Regulation of Ca\textsuperscript{2+} Transport by Platelet-Derived Growth Factor-BB in Rat Vascular Smooth Muscle Cells

Massimo Cirillo, Stephen J. Quinn, Jose R. Romero, and Mitzy L. Canessa

The present study investigates the effects of platelet-derived growth factor (PDGF) isoform BB (PDGF-BB) on cytosolic Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]i), Ca\textsuperscript{2+} transport, and Ca\textsuperscript{2+} pools in rat vascular smooth muscle (VSM) cells. VSM cells from thoracic aorta of Milan normotensive rats were enzymatically dispersed, cultured in 10\% serum medium, and made quiescent by 72 hours in 0.3\% serum medium. [Ca\textsuperscript{2+}]i, Ca\textsuperscript{2+} influx, Ca\textsuperscript{2+} efflux, and exchangeable cell Ca\textsuperscript{2+} pool were evaluated by ratiometric fluorescent and radioisotope techniques. Ca\textsuperscript{2+} transport showed time-dependent changes during stimulation with PDGF-BB. The initial early responses to this peptide were a transient rise in [Ca\textsuperscript{2+}]i, a 30\% decrease in Ca\textsuperscript{2+} influx, and a 3.6-fold increase in the rate constant for active Ca\textsuperscript{2+} efflux. Stimulation of Ca\textsuperscript{2+} efflux and inhibition of Ca\textsuperscript{2+} influx were associated with a substantial 30\% reduction in the cell Ca\textsuperscript{2+} pool. This initial stimulation of Ca\textsuperscript{2+} efflux is concomitant with Ca\textsuperscript{2+} mobilization into the cytosol and is due to activation of Na\textsuperscript{+}-independent Ca\textsuperscript{2+} influx via the Ca\textsuperscript{2+} pump. After a 10-minute stimulation, Ca\textsuperscript{2+} influx returned to the basal value, whereas Ca\textsuperscript{2+} efflux remained 2.2-fold above control values, leading to a decline in [Ca\textsuperscript{2+}]i, below basal levels and a further decrease in the cell Ca\textsuperscript{2+} pool. Nearly half of this late Ca\textsuperscript{2+} efflux appears to be driven by Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange, as evidenced by its external Na\textsuperscript{+} dependence. After a 120-minute stimulation with PDGF-BB, nifedipine-sensitive Ca\textsuperscript{2+} influx is increased 37\% above basal levels, and Ca\textsuperscript{2+} efflux remains elevated. During prolonged stimulation by PDGF-BB, both Ca\textsuperscript{2+} influx and efflux are stimulated, resulting in a new intracellular Ca\textsuperscript{2+} homeostasis marked by the recovery of the cell Ca\textsuperscript{2+} pool but a lowered [Ca\textsuperscript{2+}]i. These final events coincide with the initiation of cell proliferation in VSM cells by PDGF-BB. (Circulation Research 1993;72:847–856)

**Key Words** • Ca\textsuperscript{2+} transport • platelet-derived growth factor • vascular smooth muscle • proliferation

Cell proliferation is a key event in tissue growth and cell differentiation. In vascular smooth muscle (VSM) cells, the initiation of the proliferative cycle and the rate of growth are controlled, in part, by autocrine and paracrine secretion of growth factors.\(^1\) Our laboratory has reported that VSM cells from genetically hypertensive rats have an increased growth rate in serum-rich medium compared with that of VSM cells from genetically normotensive rats.\(^2\) This abnormality may be responsible for the vascular hyperplasia observed in genetic models of rat hypertension and partially account for the high vascular resistance to blood flow.\(^3\) Serum-grown VSM cells from hypertensive rats are reported to have higher cytosolic Ca\textsuperscript{2+} concentrations ([Ca\textsuperscript{2+}]i) than those of normotensive rats,\(^2,4\)

suggesting that Ca\textsuperscript{2+} transport might be abnormally regulated during serum-stimulated growth.

Platelet-derived growth factor (PDGF) is a mitogen that is released into the serum by platelets, endothelial cells, and monocytes.\(^5\) The signal transduction mechanism of PDGF occurs through a complex biochemical cascade initiated by receptor binding, followed by activation of receptor-associated tyrosine kinase and phosphorylation of phospholipase C-\gamma.\(^6\) This enzyme hydrolyzes membrane phosphatidylinositol to produce diacylglycerol, which activates protein kinase C and inositol trisphosphate, which releases Ca\textsuperscript{2+} from intracellular stores. PDGF-BB is a powerful mitogenic isoform of PDGF that can initiate the proliferative cycle in VSM cells.\(^5,7\)

The present study investigates the regulation of Ca\textsuperscript{2+} transport in quiescent VSM cells during stimulation with PDGF-BB.

**Materials and Methods**

**Materials**

Tris, MOPS, bovine serum albumin, glucose, choline chloride, NaCl, KCl, CaCl\textsubscript{2}, EGTA, MgCl\textsubscript{2}, KH\textsubscript{2}PO\textsubscript{4}, LaCl\textsubscript{3}, sodium dodecyl sulfate (SDS), Na\textsuperscript{+}-K\textsuperscript{+} tartrate, CuSO\textsubscript{4}, Na\textsubscript{2}CO\textsubscript{3}, and Folin & Ciocalteu’s phenol reagent were from Sigma Chemical Co., St. Louis, Mo. Fura-2 AM and nifedipine were from Calbiochem Corp., La...
Jolla, Calif. 4Ca was from Amersham Corp., Arlington Heights, Ill. Ecoscint A was from National Diagnostics Inc., Manville, N.J. Dulbecco's modified Eagle's medium (DMEM), fetal bovine heat-inactivated serum (FBS), Na+-penicillin, Na+-streptomycin, and trypsin-EDTA were from Gibco Laboratories, Grand Island, N.Y. Human recombinant PDGF-BB was kindly provided by Dr. G. Nascimento, Chiron Co., Emeryville, Calif.

Cell Isolation and Culture

VSM cells were isolated by enzymatic dissociation from thoracic aorta of 16 normotensive 2-month-old rats of the Milan strain. Four separate preparations of four rats each were used in this study. VSM cells were cultured in 75-cm² flasks containing DMEM, 10% FBS, penicillin (100 units/ml), and streptomycin (100 µg/ml), as previously reported in this laboratory. 4 At confluence, VSM cells were harvested by trypsinization and passed 1:4 into 75-cm² culture flasks. For experiments, VSM cells at passages 6–12 were replicate-plated into 35-, 50-, and 100-mm culture dishes for measurements of Ca²⁺ fluxes and cell Ca²⁺ content or onto 22×40-mm glass coverslips for [Ca²⁺]c measurements. VSM cells were made quiescent by 72-hour incubation with 0.3% FBS-DMEM before all experiments.

Measurements of [Ca²⁺]c

[Ca²⁺]c was measured in plated VSM cells during stimulation with PDGF-BB using a modification of a microspectrofluorometric technique previously reported. 8 Quiescent VSM cells were cultured on a glass coverslip as detailed above, removed from the CO₂ incubator, washed with a balanced salt solution (BSS) containing (mM) NaCl 130, KCl 2, KH₂PO₄ 1, CaCl₂ 1.2, MgCl₂ 1, and glucose 10, along with 1 mg/ml BSA and Tris-MOPS, pH 7.4, and equilibrated for 120 minutes at 37°C in the same solution. The VSM cells were loaded with fura-2 AM (3 µM for 30 minutes) starting at the 60-minute time point of equilibration. The fura-2 AM-rich solution was replaced with fresh BSS at the 90-minute time point. At the end of the 120-minute equilibration, the glass coverslip was then placed on an inverted microscope (Nikon) and superfused with BSS at 37°C. A dual wavelength excitation light source (Photon Technology International, Princeton, N.J.) was used to alternate excitation wavelengths from a pair of monochromators centered at 350 and 380 nm with a bandwidth of 4 nm. The inverted microscope was equipped with an ultraviolet-transmitting objective (UVF series ×100, Nikon), an epifluorescence dichroic mirror centered at 430 nm, and a long-pass barrier filter transmitting beyond 480 nm. The emission light detection system consisted of a photometer with a photon-counting photomultiplier tube mounted to the side camera port of the microscope. The computer synchronized the photomultiplier signal collection with alternation of the excitation wavelength to separate the two emission signals. The emission ratio after background subtraction was used to estimate changes of [Ca²⁺]c over time. All [Ca²⁺], recordings were digitally filtered with a 25-point Savitsky-Golay smoothing routine.

Measurements of Ca²⁺ Influx

Quiescent VSM cells were removed from the CO₂ incubator, washed, and equilibrated for 120 minutes at 37°C with BSS. 4Ca influx was initiated by replacing the BSS with a 4Ca-rich BSS (5 µCi/ml). To stop influx, the 4Ca-BSS was removed, and the dishes were rapidly washed with ice-cold washing solution containing (mM) LaCl₃ 10, MgCl₂ 90, and Tris-MOPS, pH 7.4 (La²⁺-rich WS), followed by three additional washes with ice-cold solution containing 100 mM MgCl₂ and Tris-MOPS, pH 7.4 (La²⁺-free WS). VSM cells were extracted with 0.1% SDS for radioactivity counting in Ecoscint A, and duplicate protein content was determined by the Lowry procedure. 9 At each time point, the value for cell-associated counts per minute was divided by the specific activity of the 4Ca-rich BSS and normalized per milligram protein. The initial rate of Ca²⁺ influx was determined by the slope of the regression line of the cell-associated Ca²⁺ (nanomoles per milligram protein) versus time. The correlation coefficient of this regression was always higher than 0.985.

Measurement of Ca²⁺ Efflux

Quiescent VSM cells, plated onto 35-mm dishes, were removed from the CO₂ incubator, washed with BSS, and equilibrated for 120 minutes at 37°C in BSS plus 4Ca (5 µCi/ml). At equilibrium, the 4Ca-rich BSS was removed by suction, and dishes were rapidly rinsed with ice-cold WS, followed by three additional washes with ice-cold solution containing 0.1 M MgCl₂ and Tris-MOPS, pH 7.4. To study the late effects of PDGF, the peptide was added to the 4Ca-rich BSS in the last 10 minutes of the equilibrium procedure, and the desaturation curve was performed in isotope-free BSS containing the same concentration of PDGF. Efflux was started by addition of isotope-free BSS at 37°C, which was replaced every 30 seconds for up to 5 minutes. At the end of the experiment, VSM cells were extracted in 0.1% SDS for measurement of radioactivity by scintillation counting and duplicate protein content determination. The radioactivity in each aliquot of the efflux medium was added to the radioactivity remaining in the cells at the end of experiments to calculate the cell-associated radioactivity at the beginning of the efflux. The cell-associated radioactivity at each time point was obtained by sequentially subtracting the radioactivity of each efflux period from the cell-associated radioactivity of the previous time point. This cell-associated radioactive activity at each time point was divided by the specific activity of the 4Ca-rich BSS to determine the cell-associated Ca²⁺ in millimoles per dish and normalized to the protein content.

Measurement of the Exchangeable Ca²⁺ Pool

Quiescent VSM cells, plated on 35-mm dishes, were washed and equilibrated for 120 minutes in BSS containing 4Ca (5 µCi/ml) at 37°C. At equilibrium (120 minutes), the 4Ca-rich BSS was removed by suction, and dishes were rapidly rinsed with ice-cold La²⁺-rich and La²⁺-free WS as detailed above. VSM cells were extracted with 0.1% SDS for radioactivity counting and duplicate protein content determination. The cell-associated radioactivity at equilibrium was divided by the specific activity of the 4Ca-rich BSS to give the cell-associated radioactive Ca²⁺, which was normalized to protein content.
Measurement of Total Cellular Ca\(^{2+}\)

Quiescent VSM cells, plated on 100-mm dishes, were equilibrated for 120 minutes in BSS at 37°C. PDGF-BB or vehicle was added to this solution from 200× stocks. After incubation, VSM cells were washed with ice-cold La\(^{3+}\)-rich and La\(^{3+}\)-free WS, extracted in 0.1% SDS to measure cellular Ca\(^{2+}\) content by atomic absorption spectrophotometry, and normalized to protein content.

Measurement of Na\(^{+}\) Influx

Quiescent VSM cells, plated on 35-mm dishes, were equilibrated for 120 minutes in BSS at 37°C. \(^{22}\)Na influx was initiated by replacing the BSS with a \(^{22}\)Na-rich (5 μCi/ml) solution containing 50 mM Na\(^{+}\) as described previously.\(^2\) To stop influx, the \(^{22}\)Na-BSS was removed, and the dishes were rapidly washed with ice-cold La\(^{3+}\)-free WS. VSM cells were extracted with 0.1% SDS for radioactivity counting in Ecoscint A and duplicate protein content determination by the Lowry procedure.\(^9\) At each time point, the value for cell-associated counts per minute was divided by the specific activity of the \(^{22}\)Na-rich BSS and normalized (nanomoles per milligram protein). The Na\(^{+}\) influx is calculated from the change in cell Na\(^{+}\) uptake in nanomoles per milligram protein as a function of time.

Statistical Analysis

Linear and nonlinear fitting of the data was performed using the ENZFITTER program (Biosoft, Elsevier, Cambridge, UK). Paired and unpaired Student’s t tests were used as appropriate. A value of \(p<0.05\) was accepted as statistically significant. Data are presented as mean±SEM unless otherwise indicated.

Results

Cytosolic Calcium Changes During PDGF-BB Stimulation

In small clusters of quiescent VSM cells, basal [Ca\(^{2+}\)]\(_i\) was 111±18 nM (\(n=14\)). During application of PDGF-BB (50 ng/ml), VSM cells responded with changes in [Ca\(^{2+}\)]\(_i\) in 12 of 14 experiments. In five experiments, PDGF-BB elicited a [Ca\(^{2+}\)] peak with an increase of 45±5 nM after a 90-second delay from the onset of hormone stimulation (Figure 1). After this peak transient, [Ca\(^{2+}\)] started to decline and continued to fall until the end of the 30-minute application. In the

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**Figure 1.** Tracings showing the effect of platelet-derived growth factor-BB (PDGF-BB) on cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) in quiescent vascular smooth muscle cells. Panel A: Representative [Ca\(^{2+}\)]\(_i\) tracings were taken from small clusters of vascular smooth muscle cells stimulated with 50 ng/ml PDGF-BB for 30 minutes. Panel B: Mean [Ca\(^{2+}\)]\(_i\) changes were determined for vascular smooth muscle cells, which exhibited an initial rise in [Ca\(^{2+}\)]\(_i\) (\(n=5\)) or no initial change in [Ca\(^{2+}\)]\(_i\) (\(n=9\)) during PDGF-BB stimulation. Control cells with no PDGF-BB application showed little change in [Ca\(^{2+}\)]\(_i\) (\(n=3\)). The onset of PDGF-BB stimulation is indicated by the arrow at the beginning of each tracing.
remaining experiments, [Ca\(^{2+}\)], did not demonstrate the initial transient but fell within 10 minutes of PDGF-BB application and continued to decline until the end of the 30-minute stimulation (Figure 1). The average [Ca\(^{2+}\)] response for all experiments showed a 41 nM fall below baseline to 70±8 nM by the 30-minute time point (n=14). Control with no PDGF-BB application showed little change in [Ca\(^{2+}\)], (n=3).

**Time Course of Changes in Ca\(^{2+}\) Influx During PDGF-BB Stimulation**

In quiescent VSM cells, the earliest time point (10 seconds) of \(^{45}\)Ca uptake was always higher than zero (Figure 2A), likely because of rapid Ca\(^{2+}\) binding to extracellular sites and some influx into the cell.\(^{10}\) Between 10 seconds and 3 minutes, Ca\(^{2+}\) uptake was linear, with the slope of this line measuring the rate of Ca\(^{2+}\) influx. Ca\(^{2+}\) influx in quiescent, unstimulated VSM cells averaged 0.494±0.035 nmol·mg protein\(^{-1}\)·min\(^{-1}\) (n=13). This basal Ca\(^{2+}\) influx was inhibited 44% by 10 μM nifedipine (p<0.005). The rate of Ca\(^{2+}\) influx was unaffected by extracellular Na\(^+\) (Na\(_0\)) removal, indicating that Na\(^+\)-Ca\(^{2+}\) exchange was not active as a pathway for Ca\(^{2+}\) influx.

Ca\(^{2+}\) influx was inhibited by 34% during the first 2 minutes of PDGF-BB stimulation (Figure 2B). However, Ca\(^{2+}\) influx returned to control values by 10 minutes of PDGF-BB application. Similar results were observed when Ca\(^{2+}\) influx was studied in Na\(^+\)-free BSS. With 120 minutes of PDGF-BB stimulation, Ca\(^{2+}\) influx had risen significantly (+37%). This 120-minute preincubation with PDGF-BB failed to increase Ca\(^{2+}\) influx when measured in the presence of 10 μM nifedipine (n=9).

**Activation of Two Pathways for Ca\(^{2+}\) Efflux During PDGF-BB Stimulation**

In quiescent VSM cells, the \(^{45}\)Ca loading was complete within 120 minutes, and equilibrium was stable for up to 240 minutes. The desaturation curve of \(^{45}\)Ca was best fit by a two-compartment exponential model.\(^{11}\) The two-compartment model gave the lowest residual values and the lowest standard error for the estimated parameters when compared with a one- or three-compartment model. The first compartment was smaller than the second compartment (2.36±0.16 versus 4.57±0.21 nmol/mg protein, n=28, p<0.001). The rate constant of the first compartment (\(k_1\)) was more than.

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**TABLE 1. Effect of Low Temperature and Lanthanum on the Rate Constants of \(^{45}\)Ca Desaturation Curve in Quiescent Vascular Smooth Muscle Cells**

<table>
<thead>
<tr>
<th>Rate constants</th>
<th>(k_1) (min(^{-1}))</th>
<th>(k_2) (10(^{-2}) min(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSS at 37°C</td>
<td>4</td>
<td>2.09±0.07</td>
</tr>
<tr>
<td>BSS at 4°C</td>
<td>4</td>
<td>2.10±0.14</td>
</tr>
<tr>
<td>La(^{3+}) (10 mM)</td>
<td>4</td>
<td>2.32±0.08*</td>
</tr>
</tbody>
</table>

\(k_1\), Rate constant of the first compartment; \(k_2\), rate constant of the second compartment; BSS, balanced salt solution. Values are mean±SEM.

The vascular smooth muscle cells were loaded with \(^{45}\)Ca, and \(^{45}\)Ca efflux was monitored in isotope-free standard BSS at 37°C, in BSS containing 10 mM LaCl\(_3\), or in standard BSS at 4°C. A two-compartment analysis of \(^{45}\)Ca desaturation determined the rate constant of Ca\(^{2+}\) efflux from each compartment by nonlinear regression analysis.

\(^*p<0.05\) vs. BSS at 37°C.
20-fold greater than the rate constant of the second compartment \( k_2 \) (2.14±0.08 versus 0.08±0.01 min\(^{-1} \), \( n=28, p<0.001 \)). Thus, the contribution of the first compartment would be negligible during the slow phase of desaturation between the 2- and 5-minute time points. This slow phase could be fit by a single exponential with a rate constant similar to that found for the second compartment of the two-compartment model.

Rate constant \( k_1 \) was rapid and unaffected by low temperature (4°C), suggesting that it represented Ca\(^{2+}\) displacement from extracellular binding sites (Table 1). Furthermore, La\(^{3+}\) (10 mM) produced a slight, but significant, increase in \( k_1 \), consistent with this interpretation. On the other hand, both low temperature and La\(^{3+}\) significantly slowed rate constant \( k_2 \) of \(^{45}\)Ca desaturation, indicating that \( k_2 \) reflects active Ca\(^{2+}\) efflux from an intracellular site against its electrochemical gradient.

Basal Ca\(^{2+}\) efflux was unaffected by Na\(^{+}\) removal, suggesting that Na\(^{+}\)-Ca\(^{2+}\) exchange was not a significant pathway for Ca\(^{2+}\) efflux in quiescent VSM cells (Figure 3A). The removal of extracellular Ca\(^{2+}\) (Ca\(_{\infty}\)) induced a slight elevation of \( k_2 \), indicating that Ca\(^{2+}\) entry into the cellular pool is fast enough to dilute the Ca\(^{2+}\) in the intracellular compartment (Figure 3B). To exclude nonspecific effects of EGTA on Ca\(^{2+}\) permeability, Ca\(_{\infty}\) removal experiments were also performed without EGTA and with the Ca\(_{\infty}\) concentration in the order of 5 \( \mu \)M, and similar results were found (4.68±0.03×10\(^{-2}\) min\(^{-1} \) for BSS, 5.38±0.17×10\(^{-2}\) min\(^{-1} \) for BSS and Ca\(_{\infty}\) removal without EGTA, \( n=4 \)). These data suggest that Ca\(^{2+}\)-Ca\(^{2+}\) exchange is not a substantial pathway for Ca\(^{2+}\) efflux.

To minimize the contribution of the extracellular compartment, the early (3-minute) effect of PDGF-BB on active Ca\(^{2+}\) efflux was studied by adding the growth factor at the 2-minute time point, after the first compartment was desaturated (Figure 4A). In this set of experiments, the rate constant for Ca\(^{2+}\) efflux was calculated by fitting the data points from 2 to 5 minutes with a single-compartment exponential model. PDGF-BB induced a sharp increase in Ca\(^{2+}\) efflux after a lag time of 40–60 seconds, and the mean values of \( k_2 \) for Ca\(^{2+}\) efflux increased fourfold with PDGF-BB stimulation (Table 2). The same increase was observed in Na\(^{+}\)-free BSS (Figure 4A, Table 2), with the Na\(^{+}\)-dependent fraction of \( k_2 \) for Ca\(^{2+}\) efflux not detectable. The PDGF-BB–induced stimulation of Ca\(^{2+}\) efflux was also unaffected by Ca\(_{\infty}\) removal (23.49±0.12×10\(^{-2}\) min\(^{-1} \) for BSS plus PDGF, 18.5±4.6×10\(^{-2}\) min\(^{-1} \) for BSS and Ca\(_{\infty}\) removal plus PDGF, \( n=4 \)). Thus, the initial stimulation of Ca\(^{2+}\) efflux by PDGF-BB was independent of Ca\(_{\infty}\) and Na\(^{+}\).

To study the later effects of this growth factor on Ca\(^{2+}\) efflux, VSM cells were preincubated with PDGF-BB during equilibration with the \(^{45}\)Ca-rich BSS. After a 10-minute preincubation with PDGF-BB, \( k_2 \) was increased 2.4-fold and became partially reduced by the removal of external Na\(^{+}\) (Figure 4B, Table 2). The increase of \( k_2 \) induced by PDGF-BB was dose dependent, with maximal effects found between 30 and 50 ng/ml (Figure 5). Although the reduction of \( k_2 \) in Na\(^{+}\)-free medium could result from a dilution of the specific activity of \(^{45}\)Ca within the VSM cells because of an increase in Ca\(^{2+}\) entry, measurements of the initial rates of Ca\(^{2+}\) entry after 10 minutes of PDGF-BB pretreatment showed no effect of external Na\(^{+}\) removal.
Na⁺ influx associated with Na⁺-Ca²⁺ exchange was stimulated by PDGF-BB, as seen in Figure 6. In the presence of inhibitors for the Na⁺-K⁺ pump (1 mM ouabain), Na⁺-H⁺ exchange (20 μM dimethylamiloride), and Na⁺-K⁺-Cl⁻ cotransporter (100 μM bumetanide), Na⁺ entry was tripled by the addition of PDGF-BB (47.8 ± 3.2 versus 154 ± 7.3 nmol/mg protein for 20 minutes [control versus 50 ng/ml PDGF], n = 12), consistent with the enhancement of Na⁺-dependent Ca²⁺ efflux (Figure 8). At a concentration of 50 μM, dimethylbenzamil, an amiloride analogue that selectively blocks Na⁺-Ca²⁺ exchange, inhibited 82% of this PDGF-stimulated Na⁺ influx (n = 3).

PDGF-BB can alkalinate cell pH in VSM cells through activation of Na⁺-H⁺ exchange, and these pH changes may influence Ca²⁺ efflux. To test this hypothesis, Ca²⁺ efflux was measured in the absence and presence of dimethylamiloride, a blocker of Na⁺-H⁺ exchange and the cell pH changes associated with PDGF stimulation. Basal Ca²⁺ efflux was similar in the presence of 20 μM dimethylamiloride (7.6 ± 1.4 × 10⁻⁷ min⁻¹, n = 3) compared with control values (Table 2). Figure 7 summarizes the time course of the PDGF-BB effect on the rate constants for Na⁺-dependent and -independent Ca²⁺ efflux. After 40 seconds of PDGF-BB stimulation, a peak elevation of kₑ for Ca²⁺ efflux was observed, which was entirely Na⁺ independent (Figure 7A). This was followed by a less pronounced but stable elevation of Na⁺-independent Ca²⁺ efflux observed for up to 120 minutes of PDGF-BB treatment. The Na⁺-dependent Ca²⁺ efflux showed a different time course, with no early activation and a progressive increase in the Na⁺-dependent component, which peaked at 10 minutes and remained elevated for up to 120 minutes of PDGF-BB treatment (Figure 7B).

Time Course of Changes in the Exchangeable Ca²⁺ Pool and Total Cellular Ca²⁺ During PDGF-BB Stimulation

In quiescent VSM cells, the exchangeable Ca²⁺ pool averaged 6.82 ± 0.31 nmol/mg protein (n = 25). Total cellular Ca²⁺ averaged 17 ± 10.8 nmol/mg protein (n = 12); thus, the exchangeable Ca²⁺ pool represents 41% of the total cellular Ca²⁺. These atomic absorption measurements of total cellular Ca²⁺ have a larger variation than measurements of exchangeable Ca²⁺ pools, because the total Ca²⁺ concentrations of the tissue extracts were at the low end of the detection range. Figure 8A shows the time course of this pool after prolonged stimulation with PDGF-BB. No significant change was observed in the first minute of PDGF-BB application. Between 1 and 3 minutes, the exchangeable Ca²⁺ pool was rapidly reduced and continued to fall more slowly between 3 and 20 minutes; however, this Ca²⁺ pool began to recover by 60 minutes of PDGF-BB stimulation and had returned to basal values by 120 minutes. Longer (150-minute) exposures to PDGF-BB did not induce further changes. The decrease in the exchangeable Ca²⁺ pool induced by PDGF-BB was dose dependent, with a maximal effect reached between 5 and 50 ng/ml (Figure 8B).

Discussion

The present study demonstrates that PDGF-BB induces a sequence of changes in Ca²⁺ transport, Ca²⁺-

![Figure 4](http://circres.ahajournals.org/). Graphs showing early and late stimulation of Ca²⁺ efflux by platelet-derived growth factor-BB (PDGF-BB) in quiescent vascular smooth muscle cells. Cell-associated Ca²⁺ is given in nanomoles per milligram protein (prot). Panel A: Early effect. Representative experiment shows the ⁴⁵Ca desaturation curves in the absence of PDGF-BB (filled squares) and during PDGF-BB stimulation in the absence (open circles) and presence (closed circles) of extracellular Na⁺ (Nao). PDGF-BB (50 ng/ml) was added 2 minutes after the start of desaturation to minimize the contribution of the extracellular compartment. Similar results were found in four experiments (see Table 2). Panel B: Late effect. Representative experiment shows the ⁴⁵Ca desaturation curves in the absence of PDGF-BB (open circles) and after 10 minutes of stimulation with PDGF-BB in the absence (open triangles) and presence (closed circles) of Nao. To study the late effect on Ca²⁺ efflux, PDGF-BB (50 ng/ml) was added to ⁴⁵Ca-rich balanced salt solution in the last 10 minutes of the equilibration procedure, and the desaturation curve was performed in isotope-free balanced salt solution containing the same concentration of PDGF-BB. Similar results were found in 12 experiments (see Table 2).
TABLE 2. Early and Late Effects of Platelet-derived Growth Factor-BB on the Rate Constant of Active Ca²⁺ Efflux in Quiescent Vascular Smooth Muscle Cells

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>Na⁺ independent</th>
<th>Na⁺ dependent</th>
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<tbody>
<tr>
<td>Early effect</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Basal</td>
<td>6.38±0.06</td>
<td>6.24±0.67</td>
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<tr>
<td>PDGF-BB</td>
<td>24.80±4.61*</td>
<td>25.34±0.33*</td>
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<tr>
<td>k₂ change</td>
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<td>19.10±0.30</td>
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<tr>
<td>Late effect</td>
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<tr>
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<td>6.61±0.54</td>
<td>6.20±0.62</td>
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<tr>
<td>PDGF-BB</td>
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<td>10.12±0.59*</td>
<td>4.95±0.67*</td>
</tr>
<tr>
<td>k₂ change</td>
<td>8.46±0.66</td>
<td>3.92±0.80</td>
<td>4.54±0.67</td>
</tr>
</tbody>
</table>

k₂, Rate constant of the second compartment; PDGF-BB, platelet-derived growth factor-BB. Values are mean±SEM.

The vascular smooth muscle cells were loaded with ⁴⁰Ca, and ⁴⁰Ca efflux was monitored in standard balanced salt solution and in Na⁺-free balanced salt solution in the absence (basal) and in the presence of PDGF-BB (50 ng/ml). For the early effect, PDGF-BB was added at the 2-minute time point of the ⁴⁰Ca desaturation. The data points between 2 and 5 minutes were used to calculate the rate constant of active Ca²⁺ efflux using a single-compartment exponential model. The calculated values are the mean±SEM for four experiments under each experimental condition. For the late effect, vascular smooth muscle cells were stimulated for 10 minutes with PDGF-BB during the equilibrium procedure as described in "Materials and Methods." Each value is the mean±SEM from 12 experiments. A two-compartment analysis of ⁴⁰Ca desaturation determined the rate constant of each compartment. The rate constant for the first compartment (k₁) using standard balanced salt solution was not significantly different for basal versus PDGF-BB stimulation (2.39±0.14 vs. 2.28±0.21 min⁻¹) with similar results obtained using Na⁺-free balanced salt solution. The total k₂ value was determined from the ⁴⁰Ca desaturation curve in standard balanced salt solution. The Na⁺-independent component was determined from the ⁴⁰Ca desaturation curve in Na⁺-free balanced salt solution; the Na⁺-dependent component was calculated by subtracting the k₂ Na⁺-free balanced salt solution from the k₂ in standard balanced salt solution in coupled experiments. *p<0.001 vs. basal value.

k₂, Rate constant of the second compartment; PDGF-BB, platelet-derived growth factor-BB. Values are mean±SEM.

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The transient increase in [Ca²⁺]ᵢ is slower and more prolonged during PDGF-BB stimulation than that induced by vasoconstrictors such as angiotensin II, which may be explained by the different time courses of inositol trisphosphate production for the two hormones.¹⁴,¹⁵ This difference may reflect the activation of specific isoforms of phospholipase C by PDGF-BB (γ₁) and by angiotensin II (β₁).

The transient rise in [Ca²⁺]ᵢ during PDGF-BB stimulation is associated with inhibition of Ca²⁺ influx, stimulation of Ca²⁺ efflux, and a marked reduction of the exchangeable Ca²⁺ pool, indicating that the elevated including the activation of phospholipase C and the generation of inositol trisphosphate. The early transient increase in [Ca²⁺]ᵢ is slower and more prolonged during PDGF-BB stimulation than that induced by vasoconstrictors such as angiotensin II, which may be explained by the different time courses of inositol trisphosphate production for the two hormones.¹⁴,¹⁵ This difference may reflect the activation of specific isoforms of phospholipase C by PDGF-BB (γ₁) and by angiotensin II (β₁).
[Ca\textsuperscript{2+}] is exclusively due to release of Ca\textsuperscript{2+} from intracellular stores. Previous studies hypothesized that the transient [Ca\textsuperscript{2+}] peak induced by PDGF-BB was, in part, due to stimulation of Ca\textsuperscript{2+} influx, since Ca\textsuperscript{2+} channel blockers and Ca\textsubscript{o} removal each reduced the magnitude of the [Ca\textsuperscript{2+}], transient.\textsuperscript{16,17} However, those studies did not directly measure Ca\textsuperscript{2+} influx and could not exclude the possibility that these experimental manipulations inhibited inositol trisphosphate generation or reduced the amount of releasable Ca\textsuperscript{2+} stores.\textsuperscript{18}

The early inhibitory effect of PDGF-BB on Ca\textsuperscript{2+} influx could be due to the increase in [Ca\textsuperscript{2+}] levels, given that the inhibition disappeared when [Ca\textsuperscript{2+}] returned to baseline. Two possible explanations are Ca\textsuperscript{2+} inactivation of Ca\textsuperscript{2+} channels at elevated high [Ca\textsuperscript{2+}] levels or activation of Ca\textsuperscript{2+}-dependent K\textsuperscript{+} channels, leading to membrane hyperpolarization and a reduction of inward Ca\textsuperscript{2+} current.\textsuperscript{19,20} However, a majority of experiments using small clusters of VSM cells showed no initial [Ca\textsuperscript{2+}], transient, raising the possibility that the changes in Ca\textsuperscript{2+} influx are unrelated to the [Ca\textsuperscript{2+}], level or reflect the behavior of a subpopulation of VSM cells. Alternatively, this inhibition of Ca\textsuperscript{2+} influx may be explained by modification of basally active Ca\textsuperscript{2+} channels during the early phases of transduction of the PDGF-BB signal.

By the 10-minute time point, the mean [Ca\textsuperscript{2+}] level had returned to baseline values, as had Ca\textsuperscript{2+} influx. Ca\textsuperscript{2+} efflux had decreased from the high rates reached during the peak rise in [Ca\textsuperscript{2+}], but still remained twofold higher than control rates. Consistent with these findings, the exchangeable Ca\textsuperscript{2+} pool continued to decrease as more Ca\textsuperscript{2+} was transported from than entered into the VSM cells. Both [Ca\textsuperscript{2+}], and the exchangeable Ca\textsuperscript{2+} pool further declined between 10 and 20 minutes of PDGF-BB stimulation.

At the 2-hour time point, Ca\textsuperscript{2+} influx and efflux continued to be elevated above control values, whereas the exchangeable Ca\textsuperscript{2+} pool had returned to prestimulation levels, suggesting the creation of a new Ca\textsuperscript{2+} equilibrium. The enhanced Ca\textsuperscript{2+} influx found during long-term PDGF-BB stimulation was abolished by nifedipine, indicating the activation of dihydropyridine-sensitive Ca\textsuperscript{2+} channels. This late stimulation of Ca\textsuperscript{2+} channels may play an important role in the mitogenic effect of PDGF-BB. It has been shown that thymidine incorporation in VSM cells stimulated by PDGF is inhibited by Ca\textsuperscript{2+} channel blockers.\textsuperscript{7} Moreover, the maximal response of phosphatidylinositol diphosphate hydrolysis and protein kinase C activation by growth factors is known to depend on Ca\textsuperscript{2+} influx.\textsuperscript{7,16}

The sequential effect of PDGF-BB on Ca\textsuperscript{2+} efflux in VSM cells appears to be due to two separate mechanisms: the Ca\textsuperscript{2+} pump and Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange. These two transport systems have quite different energetic and kinetic characteristics.\textsuperscript{21} The Ca\textsuperscript{2+} pump moves Ca\textsuperscript{2+} out of the cell against its electrochemical gradient, using the energy provided by ATP hydrolysis. The Na\textsuperscript{+}-Ca\textsuperscript{2+} antipporter uses the Na\textsuperscript{+} gradient to exchange one Ca\textsuperscript{2+} for three Na\textsuperscript{+}. The Ca\textsuperscript{2+} pump is a low-capacity high-affinity transport system, whereas the Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange has a very high capacity but a low affinity for [Ca\textsuperscript{2+}]. In quiescent VSM cells, we found that active Ca\textsuperscript{2+} efflux occurs exclusively via a Na\textsuperscript{+}-independent pathway, which is Na\textsuperscript{+} and temperature sensitive. This pathway, the Ca\textsuperscript{2+} pump, was activated fourfold in the first few minutes of PDGF-BB stimulation of VSM cells. This early stimulation of Ca\textsuperscript{2+} efflux is likely a substrate-dependent stimulation of the Ca\textsuperscript{2+} pump, supported by the identical time course of the rise and fall of [Ca\textsuperscript{2+}]. However, this rise in [Ca\textsuperscript{2+}], was not sufficient to increase Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange at early times of PDGF-BB stimulation.

The later, more sustained activation of Ca\textsuperscript{2+} efflux resulted from an increase in the rate constants of both

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**FIGURE 7.** Graphs showing time course of changes in the rate constant of the second compartment (K\textsubscript{2}) for Ca\textsuperscript{2+} efflux from vascular smooth muscle cells during platelet-derived growth factor-BB (PDGF-BB) stimulation. Panel A: Na\textsuperscript{+}-independent changes. Ca\textsuperscript{2+} efflux was measured in Na\textsuperscript{+}-free balanced salt solution after 2, 5, 10, and 120 minutes of stimulation with PDGF-BB (50 ng/ml). This component is an estimate of the Ca\textsuperscript{2+} pump. Each value is the mean±SEM from four to 12 experiments. Panel B: Na\textsuperscript{+}-dependent changes. Ca\textsuperscript{2+} efflux was calculated as the difference between Ca\textsuperscript{2+} efflux in standard and extracellular Na\textsuperscript{+}-free balanced salt solution from paired experiments after 2, 5, 10, and 120 minutes of stimulation with PDGF-BB (50 ng/ml). This component is an estimate of Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange. No Na\textsuperscript{+}-dependent activity was detected in vascular smooth muscle cells under control conditions and after 2 minutes of PDGF-BB stimulation. Each value is the mean±SEM from four to 12 experiments. *p<0.05 and **p<0.01 vs. control.
FIGURE 8. Graphs showing changes in the exchangeable Ca\(^{2+}\) pool of quiescent vascular smooth muscle cells during platelet-derived growth factor-BB (PDGF-BB) stimulation. Panel A: Time course of changes in the exchangeable Ca\(^{2+}\) pool by 50 ng/ml PDGF-BB. The calculated values are the mean±SEM for four to 20 experiments. The exchangeable Ca\(^{2+}\) pool was significantly reduced between 2 and 60 minutes of PDGF-BB stimulation but was not significantly different from the basal value for the 120- and 150-minute time point. During long stimulations with PDGF-BB, the balanced salt solution was changed every 20 minutes to keep constant the PDGF-BB concentration. The size of the exchangeable Ca\(^{2+}\) pool is expressed as the percentage of control values in paired experiments. Panel B: Dose–response change in the exchangeable Ca\(^{2+}\) pool during PDGF-BB stimulation. Vascular smooth muscle cells were stimulated for 10 minutes with different concentrations of PDGF-BB. The calculated values are the mean±SEM for four to 20 experiments. The size of the exchangeable Ca\(^{2+}\) pool is expressed as the percentage of control values in paired experiments.

The Ca\(^{2+}\) pump and, more important, Na\(^{+}-\)Ca\(^{2+}\) exchange. This enhanced Ca\(^{2+}\) efflux cannot be explained by increased Ca\(^{2+}\) substrate availability, since [Ca\(^{2+}\)], had returned to the basal values by 10 minutes and then began to decrease below basal levels. Therefore, the increased rate constant must reflect a lower $K_a$ for [Ca\(^{2+}\)], or an increased maximal activity of these transport systems. The increased Na\(^{+}-\)Ca\(^{2+}\) exchange is also indicated by the enhanced Na\(^{+}\) influx in the presence of PDGF-BB, which can be blocked by 50 μM dimethylbenzamil. At the same time that Na\(^{+}-\)Ca\(^{2+}\) exchange was stimulated to greater activity, most VSM cells began to show a decline from basal [Ca\(^{2+}\)] values, even for those that did not initially respond with a rise in [Ca\(^{2+}\)].

Several hypotheses can explain the late PDGF-BB–induced activation of Ca\(^{2+}\) efflux. PDGF-BB induces cell alkalization in VSM cells by the activation of Na\(^{+}-\)H\(^{+}\) exchange.\(^7,21\) An increase in cytosolic pH could be involved in the stimulation of Ca\(^{2+}\) efflux;\(^22,23\) however, dimethylamiloride at concentrations that block Na\(^{+}-\)H\(^{+}\) exchange\(^24\) was not able to inhibit the activation of Ca\(^{2+}\) efflux. Alternatively, activation of protein kinase C by PDGF-BB could elevate Ca\(^{2+}\) influx by increasing the maximal velocity of the Ca\(^{2+}\) pump, as reported in rat cultured VSM cells,\(^25\) human neutrophils,\(^26\) and human erythrocytes.\(^27\) Furthermore, Vigne et al\(^28\) showed that phorbol esters increase the maximal capacity of Na\(^{+}-\)Ca\(^{2+}\) exchange, as measured by Na\(^{+}\)-depen-

dent Ca\(^{2+}\) entry, in the A23 VSM cell line. Activation of protein kinase C may also be responsible for the late activation Ca\(^{2+}\) influx by PDGF-BB, as demonstrated in electrophysiological and transport studies in cardiac muscle cells.\(^29,30\) These observations suggest that protein kinase C activation of Ca\(^{2+}\) transport pathways may be a general phenomenon, where increased Ca\(^{2+}\) influx is coupled to sustained generation of diacylglycerol, which maintains the membrane-translocated protein kinase C in its active state.\(^31\) Thus, the late stimulation of Ca\(^{2+}\) influx and efflux induced by PDGF-BB in VSM cells may represent different effects of the same biochemical event (protein kinase C activation and its membrane translocation) occurring at the initiation of the cell proliferative cycle.

In summary, the present study provides evidence that PDGF induces time-dependent modifications of Ca\(^{2+}\) transport in VSM cells. The early increase in [Ca\(^{2+}\)], due to Ca\(^{2+}\) mobilization from intracellular stores is a transitory change, which is rapidly buffered by the stimulation of Ca\(^{2+}\) efflux and the inhibition of Ca\(^{2+}\) influx. The late sustained activation in Ca\(^{2+}\) influx and efflux are more likely involved in the long-term proliferative response initiated by PDGF and appear to be more generally shared by most VSM cells. During the later stages of PDGF stimulation, a new dynamic equilibrium is achieved by the enhancement of both Ca\(^{2+}\) influx and efflux. Of particular importance is the finding that a dormant Na\(^{+}-\)Ca\(^{2+}\) exchange is stimulated by the mito-
gen, activating a new pathway for Ca\(^{2+}\) efflux. The modulation of this transport system by PDGF may be a pivotal factor in the regulation of Na\(^{+}\)-Ca\(^{2+}\) exchange as well as a key mechanism in the control of [Ca\(^{2+}\)]\(_e\), in the proliferating VSM cells. This possibility is very intriguing, since [Ca\(^{2+}\)]\(_e\) is elevated and the growth rate is accelerated in cultured VSM cells from genetically hypertensive rats.\(^{2,24}\)

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