affinity [on average dissociation constant ( $K_d$ ) =  $3.3\text{E-}10$ ]. For cell densities higher than 70%, a trend toward a decrease of both binding sites and affinity is commonly observed, with a further decrease in density-arrested cells (on average 55,000 binding sites,  $K_d = 2.3\text{E-}10$ ).

In summary, our results suggest that the plateau phase of the increase in  $[\text{Ca}^{2+}]_i$  is essential for eliciting full PDGF-induced replicative competence in human HSC. In low-density cell cultures this mechanism appears to be hampered by the impossibility of inducing an ordered and uniform response in terms of sustained  $[\text{Ca}^{2+}]_i$  signal. Interestingly, these differences in the time-course kinetics of  $[\text{Ca}^{2+}]_i$  between low- and high-

density HSC cultures were not observed after stimulation with agonists operating through a G protein-coupled receptor, namely, thrombin, angiotensin II, and endothelin I (14). Given the complex cascade of intracellular signaling events and cytoskeletal rearrangements after stimulation with PDGF-BB and leading to cell proliferation, further studies are needed to clarify at which level(s) the sustained  $[\text{Ca}^{2+}]_i$  increase could play a functional role.

We thank Chiara Sali for expert technical assistance.

This work was supported by grants from Ministero dell'Università e della Ricerca Scientifica e Tecnologica-Progetto Nazionale Cirrosi Epatica ed Epatiti Virali (Rome, Italy) and Consiglio Nazionale delle Ricerche-Progetto Finalizzato Aging PF Aging 951612 (Rome, Italy). Financial support was also provided by Fondazione Italiana per lo Studio del Fegato (Italian Liver Foundation, Florence, Italy).

Address for reprint requests: P. Failli, Centro Interuniversitario Ipossie, Dipartimento di Farmacologia, Viale G. B. Morgagni, 65, I-50134 Florence, Italy.

Received 27 October 1994; accepted in final form 31 May 1995.

## REFERENCES

1. Cadena, D. L., and G. N. Gill. Receptor tyrosine kinases. *FASEB J.* 6: 2332–2337, 1992.
2. Casini, A., M. Pinzani, S. Milani, C. Grappone, G. Galli, A. M. Jezequel, D. Schuppan, C. M. Rotella, and C. Surrenti. Regulation of transforming growth factor- $\beta$ 1 in human fat-storing cells. *Gastroenterology* 105: 245–253, 1993.
3. Estacion, M., and L. J. Mordan. Expression of voltage-gated calcium channels correlates with PDGF-stimulated calcium influx and depends upon cell density in C3H 10T1/2 mouse fibroblasts. *Cell Calcium* 14: 161–171, 1993.
4. Estacion, M., and L. J. Mordan. Competence induction by PDGF requires sustained calcium influx by a mechanism distinct from storage-dependent calcium influx. *Cell Calcium* 14: 439–454, 1993.
5. Friedman, S. L. The cellular basis of hepatic fibrosis. Mechanisms and treatment strategies. *N. Engl. J. Med.* 328: 1828–1835, 1993.
6. Friedman, S. L., and F. J. Roll. Isolation and culture of hepatic lipocytes, Kupffer cells and sinusoidal endothelial cells by density gradient centrifugation with stractan. *Anal. Biochem.* 161: 207–218, 1987.
7. Grynkiewicz, G., M. Poenie, and R. Y. Tsien. A new generation of  $\text{Ca}^{2+}$  indicators with greatly improved fluorescence properties. *J. Biol. Chem.* 260: 3440–3450, 1985.
8. Kondo, T., F. Konishi, H. Inui, and T. Inagami. Differing signal transduction elicited by three isoforms of platelet-derived growth factor in vascular smooth muscle cells. *J. Biol. Chem.* 268: 4458–4464, 1993.
9. Maher, J. J., and R. F. McGuire. Extracellular matrix gene expression increases preferentially in rat lipocytes and sinusoidal endothelial cells during hepatic fibrosis in vivo. *J. Clin. Invest.* 86: 1641–1648, 1990.
10. Milani, S., H. Herbst, D. Schuppan, E. G. Hahn, and H. Stein. In situ hybridization for procollagen types I, III and IV mRNA in normal and fibrotic rat liver: evidence for predominant expression in nonparenchymal liver cells. *Hepatology* 10: 84–92, 1989.
11. Milani, S., H. Herbst, D. Schuppan, E. O. Riecken, and H. Stein. Cellular localization of laminin gene transcripts in normal and fibrotic human liver. *Am. J. Pathol.* 134: 1175–1182, 1989.
12. Nishimura, J., S. Kobayashi, T. Shikasho, and H. Kanaide. Platelet-derived growth factor induces *c-fos* and *c-myc* mRNA in rat aortic smooth muscle cells in primary culture without elevation of intracellular  $\text{Ca}^{2+}$  concentration. *Biochem. Biophys. Res. Commun.* 16: 1198–1204, 1992.
13. Pinzani, M., H. E. Abboud, L. Gesualdo, and S. L. Abboud. Regulation of macrophage-colony stimulating factor in liver fat-

- storing cells by peptide growth factors. *Am. J. Physiol.* 262 (*Cell. Physiol.* 31): C876–C881, 1992.
14. **Pinzani, M., P. Failli, C. Ruocco, A. Casini, S. Milani, E. Baldi, A. Giotti, and P. Gentilini.** Fat-storing cells as liver-specific pericytes: spatial dynamics of agonist-stimulated intracellular calcium transients. *J. Clin. Invest.* 90: 642–646, 1992.
  15. **Pinzani, M., A. Gentilini, A. Caligiuri, R. De Franco, G. Pellegrini, S. Milani, F. Marra, and P. Gentilini.** Transforming growth factor- $\beta$ 1 regulates platelet-derived growth factor receptor  $\beta$  subunit in human liver fat-storing cells. *Hepatology* 21: 232–239, 1995.
  16. **Pinzani, M., L. Gesualdo, G. M. Sabbah, and H. E. Abboud.** Effects of platelet-derived growth factor and other polypeptide mitogens on DNA synthesis and growth in cultured rat liver fat-storing cells. *J. Clin. Invest.* 84: 1786–1793, 1989.
  17. **Pinzani, M., T. C. Knauss, G. F. Pierce, P. Hsieh, W. Kenney, G. R. Dubyak, and H. E. Abboud.** Mitogenic signals for platelet-derived growth factor isoforms in liver fat-storing cells. *Am. J. Physiol.* 260 (*Cell Physiol.* 29): C485–C491, 1991.
  18. **Pinzani, M., S. Milani, C. Grappone, R. De Franco, A. Gentilini, A. Caligiuri, and P. Gentilini.** Expression of platelet-derived growth factor (PDGF) and its  $\alpha$  and  $\beta$  receptor subunits in normal and cirrhotic liver (Abstract). *Hepatology* 18: 206A, 1993.
  19. **Pinzani, M., S. Milani, C. Grappone, F. L. Weber, Jr., P. Gentilini, and H. E. Abboud.** Expression of platelet-derived growth factor in a model of acute liver injury. *Hepatology* 19: 701–707, 1994.
  20. **Tucker, R. W., D. T. Chang, and K. Meade-Coburn.** Effects of platelet-derived growth factor and fibroblast growth factor on free intracellular calcium and mitogenesis. *J. Cell. Biochem.* 39: 139–151, 1989.
  21. **Wang, Z., M. Estacion, and L. J. Mordan.**  $\text{Ca}^{2+}$  influx via T-type channels modulates PDGF-induced replication of mouse fibroblasts. *Am. J. Physiol.* 265 (*Cell. Physiol.* 32): C1239–C1246, 1993.

